OXYGEN FREE RADICALS AND THE TISSUE INJURY

Editors: B. Matkovics, D. Boda, H. Kalász

Akadémiai Kiadó, Budapest

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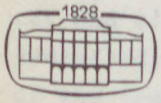
Editors

B. MATKOVICS, M. D., C. Sc.
Biological Isotope Laboratory
József Attila University
Szeged, Hungary

D. BODA, M. D., D. Sc.
Department of Pediatrics
Szent-Györgyi Albert University Medical School
Szeged, Hungary

H. KALÁSZ, Ph. D., D. Sc.
Department of Pharmacology
Semmelweis University of Medicine
Budapest, Hungary



AKADÉMIAI KIADÓ, BUDAPEST 1988

ISBN 963 05 4951 4

Printed in Hungary
by Akadémiai Kiadó és Nyomda Vállalat, Budapest

PREFACE

Researchers dealing with oxygen radicals first came together on 21-26 June 1976, in Banyuls, France, supported by the European Molecular Biology Organization, in order to survey their results, and mainly those relating to the superoxide dismutases (SOD-s; EC 1.15.1.1) and enzymatic antioxidant defence. It was only seven years before this meeting that McCord and Fridovich recognized the enzymatic nature of erythrocuprein and other cupreins, and were able to demonstrate the equality of the most important participants in the enzymatic antioxidant defence: the SOD-s and the erythrocupreins. Conferences are now generally held every two years (followd by the publication of the proceedings) by all those who deal with the SOD-s, or more broadly with the enzymatic antioxidant systems. The recognition of the role of SOD (the enzymatic antioxidant systems) and the relevant theories is often compared to the discovery by Watson and Crick, for the questions discussed shed light on certain metabolic diseases, on changes in the nervous system, the heart, the liver, the kidney and the internal system, and on the problems of ageing, tumour formation, arteriosclerosis, etc., or enable the development of conceptions ensuring progress.

Many people are currently striving to separate the chemical and biochemical results from those achieved in the biological and clinical fields. In our view, such a separation is not justified, for the results can be synthesized from the mosaic of the individual data only if they are treated as a dynamic enti-

ty. Accordingly, with a sequential ordering, we have decided to publish the results in one volume.

The lectures that appear in this publication were delivered at a colloquium in Szeged on 6-7 January 1986. This colloquium was preceded by the conference on similar topics on 10-11 January 1984 in Pécs, a meeting that we all look back on with great enthusiasm.

It is very gratifying that the organizers have decided that similar meetings should be arranged biènnaly, to allow new surveys on the latest results concerning oxygen radicals and the antioxidant systems.

Before this preface becomes too lengthy, it may be apt to cite a sentence of Mark Twain, already mentioned in the introduction to the book by W.B. Jakoby: "Even if you are on the right track, you'll get run over if you just sit there".

This publication contains the material of a conference at which the original papers presented by theoreticians, pharmacologists and clinicians provided an interdisciplinary survey of the biological significance of free radicals. By virtue of these interactions, the book may yield new perspectives for those working in these special fields.

Budapest, 1987 December

The EDITORS

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INVESTIGATION OF THE INFLUENCE OF PARACETAMOL ON THE ACID HAEMOLYSIS OF ERYTHROCYTES IN VIVO AND IN VITRO

I. ALTORJAY, B. SÁRI and L. DALMI

2nd Department of Medicine, University Medical School, Debrecen, Hungary

INTRODUCTION

Increasing attention has recently been paid again to the mode of action of paracetamol (acetaminophen), a well-known hepatotoxic agent, used therapeutically as an analgesic and antipyretic (1-5). In Hungary it is a component of a muscle relaxant, Scutamil-C, which contains 100 mg/tablet. According to Wendel (3), the phase-I metabolism of paracetamol caused a dose-dependent lipid peroxidation in the mouse, which could be prevented if the animals were pretreated with liposomally entrapped reduced glutathione. Casini et al. (4) found that paracetamol and bromobenzene form conjugates with hepatic glutathione. Under such conditions the glutathione level decreases, which makes the liver cells more susceptible to lipid peroxidation. Since the erythrocytes also contain a high level of glutathione, it seemed to be of interest to investigate whether paracetamol pretreatment or incubation with paracetamol in pharmacological doses induces erythrocyte membrane damage.

The method of acid haemolysis was earlier used mainly for the diagnosis of paroxysmal nocturnal haemoglobinuria (6). We have recently elaborated a modified method in which the haemolytic process is performed in an aggregometer and recorded in the form of a dynamic haemolysis curve. By detecting changes at the time of full haemolysis, this procedure seems to be applicable for the investigation of the membrane-damaging and cytoprotective effects of different agents. We have

already given an account of our examinations with this method on the cytoprotective effect of Catergen (7, 8). We now present the results of our examinations on the influence of paracetamol on the acid haemolysis of murine and human erythrocytes, both in vitro and in vivo.

METHODS AND RESULTS

In our modified method the haemolytic process induced by hydrochloric acid is performed at 37°C with constant stirring in a Chronolog aggregometer. The gradual increase of the transmission of the erythrocyte suspension can be registered by a potentiometric recorder in the form of a dynamic haemolysis curve. The principle of the method is outlined in Fig. 1.

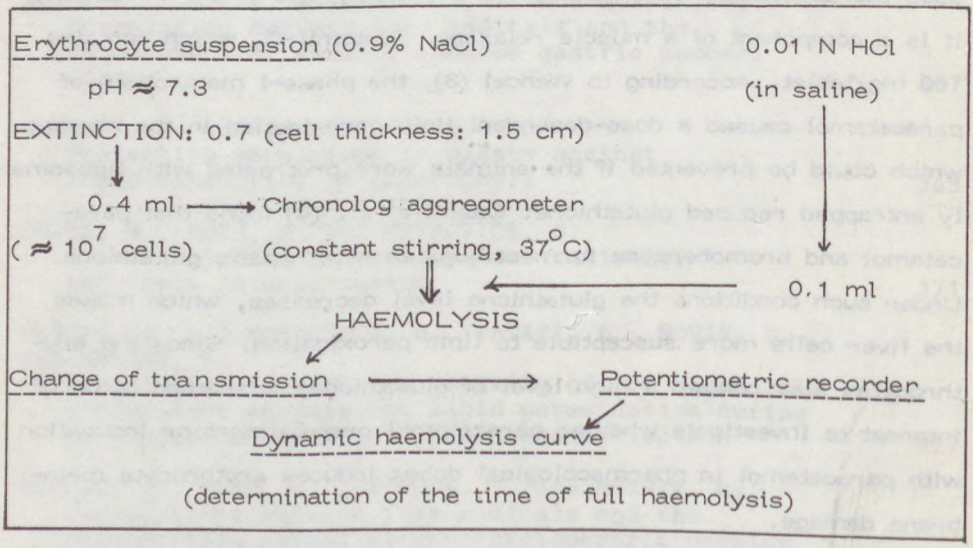

Fig. 1. Investigation of acid haemolysis in an aggregometer

The shape of a dynamic haemolysis curve and the changes in the pH during the haemolytic process are shown in Fig. 2.

In our animal experiments, a 10 mg/kg dose of paracetamol was administered orally to 12 adult mice. In parallel with this, 6 animals received the corresponding dose of Scutamil-C, dissolved in distilled

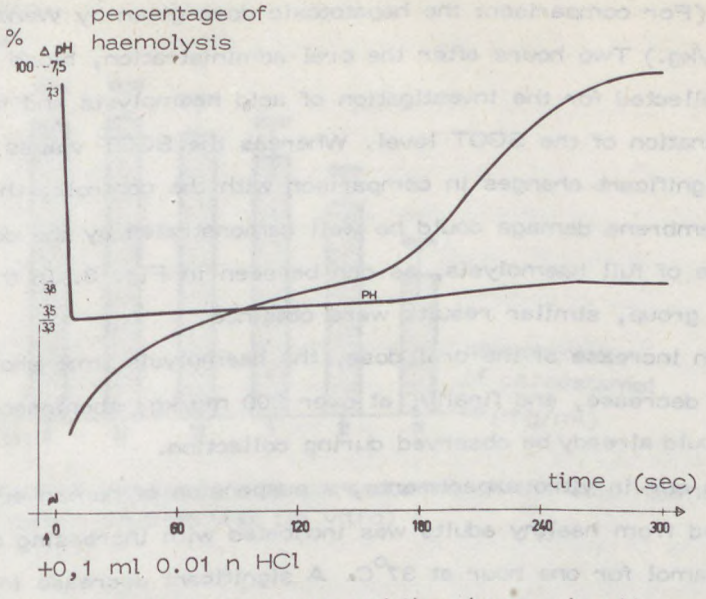

Fig. 2. Dynamic haemolysis curve and the changes in pH

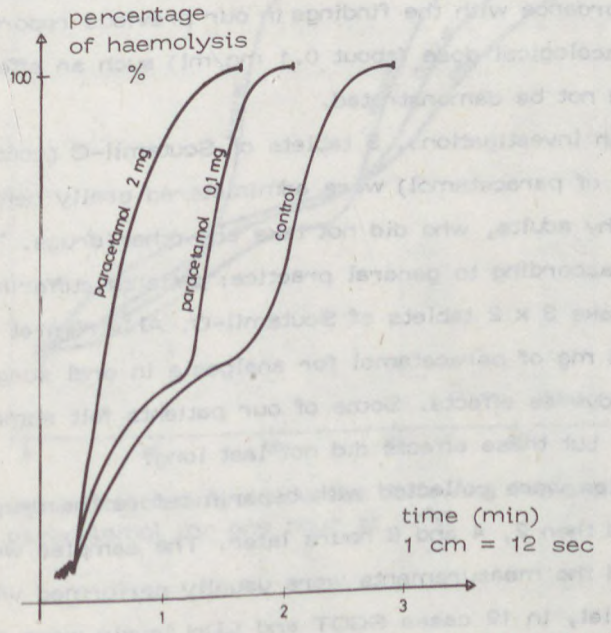

Fig. 3. The effect of paracetamol on acid haemolysis in mice, 2 hours after oral administration

water. (For comparison: the hepatotoxic dose given by Wendel was 400 mg/kg.) Two hours after the oral administration, blood samples were collected for the investigation of acid haemolysis and for the determination of the SGOT level. Whereas the SGOT values did not show significant changes in comparison with the controls, the erythrocyte membrane damage could be well demonstrated by the decrease in the time of full haemolysis, as can be seen in Fig. 3. In the Scutamil-C group, similar results were obtained.

On increase of the oral dose, the haemolysis time showed a further decrease, and finally, at over 200 mg/kg, spontaneous haemolysis could already be observed during collection.

In our in vitro experiments, a suspension of human erythrocytes collected from healthy adults was incubated with increasing doses of paracetamol for one hour at 37°C. A significant decrease in the time of full haemolysis could be observed when the solution in which the erythrocytes were incubated contained at least 5 mg/ml paracetamol. These results are shown in Figs 4 and 5. It must be stressed, however, that, in accordance with the findings in our previous report (8), in the human pharmacological dose (about 0.1 mg/ml) such an effect of paracetamol could not be demonstrated.

In our human investigations, 5 tablets of Scutamil-C (containing a total of 500 mg of paracetamol) were administered orally before meals to 33 healthy adults, who did not take any other drugs. The dose was chosen according to general practice: patients suffering from discopathy often take 3 x 2 tablets of Scutamil-C. Ahlström et al. (5) administered 1000 mg of paracetamol for analgesia in oral surgery with no serious adverse effects. Some of our patients felt somewhat dizzy and sleepy, but these effects did not last long.

Blood samples were collected with heparin before the drug was administered, and then 2, 4 and 6 hours later. The samples were stored at 4°C and the measurements were usually performed within 2 hours. In parallel, in 12 cases SGOT and LDH levels were also measured at the beginning and after 6 hours. Table 1 contains the data on our patients.

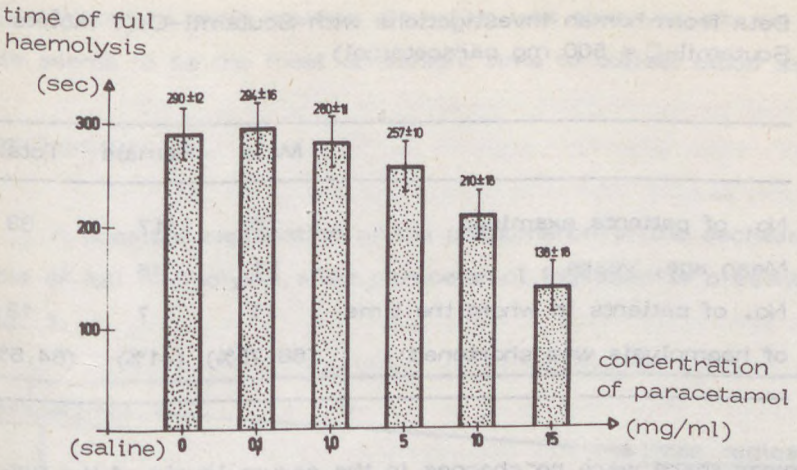

Fig. 4. Effect of incubation with paracetamol on the acid haemolysis of human erythrocytes (in vitro)

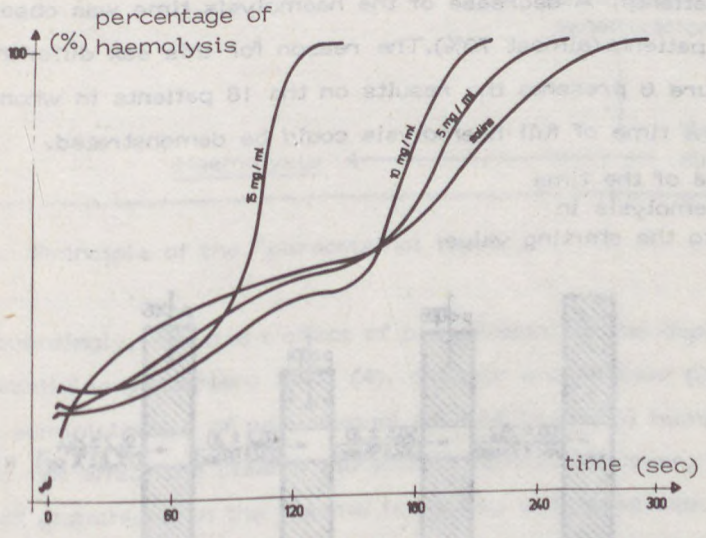

Fig. 5. Dynamic haemolysis curves following incubation with paracetamol for one hour at 37°C

Table 1 Data from human investigations with Scutamil-C (5 tablets of Scutamil-C = 500 mg paracetamol)

	Male	Female	Total
No. of patients examined	16	17	33
Mean age, years	51	46	
No. of patients in whom the time of haemolysis was shortened	11 (68.75%)	7 (41%)	18 (54.5%)

Whereas there were no changes in the serum levels of the different enzymes, the time of full haemolysis decreased significantly in 18 of the 33 patients (54.5%). The most marked shortening was observed 4 hours after the oral administration of Scutamil-C. The influence of paracetamol on the process of acid haemolysis was more obvious in the male patients. A decrease of the haemolysis time was observed in 11 of the 16 patients (almost 70%). The reason for this sex difference is not clear. Figure 6 presents the results on the 18 patients in whom shortening of the time of full haemolysis could be demonstrated.

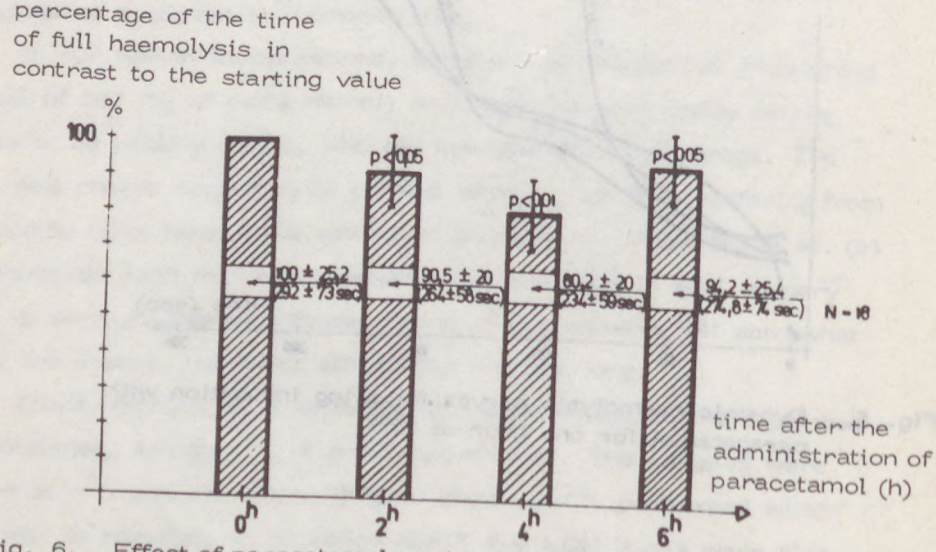

Fig. 6. Effect of paracetamol on the time of full haemolysis in vivo (following oral administration of 5 tablets of Scutamil-C = 500 mg paracetamol)

Since the most marked alteration was observed after 4 hours, this seems to be the most convenient time to collect blood samples.

DISCUSSION

A possible explanation of the phenomenon of the decrease in the time of full haemolysis after paracetamol ingestion is presented in Fig. 7.

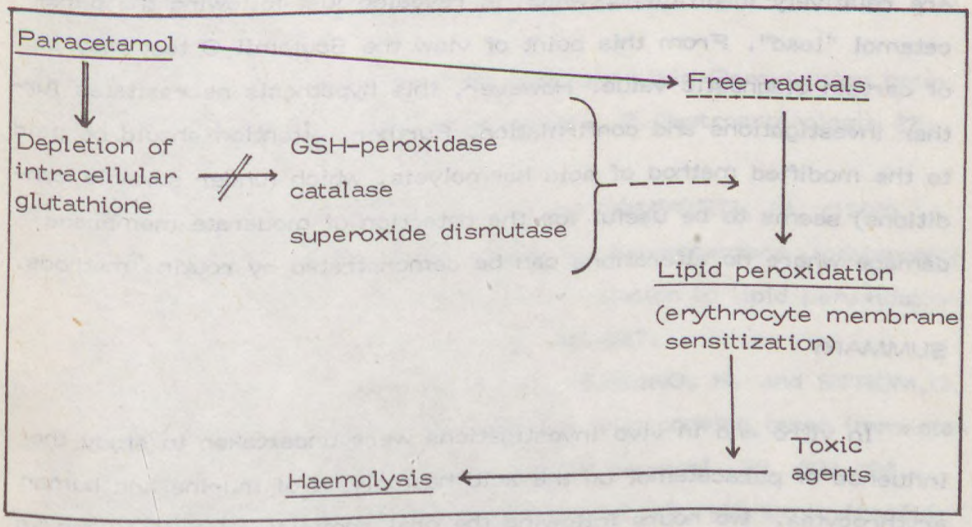

Fig. 7. Principle of the "paracetamol test"

Accordingly, the main effect of paracetamol is the depletion of the intracellular glutathione level (4). Beutler and Gelbart (9) found that the administration of paracetamol to rabbits and to human volunteers did not affect the plasma glutathione levels. However, the amount of glutathione in the plasma is minute compared with its concentration in the red cells (<0.001 of the concentration there), and therefore it cannot represent the latter. Due to the depletion of the glutathione level, the free radical-eliminating capacity of the glutathione peroxidase system is reduced. Thus, free radicals may provoke lipid peroxidation in the membrane of the erythrocytes earlier and

more easily. Subsequently, toxic factors, such as hydrochloric acid, result in a faster haemolysis.

It must be emphasized, however, that in the given dose Scutamil-C itself does not cause haemolysis (as is shown by the normal LDH levels), but in a certain percentage of the cases it may sensitize the erythrocyte membrane, which leads to faster haemolysis in response to the associated toxic influence. On the other hand, it may also be supposed that in these cases the scavenger mechanisms are relatively insufficient, which is revealed just following the paracetamol "load". From this point of view the Scutamil-C test may be of certain diagnostic value. However, this hypothesis necessitates further investigations and confirmation. Further, attention should be paid to the modified method of acid haemolysis, which (under suitable conditions) seems to be useful for the detection of moderate membrane damage where no alterations can be demonstrated by routine methods.

SUMMARY

In vitro and in vivo investigations were undertaken to study the influence of paracetamol on the acid haemolysis of murine and human erythrocytes. Two hours following the oral administration of 10 mg/kg paracetamol to adult mice, a significant decrease in the time of full haemolysis could be demonstrated. Similarly, a gradual decrease in the haemolysis time was found following a one-hour incubation of human erythrocytes with increasing concentrations of paracetamol at 37°C.

Examinations were also carried out after the oral administration of 5 tablets of Scutamil-C to 33 healthy adults. Four hours later, a significant shortening of the time of full haemolysis could be demonstrated in almost 55% of the patients. This effect can probably be explained by the decreased protection of the erythrocytes against free radicals, as a consequence of paracetamol-induced membrane damage.

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Oxygen Free Radicals and the Tissue Injury
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COMPARISON OF LUMINOL AND LUCIGENIN-ENHANCED CHEMILUMINESCENCE OF GRANULOCYTES. STUDY OF ANTIINFLAMMATORY DRUGS

M. ANTAL, Z. ROZSNYAY, GY. GENTI and K. MERÉTEY

National Institute of Rheumatology and Physiotherapy, Budapest, Hungary

INTRODUCTION

The chemiluminescence (CL) of granulocytes evoked by many surface and phagocytosis-inducing stimuli is a widely studied phenomenon in immunological and drug research (8-10). The oxygen radicals produced by phagocytes can be measured indirectly by this CL assay. The introduction of luminol (1) and more recently of lucigenin (12) as agents oxidized by different oxygen radicals has widened the use of CL in the research into the membrane metabolism and signal processing.

In our present studies we have compared the two CL systems by using two surface stimuli and different nonsteroidal antiinflammatory drugs.

MATERIALS AND METHODS

Separation of granulocytes

Heparinized blood was taken from healthy volunteers. The granulocytes (purity > 90%) were separated by dextran sedimentation (1% dextran T 150, Pharmacia) at room temperature for 30 minutes, the pellet of a Ficoll-Uromiro separated granulocyte-red blood cell suspension being applied (3). After three washings, the cell suspension

was adjusted to 2 x 10^5/ml. The final concentration of luminol was 32 μM and that of lucigenin was 6.25 μM, determined as optimal in preliminary experiments. (The compounds were purchased from Sigma Co., USA.) Con A, PHA, indomethacin, phenylbutazone and piroxicam were dissolved in phosphate-buffered saline (PBS). The concentrations are shown in the details on the different experiments. The reaction volume was 1.2 ml. The CL response was detected by a Beckmann LS 100 scintillation counter in mode of coincidence out. The cpm values were detected every 5 minutes for one hour. The CL reaction was evaluated by summarizing the cpm values obtained 5, 10 and 15 minutes after the initiation of the reaction.

RESULTS

CL responses of granulocytes to different doses of PHA and Con A in the presence of luminol. The granulocytes were stimulated by 0.1, 1, 10 or 100 μg of PHA or Con A (Fig. 1). The luminol-dependent response was much higher to PHA than to Con A. Con A induced a CL reaction only in the highest concentration.

Lucigenin-enhanced CL reaction to different doses of PHA and Con A. The reaction to the same stimuli showed a strikingly different pattern when lucigenin was used as amplifier (Fig. 2). While PHA was not an inducer in all doses, Con A proved to be a very active stimulant in all doses studied. A dose-response relationship was observed.

Drug-induced changes in the mitogen-induced CL responses. As the CL response to PHA in the lucigenin system and to Con A in the luminol system was very low, these experimental systems could not be used in a pharmacological study. The nonsteroidal antiinflammatory drugs were present in the concentration range from 10^{-4} - 10^{-8} M (Fig. 3). In the luminol system, all drugs dose-dependently inhibited the production of oxygen radicals. However, in the lucigenin system a

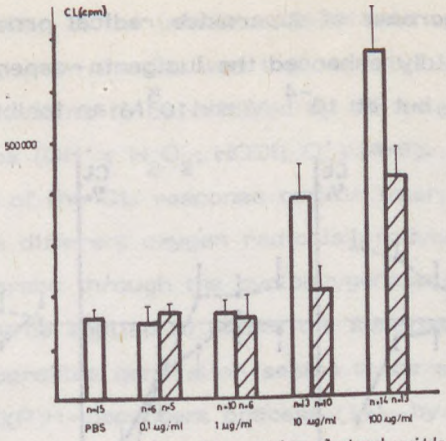

Fig. 1. CL response of granulocytes stimulated with PHA or Con A in the presence of luminol.
The results show the summed counts detected 5, 10 and 15 minutes after the initiation of CL. Empty columns represent the PHA CL reactions, and hatched columns the Con A-induced CL. The numbers of experiments are indicated under the columns. The bars denote the means and SE of the results.

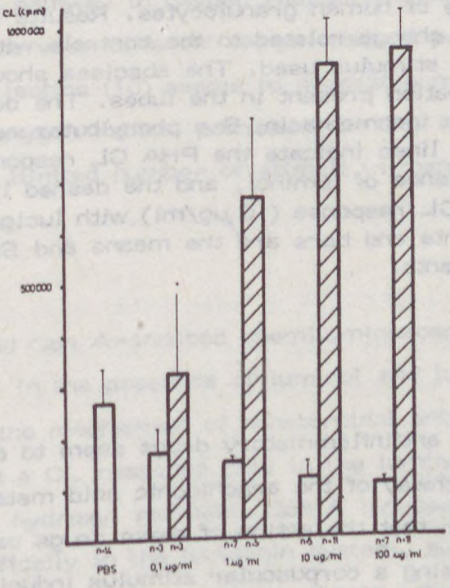

Fig. 2. CL response of human polymorphonuclear blood cells with lectins as stimuli and lucigenin as amplifier.
The results show the summed counts detected 5, 10 and 15 minutes after the initiation of CL. Empty columns represent the PHA CL reactions, and hatched columns the Con A-induced CL. The numbers of experiments are indicated under the columns. The bars denote the means and SE of the results.

dose-dependent increase of superoxide radical production was observed. Phenylbutazone mildly enhanced the lucigenin-dependent CL at the lower concentrations, but at 10^{-4} M and 10^{5} M an inhibition of CL could be detected.

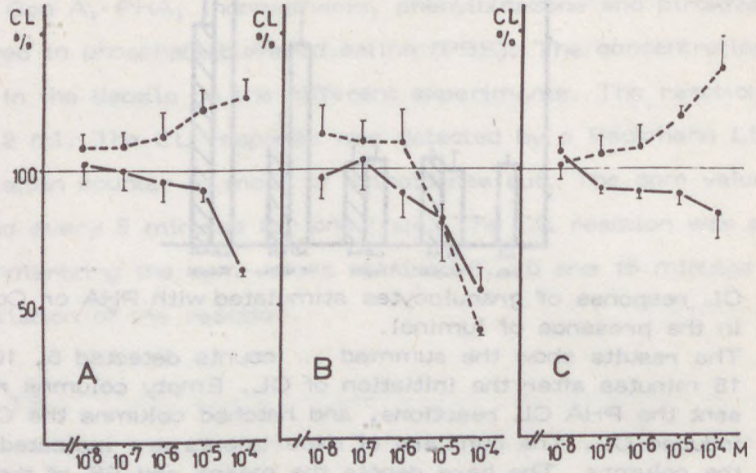

Fig. 3. Effects of nonsteroidal antiinflammatory drugs on the CL response of human granulocytes. Results are expressed as relative change related to the controls without the drugs but with the stimulus used. The abscissa shows the actual drug concentration present in the tubes. The dose-response curves are: A = indomethacin; B = phenylbutazone; C = piroxicam. The full lines indicate the PHA CL response (10 µg/ml) in the presence of luminol, and the dashed lines the Con A-evoked CL response (10 µg/ml) with lucigenin as amplifier. The points and bars are the means and SE values of 3-5 experiments.

DISCUSSION

Nonsteroidal antiinflammatory drugs seem to act by inhibiting the cyclooxygenase pathway of the arachidonic acid metabolism (7). In vitro studies have shown that the action of these drugs can be tested by the inhibition of CL using a corpuscular stimulus inducing phagocytosis in the granulocytes (13). In our present studies two surface stimuli, PHA and Con A, were used for this purpose.

We have found a dose-dependent inhibition of the detectable oxygen radicals in the luminol system by indomethacin, phenylbutazone

and piroxicam, similar to the inhibition found earlier with indomethacin and phenylbutazone, when the CL was induced with a corpuscular stimulus (2). Luminol seems to be oxidized by the interaction of different oxygen intermediates ($OH^{\cdot}$; H_2O_2; HOCl; $O_2^{\cdot}$) (4-6).

The decrease of the CL response can be interpreted as reflecting the inhibition of the different oxygen radicals produced intracellularly or within the membrane through the cyclooxygenase pathway.

Lucigenin may be applied to detect the superoxide anion specifically (12). As superoxide generation seems to be a membrane-associated and NAD(P)H-dependent process (11), by using this system we are able to monitor the first events in the oxygen radical pathway. As the nonsteroidal antiinflammatory drugs do not inhibit, but rather enhance the lucigenin-dependent CL, we have to consider that the produced superoxide anions cannot be transformed to other radicals through the usual metabolic chain reaction. However, the participation of the lipoxygenase pathway in oxygen radical production has not yet been elucidated. The very different oxygen radical production after stimulation by two lectins (10) seems to suggest a more complicated mechanism of the oxygen radical pathway than that suggested from the use of only a very limited number of stimuli in many of the reported experiments.

SUMMARY

The PHA- and Con A-induced chemiluminescence response of human granulocytes in the presence of luminol and lucigenin amplifiers was used to study the mechanism of nonsteroidal antiinflammatory drugs. PHA caused a CL response only in the luminol system, showing a dominance of hydroxyl radicals. Con A induced the appearance of CL nearly specifically in the lucigenin system, suggesting the preference for superoxide production. Indomethacin, phenylbutazone and piroxicam dose-dependently inhibited the PHA CL. The Con A-induced CL was enhanced by all doses studied (10^{-4} -10^{-8} M) in the cases of indomethacin and piroxicam, but phenylbutazone caused an enhancement in low doses and inhibition in a high dose (10^{-4} -10^{-5} M).

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Oxygen Free Radicals and the Tissue Injury
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THE SEARCH FOR IDEAL ANTIDOTE TREATMENT IN GRAMOXONE® INTOXICATION

K. BARABÁS, L. SZABÓ and B. MATKOVICS*

Institute of Public Health and Epidemiology
University Medical School of Szeged and
*Biological Isotope Laboratory,
József Attila University of Szeged, Hungary

SUMMARY

The herbicide Gramoxone^{R}, the active ingredient of which is paraquat, actively accumulates in the lung in the mammalian organism, where it exerts its toxic effect through the generation of oxygen radicals. Efforts were made to counteract the toxic effect in experiments on mice. Its penetration into the cells was blocked with the diamines putrescine and cadaverine. The antioxidant defence was enhanced with cysteine, glutathione and D-penicillamine. The synthesis of the prostaglandins, which are responsible for the acute symptoms, was inhibited with Aspisol. Accumulation inhibition with biogenic amines is considered the most effective.

INTRODUCTION

The pulmonary toxicity of paraquat (PQ) the reagent in widely used pesticide Gramoxone^{R}, is well established. The lung specificity of the toxic effect is due to the active accumulation of PQ in type II pneumocytes (Rose 1974); the bipyridylium passes through a redox cycle in which oxygen radicals are generated from the molecular oxygen present (Bus 1976). The produced superoxide ($\dot{O}_2^-$) and hydroxyl (HO$^{\cdot}$) radicals are not only membrane destructive, but also start an arachidonic acid cascade and stimulate prostaglandin synthesis, there-

by leading to acute poisoning, which is often lethal even in this early phase. PQ has a specific PGF_2 synthesis-enhancing effect, which results in an increased fluid outflow to the intracellular space, leading to pulmonary oedema (Lindenschmidt 1983) (Fig. 1).

A deeper knowledge of the details of this mechanism enabled us to draw up possible strategies to prevent the toxic effect:

1. To avoid the entry of PQ into the pneumocytes.
2. To eliminate the generated oxygen radicals and to intensify the antioxidant effect.
3. To inhibit the PG synthesis responsible for the first clinical symptoms.

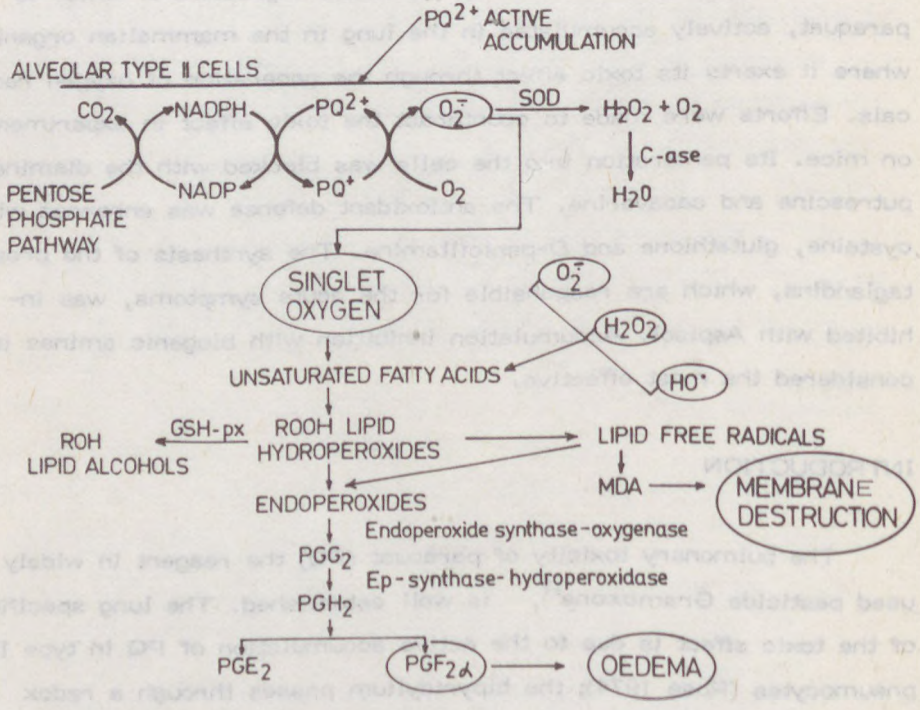


Fig. 1. Biochemical mechanism of paraquat toxicity in alveolear type II cells

The present paper is a summary of our investigations in mice relating to these strategies.

MATERIALS AND METHODS

Male CFLP mice of the same age, weighing 30 ± 2 g, obtained from the Laboratory Animal Breeding Centre, Gödöllő, Hungary, were used. Experimental groups contained 10 animals and all experiments were repeated three times. Reported results are average values calculated from the three examinations.

Gramoxone^{R} was administered orally while antidote injections were given intraperitoneally (ip).

The following chemical materials were applied: A Gramoxone^{R} (ICI, UK) preparation containing 25% PQ, in a dose of 120 mg/kg.

0.467 nmol of putrescine dihydrochloride (PU) was added to PQ in equivalent quantity, as was cadaverine dichloride (CA) Sigma.

Reduced glutathione (GSH) and cysteine (CYS) (Calbiochem, Switzerland) and D-penicillamine (D-PA) (Metalcaptase^{R} HCl; Knoll AG, FRG) were applied in doses of 50 mg/kg to eliminate the generated oxygen radicals and to intensify the antioxidant effects.

To inhibit prostaglandin synthesis, aspirin-containing ASPISOL (Bayer, FRG) was tested; the effective dose was found to be 15 mg/kg.

It had been proved in a series of preliminary experiments that none of the materials used in the above doses were toxic in themselves.

Three parameters were studied in our present work: the survival rate, the lung weight, and the PQ contents of the lung, liver, kidney and plasma, by the methods of Jarvie (1983).

The activities of various enzymes were examined by the following methods:

Superoxide dismutase (SOD) activity was determined by the epinephrine-adrenochrome method (Misra et al. 1972, Matkovics et al. 1977).

The spectrophotometric method of Beers et al. (1952) was applied to measure catalase (C-ase) activity.

Glutathione peroxidase (GP-ase) determination was carried out as described by Sedlak et al. (1968) with cumene hydroperoxide as substrate. The reduced glutathione residue was measured by the method of Sedlak et al. (1968).

The method of Placer et al. (1966), based on a colorimetric thiobarbituric acid reaction, was used for quantitative measurement of the tissue lipid peroxidation (LP).

Statistical analysis was performed with a two-sample t test.

RESULTS

The first experiments were focussed on the distribution of Gramoxone^{R} given in a dose of 120 mg/kg in mice. 24 hours after administration the PQ was accumulated in the kidneys and the lungs, with a minimal quantity in the plasma and no detectable amount in the liver (Fig. 2).

The survival data on diamine-treated control animals are shown in Fig. 3. The 72-hour survival of PU and CA-treated mice was 100%.

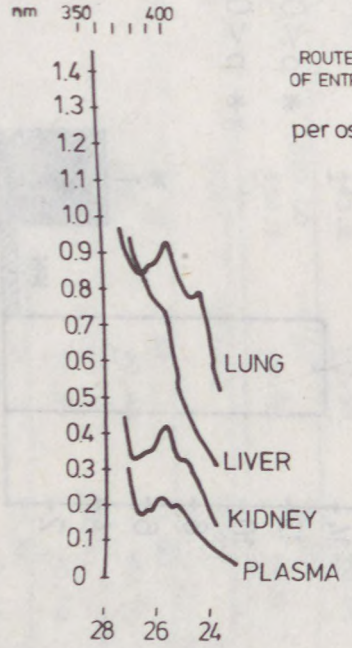
No statistically significant difference in the lung weight/body weight ratio can be seen between animals treated with PQ only and those given other drugs as well.

The rate of PQ assimilation is notably reduced by diamine in homogenized mouse lung tissues (Fig. 4).

CYS, GSH and D-PA administration did not improve survival rate as effectively as did diamine treatment (Fig. 5).

The influence on the protective enzymatic activities was similar for all three drugs. The SOD activity was increased. More intensive C-ase activity was observed only in the homogenized liver and lung tissues. PQ enhanced the enzymatic activity in the haemolysed tissues, while D-PA, GSH and CYS decreased it. The GP-ase activity was increased by PQ, D-PA, GSH and CYS, and approached the control value (Table 1).

SPECTRUM OF PARAQUAT IN TISSUES (24h AFTER TREATMENT)



PARAQUAT DISTRIBUTION IN TISSUES

ROUTE OF ENTRY	DOSE	SPECIES	TIME AFTER TREATMENT	TISSUE	CONCENTRATION
per os	120 mg/kg	MOUSE	24h	PLASMA	0.9 ± 0.01 mg/l
				LUNG	13.6 ± 3.1 mg/kg
				KIDNEY	6.2 ± 2.3 mg/kg
				LIVER	—
			48h	PLASMA	0.1 ± 0.01 mg/l
				LUNG	9.4 ± 2.4 mg/kg
				KIDNEY	1.0 ± 1.5 mg/kg
				LIVER	—
			72h	PLASMA	—
				LUNG	4.0 ± 2.10 mg/kg
				KIDNEY	1.0 ± 1.80 mg/kg
				LIVER	—

Fig. 2. Paraquat distribution

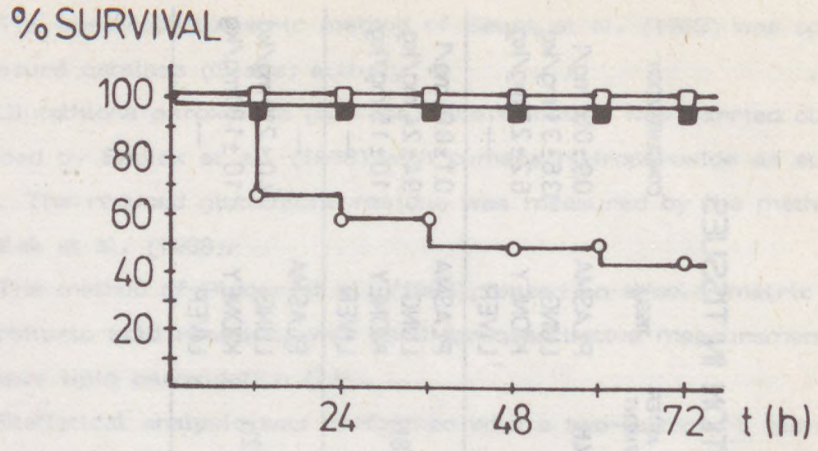


Fig. 3. Cadaverine (C □—□) and putrescine (P ■—■) treatment of gramoxone poisoning (PQ LD_{50} o—o)

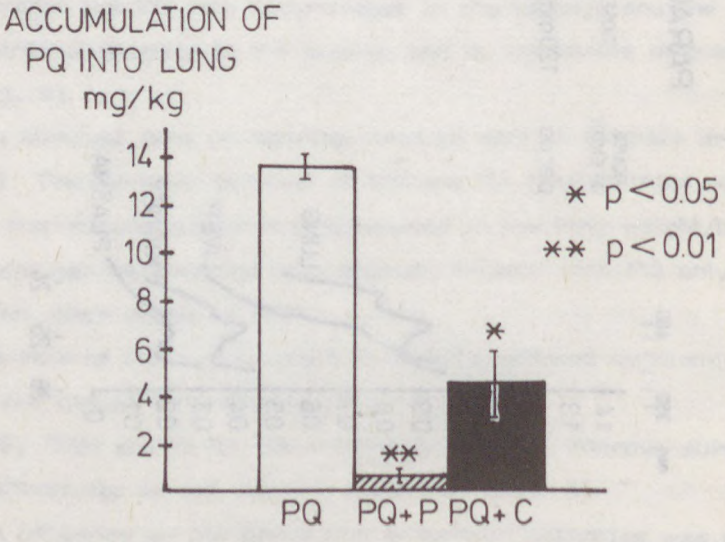


Fig. 4. Paraquat concentration in mouse lung after oral administration of PQ (□) and putrescine (▨) and cadaverine (■)

Table 1. Changes in antioxidant enzymatic activity following PQ, D-PA, CYS and GSH treatment (Changes in LP values)

Tissue	Treatment	ANTIOXIDANT ENZYMATIC ACTIVITY SOD U/g w.t.w	C-ase BU/g w.t.w	GSM-px U/g w.t.w	LP values nmol MDA/g w.t.w
LIVER	Control	2400.70 ±301	5.00 ±0.40	11.31 ±0.12	29.40 ±5.30
	PQ	2800.50 ±271	3.50 ±0.50	16.30 ±0.09	32.45 ±4.80
	D-PA	3010.25 ±375	4.90 ±0.60	12.00 ±0.07	20.30 ±3.20
	GSH	2250.00 ±180	4.85 ±0.70	11.71 ±0.09	29.75 ±4.20
	CYS	2000.00 ±200	4.20 ±0.30	12.08 ±0.10	33.10 ±7.10
LUNG	Control	350.37 ±70.2	5.53 ±0.42	10.35 ±0.70	39.30 ±4.20
	PQ	245.42 ±30.9	4.70 ±0.53	13.71 ±3.08	52.15 ±3.90
	D-PA	339.35 ±29.3	5.37 ±0.40	9.30 ±0.09	42.30 ±6.10
	GSH	362.49 ±43.2	5.21 ±0.30	10.30 ±3.93	45.70 ±5.7
	CYS	370.00 ±39.7	4.98 ±0.35	10.90 ±1.0	40.30 ±4.1
		U/ml	BU/ml	U/ml	nmol MDA/ml
Haemo-lysate	Control	710.35 ±62.0	5.42 ±0.40	11.90 ±0.10	45.70 ±5.2
	PQ	580.78 ±60.7	9.01 ±0.57	25.50 ±0.18	62.30 ±3.90
	D-PA	809.10 ±81.9	5.30 ±0.31	10.55 ±0.10	50.30 ±2.90
	GSH	740.32 ±39.0	6.42 ±0.49	10.00 ±0.13	49.40 ±3.20
	CYS	802.70 ±63.20	5.40 ±3.50	11.30 ±0.20	55.7 ±4.30

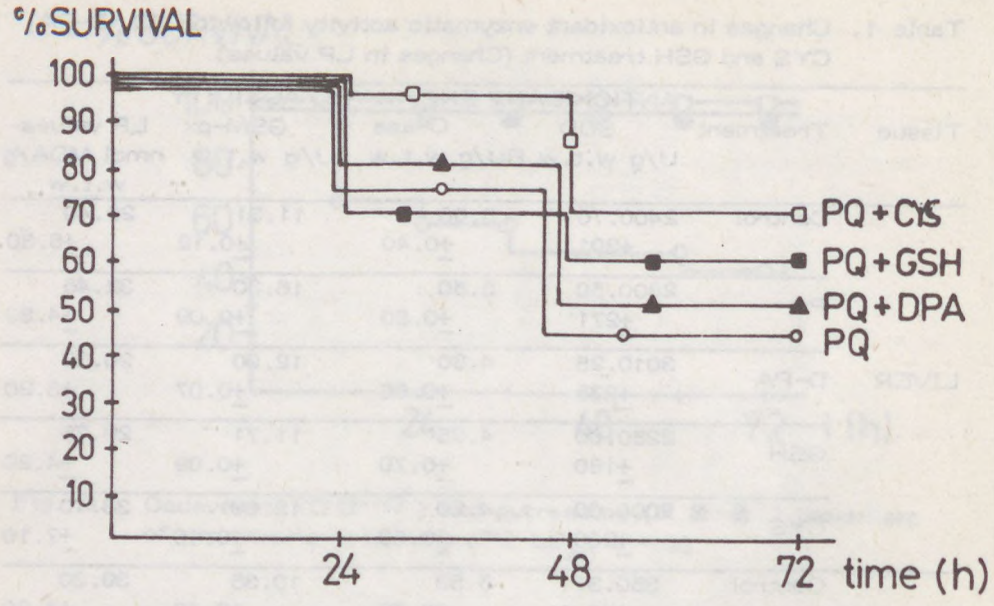


Fig. 5. Cysteine (Cys □—□) glutathione (GSH ■—■) and D-penicillamine (DPA ▲—▲) treatment of Gramoxone (PQ o—o) poisoning

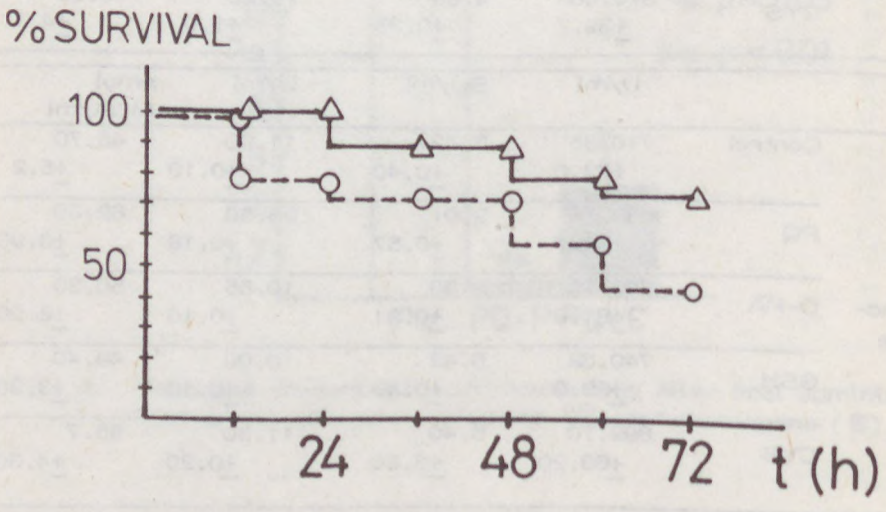


Fig. 6. Aspisol (ASP ▲—▲) treatment of Gramoxone poisoning (PQ o--o)

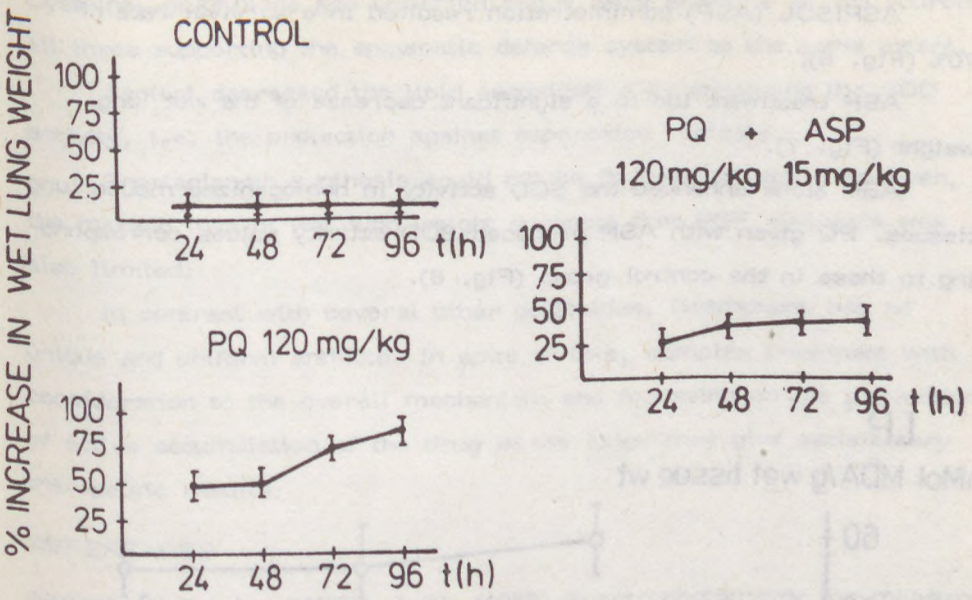


Fig. 7. Effect of Aspisol in lung. Lung weight changes based on percentage increase over initial wet weight

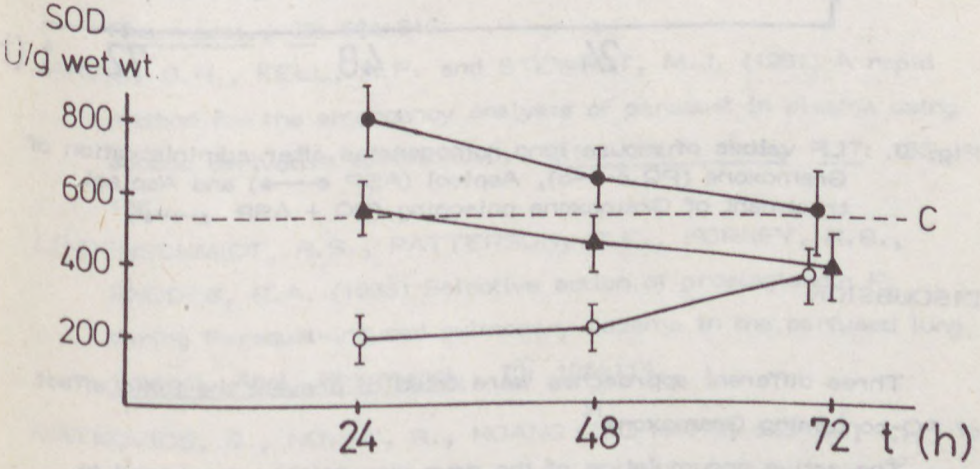


Fig. 8. Total SOD activities in mouse lung homogenates after administration of Gramoxone (PQ o—o) and Aspisol (ASP ●—●) and Aspisol treatment of Gramoxone poisoning (PQ + ASP ▲—▲)

ASPISOL (ASP) administration resulted in a survival rate of 70% (Fig. 6).

ASP treatment led to a significant decrease of the wet lung weight (Fig. 7).

ASP alone enhanced the SOD activity in homogenized mouse lung tissues. PQ given with ASP produced SOD activity values corresponding to those in the control group (Fig. 8).

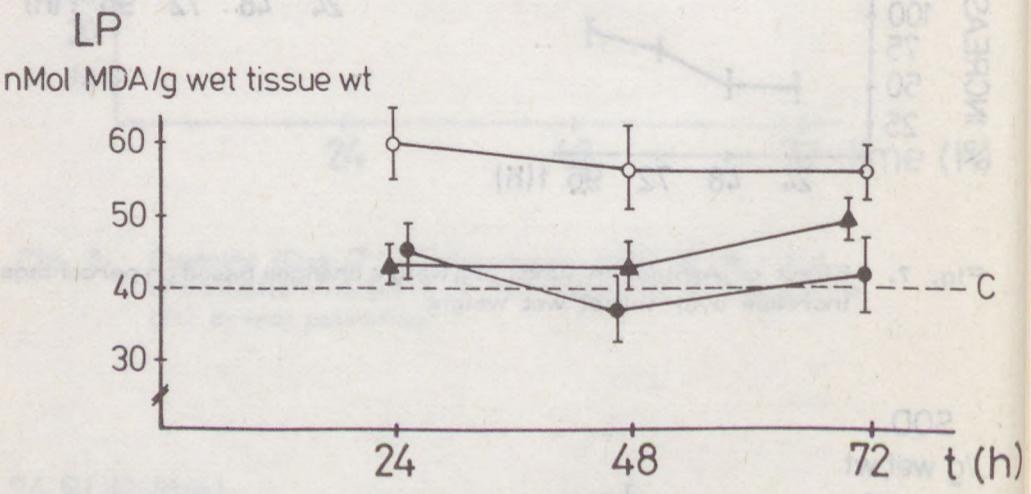


Fig. 9. LP values of mouse lung homogenates after administration of Gramoxone (PQ o—o), Aspisol (ASP ●—●) and Aspisol treatment of Gramoxone poisoning (PQ + ASP ▲—▲)

DISCUSSION

Three different approaches were tried to prevent the toxic effect of PQ-containing Gramoxone^{R}.

The active accumulation of the drug was notably decreased in mice when diamines, putrescine and cadaverine were used, in all probability due to their competitive effect.

Cysteine, glutathione and D-penicillamine were effective as SH sources, all three supporting the enzymatic defence system to the same extent.

Aspisol decreased the lipid peroxidation by enhancing the SOD activity, i.e. the protection against superoxide radicals.

Prostaglandin synthesis could not be followed directly; however, the marked loss of wet lung weight suggests that PGF synthesis was also limited.

In contrast with several other pesticides, Gramoxone has no unique and uniform antidote. In spite of this, complex treatment with consideration to the overall mechanism and focussing on the prevention of active accumulation of the drug in the lungs may give satisfactory therapeutic results.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

STUDIES ON THE BIOCHEMICAL CORRELATIONS OF THE STRUCTURE AND MECHANISM OF ACTION OF DIHYDROQUINOLINE-TYPE, WATER- AND LIPID-SOLUBLE SYNTHETIC RADICAL SCAVENGERS

V. BÄR, B. TÖRÖK*, E. RŐTH* and I. ROZSNYAI

Material Chemical Works,
Budapest, Hungary
*Department of Experimental Surgery
University Medical School,
Pécs, Hungary

In view of the increasing number and quantity of chemicals polluting the environment, the rising drug consumption and the longer average duration of life (mainly in the industrialized countries), rapidly increasing amounts of peroxides and free radicals have to be reckoned with. Recent investigations have revealed that xenobiotic-induced lipid peroxidation affects the integrity of the cellular and subcellular membranes, and free radicals initiate chain reactions in them. The search for and use of radical scavengers of high potency and good tolerance in human beings is therefore indicated.

The aim of our studies was to develop amine-type radical scavengers for clinical practice. Such scavengers were chosen because their chain-terminating effect occurs not only via chain transfer, but also via recombination. These compounds readily decompose the hydroperoxides formed during lipid peroxidation. They also inactivate the radicals formed during oxidative degradation.

While planning the structure of these compounds, we took into consideration the fact that the increase of the molecular weight and the substituent at position 6 are of decisive importance as concerns the toxicity properties.

Our team developed the lipid-soluble compound 6,6'-methylene--bis(2,2,4-trimethyl-1,2-dihydroquinoline) (1). Its trade name is Sensorad, and its code name is MTDQ. Its structure is shown in Fig. 1.

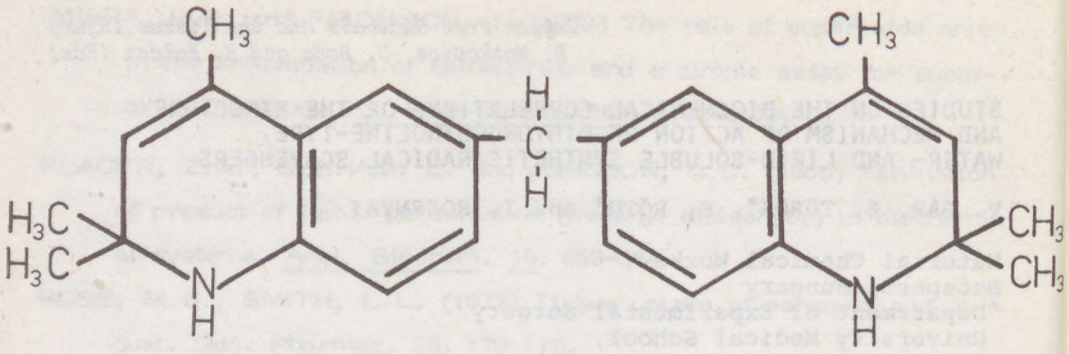

Fig. 1. The chemical structure of 6,6´methylene-bis(2,2,4-trimethyl-1,2-dihydroquinoline)

A water-soluble derivative of this compound has also been developed (2): 6,6´-methylene-bis(2,2-dimethyl-4-methanesulfonic acid sodium-1,2-dihydroquinoline). Its trade name is Kontrad, and its code name is MTDQ-DA.

Both compounds contain 3 functional groups. These are the mobile H atoms contained in the NH groups, which are responsible for the radical scavenger effect, ard the methylene group, which exerts peroxide-decomposing action in addition to its reaction with ROH, which results in hydrole formation (9).

Sensorad has been found suitable for use in oncoradiological practice to improve the effectivity of the fractionated telecobalt therapy of patients with stage III and IV breast cancer and head-and-neck malignancies. The favourable tolerability of the compound is of special importance (5, 6, 7, 10).

The intensity of the antioxidant action of MTDQ has been compared with those of butylhydroxytoluene (BHT) and vitamins E and A by means of the AOM method in vitro (Fig. 2.).

The intensity of the antioxidant effect of MTDQ-DA has been compared with those of D-penicillamine, ascorbic acid and glutathione (Fig. 3.).

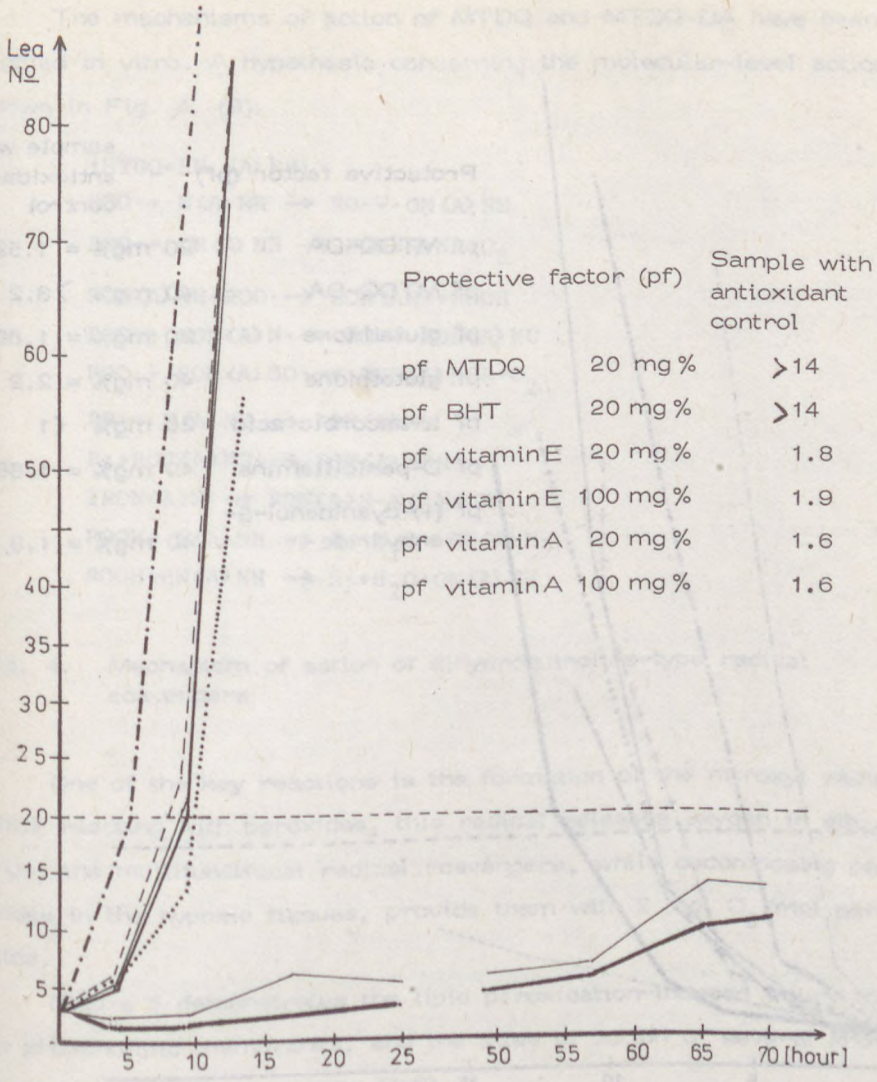

Fig. 2. AOM (Lea No.) of MTDQ, BHT, vitamin E and vitamin A

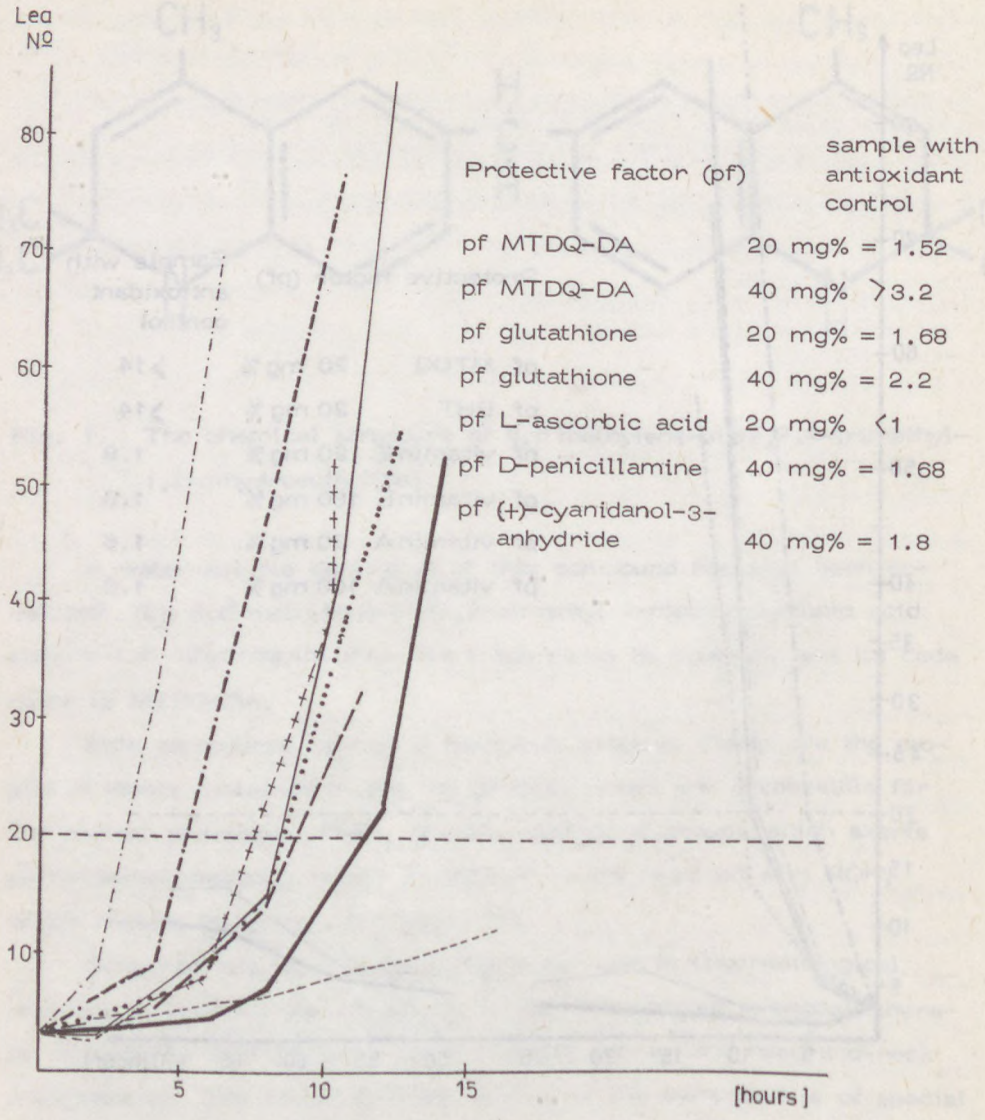

Fig. 3. AOM (Lea No.) of MTDQ-DA, D-penicillamine, ascorbic acid and glutathione

The mechanisms of action of MTDQ and MTDQ-DA have been studied in vitro. A hypothesis concerning the molecular-level action is shown in Fig. 4. (9).

(MTDQ=HN ⟨A⟩ NH)

$ROO\cdot + \cdot N\langle A\rangle NH \rightarrow RO\cdot + \cdot ON\langle A\rangle NH$

$ROO\cdot + \cdot ON\langle A\rangle NH \rightarrow RON\langle A\rangle NH + O_2$

$RON\langle A\rangle NH + ROO\cdot \rightarrow RON\langle AN\cdot + ROOH$

$ROO\cdot + RON\langle A\rangle N\cdot \rightarrow RO\cdot + RON\langle A\rangle NO$

$ROO\cdot + RON\langle A\rangle NO\cdot \rightarrow RON\langle A\rangle NOR + O_2$

$RO\cdot + \cdot N\langle A\rangle NH \rightarrow RON\langle A\rangle NH$

$R\cdot + RON\langle A\rangle NO\cdot \rightarrow RON\langle A\rangle NOR$

$2RON\langle A\rangle N\cdot \rightarrow RON\langle A\rangle N-N\langle A\rangle NOR$

$ROOH + \cdot N\langle A\rangle NH \rightarrow R\cdot + H_2O + \cdot ON\langle A\rangle N\cdot$

$ROOH + HN\langle A\rangle NH \rightarrow R\cdot + H_2O + ON\langle A\rangle NH$

Fig. 4. Mechanism of action of dihydroquinoline-type radical scavengers

One of the key reactions is the formation of the nitroxyl radical. While reacting with peroxides, this radical releases oxygen in situ. Thus, the multifunctional radical scavengers, while decomposing peroxides in the hypoxic tissues, provide them with 2 mol O_2/mol peroxide.

Figure 5 demonstrates the lipid peroxidation-induced injury in the phospholipid membranes, and the sites of action of several known drugs and radical scavengers (3-5).

Flohe found that lipoxygenase catalyses the formation of hydroxy fatty acid from arachidonic acid, which exerts a chemotactic effect on the polymorphonuclear leukocytes. Lipoxygenase is complementary with the cyclo-oxygenase system. Thus, it takes part in thrombocyte aggregation. Lipoxygenase is responsible for the peroxidation of mitochondrial lipids, and for inhibition of the activity of several enzymes of the respiratory chain (6). 15-HPETE is a potent inhibitor of prostacyclin synthetase. Recent studies revealed that the above substance is involv-

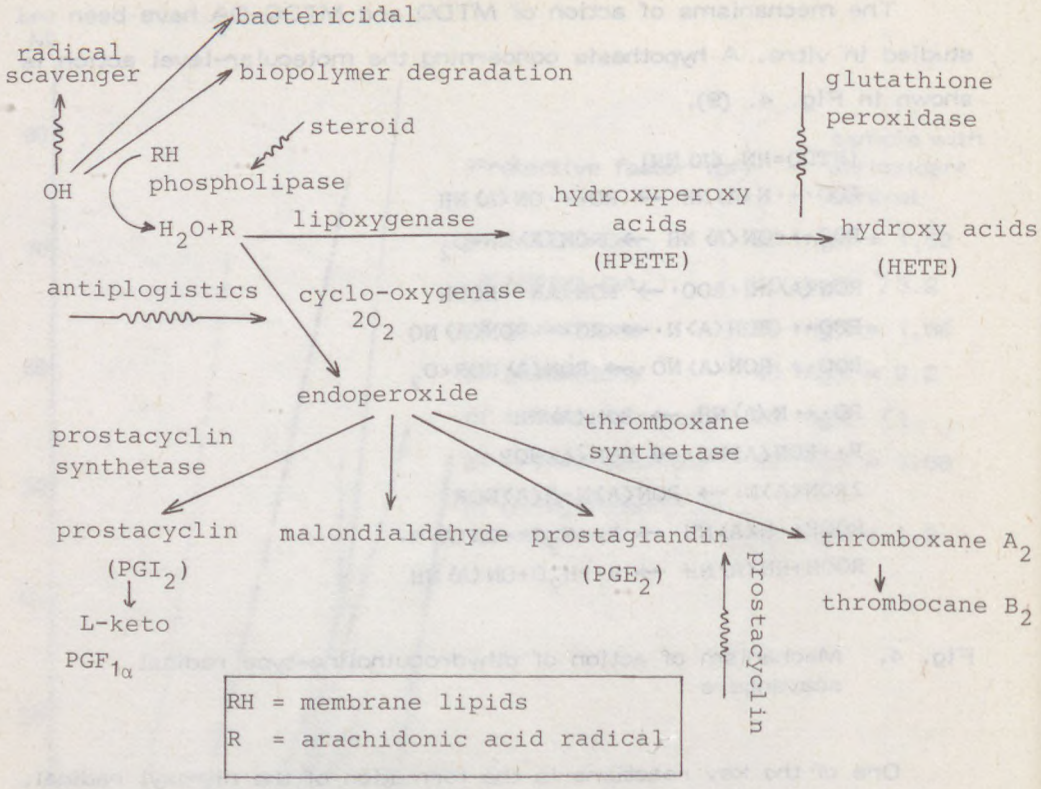

Fig. 5. Lipid peroxidation-induced injury in the phospholipid membranes, and the sites of action of several known drugs and radical scavengers

ed in tissular autoxidation, accompanied by an increased production of thiobarbituric acid-reactive substances, as shown by increased malondialdehyde production. However, it is common knowledge that the thiobarbituric acid-reactive substances are endoperoxides and precursors of malondialdehyde (4, 6). The formation and types of reactions of malondialdehyde during tissular autoxidation are demonstrated in Fig. 6.

It must be considered that platelet aggregation on the arterial wall is one of the events contributing to plaque formation. HHT and MDA give TXA_2, a vaso- and bronchoconstrictor agent responsible for thrombocyte aggregation. Prostacyclin, synthesized by prostacyclin synthetase in the endothelial cells and strongly inhibited by lipid per-

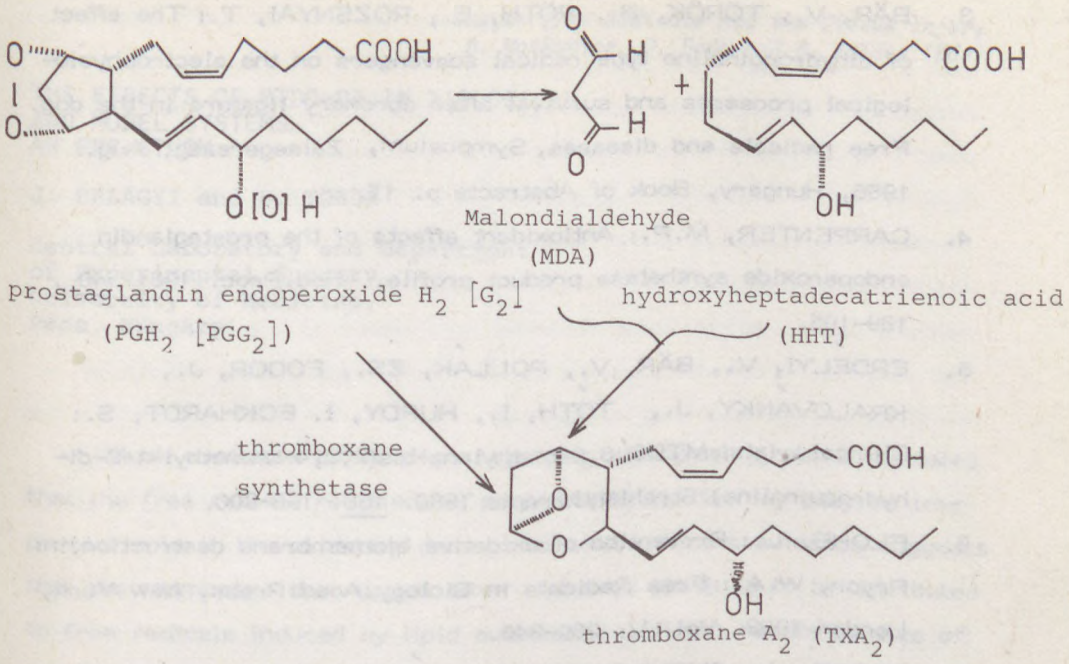

Fig. 6. Formation and types of reactions of malondialdehyde during tissular autoxidation

oxides, is a most potent inhibitor of platelet aggregation, and also a highly potent vaso- and bronchodilator agent; however, its continuous production, provided by or complemented with non-toxic synthetic radical scavengers, is of promise for the therapy of free radical-induced pathological processes.

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Oxygen Free Radicals and the Tissue Injury
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THE EFFECTS OF MTDQ-DA IN BIOLOGICAL AND MODEL SYSTEMS. AN EPR STUDY

J. BELÁGYI and B. TÖRÖK

Central Laboratory and Department
of Experimental Surgery,
University of Medicine,
Pécs, Hungary

Much available information regarding biological systems indicates that the free radical reactions of peroxidizing lipids may play an important role in the oxidative damage to cells. Recent evidence suggests that the membrane destruction after ischemic shock is to be attributed to free radicals induced by lipid autoxidation. From recent studies of the interaction mechanisms in complex biological systems, it is also known that a dihydroquinoline derivative, MTDQ-DA, can strongly influence the biological processes.

We have adapted the technique of electron paramagnetic resonance (EPR) for the detection of molecular motions induced in supramolecular assemblies by MTDQ-DA and to allow the direct identification of free radical species in the presence of MTDQ-DA in different experimental situations.

I. It is widely accepted that the structure and dynamics in a supramolecular assembly determine the inherent behavior of biological systems. In biological membranes, the conformational changes induced in proteins by different drugs are presumably not attenuated immediately at the annular lipid region, but are transferred via the lipid-protein interactions, to the bulk lipid region, modifying the arrangement and mobility of the phospholipid chains. In turn, any perturbation in the lipid region of the membranes probably affects the packing of the annular lipids around the proteins, which leads to a steric rearrangement of the protein segments and/or protein domains, thereby influencing the biological functions.

By means of conventional and saturation transfer EPR techniques and spin-labeling, experiments were performed on isolated sciatic nerves (n. ischiadicus) from frog, Rana esculenta. The spin labels used in the experiments were the fatty acid label [2-(3-carboxypropyl)-4,4'-dimethyl-2-tridecyl-3-oxazolidinyloxyl] or the maleimide spin label (4-maleimido-2,2,6,6-tetramethylpiperidinoxyl). A rapid and effective method was developed to incorporate fatty acid probes into the lipid regions and to attach the maleimide spin labels to the thiol sites of the proteins of the membrane (1). The maleimide probe molecules were located in a strong hydrophobic microenvironment (polarity index $\underline{h}$ = 1.2) and attached rigidly to a protein domain which rotates on the submicrosecond time scale (rotational correlation time $\tau_2 = 0.12\,\mu$) as estimated from the ST-EPR spectra.

In the presence of MTDQ-DA (10 mg/ml in Ringer solution), a significant increase (0.04 mT) was obtained in the hyperfine splitting constant ($2A'_{zz}$ = 5.898 mT), which indicates increases of the order-parameter, S, and the rotational correlation time. The probe molecules are located in a strong hydrophobic region of the membrane ($\underline{h} \sim 1.0$), and therefore the ring system should penetrate deeply into the hydrophobic region, producing a strong perturbation, which influences the internal mode of motion of the protein domains that hold the labels (Fig. 1).

The treatment of the frog nerve with MTDQ-DA led to structural alterations similar to those obtained in membranes after the incorporation of cholesterol. The partition coefficient of MTDQ-DA in the nerve membrane is not yet known, therefore the effect of MTDQ-DA cannot be compared with those of known membrane perturbants.

The experiments carried out on liposomes resulted in an increase of the hyperfine splitting constant of the fatty acid probes. It follows that MTDQ-DA might act as a non-specific membrane perturbant; the effect of MTDQ-DA, which influences primarily the lipid region, is efficiently transferred to the spin-labeled membrane proteins and leads to restricted rotational motion and lateral diffusion.

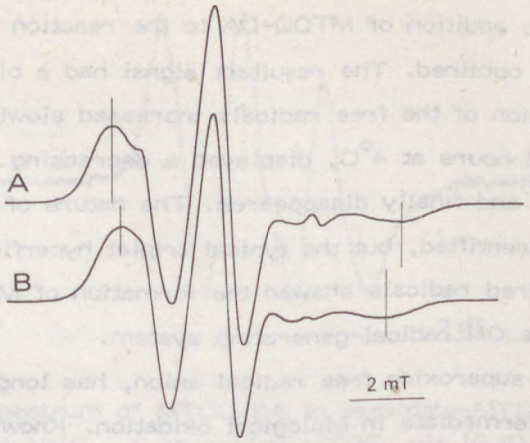

Fig. 1. EPR spectra of maleimide spin-labeled nerve fibers.
A: after MTDQ-DA treatment for 15 min at 4°C;
B: control nerve fibers

II. In biological systems, the generation and the recombination of free radicals have been found in several biochemical reactions. The technique of EPR has allowed the detection of the free radical intermediates in many metabolic pathways, involving both naturally-occurring and foreign compounds. However, this was so only in cases where the free radicals had relatively long lifetimes and the steady-state concentration of the free radicals was comparatively high. In most biological systems these conditions are not fulfilled. However, in some cases a compound which is reactive with free radicals produces a long-lived free radical product which can be detected by EPR.

MTDQ-DA contains a sterically hindered amine, therefore the formation of a long-lived free radical species on the nitrogen atom is to be expected in model and biological systems.

The measurements were carried out in a hydroxyl radical-generating system in the presence of 1-50 mg/ml MTDQ-DA, 0.15 M KCl, 0.5% H_2O_2, 0.4 mM EDTA and 0.2 mM Fe^{2+}, the mechanism being that of the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \dot{O}H$$

After the addition of MTDQ-DA to the reaction mixture, an EPR spectrum was obtained. The resultant signal had a biphasic character: the concentration of the free radicals increased slowly, reached a maximum in 2 hours at 4°C, displayed a decreasing signal amplitude for 72 hours, and finally disappeared. The nature of the radical has not yet been identified, but the typical triplet hyperfine structure of nitrogen-centered radicals showed the formation of MTDQ-DA free radicals in the OH radical-generating system.

$\dot{O}_2^-$, the superoxide free radical anion, has long been regarded as a potential intermediate in biological oxidation. Knowles and coworkers (2) demonstrated that in the purely inorganic system

$$IO_4^- + H_2O_2 \longrightarrow \dot{O}_2^- + \ldots$$

the signal-giving species is $\dot{O}_2^-$, which has a relatively long lifetime in the alkaline pH region ($t_{1/2}$ 0.01 sec, pH 10.0). This conclusion was based on the observation that in a much better understood system, an oxygen-saturated aqueous solution of $Ba(OH)_2$, it proved possible to obtain an identical EPR signal after irradiation with 4 MeV electrons. The pulse-radiolysis experiments were later extended to the mechanism of action of superoxide dismutase (3).

The EPR experiments performed on periodate-MTDQ-DA and periodate/peroxide-MTDQ-DA systems showed the formation of stable free radicals in both systems (18.0 mM $NaIO_4$, 6.25 mg/ml MTDQ-DA and 0.062% H_2O_2; Fig. 2). Although the whole of the hyperfine structure is not well resolved, there is clear evidence for the presence of the typical triplet hyperfine structure of nitrogen-centered radicals, which could only arise from MTDQ-DA. It may be concluded from the experiments that MTDQ-DA not only participates as an active species in the superoxide-generating system, but is very probably involved directly in the biological oxidation-reduction processes.

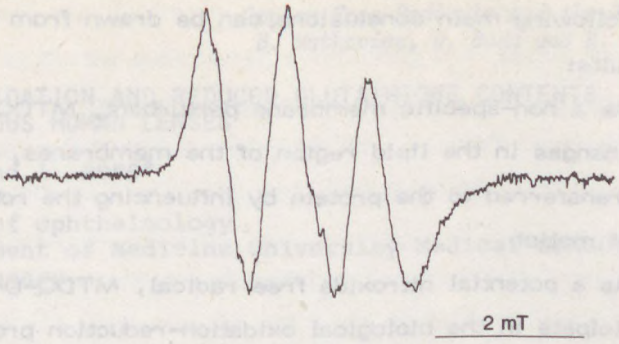

Fig. 2. EPR spectrum of MTDQ-DA in periodate-MTDQ-DA system (18 mM $NaIO_4$, 6.25 mg/ml MTDQ, pH 10.5)

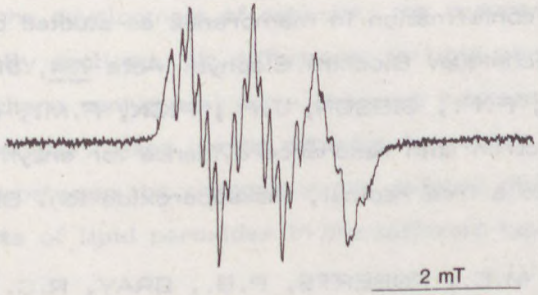

Fig. 3. EPR spectrum of MTDQ-DA in superoxide radical-generating system (50 mg/ml MTDQ-DA, 10 mg/ml KO_2, EDTA-Na_2CO_3 buffer, pH 10.2, 0°C)

It is known that superoxide radicals are generated in the system KO_2 in EDTA-Na_2CO_3 buffer, pH 10.2. When MTDQ-DA was added to the reaction mixture, an EPR signal was obtained (Fig. 3).

The well-resolved superhyperfine structure superimposed on the triplet hyperfine structure of nitrogen-centered radicals shows the contribution of MTDQ-DA to the EPR signal detected.

The following main conclusions can be drawn from the experimental results:

1. As a non-specific membrane perturbant, MTDQ-DA induces changes in the lipid region of the membranes, which are transferred to the protein by influencing the rotational modes of motion.
2. As a potential nitroxide free radical, MTDQ-DA might participate in the biological oxidation-reduction processes.
3. As a synthetic radical scavenger, MTDQ-DA might act in the superoxide free radical anion-generating system.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

LIPID PEROXIDATION AND REDUCED GLUTATHIONE CONTENTS IN CATARACTOUS HUMAN LENSES

A. FACSKÓ and I. ÉDES*

Department of Ophthalmology
*2nd Department of Medicine, University Medical School,
Szeged, Hungary

SUMMARY

The amounts of reduced glutathione content and lipid peroxides have been measured in cataractous human lenses during cataract formation. With the development of cataract, the reduced glutathione content gradually declined. No differences in lipid peroxide content (formation of diene conjugates) were observed between the different types of cataractous lenses during cataract formation. No correlation could be found between the changes in the reduced glutathione content and the amounts of lipid peroxides in the different types of cataractous lenses.

INTRODUCTION

Many of the changes observed in the lens during senile cataract development are due to oxidative processes, particularly in the nuclear region. These include the oxidation of different amino acids (6, 12, 13, 14), and reduced glutathione (GSH) (1, 12). Moreover, the protein sulfhydryl content of the lens is considerably decreased, while both protein-protein and protein-glutathione disulfide contents are increased (8, 13). It is commonly believed that the dramatic increase in the oxidative processes in the lens is due mainly to photo-oxidation of the proteins. Fecondo and Augusteyn (7) measured the activities of

the antioxidant enzymes during cataract formation and found that both superoxide dismutase and glutathione peroxidase activities were decreased in the nuclear region. They suggested that the inactivation of these enzymes may result in an elevation of the H_2O_2 and superoxide radical (O_2^-) contents and that this may contribute to the oxidative damage.

The present study was undertaken in an attempt

- to gain information about the in vivo lipid peroxidation in the different types of cataractous lenses;
- to establish any correlation between the changes in the GSH content and the amounts of lipid peroxides during cataractogenesis.

MATERIALS AND METHODS

Cataractous lenses were obtained during surgery at the Department of Ophthalmology and were classified into types I-IV according to colour, as described by Pirie (9). The lenses from patients with diabetic, traumatic, steroid, congenital or complicated cataracts were excluded from subsequent analysis.

The lipids were extracted according to the method of Bligh and Dyer (2). The lipid peroxide content (the formation of diene conjugates) was evaluated by measuring the optical density (OD) of the lipids at 232 nm (11) in a UV-VIS double-beam spectrophotometer. The lipids were measured in cyclohexane and the OD/lens value was then calculated.

For the GSH determinations, the lenses were homogenized in ice-cold 0.2 M sulfosalicylic acid. Following centrifugation the non-protein sulfhydryl content was measured with 5,5´-dithiobis-(2-nitrobenzoate) at 412 nm, according to Boyne and Ellman (3).

Statistical evaluation of the results was performed with Student´s t test. Values with $P < 0.05$ were regarded as statistically significant.

RESULTS

The lipid peroxide (formation of diene conjugates) contents of different types of cataractous lenses are given in Table 1. The data are expressed as OD at 232 nm/lens. Relatively low values were found in all cataractous lenses investigated. No significant difference could be demonstrated between the different groups of lenses during cataractogenesis.

Table 1. GSH and diene conjugate contents in cataractous lenses

Lens type	Mean age	Diene conjugates (optical density at 232 nm/lens)		Mean age	GSH (nmol/lens)	
Nuclear cataracts						
Type I	70	1.1±0.5	(20)	70	247.0±149.0	(10)
Type II	74	1.2±0.6	(22)	74	182.5±173.4	(10)
Type III	72	1.0±0.5	(22)	75	76.7± 70.1*	(10)
Type IV	76	0.9±0.5	(19)	75	105.7± 95.5*	(10)

* Significantly different from the value found in the type I lenses; $P < 0.05$. The numbers of determinations are given in brackets.

Wide variations in GSH content were observed in cataractous human lenses. This is illustrated by the large standard deviations. However, types III and IV contained significantly lower amounts of GSH as compared to the group I lenses. No correlation was found between the amounts of GSH and the in vivo lipid peroxide content in the different types of cataractous lenses.

DISCUSSION

The oxidants derived from the univalent reduction of oxygen are responsible for a variety of pathological phenomena. These include radiation damage, aging, the oxidation of proteins and lipid peroxidation (4), which might play an important role in cataractogenesis. The lipid peroxide content was measured in 83 cataractous lenses in the course of cataract formation. Initial cataractous lenses (type I) were used as a control for our measurements. An attempt was made to determine the diene conjugate content of normal lenses. Unfortunately, due to the limited number of normal lenses and to the post mortem modifications, conflicting results were found. Theoretically, fresh normal human lenses would be advantageous as a control for the lipid peroxidation and GSH measurements.

Fecondo and Augusteyn (7) found that the activities of glutathione peroxidase and superoxide dismutase are decreased in cataractous lenses, and concluded that free radicals may be responsible for the oxidative modifications of the lens proteins. However, we could not demonstrate significant differences in lipid peroxide content between the type I-IV cataracts. In advanced types (III and IV), the amounts of lipid peroxides were in fact slightly but not significantly lower. Corongiu et al. (5) failed to detect an enhanced diene conjugate content in cataractous lenses from normal and glucose-6-phosphate dehydrogenase-deficient patients. However, they did not investigate the diene conjugate content during the course of cataract formation. It is not clearly understood why no correlation could be found between the changes in the GSH contents and the amounts of lipid peroxides. One possible explanation is that the overproduction of free radicals due to inactivation of the scavenger enzymes affects mainly the proteins and GSH. This concept is supported by the unusually low concentration of polyunsaturated fatty acids found in the plasma membrane of normal and cataractous human lenses (10) which might explain the relatively high resistance of the lipids towards peroxidation.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

FREE RADICALS IN RHEUMATIC DISEASES

Gy. GENTI, Mariann ANTAL, Eszter HORVÁTH, G. BÁLINT and Katalin MERÉTEY

National Institute of Rehumatology and Physiotherapy, Budapest, Hungary

SUMMARY

There is substantial evidence which implicates the oxygen derived free radicals in the pathogenesis of inflammatory joint diseases such as rheumatoid arthritis or other inflammatory arthropathies. Free radicals are produced by polymorphonuclear leukocytes and other inflammatory cells, the most toxic species being the hydroxyl radical. Its formation requires transition metal (iron, copper) ions. Inflammatory rheumatic diseases are characterized by the extracellular generation of free radicals. Proteoglycans, collagen and hyaluronic acid are not very well protected against free radicals. Therapeutic possibilities against free radical injury are also discussed.

In the past few years, the interest of researchers has increasingly been attracted by a group of extremely reactive chemical species, the oxygen free radicals (FR). Experimental and clinical data have accumulated suggesting that these FR might be involved in the pathogenesis of a number of rheumatic diseases characterized by either acute or chronic inflammation (3, 8, 9, 11-13, 19, 20, 32). Polymorphonuclear leukocytes play a central role in the inflammatory reaction. During phagocytosis (4, 9, 10, 18, 32), granulocytes and macrophages produce large amounts of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), which, in the presence of iron as a

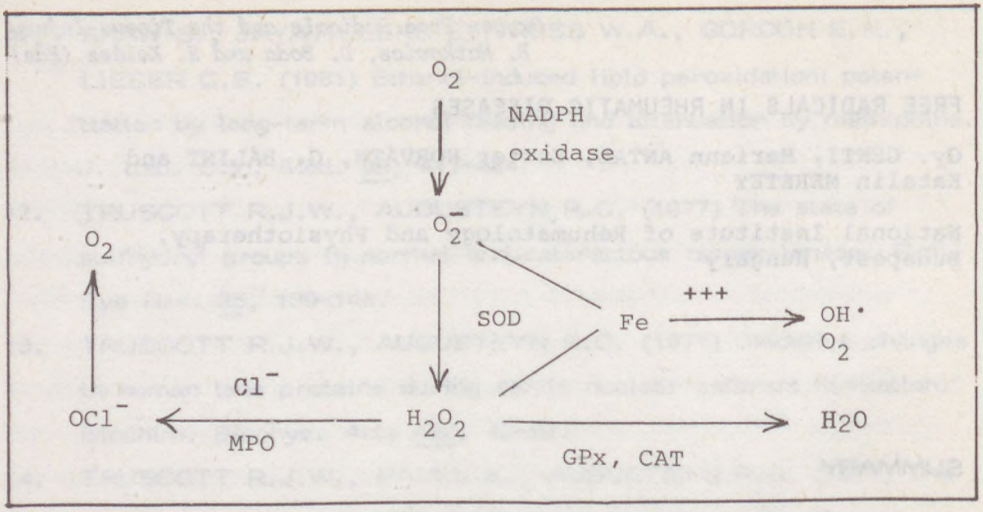

Fig. 1. Schematic illustration of generation and enzymatic elimination of free radicals (SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, MPO: myeloperoxidase)

catalyst, form the highly reactive hydroxyl radicals ($OH^{\cdot}$) (Fig. 1). These FR are able to attack and destroy lipid-containing membranes (lipid peroxidation) and connective tissue (13, 14, 20, 25, 33). Protective and controlling mechanisms against FR include enzymes, such as superoxide dismutase (6, 24, 25), glutathione peroxidase, catalase and peroxidases (5, 7, 22, 23, 29), and non-enzymatic compounds (vitamins A and E, ascorbic acid, ceruloplasmin and transferrin) (11, 20, 33). Inflammatory rheumatic diseases are characterized by the extracellular generation of FR (Table 1), and they may play a role in pathogenesis of these disorders due to the combination of four factors (11):

1. the presence of inflammatory cells (granulocytes, monocytes and macrophages);
2. stimuli to O_2^- and H_2O_2 generation;
3. the presence of chelated metal complexes;
4. low levels of scavenging enzymes in the synovial fluid.

Table 1. Free radical classification of disease states (10)

1. Increased intracellular generation
   a) hypo- and hyperoxygenation syndromes
   b) chemicals
   c) hemolytic anemias
   d) vitamin A or E deficiency
   e) aging
2. Increased extracellular generation
   a) inflammatory states
      acute
      chronic: rheumatoid arthritis, connective tissue disorders, vasculitis
   b) immune diseases
3. Increased intra- and extracellular generation
   a) radiation
   b) chemical carcinogens

In inflammatory arthropathies, granulocytes accumulate in the synovial fluid and are exposed to different stimuli: in rheumatoid arthritis (RA), immune complexes and complement components may induce the respiratory burst of granulocytes and the generation of FR. The interaction of monosodium urate, calcium pyrophosphate dihydrate and hydroxyapatite crystals with polymorphonuclear leukocytes in gout, chondrocalcinosis and hydroxyapatite synovitis is well known. Phagocytosis of these crystals results in the activation of many mediators, including FR (28). Although evidence of FR involvement in osteoarthrosis is less strong, there are some indications that FR might be implicated (11). The formation of hydroxyl radical ($OH^{\cdot}$) requires iron salts (8, 19). There is an increased deposition of iron in the synovial membrane (8), and "free" iron and catalytic iron complexes can be found in the pathogenesis of RA (26, 32). The role of copper in $OH^{\cdot}$ generation has recently been described (15).

There are very few and conflicting data in the literature on the scavenging enzymes present in the synovial fluid. In RA, low contents

of superoxide dismutase (SOD) have been found (6, 7, 24). Blake et al. (7) measured low concentrations of catalase (CAT) in RA synovial fluid, while others (5, 6) found elevated CAT and glutathione peroxidase (GPx) activities compared with the synovial fluid of traumatic and osteoarthritic patients. Our results showed marked increases of CAT and PO activities and decreases of SOD and GPx activities compared with osteoarthrosis (3). When we arbitrarily divided our RA patients into two groups (patients with a high serum and synovial malonaldehyde (MDA) level (>7 µmol/l), and patients with a low serum and synovial MDA level (<7 µmol/l), significant differences were found between the two groups in respect of the enzyme activities in the synovial fluid. However, similar differences could not be detected in the serum (Table 2). These data indicate that the synovial levels of scavenging enzymes probably much better reflect the inflammatory status than the serum levels. Evidence is accumulating that RA can be considered to be a "free radical disease", as shown in Table 3.

If we accept the hypothesis that FR may promote inflammation, the question arises as to how we can diminish or prevent the damage caused.

1. Use of FR scavengers: SOD has been used for the treatment of RA and osteoarthrosis, but convincing data are still lacking (21). The combination of SOD and CAT with the intramuscular injection of GPx has been claimed to have an anti-inflammatory effect, but this has not been fully proved yet (31). It is clear that the efficient removal of O_2^- and H_2O_2 will minimize the possibility of $OH^\cdot$ formation.
2. Chelation of iron and copper: It has been shown that desferrioxamine (Desferal®) may have a beneficial effect in RA (8). Desferrioxamine is not only an inhibitor of $OH^\cdot$ formation, but also an effective radical scavenger. Adverse ocular and cerebral reactions can be avoided by giving lower doses. D-Penicillamine can directly chelate the potentially toxic copper and this complex has SOD-like activity (8).

Table 2. *Malondialdehyde and enzymatic scavengers in rheumatoid serum and synovial fluid (MDA = malondialdehyde, GPx = glutathione peroxidase, SOD = superoxide dismutase, CAT = catalase, PO = peroxidase)*

SERUM		MDA µmol/l	GPx x 10^3 U/g prot.	SOD U/g prot.	CAT U/mg prot.	PO x 10^{-3} U/mg prot.
Group 1						
MDA > 7 µmol/l	$\bar{x}$	13.11	1.94	33.89	0.29	2.89
n=13	S.E.	0.77	0.14	3.99	0.10	0.48
Group 2						
MDA < 7 µmol/l	$\bar{x}$	10.57	1.73	21.60	0.20	3.33
n=10	S.E.	0.62	0.19	7.57	0.05	0.71
p		< 0.002	n.s.	n.s.	n.s.	n.s.
SYNOVIAL FLUID						
Group 1						
MDA > 7 µmol/l	$\bar{x}$	9.32	1.83	45.54	2.43	19.65
n=27	S.E.	0.31	0.11	4.92	0.54	4.12
Group 2						
MDA < 7 µmol/l	$\bar{x}$	5.70	2.19	57.83	0.74	9.62
n=21	S.E.	0.17	0.13	10.11	0.23	2.91
p		< 0.001	< 0.05	n.s.	< 0.01	< 0.05

Table 3. Rheumatoid arthritis as a "radical disease"

Clinical features
signs of chronic inflammation (dolor, calor, tumour, functio laesa), slow progression
Synovial fluid
low viscosity, accumulation of polymorphonuclear leukocytes, elevated MDA content, low level of SOD, immune complexes, elevated non-protein-bound iron
Iron
low serum iron and haemoglobin levels, catalytic iron complexes in synovial fluid, iron deposits in synovial membrane, relationship between clinical activity and iron metabolism

3. Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used in the treatment of the inflammatory rheumatic diseases. Their mode of action is not fully understood. It is clear now that their antiinflammatory effect is not only connected with the inhibition of prostaglandin synthesis; many of them (indomethacin, phenylbutazone and piroxicam) are also FR scavengers (2, 30). Antal et al. (2) found a good correlation between the antiinflammatory effect of NSAIDs and their ability to inhibit the chemiluminescence of granulocytes. This provides a useful indirect method for the measurement of FR generation (1, 2, 27).

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ACKNOWLEDGEMENT

This paper was supported by the Hungarian State Office for Technical Development.

Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

EFFECTS OF DIHYDROQUINOLINE ANTIOXIDANTS ON HEPATIC MICROSOMAL ENZYME ACTIVITY

Anna BLÁZOVICS, Anikó SOMOGYI* and J. FEHÉR*

Arteriosclerosis Research Group
*2nd Department of Medicine,
Semmelweis University of Medicine
Budapest, Hungary

SUMMARY

The antioxidant and antiatherogenic effects of CH 402 [Na(2,2-dimethyl-1,2-dihydroquinolin-4-yl)methanesulphonate] and MTDQ-DA 6,6'-methylene-bis-(2,2-dimethyl-4-methanesulphonic acid)Na-1,2-dihydroquinoline on the microsomal monooxygenase system were studied in in vitro and in vivo experiments in normo- and hyperlipidaemic rat liver microsomes. These drugs decreased the induced lipid peroxidation in vitro, but failed to affect the activity of the monooxygenase system in vitro in both normo- and hyperlipidaemic rats. The two dihydroquinoline derivatives showed different effects in hyperlipidaemic rats in vivo. MTDQ-DA increased the activities of NADPH cytochrome c reductase, NADH ferricyanide reductase, p-hydroxylase and N-demethylase in vivo in hyperlipidaemic rats, and influenced the pathogenic lipid metabolism.

INTRODUCTION

The aim of the present work is to survey the effects of recently developed dihydroquinoline antioxidants on the cytochrome P_{450}-related, cytochrome P_{450} reductase and cytochrome b_5 reductase activities in rat liver, to elucidate the mechanisms of action and to assess whether the ultrastructural morphological changes correlate with the biological

parameters of the monooxygenase system. In previous studies we found that CH 402 and MTDQ-DA completely inhibited the enzymic and non-enzymic induced lipid peroxidation in different enzyme systems in vitro in the liver and in the brain, and also had membrane-protecting effects (1, 2, 4). In in vivo experiments, MTDQ-DA treatment caused regression in steatosis (3, 7).

MATERIALS AND METHODS

Young male Wistar albino rats weighing 150-200 g were used. The animals in the first group were kept on normal LATI chow; the second group was treated with MTDQ-DA or CH 402 in a dose of 200 mg/kg b.w./day for 5 days; the third group was fed with the fat-rich diet for 8 days; and the fourth group was fed with the fat-rich diet and treated via a gastric tube with CH 402 or MTDQ-DA in a dose of 200 mg/kg/b.w./day for 5 days from the third day on. The fat-rich diet contained 2% cholesterin, 0.5% cholic acid and 20% sunflower oil.

Liver microsomes were prepared by ultracentrifugation methods. The procedure was carried out in 0.15 M KCl solution. Lipid peroxidation was studied by the method of Jansson and Schenkman (1977). Malondialdehyde production was assayed by the thiobarbituric acid test of Ottolenghi (1959). NADPH cytochrome c reductase, NADH ferricyanide reductase, p-hydroxylase (aniline) and N-demethylase (amidazophen) were measured by standard methods (Jansson and Schenkman, 1977; Schenkman et al., 1967; Nash, 1953). The protein contents of the preparations were determined by the procedure of Gornall et al. (1949).

Glucose-6-phosphate-dehydrogenase and serum bovine albumin were obtained from Calbiochem AG (Lucerne, Switzerland), NADPH and NADH from SIGMA (St. Louis, USA), and all other reagents from Reanal (Budapest).

Statistical analysis was performed according to the t test, with the confidence limits: $h_1;h_2 = \bar{x} \pm t_{P_{95\%}} s_{\bar{x}}$. All data are mean values obtained from 10 experiments.

For each experiment, microsomal fractions were used from 5 different rats.

RESULTS

The NADPH + Fe^{3+}-induced lipid peroxidation was studied in vitro on liver microsome preparations from normo- and hyperlipidaemic rats. The NADPH cytochrome P_{450} reductase activity was decreased, when the rats were fed with the fat-rich diet. Both CH 402 and MTDQ-DA decreased the induced lipid peroxidation in time- and concentration-dependent manners. The lipid peroxidation (expressed as malondialdehyde generated) was inhibited in controls and in animals on the fat-rich diet by in vivo treatment with CH 402. MTDQ-DA treatment in vivo inhibited the microsome-induced lipid peroxidation of the normolipidaemic rats, and stimulated it in animals fed with the fat-rich diet, in contrast with the control diet (Figs 1 and 2).

Both CH 402 and MTDQ-DA significantly increased the microsomal aniline hydroxylase activity in normolipidaemic rats, but no other changes were observed (Table 1). CH 402 did not have a significant effect on the enzyme activities in steatosis, but MTDQ-DA increased the activities of all the enzymes of the monooxygenase system (Table 2).

DISCUSSION

In our experiments neither CH 402 nor MTDQ-DA had an effect on the activity of the microsomal monooxygenase system in vitro. Therefore, it can be stated that these drugs do not influence the enzyme functions. When the rats were kept on a normal diet for 8 days and were treated with 200 mg/kg/b.w./day of these drugs from the third day on, CH 402 and MTDQ-DA did not have significant effects on the activities of N-demethylase, NADPH cytochrome c reductase or NADH ferricyanide reductase.

Both drugs significantly increased the activity of aniline hydroxylase. When the animals were kept on the fat-rich diet for 8 days, the enzyme activities were less than those in the control group. When the rats were treated with CH 402 and kept on the atherogenic diet,

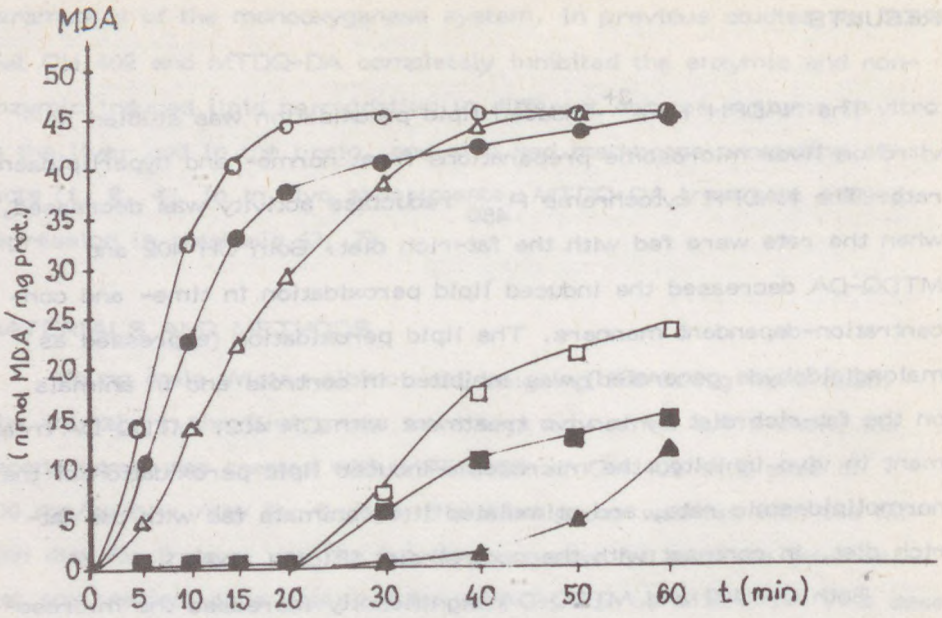

Fig. 1. In vitro effects of CH 402 and MTDQ-DA on the NADPH and Fe^{3+} induced lipid peroxidation in rat liver microsomes in normo- and hyperlipidaemic animals

- Control diet
- Control diet + 5.10^{-5} M CH 402
- Control diet + 5.10^{-5} M MTDQ-DA
- Fat-rich diet
- Fat-rich diet + 5.10^{-5} M CH 402
- Fat-rich diet + 5.10^{-5} M MTDQ-DA

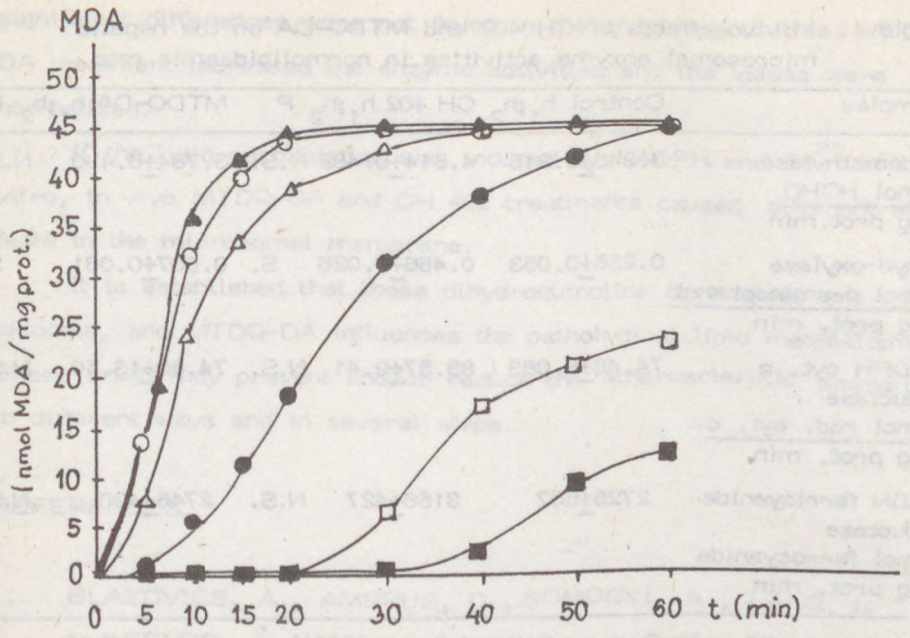

Fig. 2. In vivo effects of CH 402 and MTDQ-DA on the NADPH + Fe^{3+}-induced lipid peroxidation in rat liver microsomes in normo- and in hyperlipidaemic animals

- Control diet
- Control diet + CH 402
- Control diet + MTDQ-DA
- Fat-rich diet
- Fat-rich diet + CH 402
- Fat-rich diet + MTDQ-DA

Table 1. In vivo effects of CH 402 and MTDQ-DA on the hepatic microsomal enzyme activities in normolipidaemic rats

Samples	Control $h_1;h_2$	CH 402 $h_1;h_2$	P	MTDQ-DA $h_1;h_2$	P
N-demethylase $\frac{\text{nmol HCHO}}{\text{mg prot.min}}$	3.846±0.215	4.514±0.482	N.S.	3.784±0.490	N.S.
p-hydroxylase $\frac{\text{nmol p-aminophenol}}{\text{mg prot. min}}$	0.255±0.053	0.486±0.026	S.	0.567±0.061	S.
NADPH cyt. c reductase $\frac{\text{nmol red. cyt. c}}{\text{mg prot. min}}$	74.48±8.083	83.57±9.41	N.S.	74.80±13.50	N.S.
NADH ferricyanide reductase $\frac{\text{nmol ferrocyanide}}{\text{mg prot. min}}$	2725±667	3156±427	N.S.	2745±420	N.S.

S. = significant, $p < 0.05$
N.S. = not significant, $p > 0.05$

Table 2. In vivo effects of CH 402 and MTDQ-DA on the hepatic mircosomal enzyme activities in hyperlipidaemic rats

Samples	Atherogenic control $h_1;h_2$	CH 402 $h_1;h_2$	P	MTDQ-DA $h_1;h_2$	P
N-demethylase $\frac{\text{nmol HCHO}}{\text{mg prot.min}}$	2.260±0.348	2.003±0.479	N.S.	3.579±0.561	S.
p-hydroxylase $\frac{\text{nmol p-aminophenol}}{\text{mg prot. min}}$	0.275±0.056	0.248±0.018	N.S.	0.533±0.041	S.
NADPH cyt. c reductase $\frac{\text{nmol red. cyt.c.}}{\text{mg prot. min}}$	47.34±15.375	41.00±5.200	N.S.	127.80±8.15	S.
NADH ferricyanide reductase $\frac{\text{nmol ferrocyanide}}{\text{mg prot. min}}$	1200±321	980±340	N.S.	1960±390	S.

S. = significant, $p < 0.05$
N.S. = not significant, $p > 0.05$

significant differences were not found in the enzyme activities. MTDQ-DA treatment increased the enzyme activities and the values were normalized.

If the lipid peroxidation was induced by NADPH and Fe^{3+} in vitro, in vivo MTDQ-DA and CH 402 treatments caused different effects in the microsomal membrane.

It is established that these dihydroquinoline derivatives are antioxidants, and MTDQ-DA influences the pathological lipid metabolism. These drugs may prevent and/or reduce the atherosclerotic alterations in different ways and in several steps.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

EFFECTS OF IN VIVO THERAPY WITH 4-AMINO-5-IMIDAZOLE-CARBOXAMIDE-PHOSPHATE (AICA-P) ON CELLULAR IMMUNOREACTIVITY*

Gy. DEÁK, K. NÉKÁM, Gy. MÜZES, A. ROMÁNY, A. VERECKEI, I. LÁNG and J. FEHÉR

Second Department of Medicine
Semmelweis University of Medicine,
Budapest, Hungary

INTRODUCTION

Oxygen stress, liver cell membrane disorganization and certain cellular immune processes are involved in the pathogenesis of chronic liver diseases. Decreasing the effects of oxygen stress, membrane protection and immunomodulation are important factors in the treatment of these disorders.

Free radical scavenger, membrane-stabilizing hepatoprotective drugs are widely used in the treatment of chronic hepatic diseases, and their role in immune reactions is also well known. We have therefore studied the effects of the new free radical scavenger hepatoprotective drug Aica-P on some important indicators of cellular immunoreactivity. The clinical data on the patients and the beneficial effects of the treatment have been described elsewhere by Fehér et al. (1978).

PATIENTS, MATERIALS AND METHODS

Twenty-two patients suffering from chronic alcoholic liver disease were treated with 3 x 200 mg Aica-P (Chinoin, Budapest) daily for 4 weeks. Whole blast lymphoblast transformation tests were performed by the modified method of Bicker et al. (1983) immediately before and after treatment. In brief: heparinized venous blood was diluted 10-fold

* This work was supported by grants from the Hungarian Academy of Sciences and the Hungarian Ministry of Health.

in TC-199 medium with 10% heat-inactivated fetal calf serum (Human, Budapest), antibiotics, and 25 mM Hepes (Serva) to a final volume of 200 µl. Five replicates were placed in flat-bottomed microplates (Greiner). Con-A (Calbiochem) or PHA (Pharmacia) was added to give a final concentration of 25 µg/ml or 2 µg/ml and 10 µg/ml, respectively. The cultures were kept in a humidified atmosphere at 37°C for 72 h. Eight hours before harvesting, 0.5 µCi ^{3}H-thymidine (UVVVR, Czechoslovakia) was added to each well, and the cultures were sucked off onto glass fibre filters (Whatman GF/c). Isotope determination was performed with a Nuclear Chicago Isocap 300 counter and the results were expressed in cpm.

The ADCC assay was performed according to the original method of Perlmann and Perlmann (1970), with slight modifications: ^{51}Cr-labelled chicken RBC (2.5×10^{4}) were added to plastic test-tubes and mixed with an appropriate amount of effector cells to give the desired effector/target cell ratio (10:1, 5:1 and 2.5:1). Anti-CRBC serum (final dilution 1:10,000) was added and the volume was made up to 0.125 ml with medium. Appropriate controls for spontaneous release were included in each experiment. After incubation for 4 h at 37°C, the radioactivities of the pellet and the supernatant were determined. Cell damage was expressed as per cent isotope release into the supernatant. The spontaneous release never exceeded 5%. The maximum isotope release was about 90%, as determined by treatment of the target cells with 1% saponin.

Cytotoxicity was expressed as:

$$\frac{\text{test release} - \text{spontaneous release}}{\text{saponin-induced release} - \text{spontaneous release}} \times 100$$

Natural killer (NK) assays were performed by the method of Jondal and Pross (1975). Briefly, 2×10^{4} ^{51}Cr-labelled K-562 targets were mixed with PBMC at an effector/target ratio of 50:1, 25:1 or 12.5:1, and incubated at 37°C for 4 h. The reaction was terminated by the addition of cold medium, and the tubes were spun at 1,500 rpm

for 5 min. The radioactivities of the pellet and the supernatant were measured in an automatic gamma counter. The target cell damage was expressed as test-tube isotope release minus spontaneous release. The spontaneous release was 12-15%.

The percentages of $T4^+$ and $T8^+$ cells were determined simultaneously, using OKT4 and OKT8 antibodies.

RESULTS AND DISCUSSION

The effects of Aica-P treatment on the ADCC and NK activities of the lymphocytes are shown in Table 1. Aica-P did not exert a significant effect on the killer and natural killer activities.

Table 1. Effects of Aica-P treatment on ADCC and NK activities of lymphocytes from patients with chronic alcoholic liver disease

	before treatment	after
ADCC (%)	26.9±3.5	31.7±3.2
NK (%)	33.7±3.1	30.7±2.7

The effects of a four-week treatment with 600 mg Aica-P daily on the 2 μg/ml (suboptimal dose) PHA-induced blastogenesis of lymphocytes from patients with chronic alcoholic liver disease are shown in Fig. 1.

Depending on the pretreatment values, these patients could be divided into two groups: those with originally low responses and those with normal ones. In vivo Aica-P treatment significantly enhanced the lymphoblast transformation of patients with originally low values (central pair of columns). On the other hand, the lymphoblast transformation of patients with originally normal values decreased significantly (right pair of columns). As concerns the average values of the two groups, no significant change was seen (left pair of columns).

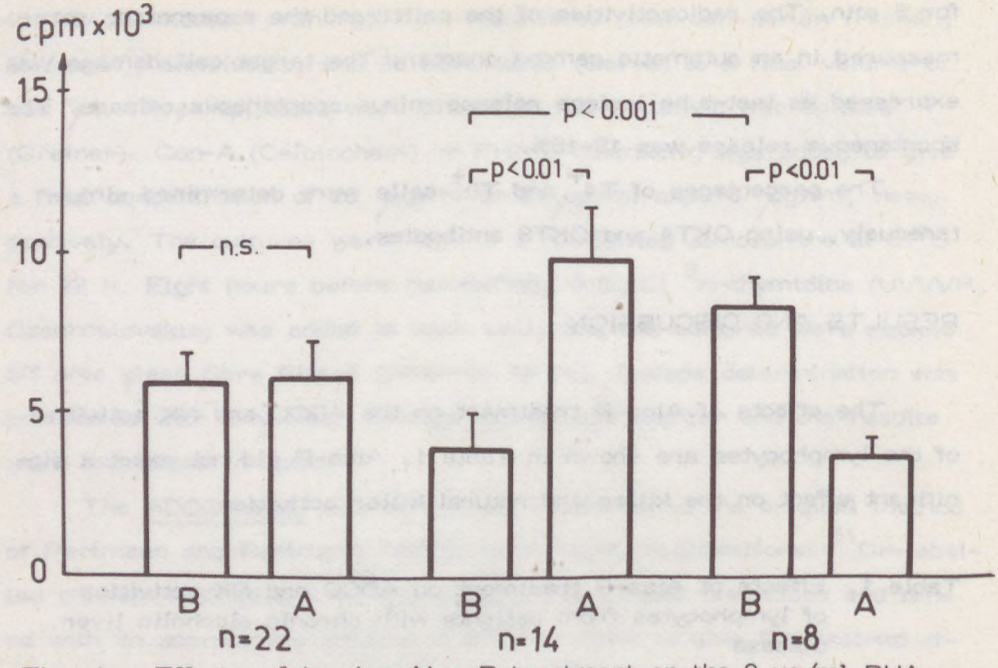

Fig. 1. Effects of in vivo Aica-P treatment on the 2 µg/ml PHA-induced blastogenesis of lymphocytes from patients with chronic alcoholic liver disease

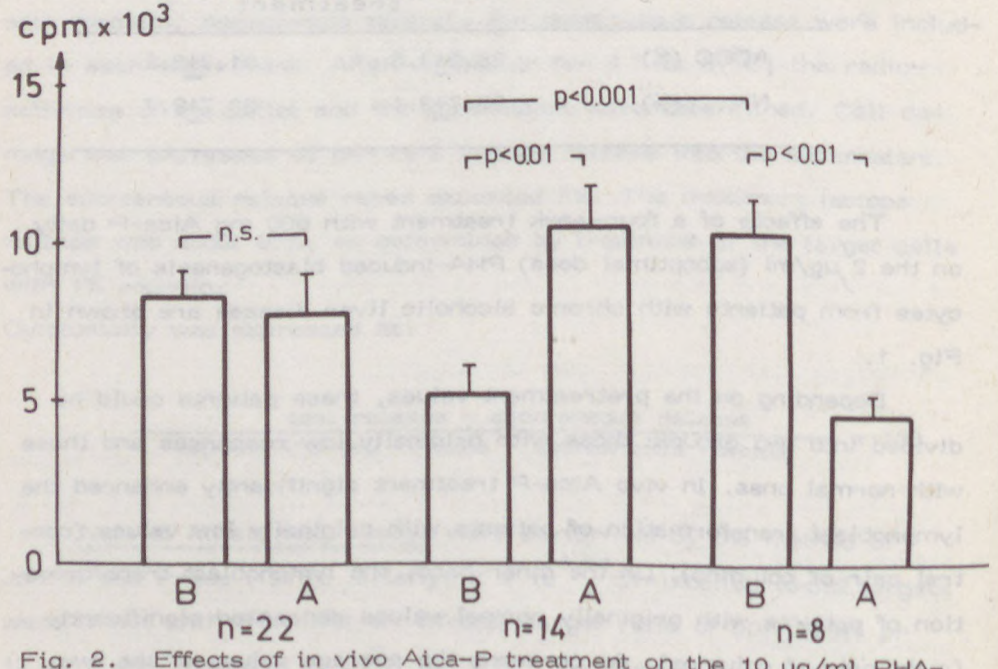

Fig. 2. Effects of in vivo Aica-P treatment on the 10 µg/ml PHA-induced blastogenesis of lymphocytes from patients with chronic alcoholic liver disease

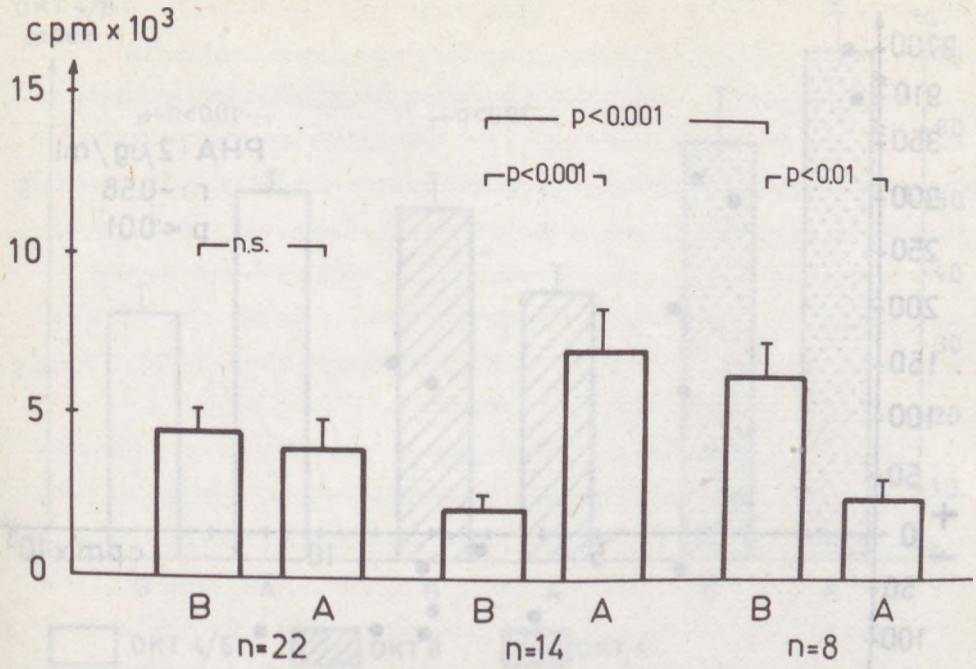

Fig. 3. Effects of in vivo Aica-P therapy on the 25 µg/ml Con-A-induced blastogenesis of lymphocytes from patients with chronic alcoholic liver disease

Similar results were found when 10 µg/ml PHA and 25 µg/ml Con-A were used for stimulation (optimal doses) (Figs 2 and 3).

The changes presented in Figs 1-3 suggested an inverse relation between the original blast transformation values and the rate and direction of the change following in vivo treatment with Aica-P. This is demonstrated in Fig. 4. There is a significant ($p < 0.01$) negative correlation between the ^{3}H-thymidine incorporation values before treatment and the rate and direction of the change following in vivo therapy for one month.

In connection with this negative correlation, we studied the effects of in vivo Aica-P therapy on the helper and suppressor cells in alcoholic patients with originally low values of lymphoblast transformation (Fig. 5). The average $OKT4^{+}/OKT8^{+}$ index of the patients was 1,27, indicating an increased number of suppressor cells (empty columns). In fact, the percentage of $OKT8^{+}$ cells in these patients was

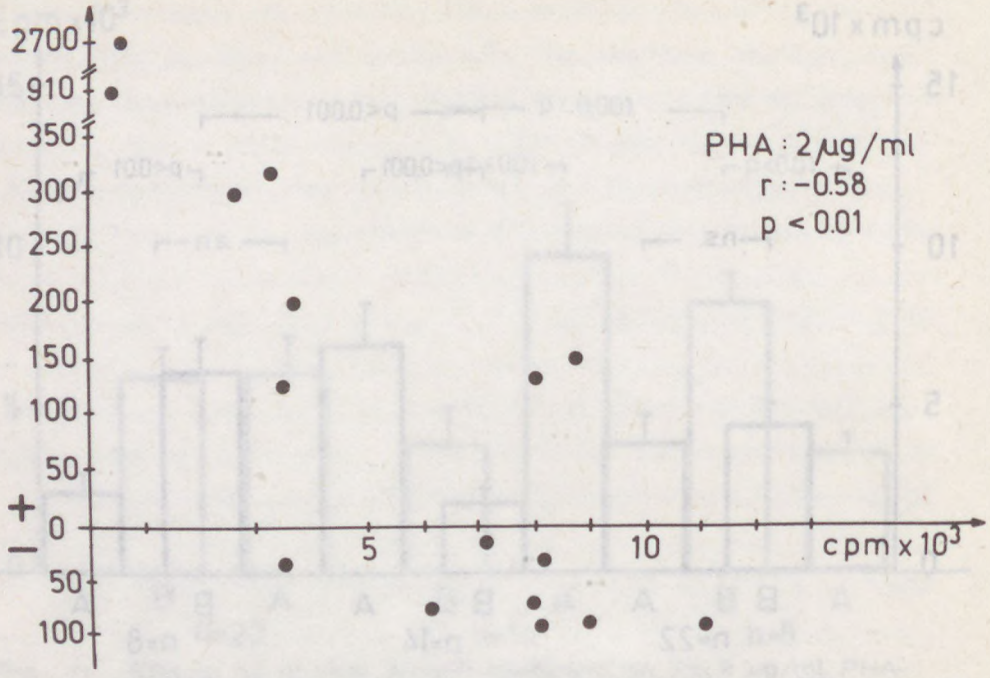

Fig. 4. Correlation between the original blast transformation values and the rate and direction of the change after in vivo Aica-P therapy

higher than normal (47.8±4.8), supporting the view that one of the reasons for the decreased blast transformation of the patients' lymphocytes may be an increase in the number of suppressor cells (left central column).

Considering the fact that the enhancement of blastogenesis was followed by a significant decrease in the number of suppressor cells after in vivo Aica-P therapy, one may argue that Aica-P exerted its effect on the lymphoblast transformation primarily by decreasing the number of $OKT8^+$ cells.

The immunomodulatory effects of Aica-P treatment are in accordance with the results of Fantone et al. (1982), Kendall-Taylor (1984), Kraut et al. (1981), and Weetman et al. (1983) indicating the important role of free radicals in the regulation of the immune response. In

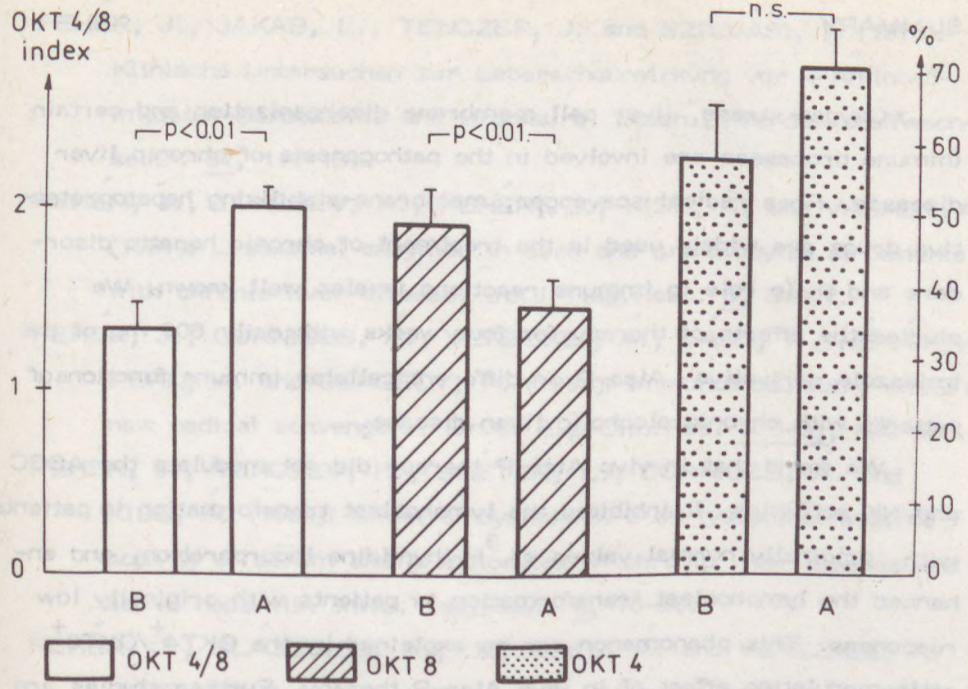

Fig. 5. Effects of in vivo Aica-P treatment on the $OKT4^+$ and $OKT8^+$ cells

previous studies we demonstrated the immuno-modulatory effects of antioxidants with different structures on various immune functions (Fehér et al. 1978).

Therefore, it seems possible that, besides their antioxidant and membrane-stabilizing functions (Balla et al. 1982, Fehér et al. 1981, 1984, 1985, Toncsev et al. 1982), immunomodulation can be an additional factor in the mechanism of action of Aica-P.

SUMMARY

Oxygen stress, liver cell membrane disorganization and certain immune processes are involved in the pathogenesis of chronic liver diseases. Free radical scavenger, membrane-stabilizing hepatoprotective drugs are widely used in the treatment of chronic hepatic disorders and their role in immune reactions is also well known. We studied the effects of therapy for four weeks with daily 600 mg of the imidazole derivative Aica-P on different cellular immune functionsof patients with chronic alcoholic liver disease.

We found that in vivo Aica-P therapy did not modulate the ADCC and NK activities. It inhibited the lymphoblast transformation in patients with originally normal values of ^{3}H-thymidine incorporation, and enhanced the lymphoblast transformation in patients with originally low responses. This phenomenon can be explained by the $OKT4^{+}/OKT8^{+}$ ratio-modulating effect of in vivo Aica-P therapy. Further studies are under way to elucidate the possible clinical value of this effect in the treatment of patients with chronic hepatic disorders.

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COMPARISON OF THE CHANGES OF TISSUE REDOX-STATE POTENTIAL AND LIPID PEROXIDATION IN DIFFERENT MUSCLES OF THE FROG

M. DELY, A. ZSOLDOS*, A. PUPPI and A. TIGYI*

Central Laboratory for Animal Experiments,
University Medical School,
Pécs, Hungary
*Institute of Biology,
University Medical School,
Pécs, Hungary

In the course of our work we have succeeded in demonstrating a close interconnection between the actual tissue redox-state potential (RSP) and the excitatory process (1, 2). According to data in the literature (3-6), there are also connections between the redox effect we observed and the extent of lipid peroxidation.

During our recent investigations, the concentration of malondialdehyde (MDA) was measured in different muscles of the frog (Rana esculenta), in the normal state, and in the presence of methylene blue (MB) as oxidant, or of ascorbate (ASC) as reductant. These redox agents were used in our earlier work. The MDA concentrations observed were compared to the RSP levels.

The isolated rectus abdominis muscles, the stomach smooth muscles and the hearts were preincubated for 10 minutes either in frog Ringer, or in Ringer containing MB_1 (7.7×10^{-5} M), MB_2 (2.7×10^{-4} M), ASC_1 (5×10^{-4} M) or ASC_2 (5×10^{-3} M) solutions.

The stomach smooth muscles were cleaned off the connective tissue and mycoderm, and the blood was also removed from the heart. The MDA concentrations were determined by the method of Fong et al. (7), as earlier modified (8). The homogenizing medium was 0.119 M NaCl solution. To eliminate the non-specific absorption, the light absorption measured at 532 nm was corrected by the value measured at 600 nm (9). For the determination of the extent of lipid peroxidation, the concentration of MDA was used, calculated from the extinction co-

efficient ε_{530} = 1.56 x 10^5 cm^2/mmol. The RSP was measured potentiometrically by the method of Cater (10), as modified previously (11).

The RSP values for the rectus (+171 mV) and the stomach smooth muscle (+166 mV) do not differ significantly, but the RSP of the heart is significantly lower (112 mV, $p < 0.001$) (see Fig. 1). The RSPs of the different tissues were increased after MB_1, but decreased following ASC_1 preincubation (Fig. 2).

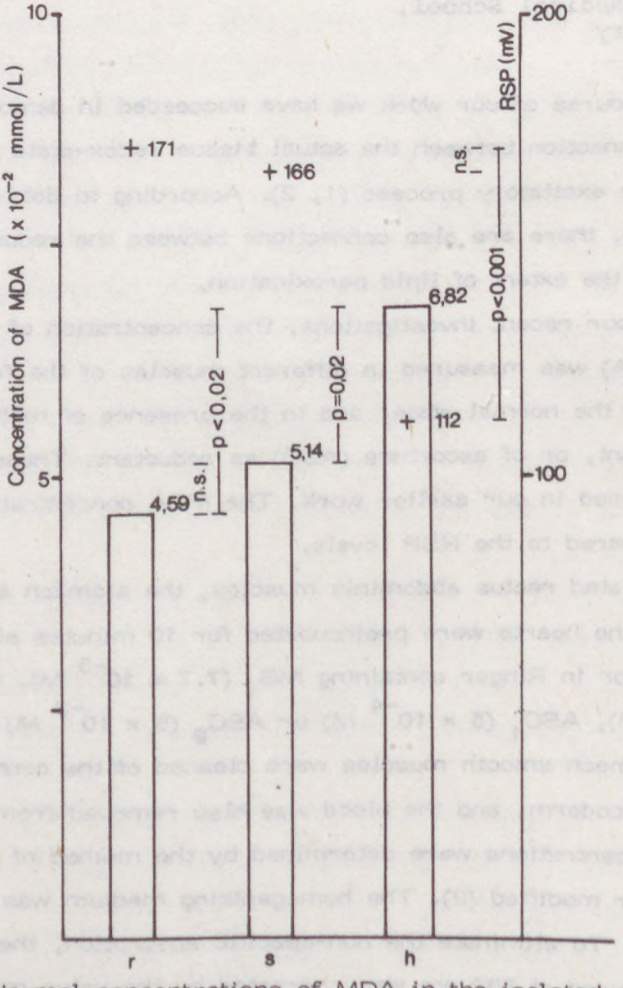

Fig. 1. Normal concentrations of MDA in the isolated rectus abdominis muscle (column r), in the stomach smooth muscle (column s) and in the heart (column h) of the frog, Rana esculenta, and the RSP values (+) for the same organs

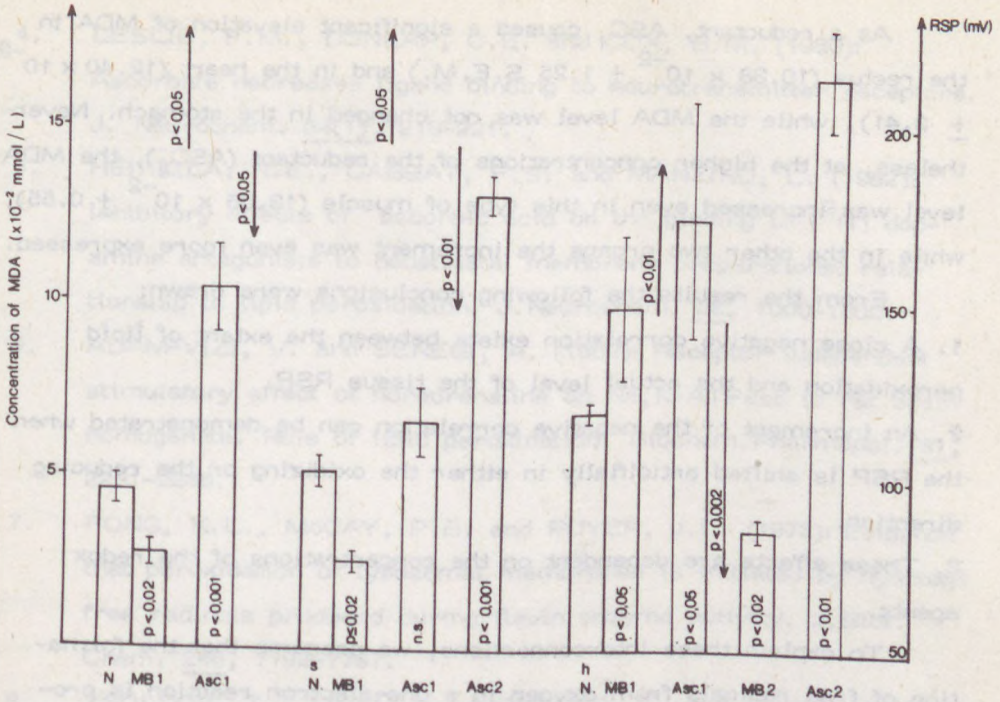

Fig. 2. Concentrations of MDA in the rectus abdominis muscle (column r), in the stomach smooth muscle (column s) and in the heart (column h) of the frog (N) in the presence of 7.7×10^{-5} M (MB_1) or 2.7×10^{-4} M (MB_2) MB, or 5×10^{-4} M (ASC_1) or 5×10^{-3} M (ASC_2) ASC. The arrows indicate changes in the RSP values in the cases of treatment with MB_1 and ASC_1.

The MDA concentrations (in mmol/L) were found to be $4.59 \times 10^{-2} \pm 0.43$ S.E.M. in the rectus muscle, and $5.14 \times 10^{-2} \pm 0.45$ S.E.M. in the stomach muscles; thus, these values are both lower than that for the heart ($6.82 \times 10^{-2} \pm 0.29$) (Fig. 1).

As may be seen in Fig. 2, the formation of MDA decreased significantly in the rectus ($2.76 \times 10^{-2} \pm 0.38$ S.E.M.) and in the stomach muscles ($2.56 \times 10^{-2} \pm 0.30$ S.E.M.) after MB_1 preincubation, while in the heart this parameter increased slightly ($p < 0.05$). In response to a higher concentration of the oxidant (MB_2), the MDA level showed a reduction in the heart too. Accordingly, conclude that the higher reducing capacity of the heart tissue is still capable of overcoming the action of the lower concentration of the oxidant.

As a reductant, ASC_1 caused a significant elevation of MDA in the rectus ($10.38 \times 10^{-2} \pm 1.25$ S.E.M.) and in the heart ($12.40 \times 10^{-2} \pm 3.41$), while the MDA level was not changed in the stomach. Nevertheless, at the higher concentrations of the reductant (ASC_2), the MDA level was increased even in this type of muscle ($13.06 \times 10^{-2} \pm 0.55$), while in the other two organs the increment was even more expressed.

From the results the following conclusions were drawn:

1. A close negative correlation exists between the extent of lipid peroxidation and the actual level of the tissue RSP.
2. An increment of the negative correlation can be demonstrated when the RSP is shifted artificially in either the oxidizing or the reducing direction.
3. These effects are dependent on the concentrations of the redox agents.

To explain these interconnections, we presume that the formation of free radicals from oxygen in a one-electron reaction is promoted by the decrease of the RSP, but inhibited following the increase of the RSP. This action might be influenced to some extent by subsequent redox processes (see also 12, 13).

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EFFECTS OF CHRONIC ALCOHOL INGESTION ON THE MYOCARDIAL LIPID PEROXIDATION AND GLUTATHIONE METABOLIZING ENZYME CONTENTS IN RATS

I. ÉDES, Gy. PIROS and M. CSANÁDY

2nd Department of Medicine, Division of Cardiology,
University Medical School,
Szeged, Hungary

SUMMARY

The effects of a chronic intake of dietary alcohol upon the myocardial lipid peroxidation and glutathione metabolism were studied in rats. As compared to the controls, the left ventricle of the alcoholic animals had an increased diene conjugate content (5.4±0.5 vs. 4.3±0.6 optical density/g wet weight) and a slightly, but not significantly decreased reduced glutathione (GSH) content. To assess the glutathione metabolism in the myocardium, the following enzymes were measured: glutathione reductase (GSSG-R), glutathione peroxidase (GSH-P) and glutathione-S-transferase (GSH-S-T). Significant increases were found in the activities of GSSG-R and GSH-P following 6 weeks on alcohol.

Thus it is apparent that in the myocardium of rats treated chronically with ethanol the observed enhanced lipid peroxidation is not necessarily associated with severe GSH depletion and an increase in the activity of GSSG-R might be, at least in part, responsible for the preservation of GSH.

INTRODUCTION

The role of oxidative damage is becoming increasingly apparent in the aetiology of ethanol-induced liver pathology (6, 13). Oei et al. (8) measured the activities of antioxidant enzymes in rats and conclud-

ed that chronic alcohol exposure leads to increased oxidative stress in the liver. They found similar enzymatic changes in the myocardium and suggested that reactive oxygen radicals might be involved in the ethanol-induced cardiac pathology. The present study was undertaken in an attempt to obtain direct evidence on the presumed lipid peroxidation in the myocardium following chronic alcohol consumption, and to investigate the effects of ethanol on the glutathione metabolizing enzyme contents.

METHODS

Studies were performed on 24 CFY male rats weighing 160 to 180 g. They were randomly assigned into two groups and fed as follows. The alcoholic group (12 rats) was given a laboratory stock diet and separately a solution of 4.6 M alcohol in tap water for 6 weeks (9). Members of the control group (12 rats) were provided with the same laboratory stock diet and tap-water. After 6 weeks on test, all animals were killed by exsanguination. The thoracic cavity was rapidly opened and the still beating heart was removed. The biochemical examinations were made on the isolated left ventricle.

The lipids were extracted according to the method of Bligh and Dyer (2). The lipid peroxide content (the formation of diene conjugates) was evaluated by measuring the optical density (OD) of the lipids at 232 nm in a UV-VIS double-beam spectrophotometer.

For the reduced glutathione (GSH) determinations, the tissue was homogenized in ice-cold 0.2 M sulphosalicylic acid. Following centrifugation the GSH content was measured with 5,5'-dithio-bis-(2-nitrobenzoate) at 412 nm (3).

The remaining tissue samples from the isolated left ventricle were used for enzyme determinations. The homogenization was performed in ice-cold 0.02 M Tris-HCl buffer (pH 7.6) containing 3 mM EDTA and 0.025% Triton X-100. Following centrifugation of the homogenate, the clear supernatants were used for enzyme measurements.

To study the GSH metabolism the following enzymes were measured: glutathione reductase (GSSG-R, E.C. 1.6.4.2), glutathione peroxidase (GSH-P, E.C. 1.11.1.9) and glutathione-S-transferase (GSH-S-T, E.C. 2.5.1.58). The activities of GSSG-R, GSH-P and GSH-S-T were determined by the method of Rathbun et al. (11) with a UV-VIS spectrophotometer at 25°C.

Table 1. GSH and diene conjugate contents of the left ventricle of control and alcoholic rats

Group	n	GSH μmol/g wet weight	Diene conjugates optical density at 232 nm/g wet weight
Alcoholic	12	1.62±0.05	5.4±0.5*
Control	12	1.66±0.07	4.3±0.6

Mean±SD

*Significantly different from the value observed for the controls ($P < 0.05$).

Table 2. Activities of GSSG-R, GSH-P and GSH-S-T in the left ventricle of controls and alcoholic rats

Group	n	GSSG-R U/g wet weight	GSH-P U/g wet weight	GSH-S-T U/g wet weight
Alcoholic	12	0.80±0.05*	16.6±0.8*	2.23±0.22
Control	12	0.65±0.03	15.2±0.8	2.27±0.26

Mean±SD

*Significantly different from the value observed for the controls ($P < 0.05$).

Statistical evaluation of the results was performed with the unpaired Student's t test. Values with $P < 0.05$ were regarded as statistically significant.

RESULTS

The effects of chronic ethanol administration on the diene conjugates in the myocardium are shown in Table 1. The data are expressed as OD at 232 nm/g wet weight. In the myocardium of the control animals a value of 4.3±0.6 was found. The level in the alcoholic rats was significantly higher.

The myocardial GSH content was slightly depressed by chronic alcohol administration, but the decrease was not significant when compared to the controls.

A marked increase was found in the myocardial GSSG-R activity as a result of alcohol treatment: 0.80±0.05 U/g wet weight in the alcoholic group against 0.65±0.03 U/g wet weight in the controls (Table 2). High GSH-P activity was noted in the left ventricle of both groups. The activity of GSH-P was slightly, but significantly elevated in the alcoholic animals. A significant change in GSH-S-T activity was not noted in the myocardium of differently treated rats.

DISCUSSION

Cardiomyopathy resulting from oxidative damage by hyperoxia (1), antineoplastic agents(adriamycin) (7), selenium or vitamin E deficiency (5, 12), in the early phase of myocardial ischaemia (10) is well documented. The relatively high sensitivity of the myocardium towards free radicals can be explained by the lower degree of development of the defence sytems. The GSH level in the myocardium is about 5-fold lower than that in the liver (15) and the transport system for oxidized glutathione has a low capacity (4). Oei et al. (8) found that alcohol exposure markedly decreased xanthine dehydrogenase activity and significantly increased the oxidase activity in the heart. Under conditions of chronic alcohol ingestion, in which the blood acetaldehyde level is elevated (14), the high xanthine oxidase activity of the heart can become a potential source of toxic oxygen radicals. In accordance with the ob-

servation, in the present study it was found that chronic ethanol treatment was associated with an increased myocardial diene conjugate content.

The changes in the GSH concentrations slightly reflected the oxidative damage of the tissue, although the increase in lipid peroxide content in the alcoholic animals does not necessarily lead to severe GSH depletion. We assumed that some changes in the activity of metabolizing enzymes were responsible for the stable GSH level. It has been shown that the activity of GSSG-R was significantly elevated in the alcoholic myocardium. The increase in the activity of this enzyme might be very important in the preservation of GSH, since the oxidized glutathione can be reduced in one step at the expense of NADPH. Our results indicate that, in the alcoholic myocardium, the enzyme is capable of reducing 1.6-1.7 μmol oxidized glutathione/g wet weight/min at 37°C. This quantity is approximately equal to the total GSH content of the left ventricle, and theoretically it might cover the demand.

A slight increase was also found in the activity of GSH-P in the alcoholic myocardium. The increase in activity of this enzyme reflects the oxidative damage of the tissue. These observations are in good agreement with those found previously by Oei et al. (8) in the heart and liver.

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THE SIGNIFICANCE OF FREE RADICAL REACTIONS IN HUMAN PATHOLOGY

J. FEHÉR

2nd Department of Medicine
Semmelweis University of Medicine,
Budapest, Hungary

As the motto of this article I have chosen a quotation from the book by Albert Szent-Györgyi published in 1983:'The fundamental difference between the living and the non-living is the state of electron unsaturation... The living state of the protein is the unsaturated free radical state.' This definition points to the outstanding importance of this topic. Research into free radicals (FRs) is relatively new and is basic in nature. The significance of FR reactions is little known among physicians dealing with therapeutic work . One of the fundamental enzymes involved in the cellular defence against FR reactions, superoxide dismutase (SOD), was isolated in 1939 by Mann and Keilin from bovine erythrocytes. Because of its copper content and its localization, the protein was originally named erythrocuprein, but its enzymatic nature was not recognized at that time. This nature, together with the reaction this enzyme catalyzes, was discovered only in 1969, by McCord and Fridovich. In fact, the assumption of its role in FR pathology dates from that time (1).

After a brief chemical introduction, I shall give an account of the role of oxygen-derived FRs and reactive oxygen intermediates (ROIs) in the pathogenesis of various human diseases.

DEFINITION OF FREE RADICALS

In most chemical substances, the electron shells are filled with paired electrons with opposite spins. The FRs are molecules or

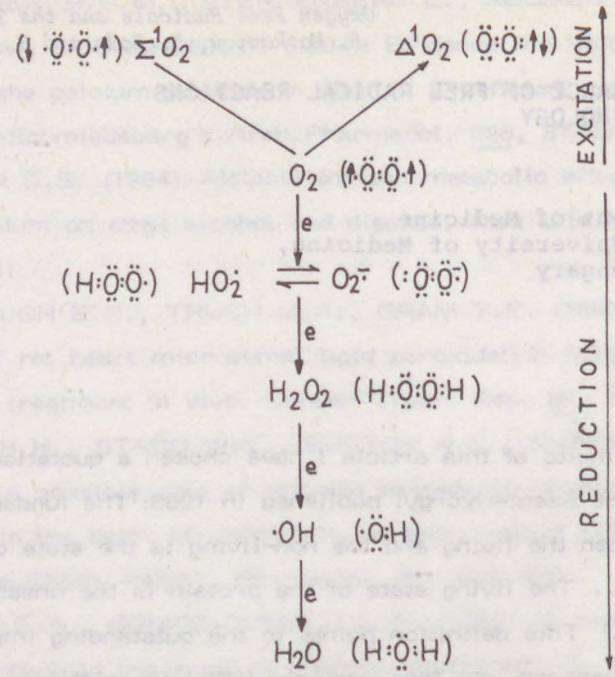

Fig. 1. Reactive oxygen intermediates formed during reduction and oxidation of oxygen (based on (2))

molecular species which contain an unpaired electron in the outermost electron shell. Since such electrons display a tendency to pair formation, the presence of an unpaired electron makes the FRs very reactive chemically, and accordingly they often have a very short lifetime. During the excitation or reduction of oxygen, the following ROIs are formed (2): on excitation: sigma and delta singlet oxygen; on reduction: the superoxide anion, the perhydroxy radical, hydrogen peroxide and the hydroxyl radical (Fig. 1).

FREE RADICAL REACTIONS AND LIPID PEROXIDATION

The FR reactions are generally chain reactions. In these chain reactions, the FRs are formed in the initiation step(s), then react in

a series of propagation steps, during which numerous FRs are preserved, and finally the FRs are destroyed in some termination process. Physiologically, the autoxidation of the lipids in the living organism is a slow, complicated process. However, if an initiator FR converts the lipid into a lipid FR by extracting a hydrogen atom from it, the lipid FR can react more easily with molecular oxygen, a peroxide FR being formed in the process. This whole process is known as lipid peroxidation (LP). The nomenclature is a little misleading, insofar as it is not restricted merely to lipids: under pathological conditions, all of the basic biomolecules (proteins, nucleic acids and carbohydrates) can be damaged. Of the lipids, the polyunsaturated fatty acids are the most sensitive to peroxidative damage. Since the proteins and lipids are constituents of the membranes, the LP produces extensive damage in the membranes. The damage of the proteins may also result in a decrease or the loss of activity of certain enzymes. The damage of the nucleic acids forms the basis of lethal, mutagenic or carcinogenic effects. Under physiological conditions, FR reactions take place in essentially all parts of the cells (3). For example, FRs may be formed in the mitochondrial electron transport chain (in the ubiquinone and cytochrome b_5 loci), in the microsomal electron transport chains (in the cytochrome P-450 and cytochrome b_5 loci), on the oxidation of many types of compounds (e.g. thiols, hydroquinones, catecholamines and oxyhaemoglobin), during the functioning of cytoplasmatic enzymes, in the peroxysomes, in the plasma membrane, and on the functioning of phagocytes (Fig. 2). ROIs are also formed on the functioning of intact cells. By means of these, the phagocytes destroy their target cells in such a way that, on their activation, a series of coordinated reactions providing reactive intermediates take place in them. Many agents are capable of activation, e.g. bacteria, immunoglobulins, immune complexes, complement factors and lymphokines.

FR reactions feature in several processes of multiplication, in the photoprotective effect of melanin and in prostaglandin (PG) synthesis. The ROIs influence PG synthesis by increasing the activity of

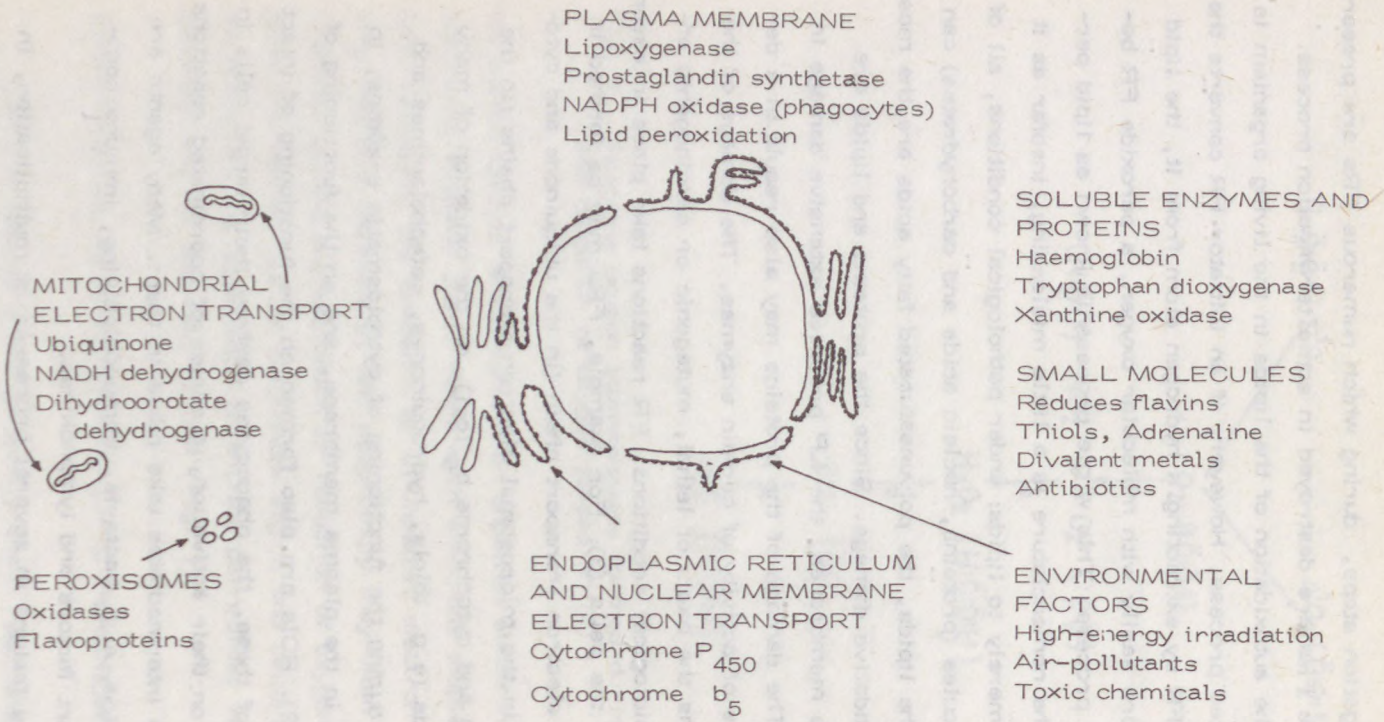

Fig. 2. Cellular sources of free radicals (based on (3))

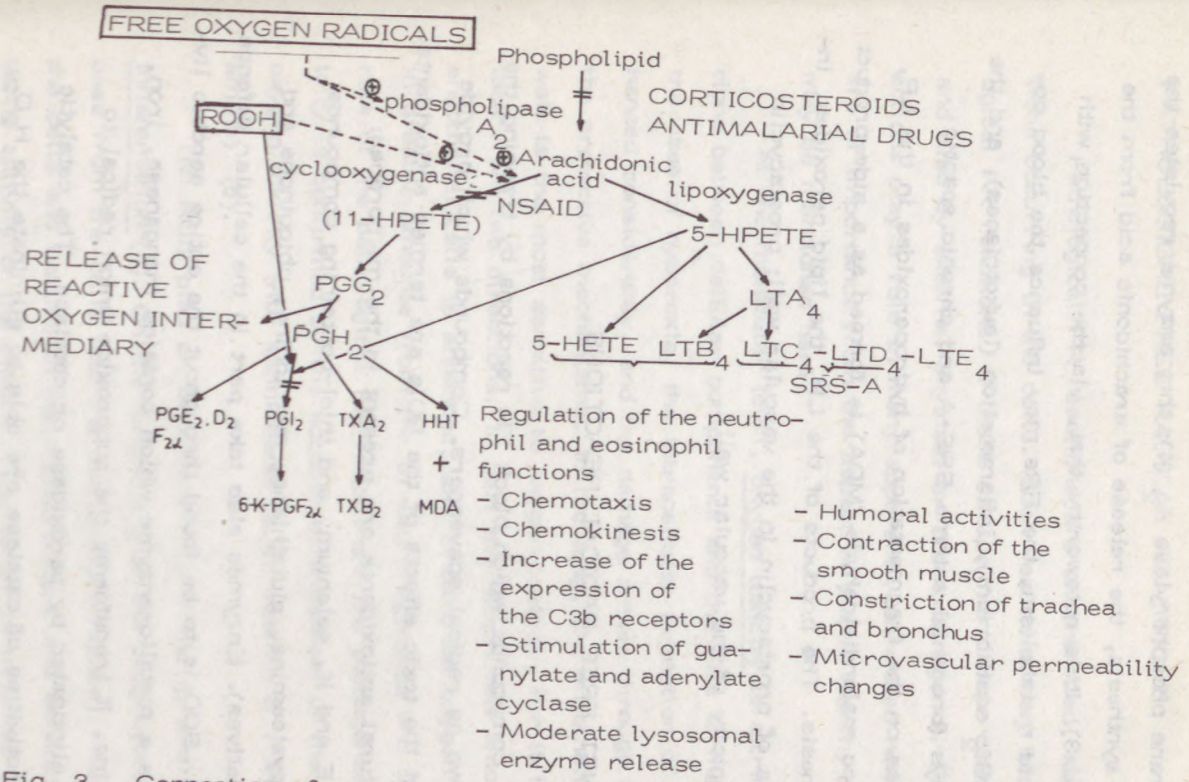

Fig. 3. Connection of reactive intermediates with prostaglandin synthesis (based on (4)).
NSAID: non-steroidal anti-inflammatory drug;
TXA_2: thromboxane A_2; TXB_2: thromboxane B_2;
HHT: (12S)-12-hydroxy-(5Z,8E,10E)-heptadecatrienic acid;
MDA: malondialdehyde; 6k-PGF_2: 6-keto-PGF_{2alpha};
HPETE: hydroperoxy-eicosatetraenic acid;
HETE: hydroxy-eicosatetraenic acid; LT: leukotriene;
SRS-A: slow reacting substance of anaphylaxis

the plasma membrane phosphorylase A_2 (4); this enzyme regulates the initial step of PG synthesis, the release of arachidonic acid from the phospholipids (Fig. 3). It is noteworthy that, via the connection with the arachidonic acid metabolism, the FRs may influence the blood coagulation (TxA_2/PGI_2 equilibrium), inflammation (leukotrienes), and the state of the airways (bronchial asthma SRS-A and chronic spastic bronchitis). Just as on the fragmentation of hydroperoxides in the LP, in PG synthesis too malondialdehyde (MDA) is formed as a side-product of the TxA_2 synthesis. The products of the LP, the lipid peroxides, inhibit the synthesis of prostacyclin in the vascular wall; prostacyclin diminishes the tonicity of the vascular wall.

DEFENCE AGAINST FREE RADICAL REACTIONS

In general, compounds which block FR reactions by trapping the radicals are known as radical scavengers. Compounds which provide protection against the toxic effects of the ROIs are termed antioxidants. The following natural antioxidants are present in the membranes: vitamins C, A, E and K, selenium, and thiol-containing compounds (e.g. cysteine, cysteamine, glutathione, methionine, ubiquinone and gallic acid derivatives). Enzymes also take part in the cellular defence against the ROIs. SOD is to be found throughout the entire aerobic living world; this is a metallo-enzyme which contains manganese, iron, or copper and zinc. It transforms the superoxide anion radical to H_2O_2, which is eliminated by peroxidase or catalase. The catalytic and peroxidase activities of catalase are able to eliminate the H_2O_2 formed during fatty acid CoA oxidation. The extracellular space is relatively less well protected against the FR reactions. The defence against the ROIs here is provided by the antioxidant activities of the LP-promoting iron and copper transport proteins, coeruloplasmin and transferrin, of urate and (to a lower extent) of glucose. Apart from these, an extracellular tetrameric SOD also protects the extracellular space from the pathological FR reactions.

PATHOLOGICAL FREE RADICAL REACTIONS

Pathological FR reactions are uncontrolled, abnormal FR reactions occurring in the cells. Such reactions may be induced by physiological FR reactions if these escape from their control mechanisms, and also by external physical or chemical agents. Pathological FR reactions play a role in many apparently unrelated human diseases that are of importance from the aspect of public health; only a few points may be mentioned here.

At the molecular level, oxygen toxicity is attributed to the reactions between cellular components and the FRs formed from oxygen. In the case of hyperoxia, the intracellular formation of these FRs is enhanced considerably, and the damage arises through the overcoming of the protective mechanisms. In the lung, the endothelium of the vessel wall is the most sensitive to oxygen toxicity, for it is exposed to attack from two directions. In part the superoxide anion formation in the endothelial cells is enhanced, and in part the oxygen FRs are released in greater amounts from the in situ phagocytes and thrombocytes. The role of the pathological FR reactions in the pathogenesis of oxygen toxicity is confirmed by the successful treatment of the consequences of oxygen toxicity with antioxidants (e.g. vitamin E , SOD and D-penicillamine) (5).

The ROIs play an important role in the general mechanism of inflammation (4). On the functioning of the phagocytes, O_2 and H_2O_2 pass from the cells into the extracellular space, causing damage to the cells and tissues in their environment and enhancing the chemotactic activity of the plasma. The latter is inhibited by SOD. The earlier-mentioned connection with the PG synthesis likewise explains the important role of the FRs in inflammation (PG derivatives and leukotrienes). The pathological FRs also influence the antibody-mediated cellular cytotoxicity (6).

A decisive role is ascribed to the pathological FR reactions in the induction of aging (7). This is confirmed by the effect of the antioxidants in lengthening the average life span in animal experiments,

and by the similar effect of a diet minimizing the FR reactions (a diet containing low copper, low unsaturated fatty acid and high natural antioxidant contents besides the essential ingredients). Two means are available for influencing the potential lifetime: a strict calorie restriction and decrease of the mantle temperature of the body. In the FR theory of aging, the aging results from the cumulative damage to the mitochondria; the death of the organism occurs if the mitochondrial function in the whole of the organism, or in a vital part such as the central nervous system, falls below a critical level.

The FR reactions have a primary causal role in the development of atherosclerosis. With the advance of age, the serum iron and copper levels become higher, the antioxidant defence of the serum decreases, and the quantity of peroxidation-sensitive unsaturated fatty acids increases in the serum and in the lipids of the wall of the arteries. A number of authors have confirmed the LP occurring in the serum and in the wall of the arteries (ceroid). The lipid peroxides selectively inhibit prostacyclin synthetase. The thromboxane - prostacyclin equilibrium is displaced in favour of the former. Accordingly, the aggregation of the thrombocytes and their adhesion to the vessel wall are enhanced. The reason why the FR theory aroused interest among those engaged in atherosclerosis research was that it combines the previously apparently independent and opposing theories of atherosclerosis (the lipid theory and the thrombogen theory) into a uniform hypothesis. Similar vessel wall-damaging LP reactions may also feature in the pathogenesis of essential hypertension (7).

When hypoxia develops as a consequence of ischaemia, ATP is dephosphorylated and AMP is formed. AMP is then further catabolized to adenosine, inosine and finally hypoxanthine. For the subsequent transformation of hypoxanthine, xanthine oxidase requires an additional substrate, molecular oxygen; this largely enters the ischaemic tissue through reperfusion, and initiates O_2^- formation via the activity of the enzyme. This explains why more severe damage is observed on reperfusion (8).

The causal role of the FR reactions is also possible in the process of carcinogenesis (9). One of the end-products of the LP, MDA, displays carcinogenic and mutagenic effects. This is not surprising, for its structure resembles those of other carcinogens applied in animal experiments. It was earlier demonstrated that two isomeric compounds, glycyl aldehyde and beta-propiolactone, exert carcinogenic action. Some authors attribute the tumour formation to the damaging effect of the extra amounts of O_2 released from the mitochondria because of the decreased or absent MnSOD (10).

The FR reactions are involved in the induction of several liver diseases. FR reactions may give rise to all of the phases (steatosis, alcoholic hepatitis and cirrhosis) in alcoholic liver damage. Numerous experimental data demonstrate that the liver-damaging effect of ethanol is attained via enhancement of the LP (11).

The pathological FR reactions also play an important part in many other types of disease, such as autoimmune diseases, diseases of the central nervous system, haemolytic anaemias, etc. (11). I do not wish to deal with these in the present article; I shall merely outline some of our observations from recent years.

OUR OWN INVESTIGATIONS

We first studied the mechanisms of action of drugs and chemicals that are able to inhibit FR reactions. Most examinations were performed with cyanidanol-3, MTDQ, MTDQ-DS and Aica-phosphate, with respect to their cytoprotective effects, their ability to block the LP, and their immunoregulatory roles, primarily with a view to whether these properties can be utilized in the treatment of chronic liver diseases.

HUMAN EXAMINATIONS

We studied the effects of Catergen and Aica-phosphate. During a 3-month treatment Catergen restored the lysosomal enzymatic ac-

tivity in both the serum and the granulocytes (12). The effects of Aica-phosphate were examined versus placebo controls. After treatment for one month, the biochemical changes indicative of the liver damage were improved considerably. The PHA and Con-A-induced blastic transformation of the lymphocytes of the patients was increased, and the ADCC and NK activities were enhanced (6).

ANIMAL EXPERIMENTS

MTDQ treatment significantly decreased the CCl_4- induced acute liver damage, while MTDQ-DS reduced the SGOT elevation due to galactosamine. The increased MDA content of the liver is indicative of FR reactions (13). MTDQ-DS prevented the doxorubicin-induced liver damage. Under in vitro conditions it exerted a membrane-stabilizing effect in mice (14).

The effect of MTDQ-DS on experimental atherosclerosis was investigated during feeding with cholesterol for 12 weeks. The serum cholesterol and triglyceride levels were decreased, while that of HDL-cholesterol was increased. The beta-glucuronidase contents of the liver and the aorta were changed in the normal direction (15).

IN VITRO STUDIES

Two types of methods were applied under in vitro conditions: lymphocyte function studies and LP measurements.

All of the four compounds examined influenced the lymphocyte functions. In healthy subjects, Catergen considerably decreased the blastic transformation induced with lectin. The effect of Aica-phosphate could be detected only in the case of the PHA, MTDQ too exerted only a moderate effect, while MTDQ-DS treatment did not lead to a significant change (6).

MTDQ treatment inhibited the Con-A-induced LIF production to a considerable extent; it did not influence the spontaneous migration.

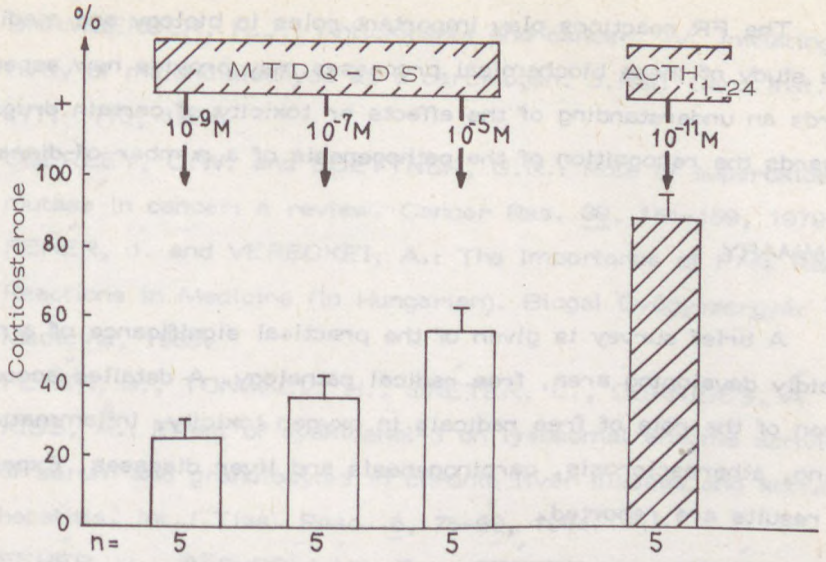

Fig. 4. In vitro effect of MTDQ-DS on corticosteroid biosynthesis in isolated rat adrenocortical cell system

MTDQ-DS similarly inhibited the Con-A-induced LIF production, and it significantly increased the spontaneous migration of the leukocytes. Catergen treatment was effective in a higher dose. It did not change the spontaneous migration in Con-A-induced LIF production. MTDQ and MTDQ-DS were ineffective on the ADCC and NK activities (6).

In vivo Aica-phosphate treatment appreciably improved the pathologically decreased lymphocyte functions (6).

Among the animal species, there are differences in the liver microsomal preparations as concerns both the non-enzymatic and the enzymatic LP. MTDQ-DS dose-dependently inhibited the ascorbic acid-induced LP in control animals and also during cholesterol feeding. The LP enhanced by cholesterol administration was diminished considerably by MTDQ-DS treatment. MTDQ-DS treatment averted the LP elevation in rats kept on the atherogenic diet (15).

An LP enhancement could be induced with ascorbic acid in a dose-dependent manner in brain homogenate, plasma membrane and microsome fractions. Treatment with cyanidanol eliminated this increase (16).

Under in vitro conditions, MTDQ-DS also elevated steroid synthesis (Fig. 4).

The FR reactions play important roles in biology and medicine. The study of these biochemical processes may provide new aspects towards an understanding of the effects or toxicity of certain drugs, and towards the recognition of the pathogenesis of a number of diseases.

SUMMARY

A brief survey is given of the practical significance of a new, rapidly developing area, free radical pathology. A detailed account is given of the role of free radicals in oxygen toxicity, inflammation, aging, atherosclerosis, carcinogenesis and liver diseases. Experimental results are reported.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

THE EFFECT OF FREE RADICALS ON THE LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY

K. FERDINANDY

Department of Infectious Diseases,
Municipal Hospital,
Békéscsaba, Hungary

SUMMARY

The E. coli 0 55 endotoxin was shown to elevate the nitroblue tetrazolium reduction and to decrease the leukocyte alkaline phosphatase activity in human neutrophils in vitro. Superoxide dismutase produced opposite effect in the two tests. We suggest that free radicals may inactivate the leukocyte alkaline phosphatase activity in the phagolysosomes.

INTRODUCTION

An altered leukocyte alkaline phosphatase (LAP) activity accompanies not only haemotological disorders, but also a number of pathological conditions (5). During phagocytosis, neutrophils generate free radicals, which can be detected with the nitroblue tetrazolium (NBT) test (1). Recent investigations have demonstrated a relationship between the LAP activity and the result of the NBT test in granulocytes (6).

Our work was undertaken to determine whether the free radicals derived from neutrophils has an effect on the LAP activity in vitro.

MATERIALS AND METHODS

Heparinized blood was drawn from healthy donors. Blood samples were incubated with or without 30 μg/ml E. coli 0 55 endotoxin at 37°C for 30 min. LAP activity was assayed by the cytochemical method of Hayhoe and Quaglino (2) and the NBT reduction was determined quantitatively according to Segal and Peters (4). In other samples the tests were also performed in the presence of 0.002 mg/ml superoxide dismutase (SOD). A paired t test was used for statistical analysis.

RESULTS

Samples incubated without the endotoxin showed a moderate decrease in LAP activity and a slight increase in NBT reduction after

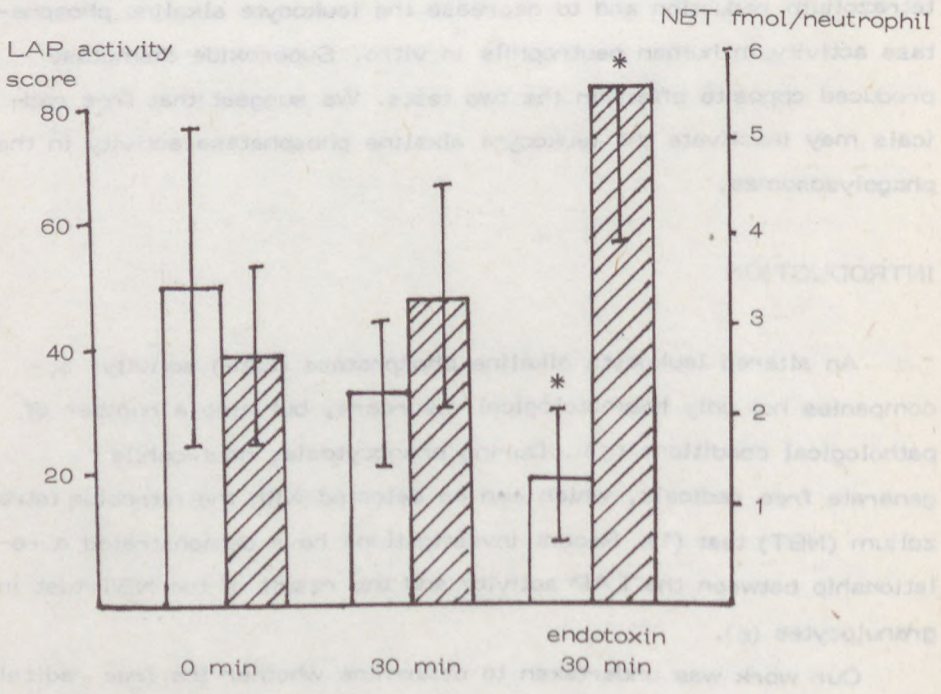

Figure 1. Effect of the endotoxin on the LAP activity and NBT reduction.
Empty column: LAP activity. Hatched column: NBT reduction. Each column represents the mean ± SD of the results obtained in 10 experiments.
* : statistically significant difference ($p < 0.05$) as compared to the control (0 min).

30 min (Fig. 1). The fall in LAP activity and the elevation in NBT reduction were more pronounced in response to the endotoxin stimulus.

SOD diminished the NBT reduction and increased the LAP activity both in the presence and in the absence of the endotoxin (Fig. 2).

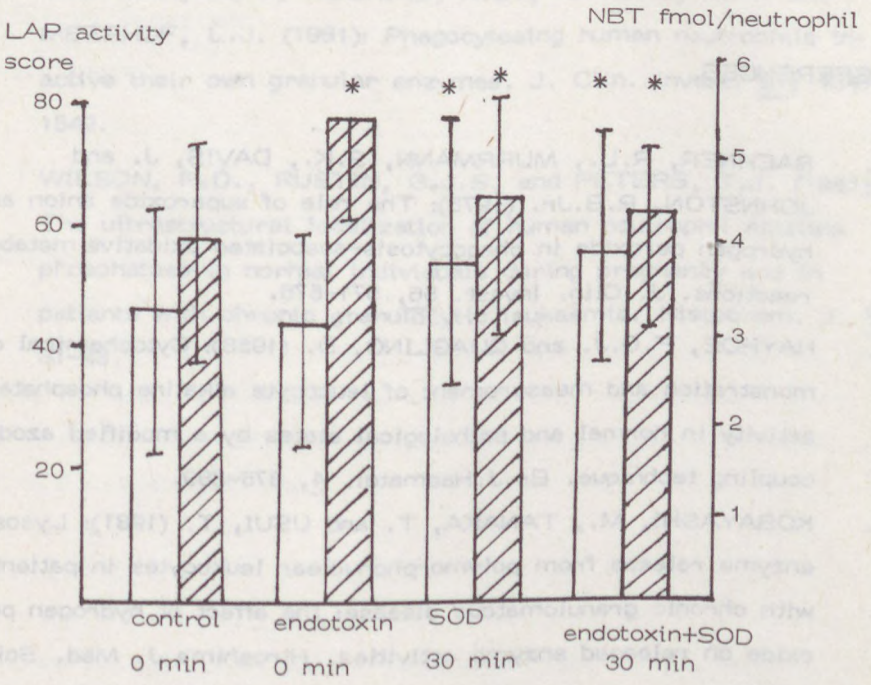

Figure 2. Effect of SOD on the endotoxin-stimulated NBT reduction and LAP activity.
Each column represents the mean ± SD of the results obtained in 8 experiments. For other details see Fig. 1.

DISCUSSION

It has been demonstrated that some lysosomal enzymes (lysozyme, acidphosphatase, alpha-mannosidase, beta-glucuronidase, alpha-fucosidase) are inactivated by free radicals, either after they have leaked into the extracellular space, or within the phagolysosomes (3, 8). According to recent studies, the LAP activity is located not only in the cytoplasmic granules, but also in the plasma membrane (7, 9).

The respiratory burst is induced by activation of NAD(P)H oxidase which is also found in the plasma membrane, so the generated free radicals might inactivate LAP within the phagosomes. The present experiments with SOD seem to prove this assumption.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

PHYSIOLOGY AND STRUCTURE OF CATALASE AND ITS DETERMINATION IN CLINICAL PRACTICE

L. GÓTH

Laboratory Department,
Municipal Hospital,
Sümeg, Hungary

SUMMARY

A short review of catalase is given, describing new aspects of catalase physiology, heterogeneity and kinetics. Methods for the determination of serum catalase activity and their application in clinical practice are also discussed.

INTRODUCTION

Catalase (EC 1.11.1.6) has been known since 1818, when Thenard (1) discovered that H_2O_2 was destroyed by animal tissues. The liberated gas was found to be oxygen by Schönbein (2). The enzyme was named catalase by Loew (3) in 1901. Sumner and Dounce (4) crystallized catalase from bovine liver in 1937.

BIOCHEMISTRY

Catalase can be found in plant, animal and human tissues. Its concentration is the highest in the liver, kidney and erythrocytes, and the lowest in the connective tissue and blood serum. It is present in aerobic microorganisms, but not in obligate anaerobic ones. In the tissues, catalase is present in the mitochondria and the matrix of the peroxisomes, and in soluble form in the erythrocytes (5). In the erythrocytes, the catalase may be bound to the membranes (6).

Its inactive monomer is synthesized on ribosomes. This monomer with a heme group forms a holomonomer. Two holomonomers form a dimer, and the active enzyme consists of two dimers. The iron in catalase is trivalent. Catalase has a molecular mass of 240 000 dalton, its isoelectric point is 5.8, its pH optimum is 7, and its T optimum is 30–40 °C.

Heterogeneity

Catalase has the following complex heterogeneity (5):

1. Genetic variants: a) catalase with decreased activity (7); b) Catalase with decreased stability (8).

2. Secondary variants, having the same molecular mass. These can be separated by various analytical methods (electrophoresis, isoelectric focusing, chromatography).

3. Variants with different molecular masses. These can be detected in different sources (plant, bacteria, human tissue), and subunits and associated forms may be present.

4. Different substances may be bound to catalase, thereby altering its molecular mass. Such substances may be other proteins, cell membrane particles, lipids or antibodies (9).

REACTION KINETICS

Catalase exhibits special kinetics, with the following characteristics: a very high turnover number, complex kinetics, a double function (catalase and peroxidase) and substrate inhibition; the catalase variants have different kinetics.

Catalase decomposes hydrogen peroxide into oxygen and water (catalase function). When the hydrogen peroxide concentration is low

(10^{-7} mol/l) and the oxygen donor (e.g. methanol, ethanol) concentration is high, catalase oxidizes H donors (peroxidase activity). If the hydrogen peroxide concentration is above 0.1 mol/l, the inactive enzyme substrate complexes II and III are formed.

PHYSIOLOGY

The physiological function of catalase is still obscure (5). Most papers underline its protective role against toxic hydrogen peroxide. The hydrogen peroxide metabolism is known: it is generated by oxidases and pathologically by the defence system and superoxide dismutase from superoxide radicals. Hydrogen peroxide serves as a substrate for peroxidases. At low hydrogen peroxide concentration (10^{-8} mol/l) it is a substrate for glutathione peroxidase, especially in erythrocytes. When the hydrogen peroxide concentration is about 10^{-7} mol/l, catalase removes the excess hydrogen peroxide through its peroxidase function, while at higher hydrogen peroxide concentrations it does so through its stronger catalase function. The protective function of catalase against toxic hydrogen peroxid generated from free radicals can be demonstrated via the following compounds, tissues or tissue particles: haemoglobin, chromosomes, erythrocytes, cell membrane, lung, heart, liver, brain, pancreas tissues, thrombus formation, DNA, eye lens.

Catalase takes part via its peroxidase function in the ethanol metabolism of the liver and heart. Catalase participates in the lipid metabolism and can influence the activities of other enzymes (tryptophan oxygenase and myeloperoxidase).

Recent findings suggest a new function of catalase: the uptake of metallic Hg from the air (10-13). Different plant, animal and human tissues can adsorb metallic Hg and oxidize it into Hg^{2+}. This oxidation is stimulated by hydrogen peroxide. The uptake of Hg depends on the catalase activity of the tissues. It has been shown that different catalase inhibitors (NaN_3, KCN, AT) decrease the Hg uptake from the air.

These new findings on the mechanisms of free radicals have enhanced our knowledge on the physiology of catalase, but the full details of its physiology are still obscure.

CATALASE IN CLINICAL PRACTICE

Possibilities for the determination of catalase activity (e.g. hydrogen peroxide substrate, assays for the determination of decomposed hydrogen peroxide or libarated oxygen) were described earlier, and papers on liver and blood (erythrocyte) catalase were published in the first decades of this century.

Erythrocyte catalase

The first findings showed a decreased blood catalase activity in patients with tumours (14). This test was then used to detect anaemias (15). In 1947, Takahara (7) found a catalase deficiency in human erythrocytes, which he named acatalasia. This phenomenon may affect the catalase activity in other tissues too. Two forms of decreased catalase activity are known. The first form (Japanese type) exhibits a very low catalase activity: 0-1% in homozygotes and 1-30% in heterozygotes (7). The second form (Swiss type) displays a 2-5% activity in homozygotes and a 50-60% activity in heterozygotes (8). This low activity stems from the decreased stability of catalase. Acatalasia is a rare disease with no symptoms and without therapy. In Switzerland, 73,661 patients were examined and only 3 patients showed acatalasia (8). Similar patients have been found in Korea, Israel, Sweden, FRG and the U.S.A.

I would suggest studies of the effects of free radicals in patients with a decreased defence system (acatalasia), as these results could contribute to a better understanding of catalase physiology.

Liver catalase

A decreased activity of liver catalase was reported in 1910 by Blumenthal (16). The factor responsible for the decreased catalase activity was named toxohormone by Nakahara (17). Uenoyama and Ono (18) discovered that the decreased liver catalase activity was caused by the altered equilibrium of two factors (activator, inhibition) responsible for the regulation of catalase synthesis. Catalase activity is currently examined in liver needle biopsy samples for diagnostic purposes.

Serum catalase

Serum catalase activity has been examined in haemotological, liver, pancreas and tumour diseases. We developed a polarographic method for the determination of serum catalase activity (19, 20). The optimum conditions for the determination are as follows: substrate: 65 µmol/ml H_2O_2 in 0.05 mol/l phosphate buffer (pH 7.4); reaction time: 60 seconds; temperature: 37°C; sample volume: 200 µl. The catalase reaction is stopped with 8.5 mol/l 3-amino-1,2,4-triazole. Hydrogen peroxide concentration is determined by a polarographic method. Polarograms are taken every 40 seconds with a programmable polarograph.

We found an increased serum catalase activity in the following diseases: a) $0.1\% < p < 1\%$: compensated form of cardiac congestion, cholelithiasis, drug abuse, diseases with different types of haemorrhage, obstructive jaundice, cholecystitis and fatty liver; b) $p > 0.1\%$: myocardial infarction, alcohol intoxication, polyglobulism, decompensated form of cardiac congestion, Paraquat toxicity, acute pancreatitis, polycythaemia vera, acute yellow atrophy, toxic hepatitis, pernicious anaemia, Zieve syndrome and haemolytic anaemia.

CONCLUSION

New results from studies on free radicals may cause a renaissance of experiments on catalase, which has been neglected in physiology and diagnostics for some years.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

INFLUENCE OF FREE RADICALS ON BIOCHEMICAL PARAMETERS OF SYNOVIAL FLUID IN RHEUMATOID ARTHRITIS AND OSTEOARTHROSIS

E. HORVÁTH, M. ANTAL, G. BÁLINT and Gy. GENTI

National Institute of Rheumatology and Physiotherapy,
Budapest, Hungary

SUMMARY

The malondialdehyde content and the glutathione peroxidase, superoxide dismutase, catalase and peroxidase activities were measured in the synovial fluid from 18 patients with rheumatoid arthritis (RA) and 13 patients with inflammatory osteoarthrosis (OA). As concerns the activity of free radicals, the rheumatoid patients were divided into "active" and "moderately active" groups on the basis of the malondialdehyde level. In the "active" group the high malondialdehyde content was accompanied by a low glutathione peroxidase activity, which reflects the inefficacy of the enzymatic detoxifying capacity. The lowest superoxide dismutase activity was also measured in this group. Highly elevated catalase and peroxide activities were found in the "active" group. There was no significant difference between the "moderately active" RA and inflammatory OA groups. All biochemical parameters in the "active" RA group differed significantly from those for the control OA patients. The common change of the selected parameters may be considered disease- and state-specific in increased inflammation in RA.

INTRODUCTION

It is evident that free radicals, such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), are involved in the pathogenesis of inflammatory arthritis (2, 8, 14, 15). On activation of the respiratory burst, neutrophils and macrophages excrete these radicals into the extracellular space.

Different enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (PO) and glutathione peroxidase (GPX), and non-enzymatic compounds, such as vitamin A, ascorbic acid, ceruloplasmin, transferrin, etc. (7), are involved in the protection against the toxic damage of the free radicals.

Studies in several laboratories have already proved that the defence of the extracellular space (joint cavity) against free radical toxicity is relatively weak, although it is rich in sources (neutrophils, monocytes and macrophages) producing oxygen-derived radicals (3, 4, 8, 14).

The aim of our paper was to collect more information about the changes induced in the synovial fluid by free radicals, and the defence against them.

MATERIALS AND METHODS

Synovial fluid was obtained from 13 patients with osteoarthrosis (OA) of the knee (10) and from 18 patients with definite rheumatoid arthritis (RA), determined by ARA criteria (18). The synovial fluid was centrifuged for 10 minutes at 1000 g and the supernatants were stored at -20°C.

Malondialdehyde: The MDA was measured by the method of OTTOLENGHI (16).

Glutathione peroxidase: The GPX activity was determined by the method of CHIU et al. (6) and SEDLAK et al. (19), in combination with the measurement of GSH, using cumene hydroperoxide as substrate.

Catalase: CAT was measured by following the degradation of H_2O_2 spectrophotometrically, according to the method of AEBI (1).

Peroxidase: PO was determined by the slightly modified method of VOLLER (20), with orthophenylenediamine as substrate.

Superoxide dismutase: The SOD activity was measured by following the inhibition of the adrenaline-adrenochrome transformation, using the method of MISRA (13). CAT and PO were blocked with NaN_3.

RESULTS

As concerns the activity of free radicals, the rheumatoid patients were divided into "active" (MDA > 7 μmol/l) and "moderately active" (MDA < 7 μmol/l) groups. The average level of MDA in the "active" group was significantly higher than in inflammatory OA (Fig. 1). The lowest GPX and SOD activities were found in the "active" RA group (Figs 2 and 3). The highest CAT and PO activities were significantly increased in the "active" RA synovial fluid as compared with the moderately active" group and the inflammatory OA patients (Figs 4 and 5).

There was no significant difference between the inflammatory OA and the "moderately active" RA groups in respect of the biochemical parameters measured.

DISCUSSION

Free radicals are capable of inducing a wide variety of tissue damage. The lipid-containing membranes are the main target of the attack and the MDA level in the synovial fluid reflects the degree of lipid peroxidation.

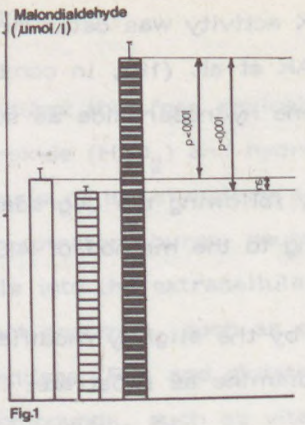
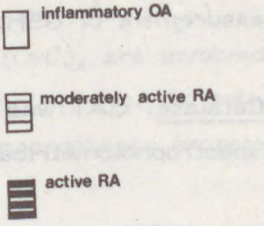


Fig.1



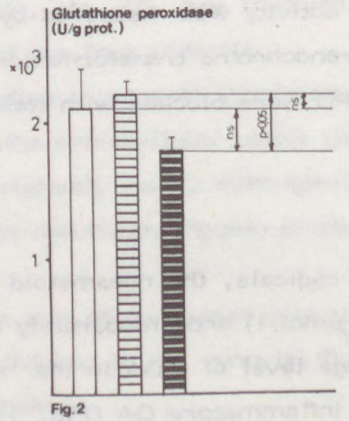


Fig.2

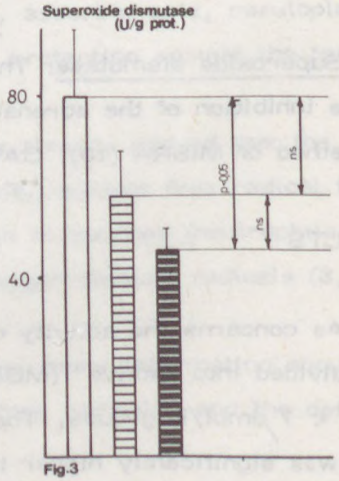


Fig.3

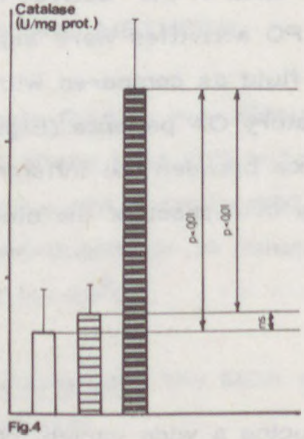


Fig.4

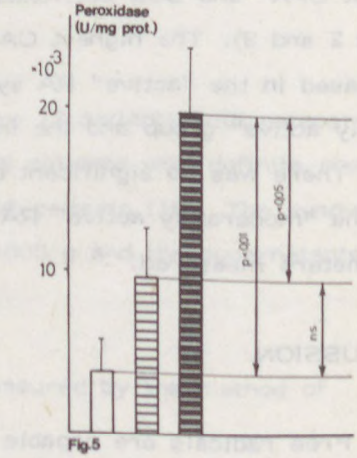


Fig.5

Figs 1-5
Changes of biochemical parameters of synovial fluid in inflammatory OA, "moderately active" RA and "active" RA

In the present study there was a significant difference between the RA group having a high synovial MDA level and the group of inflammatory OA patients. This is in accordance with the results reported by LUNEC et al. (11). Elevation of the synovial MDA level was accompanied by a low activity of GPX in the "active" RA group.

Experiments on animals with a Se deficiency showed that a decrease of the GPX activity may enhance the amount of H_2O_2 and other peroxides in the extracellular space, resulting in expanding inflammation (17). It can be predicted that an increased generation of free radicals will damage the GPX, and its low activity may boost lipid peroxidation. In contrast with the finding of BLAKE (4), we could demonstrate SOD activity in the rheumatoid synovial fluid. The lowest SOD activity was found in the "active" RA group, and the highest in the OA group. This result is in contrast with the observation of IGARI (9).

Very high CAT and PO activities were found in the "active" RA group as compared with the OA group, in accordance with the results of BIEMOND et al. (3). We also detected significant differences between the "active" and "moderately active" RA groups.

These changes in enzyme activity can be interpreted in terms of the in vitro results of MICHELSON (12), who found that a decrease in GPX activity was caused by the $O_2^{\cdot -}$ radical, whereas SOD and CAT appeared to be resistant. He stated that SOD was sensitive to the presence of CO_3^{2-}, while CAT was not. The decreased SOD activity in the "active" RA group can be explained by the high synovial pCO_2 (5).

We were not to able to show significant differences between the "moderately active" RA and OA groups, but significant differences between the "active" RA and inflammatory OA groups were observed in all parameters.

We favour the hypothesis that the changes in the selected biochemical parameters may be specific for the disease and the state in acute exacerbation of RA.

The pathomechanism of inflammatory arthritis cannot be explained by free radical generation only, free radicals may also contribute to the prolongation and progression of the diseases. The biochemical parameters of free radical activity and of the scavenging mechanism may help in the selection of anti-inflammatory drugs for evaluations of their efficacy.

This work was supported by the Hungarian State Office for Technical Development.

The authors express their thanks for the technical help of Mrs. L. Ujvári and Mrs. J. Bariska.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

PHYSICAL AND PHYSICO-CHEMICAL DETECTION OF FREE RADICALS

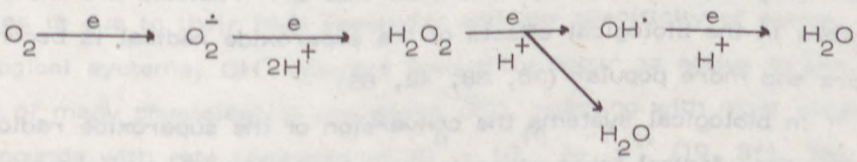
Zofia JÓŹWIAK and Wanda LEYKO

Department of Biophysics,
University of Łódź,
90-237 Łódź, Poland

I. INTRODUCTION

Animal tissues contain $5 \times 10^{14} - 5 \times 10^{15}$ radicals/g tissue (61, 100). The radical content of individual tissues depends on the metabolic state of the cells (59). In the majority of cases, malignant tissues contain a much lower concentration of radicals than normal tissues (60, 96). From among the latter, the highest concentrations of free radicals were found in the liver, kidneys and heart (61).

Cells are subjected to the action of both radicals formed during the metabolism of organic compounds and active oxygen species. One-electron reduction of dioxygen leads to the successive generation of the superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$) and water in the following reactions (45, 101):



The first of these reactions is endothermic, while the following steps in the reaction chain are exothermic and proceed spontaneously, with rate constants for steps 2 and 4 of 10^8 and 10^9 M^{-1} s^{-1}, respectively (8, 9, 25, 99).

In these reactions, the electron donors are components of the electron transport systems: the photosynthetic, mitochondrial and microsomal ones (22), as well as metal ions (69), organic compounds (flavins, quinones, nitroaromatic compounds, melanins, thiol compounds) (64, 70, 93), substrates of autoxidizable enzymes: aldehyde oxidase, xanthine oxidase, NADPH oxidase, NADPH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase (2, 6, 33, 53, 74) and proteins, cytochrome P-450 and hemoglobin (93).

The product of the first step of oxygen activation, the superoxide radical anion, $\dot{O}_2^-$ (or its corresponding acid, the perhydroxyl radical $HO_2^{\cdot}$) is a nucleophilic reactant with the properties of both an oxidant and a reductant (88). In the reactions of spontaneous dismutation and of dismutation catalyzed by superoxide dismutase (SOD), the superoxide radicals generate H_2O_2 (9, 27, 34). The course of further reactions involving the superoxide radical and H_2O_2 depends on the properties of the systems in which these products are generated. In systems containing no metal ions, both products of oxygen reduction, the superoxide radical and H_2O_2, have low reactivity and do not constitute a significant danger for the majority of cell constituents (22, 41, 57, 68, 86). Though the exact mechanism of cell damage by $\dot{O}_2^-$ is not yet known in detail, it has been suggested that the superoxide radical may either interact with critical biomolecules directly (35, 84), or initiate a chain of reactions leading to the formation of $OH^{\cdot}$ radicals (14, 40, 41, 43, 71). The view that $OH^{\cdot}$ radicals are the real factors in the biological effects of the superoxide radical is becoming more and more popular (36, 38, 42, 65).

In biological systems the conversion of the superoxide radical to the $OH^{\cdot}$ radical takes place in superoxide radical-driven Fenton reactions (11, 18), catalyzed by metal ions or their complexes:

$$O_2^{\cdot-} + H^+ \rightleftharpoons HO_2^{\cdot}$$

$$HO_2^{\cdot} + O_2^{\cdot-} + H^+ \longrightarrow H_2O_2 + O_2$$

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

$$3\ O_2^{\cdot-} + 2\ H^+ \longrightarrow 2\ O_2 + OH^{\cdot} + OH^-$$

Theoretically, it results from the above reactions that under optimum conditions at pH 4.8, one $OH^{\cdot}$ radical is formed from each three $O_2^{\cdot-}$ produced (1). Under physiological conditions, the efficiency of generation of $OH^{\cdot}$ radicals in the Fenton reaction is much lower and hard to calculate, due to the changes in the environmental pH and the decay of superoxide radical anions in the dismutation reaction with diffusion-controlled constants (85).

The $OH^{\cdot}$ radical is a very weak acid and in aqueous media dissociates to yield the alkali form O^- (76, 97):

$$OH^{\cdot} + OH^- \xrightarrow{pK\ 11.85} O^- + H_2O$$

The $OH^{\cdot}$ radical is one of the strongest oxidizing agents. At $25^{\circ}C$ its standard electrode potential is $E^{\circ} = 1.83$ V (91).

The high toxicity of $OH^{\cdot}$ radicals in reactions with macromolecules is due to their high reactivity and low specificity of action. In biological systems, $OH^{\cdot}$ radicals frequently occur as active intermediates of many physiological processes (20), reacting with most organic compounds with rate constants of $10^9 - 10^{10}\ M^{-1}s^{-1}$ (19, 21). The accumulation of free radicals and of products of their reactions in the cells in the course of the normal metabolism and as a result of the action of physical and chemical agents on the cells, therefore leads to the irreversible damage to the cell.

II. METHODS OF IDENTIFICATION OF ACTIVE OXYGEN SPECIES

In recent years a series of methods have been elaborated for the identification of active oxygen species.

1. Direct methods

1.1 Determination of superoxide radical anions

Direct observation of the process of generation of oxygen radicals is possible only in the case of the superoxide radical anion. In direct determination methods, the rate of decay of the $O_2^{\dot{-}}$ generated by pulse radiolysis or H_2O_2 photolysis is measured.

In the method of pulse radiolysis, the superoxide radical can be produced during the passage of an electron beam through an oxygenated aqueous solution of the following formate (24):

$$H_2O \rightsquigarrow H_2O^* \longrightarrow OH^{\cdot} + H^+ + e^-_{aq}$$

$$e^-_{aq} + O_2 \longrightarrow O_2^{\dot{-}}$$

$$OH^{\cdot} + HCO_2^- \longrightarrow H_2O + CO_2^{\dot{-}}$$

$$CO_2^{\dot{-}} + O_2 \longrightarrow O_2^{\dot{-}} + CO_2$$

In the method of photolysis of H_2O_2, the superoxide radical is produced in aqueous solution by the photolysis of H_2O_2 with ultraviolet radiation (3). The sequence of events which occurs is as follows:

$$H_2O_2 \xrightarrow{h\vartheta} 2\,OH^{\cdot}$$

$$OH^{\cdot} + H_2O_2 \longrightarrow H_2O + HO_2^{\cdot}$$

$$HO_2^{\cdot} \longrightarrow O_2^{\dot{-}} + H^+$$

Both these methods permit the production of considerable superoxide radical concentrations, of the order of 200 μM (24). The radicals

generated are identified on the basis of measurements of their UV spectrum and estimation of the rate of spontaneous superoxide radical decay, due to their disproportionation to oxygen and H_2O_2 (7, 66, 67, 98).

Direct detection of the superoxide radical is also possible by the ESR method combined with the rapid freezing technique (13). Superoxide, trapped by the rapid freezing method in water or in aqueous dimethyl sulfoxide (DMSO), exhibits an axial ESR spectrum, which, owing to fast relaxation, is detectable only at temperature below about 220 K. This creates problems in the performance of determinations and complicates the interpretation of the spectra obtained.

The ESR permits detection of radicals at concentrations of 10^{-8} - 10^{-6} M in aqueous solutions (10). In practice, this means a possibility for the detection only of stable free radicals, which must accumulate until the required concentration of unstable radicals is reached; a sufficiently high steady-state concentration may be attained. There are many examples of such radicals being detected directly in biological systems, including flavin radicals (5), quinone radicals, amino and nitroxide radicals, as well as phenothiazine cation radicals (62).

Highly reactive free radicals, among them the $OH^{\cdot}$ radical, do not attain high concentrations and their direct detection in biological systems appears almost impossible.

2. Indirect methods

2.1 Determination of hydroxyl radicals

The hydroxyl radical is usually detected by observing the formation of a reaction product of the $OH^{\cdot}$ radical with a second chemical species. Methods employed for the detection of $OH^{\cdot}$ radicals include: changes in tryptophan absorbance at 278 nm (65), the production of diphenols from phenols such as salicylic acid (71), the formation of thiobarbituric acid-reactive products on reaction with deoxyribose (39),

the bleaching of p-nitrosodimethylaniline (23) and the formation of spin-trapped adducts with nitrones (15).

Beauchamp and Fridovich (4) applied the reaction of the oxidation of methional (β-mercaptopropanol) to ethylene for the detection of hydroxyl radicals. The ethylene content was estimated by gas chromatography.

The oxidizing properties of $OH^{\cdot}$ radicals also constitute the principle of methods based on the ability of $OH^{\cdot}$ radicals to hydroxylate aromatic compounds. The hydroxyl radicals bind to the aromatic ring with an almost diffusion-controlled rate (78). The hydroxylated aromatic species can be determined by colorimetric methods or by gas/liquid chromatography (GLC). Colorimetry has a low sensitivity and, moreover, does not permit identification of the various isomeric forms of hydroxylated aromatic compounds (40). On the other hand, the GLC determination suffers from a lengthy derivatization procedure for the compounds to be determined (79).

Radzik et al. (77) employed liquid chromatography coupled with an electrochemical method (LCEC) for the determination of hydroxylated aromatic compounds. The principle of this method consists in the estimation of the content of hydroxylated aromatic compounds produced in a system generating free oxygen radicals. In this method, phenol and aniline are usually employed as hydroxyl radical-trapping agents in hypoxanthine-xanthine oxidase incubations catalyzed by an iron salt. In the determinations performed in the presence of phenol, the mobile phase contains 5% acetonitrile and 95% 0.1 M ammonium acetate buffer, pH 4.0. The detector potential is + 0.70 V and the column temperature is 30°C.

Figure 1 presents a typical LCEC chromatogram of phenol in an incubation mixture containing xanthine oxidase and hypoxanthine. Identification of the peaks was performed on the basis of the retention times of the individual compounds. Moreover, the separated compounds were characterized electrochemically by calculation of the half-wave potential (Table 1).

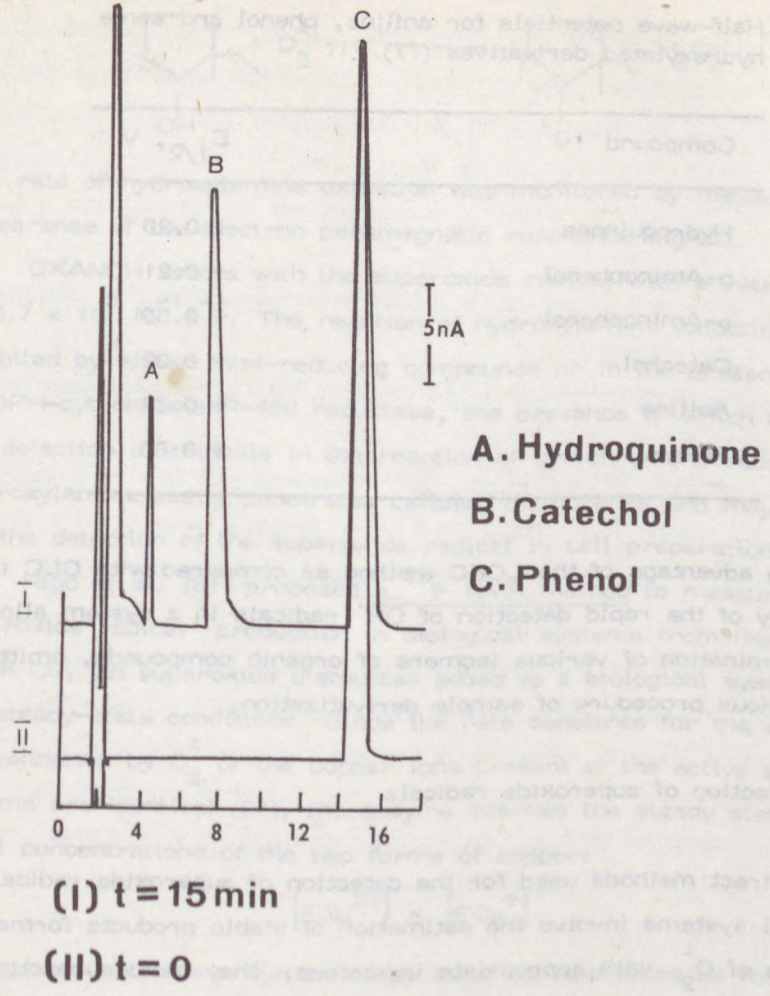


Figure 1. LCEC chromatogram of xanthine oxidase-hypoxanthine incubation with phenol (77).

Table 1. Half-wave potentials for aniline, phenol and some hydroxylated derivatives (77).

Compound	$E_{1/2}$, V
Hydroquinone	+ 0.20
p-Aminophenol	+ 0.21
o-Aminophenol	+ 0.30
Catechol	+ 0.32
Aniline	+ 0.72
Phenol	+ 0.80

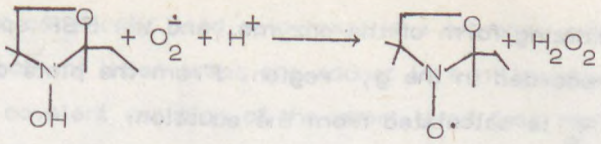
The advantage of the LCEC method as compared with GLC is the possibility of the rapid detection of $OH^{\cdot}$ radicals in a system allowing the determination of various isomers of organic compounds, omitting the laborious procedure of sample derivatization.

2.2 Detection of superoxide radicals

Indirect methods used for the detection of superoxide radicals in biological systems involve the estimation of stable products formed in reactions of $O_2^{\cdot-}$ with appropriate indicators. They include reactions in which the superoxide radical formed reduces, oxidizes or binds to an indicator present in a system.

The reducing and oxidizing properties of superoxide radicals have been applied in many chemical methods for radical detection. In the above methods, the superoxide radicals produced reduce cytochrome c, nitroblue tetrazolium and tetranitromethane, or are initiators and propagators of the autoxidation of epinephrine, pyrogallol and 6-hydroxydopamine (12, 32, 94).

The determination of superoxide radicals is also possible in the oxidation of hydroxylamine, OXANOH (2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine), to its corresponding nitroxide, OXANO (2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl) (83):



The rate of hydroxylamine oxidation was monitored by measuring the appearance of the electron paramagnetic resonance signal.

OXANOH reacts with the superoxide radical with a rate constant of 6.7×10^3 $M^{-1} s^{-1}$. The reaction of hydroxylamine oxidation is not inhibited by either thiol-reducing compounds or in the presence of NADPH-cytochrome P-450 reductase, the presence of which hampers the detection of radicals in the reaction of cytochrome c reduction. Hydroxylamine easily penetrates cellular membranes and may be used for the detection of the superoxide radical in cell preparations.

Rigo et al. (81) proposed a ^{19}F NMR method to measure the superoxide radical production in biological systems from the rate by which Cu, Zn superoxide dismutase added to a biological system attains the steady-state conditions. Since the rate constants for the reduction and oxidation by $O_2^{\cdot-}$ of the copper ions present at the active site of the enzyme are identical (27), the enzyme reaches the steady state at equal concentrations of the two forms of copper:

$$[ECu^{2+}] \approx [ECu^{+}]$$

The approach to the enzymic steady state can therefore be followed by adding a small amount of oxidized or reduced SOD to the $O_2^{\cdot-}$ producing system containing F^-, and measuring the relaxation time T_1 of $^{19}F^-$ as a function of time. The rate of superoxide generation can be calculated from the kinetics of this process (82). The relaxation rate of $^{19}F^-$ is a very sensitive and specific probe of the Cu^{2+} present in the oxidized form of the enzyme (89). However, due to the high concentrations of F^-, this method should not be applied for the detection of radicals in biological systems.

On the same principle, superoxide radicals can be detected by determining the steady state of superoxide dismutase by ESR (90). In this case, a system generating superoxide is supplemented with a

reduced or oxidizing form of the enzyme, and the ESR spectra of Cu, Zn SOD are recorded in the g, region. From the plots of $\ln [ECu^{2+}]$ versus time, R_o is calculated from the equation:

$$R_o = k [SOD]$$

where

k = first-order kinetic rate constant of the process of attainment of the steady state;

[SOD] = concentration of the enzyme added to a superoxide-generating system.

Using this method, Scarpa et al. (90) measured the rate of production of superoxide radicals in human red blood lysates, and found a value of $2.02 \pm 0.97 \times 10^{-8}$ $M^{-1}s^{-1}$ under the experimental conditions employed.

Reactions of addition of an oxygen radical to an indicator have been used in the spin-trapping methods. The method of detecting reactive short-lived free radicals by the spin-trap technique was first demonstrated in the late 1960s. Since then, several reviews have appeared containing numerous interesting data, not only on oxygen free radicals, but also on free radical metabolites of toxic compounds (16, 29, 51, 52, 55, 63).

This method involves the addition of an organic compound (spin trap) to a system generating unstable free radicals. The spin trap is capable of trapping free radicals rapidly to form a relatively stable spin adduct:

$$R^{\cdot} + ST \longrightarrow STR^{\cdot}$$

where:

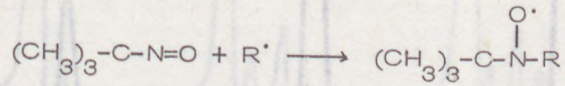
$R^{\cdot}$ = unstable radical;

ST = spin trap;

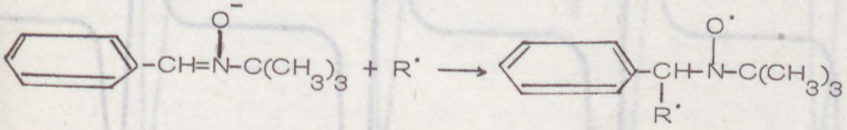
$STR^{\cdot}$ = spin adduct.

Thus, the short-lived radicals are trapped in a long-lived form, which can be observed at room temperature using conventional ESR equipment.

The most commonly used spin traps are nitrones and nitroso compounds. With both of these traps the adduct is a nitroxide-free radical formed in the covalent reaction of the short-lived free radicals with the spin trap (29, 52). In the case of nitroso spin traps, the radical studied binds directly to the nitroxide nitrogen:



With nitrone spin traps, the trapped radical is bonded to the α-carbon:

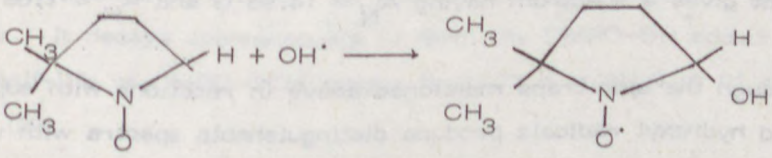


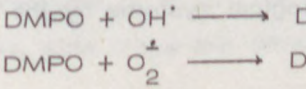
PBN (tert-butylnitrone)

Nitrones have a low specificity. In contrast to the nitroso spin traps, which react mainly with carbon radicals, nitrones trap a large number of various radicals, including carbon, hydrogen, oxygen, nitrogen and halogens (49).

The identification of oxygen free radicals, the superoxide radical and $OH^{\cdot}$, in biological systems is possible only in the presence of nitrones (31, 46, 50). In particular, two types of compounds have been commonly employed: a cyclic nitrone trap, DMPO (5,5´-dimethyl-1-pyrroline-N-oxide), and aryl nitrones: 4-POBN (α-4-pyridyl-1-oxide N-tert-butylnitrone) and PBN (tert-butylnitrone).

The short-lived hydroxyl and superoxide radicals react with DMPO to form the following radicals:





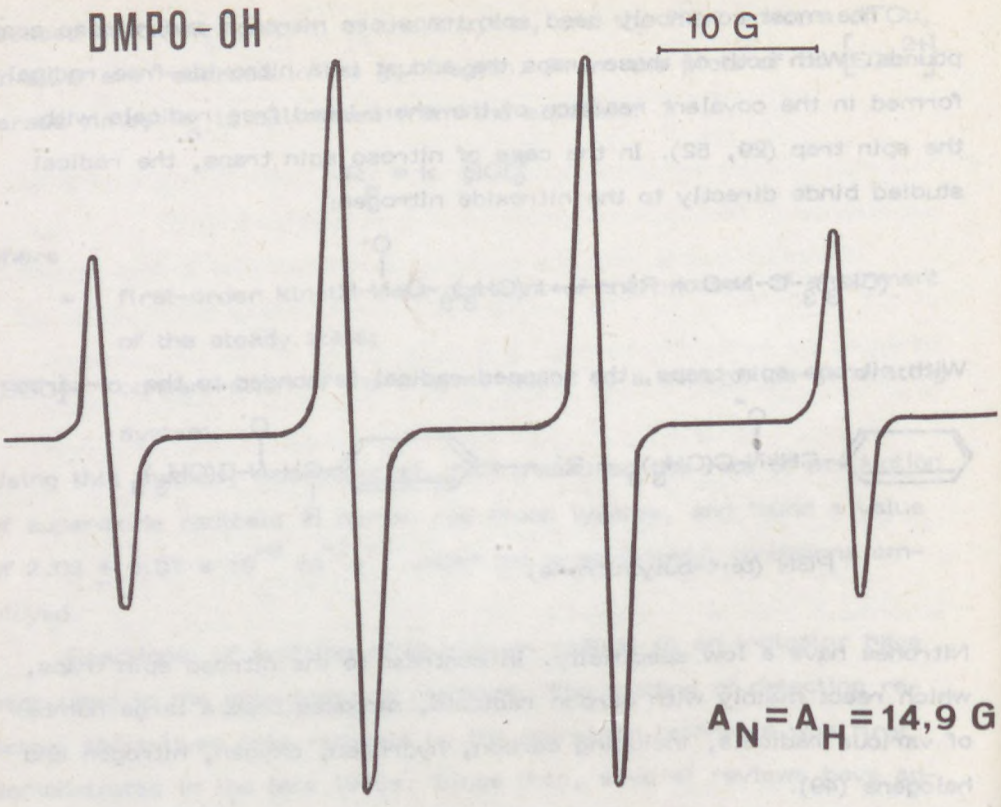


Fig. 2. Computer-simulated spectrum of DMPO-OH adduct (28)

The ESR spectrum of DMPO-OH consists of a characteristic 1:2:2:1 quartet ($A_N = A_H$ = 14.9 G) (28,30) (Fig. 2).
The trapping of superoxide radicals by DMPO gives a six-line spectrum (A_N = 14.3 G, A_H^{β} = 11.7 G, A_H^{α} = 1.25 G) (28) (Fig. 3).

The 4-POBN-OOH radical adduct (A_N = 14.16 G, A_H = 1.75 G) has a spectrum similar to that reported for DMPO-OOH. The 4-POBN-OH adduct gives a spectrum having A_N = 14.93 G and A_H = 1.69 G (28).

Though the spin traps mentioned above in reactions with superoxide and hydroxyl radicals produce distinguishable spectra with various values of the hyperfine splitting of the adduct, the identification of oxygen free radicals in biological systems by the spin trap technique is not always simple.

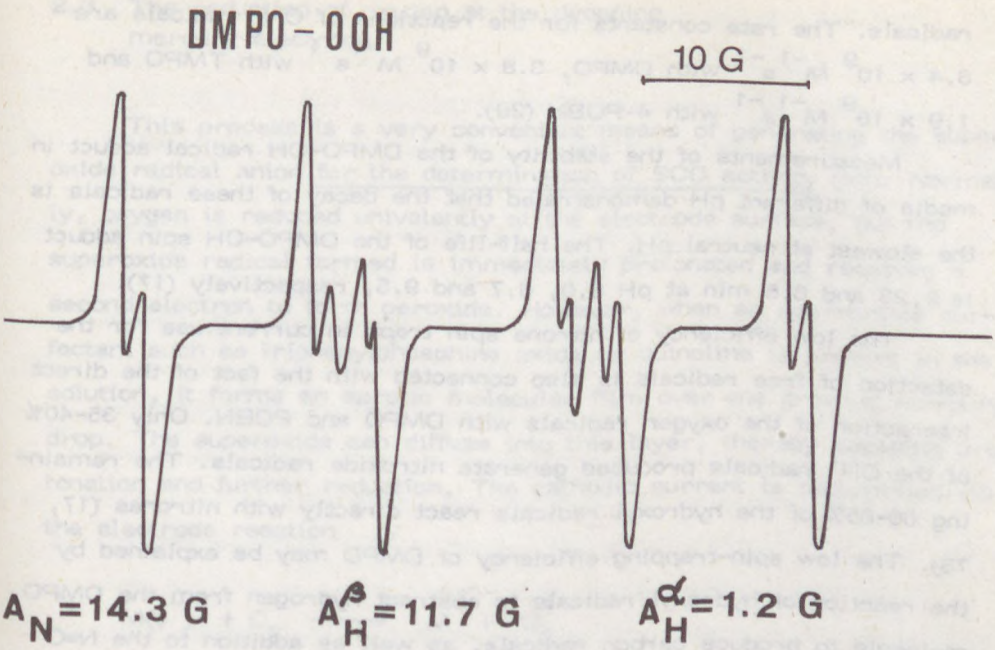


Fig. 3. Computer-simulated spectrum of DMPO-OOH spin adduct (28).

Nitrones are highly reactive compounds and in the presence of numerous substances may be reduced or oxidized into a variety of products (44). They are also easily hydrolyzed to hydroxylamine or nitroxide derivatives. The susceptibility of nitrones to hydrolysis depends on both the pH and the structure of the compound. Acyclic and aryl nitrones in a medium of low pH are especially prone to hydrolysis (44, 50).

Similarly, the stabilities of the reaction products of the superoxide and hydroxyl radicals are conditioned by many factors. Under the experimental conditions, the DMPO-OOH spin adduct is very unstable. It decays spontaneously to form the DMPO-OH adduct (29, 31). The half-life of DMPO-OOH ranges from 27 s at pH 9 to 91 s at pH 5 (29).

In contrast to the low rate constants for the reactions of nitrones with superoxide radicals (7 $M^{-1}s^{-1}$ in the presence of TMPO and 10 $M^{-1}s^{-1}$ for DMPO), spin traps are efficient detectors of hydroxyl

radicals. The rate constants for the reactions of $OH^{\cdot}$ radicals are 3.4×10^{9} $M^{-1}s^{-1}$ with DMPO, 3.8×10^{9} $M^{-1}s^{-1}$ with TMPO and 1.9×10^{9} $M^{-1}s^{-1}$ with 4-POBN (29).

Measurements of the stability of the DMPO-OH radical adduct in media of different pH demonstrated that the decay of these radicals is the slowest at neutral pH. The half-life of the DMPO-OH spin adduct is 2,23 and 6.5 min at pH 3.0, 6.7 and 9.5, respectively (17).

The low efficiency of nitrone spin traps in current use for the detection of free radicals is also connected with the fact of the direct interaction of the oxygen radicals with DMPO and POBN. Only 35-40% of the $OH^{\cdot}$ radicals produced generate nitroxide radicals. The remaining 60-65% of the hydroxyl radicals react directly with nitrones (17, 73). The low spin-trapping efficiency of DMPO may be explained by the reaction of hydroxyl radicals to abstract hydrogen from the DMPO molecule to produce carbon radicals, as well as addition to the N=C double bond to form nitroxide radicals (17). For POBN the low spin-trapping efficiency for hydroxyl radicals is explained in terms of the addition reactions of $OH^{\cdot}$ radicals to the pyridine ring (73).

The advantage of nitrones used in the studies of biological processes is the demonstrated lack of inhibition of enzymes catalyzing one-electron reactions of oxidation and reduction (NADPH-cytochrome c reductase, xanthine oxidase) in their presence (28, 54). Moreover, the spin-trapping technique has made possible for the first time the investigation of many free radicals in biological system.

In comparison with other methods of radical estimation, the spin-trap technique has turned out to be very useful for the detection of oxygen radicals in the course of many biological processes. For example, the presence of hydroxyl radicals has been detected by spin trapping in the lipid peroxidation in microsomal systems (58, 87), in the photolysis of chlorophylls (48) and in reactions involving the anthracycline and glycopeptide antibiotics (56, 72, 75, 95). The superoxide radical has been identified in microsomal systems (92), in human PMN neutrophils (37), and in the photolysis of melanin (26) and chloroplasts (47).

2.3 The reduction of oxygen at the dropping mercury electrode

This process is a very convenient means of generating the superoxide radical anion for the determination of SOD activity (80). Normally, oxygen is reduced univalently at the electrode surface, but the superoxide radical formed is immediately protonated and receives a second electron to form peroxide. However, when an appropriate surfactant such as triphenylphosphine oxide or quinoline is present in the solution, it forms an aprotic molecular film over the growing mercury drop. The superoxide can diffuse into this layer, thereby escaping protonation and further reduction. The cathodic current is determined via the electrode reaction

$$M(e^-) + O_2 \longrightarrow M + O_2^{\cdot-}$$

and the concentration of $O_2^{\cdot-}$ at the electrode surface is determined via the rate of its dismutation:

$$M(e^-)H_2O + O_2^{\cdot-} \longrightarrow M + HO_2^- + OH^-$$

which is increased in the presence of SOD, thereby elevating the value of the cathodic current for univalent oxygen reduction.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

THE FADING OF EVOKED MUSCLE POTENTIAL IN GENERAL HYPOXIA BEFORE AND FOLLOWING MTDQ-DA ADMINISTRATION IN DOGS

P. KENYERES, G. MORVAY, M. TEKERES and B. TÖRÖK

Institute of Anaesthesiology and Intensive Therapy,
Institute of Experimental Surgery,
University of Medicine,
Pécs, Hungary

INTRODUCTION

Electromyography (EMG), as a method for the measurement of the bioelectric activity of skeletal muscles, is able to express the conductive capacity of muscle tissue and it can characterize the neuromuscular membrane function via detection of the neurally evoked muscle compound action potential (CAP) (1, 3). The fading of the evoked muscle CAP therefore indicates a deterioration of this function, which can develop under pathological conditions, such as in hypoxic states (1, 6, 7, 10, 11, 13).

Free radicals are believed to play an important role in the pathogenesis of membrane function (9). In our present study, the fading of the evoked muscle CAP was investigated under hypoxic conditions before and following administration of a free-radical scavenger (MTDQ-DA) in dogs.

MATERIAL AND METHOD

The fading of the short tetanic electromyogram of the peroneal muscles, evoked neurally by tetanic stimulation at 200 Hz for 100 ms, was investigated by EMG in four dogs under 0.25 vol% Halothane anaesthesia after 5-minute hypoxic attacks caused by N_2O inhalation, before and following MTDQ-DA administration.

Such hypoxic periods lasting for 5 minutes were induced on five occasions. MTDQ-DA was administered intraarterially after the fourth suffocating attack, in a dose of 150 mg/kg body-weight. In the non-hypoxic periods the dogs were ventilated at a 16 min frequency with N_2O/O_2 in a ratio of 3:1.

The peroneal nerve, at the head of the fibula, was stimulated electrically with supramaximal rectangular stimuli of 0.2 ms duration by means of a constant current peripheral nerve stimulator and needle electrodes (2). The evoked CAPs of the peroneal muscles were recorded with surface skin electrodes on a two-channel myoscope by the polaroid technique.

The amplitudes of the initial maximal and the subsequent minimal CAPs of the tetanic electromyogram were measured and the fading response was expressed as a percentage in terms of the height of the amplitude reduction, using the well-known formula (4):

$$F\ 200\ Hz\ (\%) = 100 - [100\ \frac{\text{ampl. of minimal CAP}}{\text{ampl. of maximal CAP}}]$$

The mean arterial pressure (MAP), heart rate (HR), rectal temperature (rT), plasma ion levels (Na, K, Ca, Mg), plasma enzymes (GOT, GPT, ALP, LDH, CK, cholinesterase), plasma lactate and blood gas parameters were measured and evaluated at the end of each hypoxic period.

RESULTS

The fading of the evoked 200 Hz electromyogram showed no change after the first and the second hypoxic periods in comparison to the control (44 $\pm$ 16.8%). After the third and the fourth hypoxic manoeuvres, the fade increased gradually (52.5 $\pm$ 16.9% and 67.7 $\pm$ 13.4%, respectively), but after the fifth hypoxic attack, following MTDQ-DA administration, it diminished significantly (54.0 $\pm$ 19.2%) (Figs 1 and 2, Table 1).

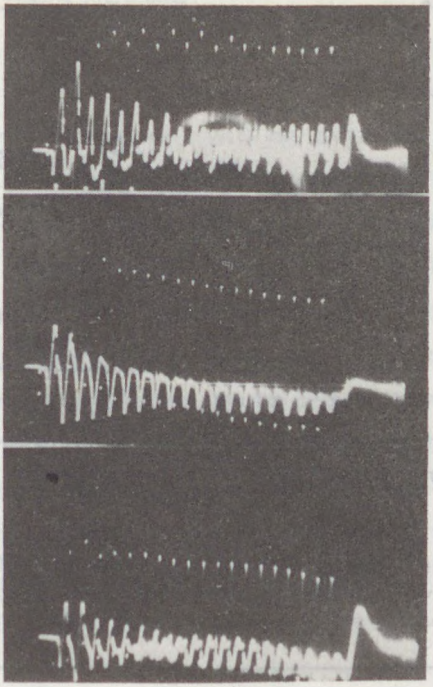

Fig. 1. Electromyograms of dog 2. Control: F 200 Hz 67% (above), S_4: F 200 Hz 81% (middle), S_5: F 200 Hz 72% (below).

Table 1. Fading of the 200 Hz electromyogram (F 200 Hz (%)) at the end of the hypoxic periods and the control measurements on 4 dogs (C = control, $S_{1...5}$ = suffocation attacks)

Dogs	C	S_1	S_2	S_3	S_4	S_5 + MTDQ-DA
1	44	50	53	60	68	25
2	67	66	67	73	81	72
3	27	20	23	37	79	73
4	38	40	36	40	43	46
Mean	44.0	44.0	44.7	52.5	67.7	54.0
SD	± 16.8	± 12.2	± 19.2	± 16.9	± 13.4	± 19.2

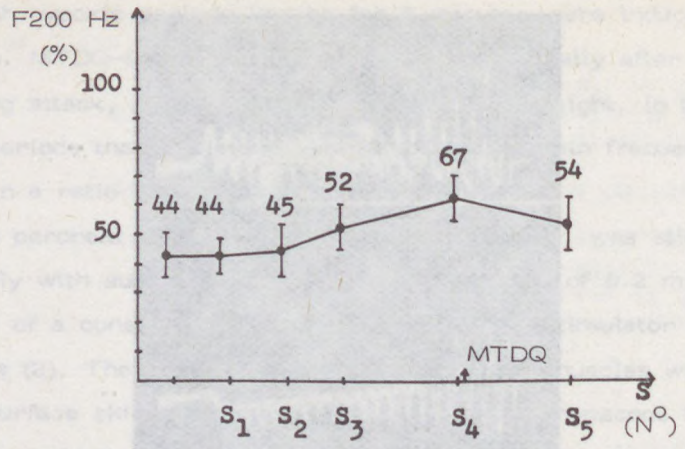

Fig. 2. The "fading-curve" for the 4 dogs (mean values)

Table 2. Parameters (mean values) on 4 dogs, indicating the progressive worsening of the general state

	K	S_1	S_2	S_3	S_4	S_5
PaO_2 (mmHg)	105	40	34	33	24	22
BE	-8,5	-7,9	-9,7	-14,7	-15,8	-17,2
Lactate (mmol/l)	1,04	2,16	2,6	3,5	4,7	5,7
MAP (mmHg)	102	75	57	53	45	38
HR (min^{-1})	174	169	138	98	93	88

The repeated hypoxic stresses caused a gradual worsening of the general state of dogs. In the course of the hypoxic attacks, the MAP, HR and PaO_2 gradually decreased and metabolic acidosis developed (s. BE and lactate), indicating the effect of the applied hypoxia (Table 2).

DISCUSSION AND CONCLUSIONS

It is evident that the conductive capacity, as a special membrane function of skeletal muscle tissue, will be damaged under pathological conditions, such as in severe hypoxia. In our experiments, the deterioration of this membrane function could be monitored by measurement of the fading of the evoked high-frequency tetanic electromyogram (8).

Under pathological conditions, both the fading (amplitude reduction) and the facilitation (increase of amplitude) of evoked muscle CAPs could be shown. Facilitation would occur, however, in the cases of high potassium and sodium, or low magnesium, hydrogen and calcium ion concentrations in the plasma, which would influence the fading effect (12). In our study, when the fading of the evoked 200 Hz electromyogram decreased significantly at the end of the fifth hypoxic attack, following MTDQ-DA administration, the above factors were not present.

In our experiments the high-frequency loading test of the conductive capacity of the skeletal muscle was applied, and the 200 Hz stimulation indicated a maximal frequency loading of motor unit membranes because of their 5 ms refractory period (1).

From the results it can be concluded that

(i) The hypoxia is manifested in a deterioration of the conductive capacity of the skeletal muscles;

(ii) MTDQ-DA, an artificial free radical scavenger, can significantly diminish the fading of the evoked muscle CAPs, i.e. the deterioration of the conductive capacity of the neuromuscular membranes;

(iii) the data corroborate the role of free radicals in the pathogenesis of the deterioration of the membrane functions in response to various stressors, and the effectiveness of artificial scavengers.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

STUDY OF LIPID PEROXIDATION IN RAGWEED POLLINOSIS

E. KOO, M. ANTAL, K. MERÉTEY and F. GERENCSÉR

National Institute of Rheumatology and Physiotherapy,
Budapest, Hungary

The inflammations and the immune reactions are always connected to some extent (6). The type of the immune response and the subsequent amplifying mechanisms determine the outcome of the reactions and the appearance of the inflammation (5).

The IgE-mediated allergic reactions of the immune system usually appear in the form of acute inflammations in the airways, intestines and blood vessels, and very often as skin reactions. The mechanism of this type of inflammatory reactions is the allergen cross-linked IgE-mediated degranulation of mast cells, the liberation of primary and secondary mediators (3).

It is very probable that the general mechanism of membrane attacks caused by the intermediates of the arachidonic acid cascade (2, 11, 7) also plays a role in the mechanism of all acute inflammatory reactions, including the allergic reactions. The aim of our investigations was to study this problem in patients suffering from ragweed pollinosis.

MATERIALS AND METHODS

Ten patients were selected who suffered from ragweed pollinosis. The diagnosis for these patients was allergic rhinitis. The patients were followed for a year. The correlation of the lipid peroxidation was tested in the period of the ragweed season, when the patients

showed the allergic clinical symptoms, and in the off-season period when the patients were symptom-free. A non-allergic control group was also included.

Blood was taken from the patients during the pollen season and the off-season and the serum samples were stored frozen until used ($-20^{\circ}C$).

The malondialdehyde (MDA) concentration was determined via the thiobarbituric acid reaction, as modified by Ottolenghi (9).

The glutathione peroxidase (GPX) was measured according to the combined technique of Chiu et al. and Sedlak et al. (1, 10). The undigested reduced glutathione was titrated with cumene hydroperoxide as substrate (5, 17).

The superoxide dismutase (SOD) activity was investigated by the method of Misra et al. (8), via inhibition of the transformation of adrenaline into adrenochrome. The interfering catalase and peroxidase activity was excluded by adding sodium azide to the system.

The technique of Hall was applied in the measurement of SH groups (4).

The statistical analysis was performed with Student's t test.

RESULTS

The results are summarized in Table 1. The MDA values are significantly higher during the pollen season than in the pollen-free period ($p < 0.005$). The activity of SH groups was significantly lower in the patients showing the allergic clinical symptoms in the season ($p < 0.02$) than in the off-season. As compared to the healthy control group, only the decrease of the SH groups in the patients during the ragweed-positive period showed a statistically significant difference.

Table 1. Serum parameters of lipid peroxidation in allergic rhinitis patients during the pollen period and off-season

Patient group	MDA µmol/l	SH µmol/l	GPX $\times 10^3$ U/g prot	SOD U/g prot
Allergic group				
During the pollen season (R+)	$\bar{x}$= 9.72	285	1.83	34.4
	SE= 0.53	18	0.07	4.2
	n = 10	10	10	10
In the symptom-free period (R-)	$\bar{x}$= 7.2	393	1.68	43.4
	SE= 0.5	28	0.11	4.7
	n = 9	10	9	9
R+ vs. R-	$p < 0.005 < 0.02$			
Control group, non-allergic (C)	$\bar{x}$= 8.7	395	1.77	34.8
	SE= 0.6	13.7	0.14	4
	n = 10	10	10	10
R+ vs. C	$p <$ 0.001			

DISCUSSION

The increased serum level of the metabolites of lipid peroxidation may come from the damaged cell membranes. Many in vivo pathological processes, e.g. allergic reactions, may cause a direct or indirect involvement of cell membranes. As a result of the membrane attack, the amounts of the metabolites of lipid peroxidation and also their decay mechanisms may be altered. Our results suggest that during the allergic processes the level of serum MDA, an accepted parameter of lipid peroxidation, is increased. At the same time, the amount of SH groups which take part in the removal of the radicals was found to be significantly lower as compared both to the healthy

controls and to the same patients in the symptom-free period. These changes seem to show an altered balance between the production of the toxic metabolites of lipid peroxidation and the scavenger activity.

As the changes appear in concordance with the allergic symptoms, we may presume that they are correlated with the inflammation caused by the allergic immune process.

SUMMARY

The serum levels of MDA and SH groups and also the activities of SOD and GPX were investigated in ten allergic patients during the pollen season and in the pollen-free period. The MDA level was increased, while the amount of SH groups was decreased significantly during the active allergic rhinitis period. The results may show the participation of lipid peroxidation in the inflammation evoked by allergic IgE-mediated processes.

ACKNOWLEDGEMENT

The authors express their gratitude to Mrs. L. Újváry for her excellent technical assistance.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

SPATIOTEMPORAL RELATIONSHIPS OF IONIZING RADIATION-INDUCED FREE RADICALS AND THEIR BIOLOGICAL EFFECTS

G.J. KÖTELES

"Frédéric Joliot-Curie" National Research Institute
for Radiobiology and Radiohygiene,
H-1775 Budapest, POB 101, Hungary

SUMMARY

Various data and examples are reviewed to demonstrate the spatiotemporal relationships of ionizing radiation-induced free radicals and their biological effects, including the formation and reactions of radicals, biological effects in small target volumes in terms of microdosimetry and the cellular defence mechanisms. Special reference is made to the radiation sensitivity of cell membranes.

INTRODUCTION

Free radicals producing cellular and tissue damage were first revealed in radiation biology (2). Since then, evidence, however indirect, has accumulated on the aetiological roles of free radicals in a series of physiological or pathological cellular processes, such as photosynthesis, inflammation, host defence mechanisms, autoimmune diseases, cell differentiation, lymphocyte mitogenesis, the life span of species, carcinogenesis, mutagenesis, and the toxicity of drugs and pesticides. The aims of relevant radiobiological studies are therefore to disclose the mechanisms through which the deleterious biological effects are caused; in this way, radiobiology provides tools for investigations involving oxygen free radicals in cellular physiology and pathology.

FORMATION AND REACTIONS OF FREE RADICALS

The process leading from the first reactions of ionizing particles of photons and the living cell to a certain "end-point" of biological effects is divided into physical, chemical and biochemical-biological phases. Although the overall time scale comprises 10^{-18} s to 10^{8} s, the various phases account for approximately 6, 12 and 8 orders of magnitude, respectively, out of the 26 orders of magnitude involved. Inorganic and organic free radicals are formed in chain reactions throughout all the physical, chemical and biochemical periods, i.e. from 10^{-18} s up to ca. 10^{4} s. Free radicals may originate from biomolecules such as DNA, e.g. guanine ($G^{+\cdot}$) and thymine ($T^{-\cdot}$) radicals ("direct action" of radiation), or they appear as products of water radiolysis ("indirect action" of radiation). The latter may happen under anoxic conditions, when the products are e^{-}_{aq}, $OH^{\cdot}$, $H^{\cdot}$ and H_2O_2, or in the presence of oxygen, when $O_2^{-\cdot}$ and $HO_2^{\cdot}$ radicals are additionally formed. The very toxic hydroxyl radical ($OH^{\cdot}$) is also generated in the presence of O_2^{-}, H_2O_2 and metal ions (4, 13). A wide selection of radical ions, such as iodide, bromide, formate and azide, can be formed from $OH^{\cdot}$ radicals, which are more selective in their reactions with organic compounds such as proteins than the initial $OH^{\cdot}$ (1, 16). The hydroxyl radical and possibly singlet oxygen ($O_2/^1\Delta$ g) may react with unsaturated lipids of biomembranes, resulting in the generation of lipid peroxide radicals ($ROO^{\cdot}$), lipid hydroperoxide (ROOH) and fragmentation products such as malondialdehyde (6, 17). Superoxide radical ($O_2^{-\cdot}$)-dependent processes are involved even in the mediation of delayed radiation damage developing in the first few hours after irradiation (40). It has been proposed that some of the O_2 generated through the reactions may be singlet oxygen, which is also an important mediator of tissue damage (14). Peroxy free radicals have been shown to damage enzymes (11). A variety of water radiolysis species react with peroxides and hydroperoxides. Many of the products of these reactions are themselves reactive or unstable species and will

take part in further reactions capable of producing chemical damage to biomolecules. Accordingly, oxygen takes part in primary and secondary reactions leading to the production of different forms of activated oxygen, including superoxide and peroxy radicals. The interconversion and formation of peroxides and other forms of activated oxygen can be promoted by ionizing radiations, causing radiobiological oxygen effects, as well as autocatalytic chain reactions such as lipid oxidation and many other biochemical effects, at various distances and even in later phases.

The radiation field where the radicals are formed is made up of a tangle of particle tracks. Via these tracks, the ionizing radiation deposits energy in the medium through which it passes, by producing secondary particles, photons and radicals. These initial, chemically reactive species are distributed inhomogeneously. A good example of the time, yield and space relations of radicals formed in a track of relatively low-energy (5 keV) electrons is given by water (38). It was pointed out that a single electron in a path not longer than 700 nm produced 385 inelastic events within 10^{-15} s from the initial energy deposition and the total number of various species of radicals was 1174 over the complete track by 10^{-11} s. The chemical development of the track was completed by 2.8×10^{-7} s (0.28 μs). The yield, as expressed by the G value (the number of species formed per 100 eV) for various reactive species (e^-_{aq}, H_3O^+, $OH^\cdot$, H_2, H_2O_2, OH^-) at 0.28 μs for selected electron energies was between 15.1 and 17.1. The radial distribution of the energy in space, i.e. within the track penumbra, is limited. Approximately 80 per cent of the electron energy between 1 and 100 keV is deposited within 1 and 100 nm (15). Under such conditions, linear energy transfers in the range of 10-50 eV per nm might occur. This seems to be quite enough to break many covalent bonds (appr. 3 eV energy) and certainly enough to disrupt and disturb a large number of much weaker secondary intermolecular bonds (0.01-0.5 eV), such as van der Waals or hydrogen-bonds, in the biochemical structures.

BIOLOGICAL EFFECTS

For an understanding of radical-induced cellular or subcellular effects, it is promising to project the events of physical energy deposition, including of course the formation of free radicals and other reactive species during the biological scenario, in the respective volumes. Due to the limited path-lengths of secondary particles and radicals, rather small subcellular volumes have to be selected to investigate the possible effects. By virtue of the introduction of small elementary targets, the influences of radiation quality and absorbed dose are separable, this separation being an important aspect in microdosimetry. With DNA as the elementary unit of the spatial structure, a cylinder 2 nm in diameter and 3.4 nm in length, corresponding to one complete turn of the double helix, is considered as the target in which the conversion of the radiation energy into primary molecular alterations can be described (3). For other radiosensitive targets, the cell membranes, it is proposed that sections containing either only lipids or proteins, as well as regions of their mutual contacts, be selected. For example, a membrane region 6 nm in length contains about 14 phospholipid units or an integral protein molecule of medium size. The linear energy of low-energy electrons, i.e. a few tens of eV per nm, can therefore also cause considerable disturbance in the molecular arrangements and consequently the functions. This approach (comparing spatial relationships of physical events and biological structure) would also allow the analysis of regional effects on cell membranes. This seems especially important when certain functions are attached to certain proteins embedded at various distances or the cooperation of various proteins and lipids (19, 20, 21).

In a consideration of the development of biological effects, it should be noted that the relevant damage is rarely produced "directly" by the initial radical processes. In biological radiation reactions, two steps are distinguished: the physical actions on biomolecules, and the subsequent biochemical repair processes. As concerns the latter,

there are several models to explain the mechanisms of development of the final damage such as sublesion interactions or the misrepair due to the formation of neighbouring lesions. Apart from the multistep biological reactions, the role of free radicals in the development of several end-points has usually been proved by indirect means, by applying scavengers. As regards the duration of existence of free radicals after irradiation, interesting data were given for Chinese hamster V79 cells (40). It was found that the treatment of cells with diethyldithiocarbamate, an efficient inhibitor of Cu, Zn-SOD in the early postirradiation period (up to 2 hours), reduced the clonogenic survival of cells, possibly through mechanisms involving O_2^- radicals interfering with potentially lethal damage. Other studies also support the idea that O_2^--dependent processes are involved in the mediation of delayed radiation damage, e.g. the SOD-protection of membranes (29) or the prevention of oncogenic transformation (27). Other examples can be taken from cellular micromorphology, where reactive oxygen metabolites caused acute and irreversible injury involving membrane ruffling, distorted microvilli, bleb formation within 15 minutes, and the cessation of cellular movements by 3 hours after exposure to free radicals; it was suggested that there was a continuous $OH^{\cdot}$ formation via O_2^--mediated transition metal conversions (36). Similar micromorphological phenomena were observed in our laboratory after various doses of X- and neutron+gamma-irradiations of human fibroblasts and blood cells (23, 24).

When we tested the breakdown and incorporation of the most radiosensitive polyunsaturated fatty acid (PUFA), arachidonic acid, it could be observed that, upon the X-irradiation of cells prelabelled with ^{14}C-arachidonic acid, only a proportion of this PUFA was degraded, while pulse-labelling after irradiation indicated a slow, delayed uptake until full regeneration. It follows from these data that one of the free radical-induced processes such as lipid peroxidation lasts for a rather long time, and the regenerative processes (which can also be influenced by delayed reactive species reactions) last for different periods of

time at various levels, e.g. the perturbed cellular surface charge distribution returns within minutes to the pre-irradiation level, while the regeneration of molecular arrangements such as receptor activities, or the replacement of injured molecules such as phospholipids, or the repair of DNA takes a much longer time (20, 21).

The extent and nature of reactions of any free radical depend to a large extent on the microenvironment in which the radical is formed. The biological effects of free radicals are therefore manifested according to the group of biomolecules with which they react. The spatio-temporal limitations of such reactions contribute to the wide varieties of biological effects on various targets at subcellular and cellular levels.

DEFENCE MECHANISMS

Defence mechanisms in a broader sense include all means of elimination of free radicals. Many cellular and extracellular compounds compete for free radicals. Reducing species, such as vitamins C and E, SH-compounds, phenols, hydroquinones, carotenoids, amines, alcohols and radioprotective compounds tend to protect by deactivating free radicals, whereas oxidizing species, which include metal ions in their oxidized states (Fe^{3+}, Cu^{2+}), oxygen, quinones and nitro compounds, tend to catalyze or promote free radical damage (12). Further, the number of biomolecules shown to react with free radicals is increasing; they include thiamine, cyanocobalamine, cytochrome C, ATP, cysteine and mixtures of these (30). Besides the large variety of molecules reacting with free radicals, the presence in the cell of enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase) determines the fate of radicals and consequently the development of cellular or tissue damage. SOD and the peroxidases are the most active components of biological defence mechanisms (8, 26). The rapidly growing immense literature on this topic can now hardly be reviewed. Again, only a few examples are given, which point to the still in-

sufficient knowledge of the role of organized biological structures in the elimination of free radicals or the protection of biomolecules against them.

In fact, the accumulating data suggest that the amount of free radical-eliminating enzymes is correlated with the radiation resistance in vivo in rats (22, 24), or even with the life-span of long-living animals (28). In certain tissues or subcellular targets, e.g. membranes, the SOD activity itself decreases upon irradiation (33, 35); the reasons for this phenomenon might be either the inhibition of enzyme activity by lipid hydroperoxides, or the radiation-induced alterations of biostructures, e.g. membranes (31). In certain biological systems, however, such as spores, neither SOD nor catalase proves significantly protective (9, 10). The complexity of the white spots in the defence mechanisms, as well as the role of cellular structures, are seen from experiments on cells the membrane system of which was enriched with radiosensitive PUFAs. Though their membranes contained 5-8 times more PUFA, murine fibroblasts proved not to be more sensitive as far as clonogenic survival is concerned than the original, unmodified cells, not even in glutathione-depleted cells. A membrane function such as the leakage of potassium, however, was more sensitive in such cells (41-43). In contrast, other authors have found that the survival of Chinese hamster V79 cells was more sensitive to ionizing radiation when they were preincubated in a PUFA-containing medium (39).

EFFECTS OF NON-IONIZING RADIATIONS

The data show that sunlight, UV-irradiation and even ultrasound (25) may initiate tissue and cell damage through free radical reactions, including lipid peroxidation. The sensitization of cells via furocoumarins and hematoporphyrins (32) is gaining more and more importance in research and clinical practice. For most of these processes the $OH^{\cdot}$ and singlet oxygen, and less so the O_2^-, seem to be responsible

(5, 7, 18, 37). The quantitative and qualitative aspects of cell damage, however, are far less known than for ionizing radiations.

ACKNOWLEDGEMENT

The interest in and support of this work by Professor L.B. Sztanyik, Director-General of the Institute, is gratefully acknowledged.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES IN ERYTHROCYTES FROM 21-TRISOMIC DOWN'S PATIENTS

Aranka LÁSZLÓ, B. MATKOVICS* and L. SZABÓ*

Department of Paediatrics, Medical University of Szeged,
*Biological Isotope Laboratory,
József Attila University,
Szeged, Hungary

SUMMARY

Investigations were made of the lipid peroxidation in the plasma and the erythrocytes and the activities of antioxidant enzymes in the erythrocytes from 21-trisomic Down's cases. An enhanced lipid peroxidation process and compensatory elevated activities of antioxidant enzymes (copper,zinc superoxide dismutase = Cu,Zn-SOD, and glutathione peroxidase) were proved. The catalase activity of the red blood cells was significantly lower in the Down's cases. The data contradict the gene dosage effect hypothesis of Cu,Zn-SOD.

INTRODUCTION

An increased Cu,Zn-SOD activity has been detected in the erythrocytes, platelets (4, 13, 14, 17), leukocytes (6) and fibroblasts (6) from 21-trisomic patients, suggesting the gene dosage effect as the most credible explanation. The Cu,Zn-SOD gene is located in chromosome 21q21-22 (3, 11, 15).

In 19 cases of 21-trisomy, investigations were made of the lipid peroxidation (LP) in the plasma and the erythrocytes, and of the activities of antioxidant enzymes in the erythrocytes, with the aim of testing the gene dosage effect hypothesis.

METHODS

The LP was measured by the TBA method (thiobarbituric acid-reactive products) of Placer et al. (12). Superoxide dismutase (SOD,

EC 1.16.1.1) activity was determined in aliquots of the chloroform-ethanol aqueous supernatant, on the basis of the inhibition of epinephrine autoxidation (8, 9). Catalase (C-ase, EC 1.11.1.6) activity was measured at 240 nm by a spectrophotometric method (1). The protein content was determined by the method of Lowry et al. (7). The results were subjected to statistical evaluation with Student's t test. All numerical data are given as means ± SD. In the enzyme activity and LP measurements, the difference between duplicate determinations was never in excess of 5%. Glutathione peroxidase (GSHPx) activity was measured by the method of Chiu et al. (2, 10).

RESULTS

The results relating to the LP and the antioxidant enzymes are given in Table 1. The amounts of TBA-positive substances (LP) in the plasma and the haemolysate of the erythrocytes of Down's patients were significantly higher than the control values, as were the SOD and GSHPx activities. The C-ase activity of the red blood cells (RBC) was significantly lower in the Down's cases.

DISCUSSION

Sinet (16) surveyed the metabolism of oxygen derivatives in Down's syndrome, pointed to an increased Cu,Zn-SOD activity, and discussed the signs suggesting increased oxidative damage in 21-trisomy, abnormalities in fibroblast cultures (16), such as early senescence of the culture, an increase in population doubling time and the signs of brain damage.

The increased GSHPx activity in 21-trisomic tissues cannot be explained by a gene dosage effect, while the gene for GSHPx activity has been assigned to chromosome 3 (3q13-3q12) (5, 18). The latter might be a secondary compensatory effect of the increased LP in the plasma and in the erythrocytes of Down's patients, similarly as for

Table 1. TBA-reactive products (lipid peroxides) and antioxidant enzymes in the red blood cells of 21-trisomic and healthy individuals

Assay	Controls (n=10)	Down's cases (21-trisomy) (n=19)	
Protein g/100 ml (haemolysate) SD±	30.33 3.41	33.77 3.78	
TBA-reactive plasma products (nmol MDA/l plasma) mean ± SD	5.5×10^3 $\pm 1.5 \times 10^3$	13.5×10^3 $\pm 7.4 \times 10^3$	$p < 0.01$
TBA-reactive products (nmol MDA/l haemol.) mean ± SD	119.2×10^3 $\pm 15.6 \times 10^3$	155.6×10^3 $\pm 57.5 \times 10^3$	$p < 0.05$
SOD (U/ml haemol.) mean ± SD	293.6 ±51.3	434.3 ±84.3	$p < 0.01$
SOD (U/mg RBC protein) mean ± SD	1.266 ± 0.273	1.453 ± 0.641	$p > 0.05$
C-ase (BU*/ml haemol.) mean ± SD	2.7 ± 0.8	1.73 ± 0.31	$p < 0.05$
GSHPx (U/mg RBC protein) mean ± SD	4.88 ± 1.08	8.49 ± 1.75	$p < 0.01$

BU* = Bergmeyer units

the increased SOD activity and the decreased C-ase activity. An increased amount of peroxides is formed and catabolized via GSHPx, as a defence against the effects of peroxide accumulation in the cells.

Our results suggest increased oxidative damage within the cells in 21-trisomy, rather than an increased gene dosage effect.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

OXIDATION OF STEROIDS BY SINGLET OXYGEN

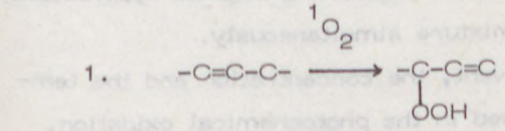
B. LOSONCZI and Á. LENGYEL

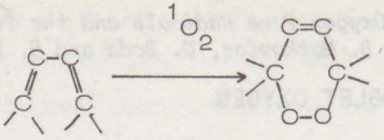
Department of Organic Chemical Technology,
Technical University,
Budapest, Hungary

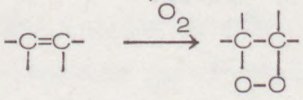
Singlet oxygen can be generated by physical and chemical methods. The classical method is the photosensitization of oxygen. The main chemical sources of singlet oxygen are the hypohalide - hydrogen peroxide reaction, and the decompositions of peroxides, peracids and ozone phosphite(1). In spite of the variety of techniques available for generating singlet oxygen, many problems may arise in preparative practice. For example, the hypohalide - hydrogen peroxide reaction involves solubility problems, and the peroxides are unstable and dangerous. The generation of singlet oxygen by the photochemical method necessitates special reactors, light sources and large amounts of solvents, but it is still the method generally used in this type of laboratory-scale oxidation.

We have developed new singlet oxygen sources (2, 3) on the basis of the reactions between organic hypohalides and organic peroxides. These are advantageous in preparative practice, solving most of the problems mentioned above.

Carbon-carbon double bonds can be oxidized by singlet oxygen. Three types of oxidation are known:



2.  (endoperoxides)

3.  (dioxetanes)

The steroids contain isolated and conjugated double bonds. It has been reported (1) that these can be oxidized by singlet oxygen. In these experiments the singlet oxygen was generated by the photochemical method, the solvent used was normally pyridine, the sensitizer was porphyrin, eosin, rose bengal or methylene blue,and the peroxide formed was reduced in the reaction mixture.

Examination of the products of the photochemical oxidation of steroids permitted the following conclusions:

1. Even in the presence of more than one oxidizable group, singlet oxygen oxidizes only at one site in the molecule. The reason for this is assumed to be the steric structure of the molecule.

2. The steric structure of the molecule changes during the oxidation only if the given group is oxidized.

3. The oxidation is often stereoselective, and the structure produced depends on the original stereochemistry.

4. After the reaction with $^1O_2$, secondary oxidation reactions often occur with $^3O_2$ or with the hydroperoxide group produced.

With our new singlet oxygen sources, we reproduced some of the photochemical oxidations of steroids described in the literature. In our experiments we used organic peroxides and organic hypohalides; these were fed into the reaction mixture simultaneously.

It was found that, if the solvent, the concentration and the temperature were similar to those used in the photochemical oxidation, the products of the oxidations were similar too. The necessary amount of oxidant was 1.5 equivalents. Experiments in progress suggest that these oxidations can be made also at higher concentrations.

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SOD LEVELS IN EARTHWORM (LUMBRICUS TERRESTRIS) PARTS

M. LOVAS*, L. SZABÓ*, B. MATKOVICS* and E. FISCHER

Department of Zoology, Janus Pannonius University, Pécs,
*Biological Isotope Laboratory, József Attila University, Szeged, Hungary

ABSTRACT

The body of earthworm (Lumbricus terrestris) was divided into three parts: the skin, intestine and chloragogen tissue, and the activities of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) were measured in these. Superoxide dismutase was separated by gel electrophoresis. The activities in the tissues displayed seasonal changes.

INTRODUCTION

The various Oligochaeta species are important members of the biocoenosis of waters and the soil. Earthworms are well known to play a role in soil formation and in the maintenance of soil productivity. It follows from the modes of living and feeding of earthworms that they are particularly exposed to the effects of xenobiotics. The Oligochaeta species are surprisingly resistant to xenobiotics; accordingly, large amounts of toxic substances may accumulate in them, and this may be dangerous for other creatures in the food chain (4). Very little is known of the detoxication mechanisms occurring in earthworms, or of the causes of the tolerance to hypoxia and antioxidants (5).

It therefore appeared justified to investigate the quantities of antioxidant enzymes (and particularly the amount and properties of superoxide dismutase) in three parts of the earthworm: the skin, intestine and chloragocytes.

MATERIALS AND METHODS

The skin or cutaneous muscle (bursa), intestine and chloragocytes were separated from earthworm (Lumbricus terrestris); the separate parts from a number of individuals were combined, and homogenized in cold 0.65% physiological saline solution during cooling. Due to the small amounts of tissue, wet weights were measured. The homogenization was followed by centrifugation, and aliquots of the resulting supernatant were used for enzyme measurements.

Enzyme activity measurements

(i) Superoxide dismutase (SOD; EC 1.15.1.1) was determined on the basis of inhibition of the epinephrine-adrenochrome transformation under the basic conditions described by Misra and Fridovich (10) or Matkovics et al. (8).

For the separation of SOD on polyacrylamide gel and for the development of the enzyme, the conditions and procedure described by Beauchamp and Fridovich (1) were applied.

(ii) Glutathione peroxidase (GP-ase; EC 1.11.1.9) activity was determined by the method of Chiu et al. (3), with cumene hydroperoxide as substrate. The reduced glutathione (GSH) residue after the action of the enzyme for the given time was measured by the method of Sedlak and Lindsay (11), using the Ellman reagent.

(iii) Catalase (C-ase; EC 1.11.1.6) activity was determined by the spectrophotometric method of Beers and Sizer (2). The C-ase activities were given in Bergmeyer units (BU). One BU is the amount of C-ase which can decompose 1.0 g H_2O_2 in 1 min.

In every case, the quantity of protein in the homogenate supernatant was estimated by the method of Lowry et al. (7).

The results were subjected to statistical evaluation with Student's t test. All numerical data are given as means $\pm$ SEM. In the enzyme activity measurements, the difference between duplicate determinations never exceeded 5%.

RESULTS

Table 1 lists the enzyme activities measured in the spring; the activity levels are highest in the chloragocytes.

Table 1. The activities of antioxidant enzymes in the parts of earthworm (May 1985)

	Total SOD U/mg prot.	C-ase BU/mg prot.	GP-ase U/mg prot.
Chloragocytes	6.6	$19.5 \cdot 10^{-3}$	0.057
	± 0.6	$\pm 1.2 \cdot 10^{-3}$	± 0.001
Intestine	5.04	$6.85 \cdot 10^{-3}$	0.065
	± 0.5	$\pm 0.7 \cdot 10^{-3}$	± 0.001
Cutaneous muscle	3.70	$3.4 \cdot 10^{-3}$	0.048
	± 0.4	$\pm 0.3 \cdot 10^{-3}$	± 0.001

Table 2. The activities of antioxidant enzymes in the parts of earthworm (July 1985)

	Total SOD U/mg prot.	C-ase BU/mg prot.	GP-ase U/mg prot.
Chloragocytes	0.67	$0.37 \cdot 10^{-3}$	$0.64 \cdot 10^{-3}$
	± 0.01	$\pm 0.01 \cdot 10^{-3}$	$\pm 0.01 \cdot 10^{-3}$
Intestine	1.27	$3.84 \cdot 10^{-3}$	$0.72 \cdot 10^{-3}$
	± 0.01	$\pm 0.20 \cdot 10^{-3}$	$\pm 0.01 \cdot 10^{-3}$
Cutaneous muscle	0.90	$1.12 \cdot 10^{-3}$	$0.23 \cdot 10^{-3}$
	± 0.01	$\pm 0.05 \cdot 10^{-3}$	$\pm 0.00 \cdot 10^{-3}$

The data from the summer measurements are given in Table 2. Here, the intestine activity levels are highest in all cases.

Figure 1 presents the results on the polyacrylamide gel electrophoresis of SOD by the method of Beauchamp and Fridovich (1). The

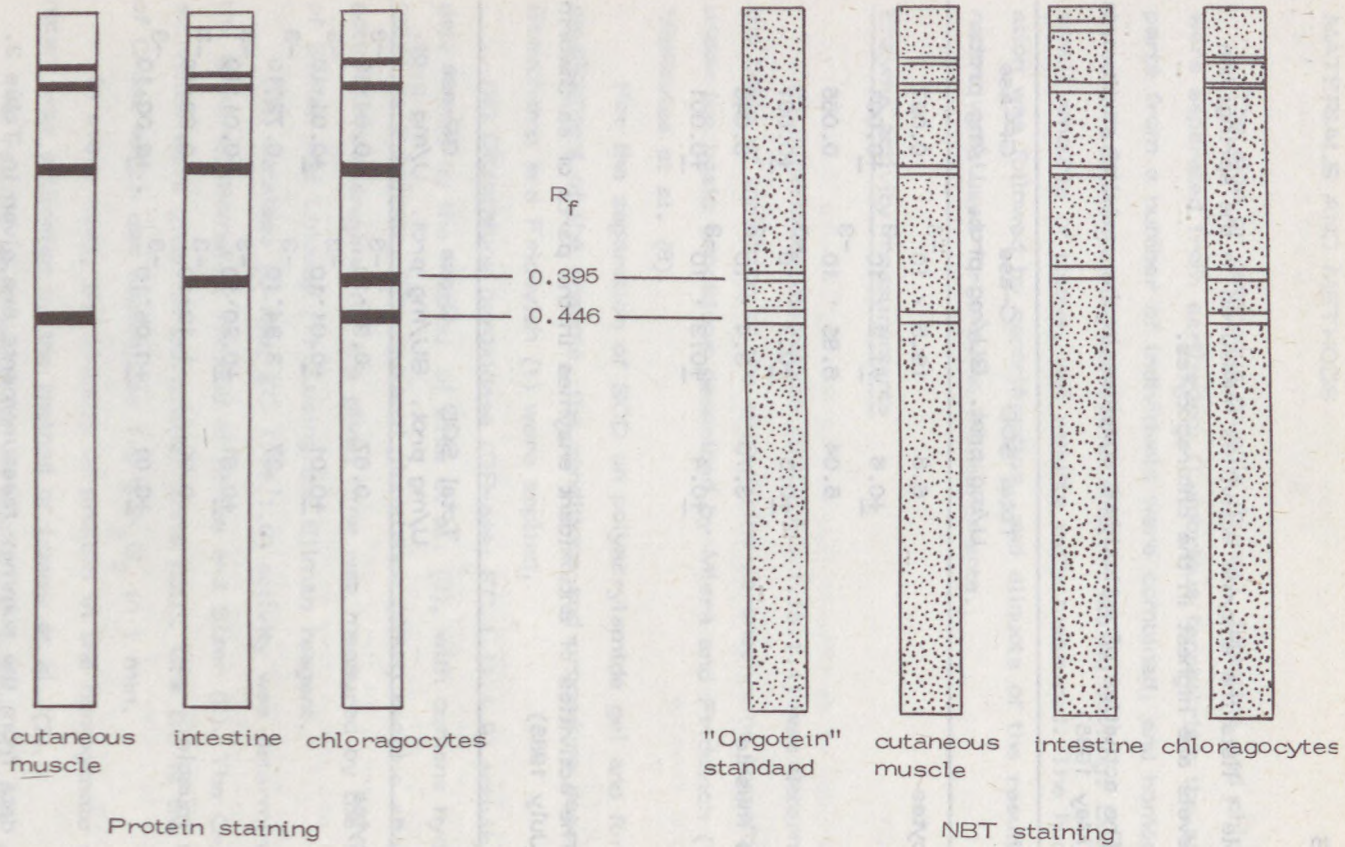

Fig. 1 PAGE of earthworm homogenates

protein was developed either with Coomassie brilliant blue or with nitroblue tetrazolium (NBT). Standard running was performed to identify SOD (9). The detected SOD could be identified as Cu,Zn-SOD, though two bands were separated in the standard, too. The upper bands could be other sorts of SODs (Mn-SOD or Fe-SOD).

DISCUSSION

It has earlier been found that the oxygen concentrations of the soil and the environment influence the size of the nucleus of the chloragocytes in earthworm and tubifex (Tubifex tubifex); these species are able to tolerate considerable changes in oxygen pressure without damage (5). This justified our investigations on the activity of the antioxidant enzyme system. Our results demonstrated that the activities of the individual antioxidant enzymes exhibited appreciable variations in the different seasons (6). This was manifested in different organ activities and in high differences in activity. This periodic change can presumably be explained in part by the variation in size of the nucleus of the chloragocytes.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

ANTIOXIDANT ENZYME CHANGES IN HUMAN BLOOD STORED AT 4-5°C

B. MATKOVICS, L. SZABÓ, E. ALFÖLDI* and Gy. GÁL*

Biological Isotope Laboratory, József Attila University, Szeged,
*Blood Center, University Medical School, Szeged, Hungary

ABSTRACT

The changes in activity of the antioxidant enzymes superoxide dismutase and catalase, and the changes in the lipid peroxidation, were studied in human blood stored at 4-5°C in various media, in order to establish whether such blood becomes unsuitable for transfusion.

INTRODUCTION

We have long been dealing with the changes in superoxide dismutase (SOD; EC 1.15.1.1) in animal and human blood under normal and pathological conditions (2, 6, 7). We have also investigated the changes in activity of other antioxidant enzymes, i.e. glutathione peroxidase (not studied in the present work) and catalase (C-ase; EC 1.11.1.6), and in the lipid peroxidation (LP), determined in the form of the total amount of thiobarbituric acid (TBA)-active substances (11).

The aims of the present study were to establish whether measurement of the two enzymes and the LP activity can be used to draw conclusions as to the efficiency of various blood-preserving agents, or as to the suitability of the preserved blood for transfusion.

MATERIAL AND METHODS

At zero time, whole blood heparinized for the purpose of measurement was used; this was taken from the blood that was later

preserved. In all cases the following general procedure was followed. The coagulation-inhibited plasma and the red blood cells (RBC) were separated. The plasma was used for LP measurements. Full blood too was used to determine the total amount of TBA-active substances. The RBC were washed 2-3 times with a 2-3-fold volume of physiological saline. The washings were followed by centrifugation. The washed RBC were haemolysed with distilled water in a ratio of 1:10 (1). Aliquots of the haemolysates, diluted if necessary, were taken for enzyme determinations.

For LP measurement (total TBA-active substances), 4.5 cm^3 10% perchloric acid TBA solution saturated with mixture was added to 0.5 cm^3 plasma or haemolysate. The resulting mixture was placed in a boiling water-bath for 20 min, and after cooling and centrifugation aliquots were used for measurements. The calibration curve was prepared with the use of malondialdehyde-dithylacetal (11).

In the determination of SOD, 1 cm^3 haemolysate was added to 0.75 cm^3 ethanol + chloroform (2:1), and aliquots of the supernatant after centrifugation were used for assays. The SOD activity was determined via the enzyme quantity-dependent inhibition of the epinephrine-adrenochrome transformation (5, 8).

The C-ase activity was determined via the rate of H_2O_2 decomposition per unit time (1, 5).

In the case of preserved blood preparations, 3.0 cm^3 aliquots were taken at intervals from blood preparations stored under sterile conditions at 4-5^{o}C and were subjected to the procedure described in preserved blood, SOD activity was measured only in the RBC. It was later observed that the air entering during the frequent opening of preserved blood samples affects the experimental results. The blood to be preserved under sterile conditions was therefore initially divided into 3 cm^3 fractions, and one such fraction was utilized for enzyme activity and LP determination after the appropriate storage time at 4-5^{o}C.

Details concerning the substances added to blood preparations in Hungary may be found in (12). The conditions used did not differ from

those in the international procedures customary for the preservation of blood. The following preserved blood samples were employed; (i) citrate-phosphate-dextrose (CPD); (ii) CPD-A,G (adenine, guanine); (iii) acid-citrate-dextrose (ACD); (iv) ACD-A,G.

For CPD and CPD-A,G the ratio was 45 cm^3 preservative to 350 cm^3 whole blood, while for ACD and ACD-A,G it was 88 cm^3 preservative to 350 cm^3 whole blood. The blood samples examined originated from individuals of both sexes and from the various blood groups. The preserved blood was stored at 4-5 ^{o}C. In general, one bottle of blood was sufficient for one series of examinations (12).

All numerical data are given as means $\pm$ SEM. In the enzyme activity and LP measurements, the differences between triplicate determinations never exceeded 5%.

RESULTS

Table 1 presents the normal values for blood treated with sodium citrate to prevent coagulation. The enzyme activities and LP results are given for both the plasma and the RBC.

The characteristic dynamics of the activity changes for SOD in Figs 1 and 2 have not previously been observed.

Figures 3 and 4 compare the changes in the C-ase activities of the RBC haemolysates in the various preservation media.

Figures 5 and 6 give the corresponding data for the plasma LP, and Figs 7 and 8 those for the LP in the RBC haemolysates.

Table 1. Superoxide dismutase and catalase activities, and thiobarbituric acid-reactive products in human plasma and red blood cells (mean ± SD)

Assay	Values
SOD U/l haemol. (n=30)	716.5×10^{-3}
mean ± SD	$\pm 92.5 \times 10^{-3}$
SOD U/g prot. (n=30)	2047.1
mean ± SD	±412.2
C-ase BU/l haemol. (n=52)	2.05×10^{-3}
mean ± SD	$\pm 0.68 \times 10^{-3}$
TBA-reactive plasma products (nmol MDA/l plasma) (n=20)	10.3×10^{-3}
mean ± SD	$\pm 3.9 \times 10^{-3}$
TBA-reactive products from haemolysate (nmol MDA/l haemol.) (n=20)	67.4×10^{-3}
mean ± SD	$\pm 15.9 \times 10^{-3}$

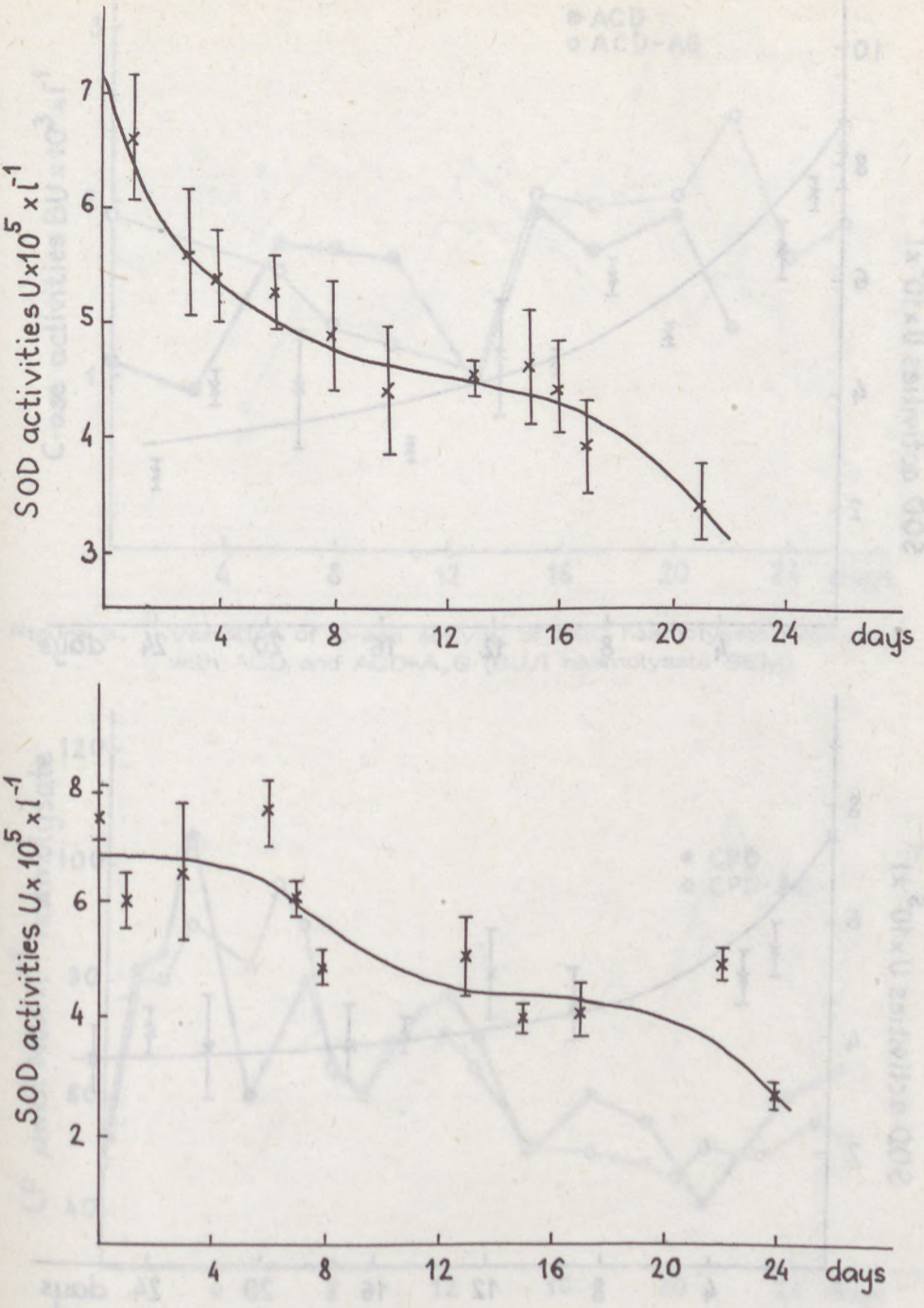

Figure 1. Variation of SOD activity of RBC preserved with CPD and CPD-A,G (U/l haemolysate ± SEM)

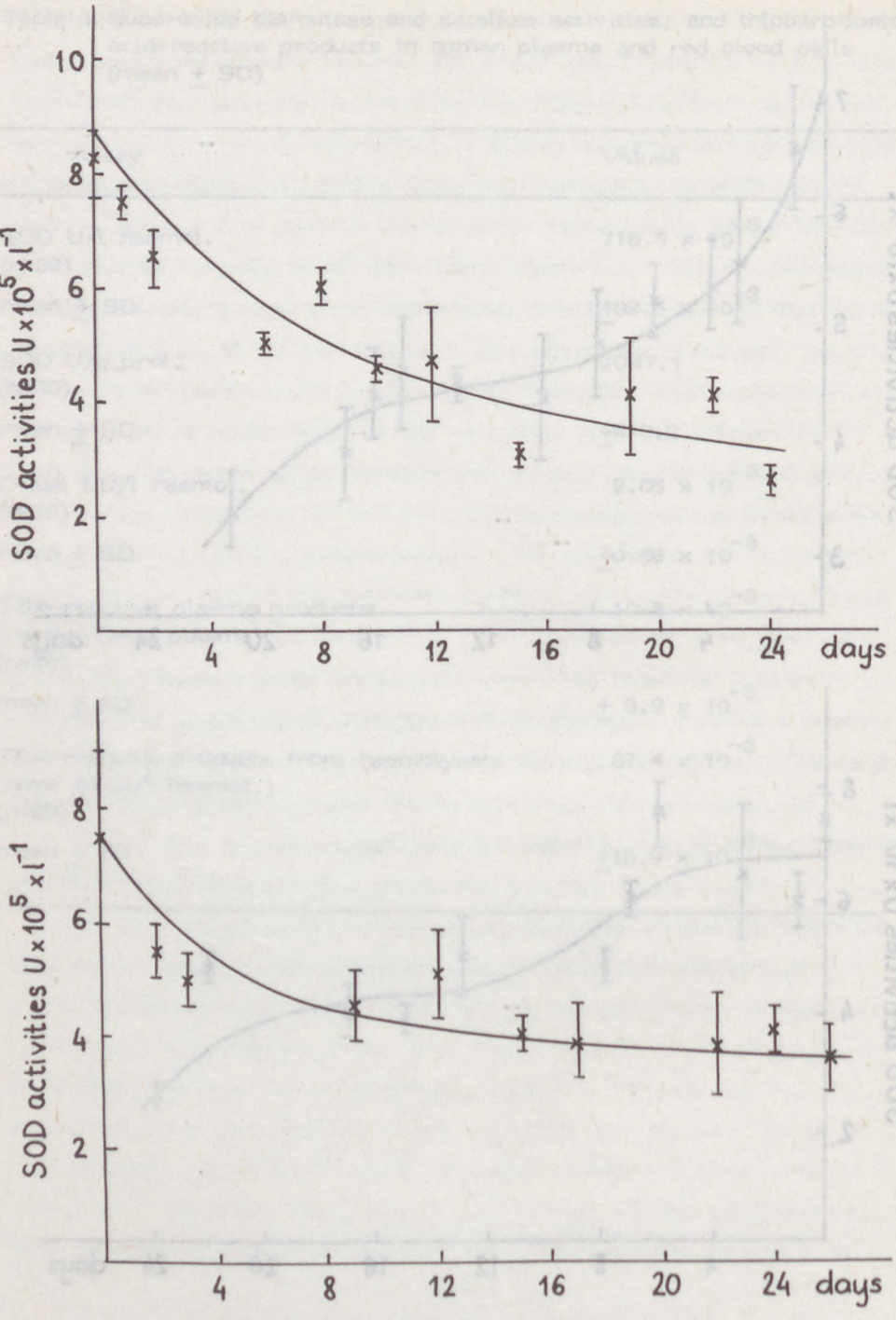

Figure 2. Variation of SOD activity of RBC preserved with ACD and ACD-A,G (U/l haemolysate ± SEM)

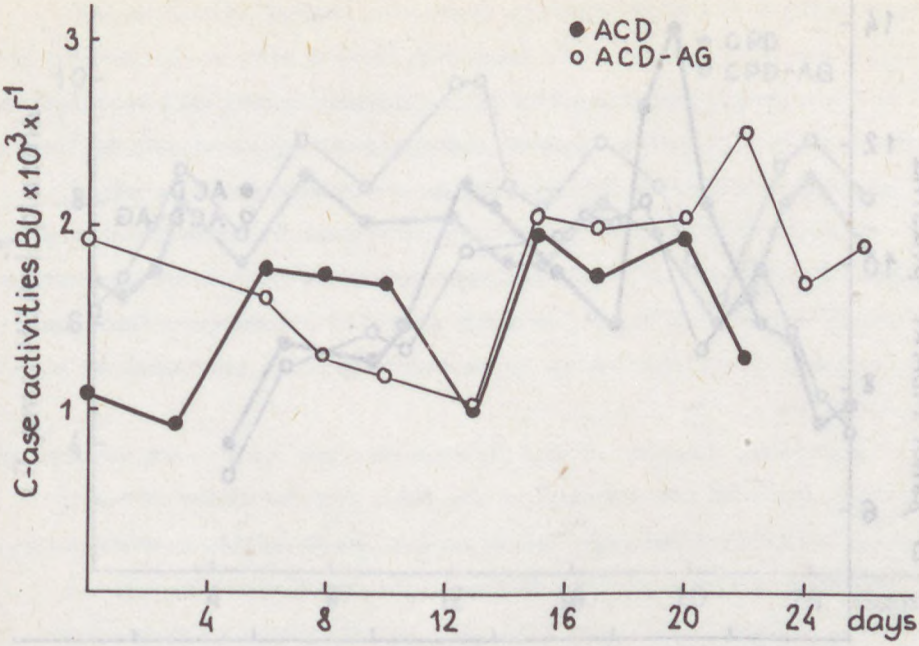

Figure 3. Variation of C-ase activity of RBC haemolysate preserved with ACD and ACD-A,G (BU/l haemolysate SEM)

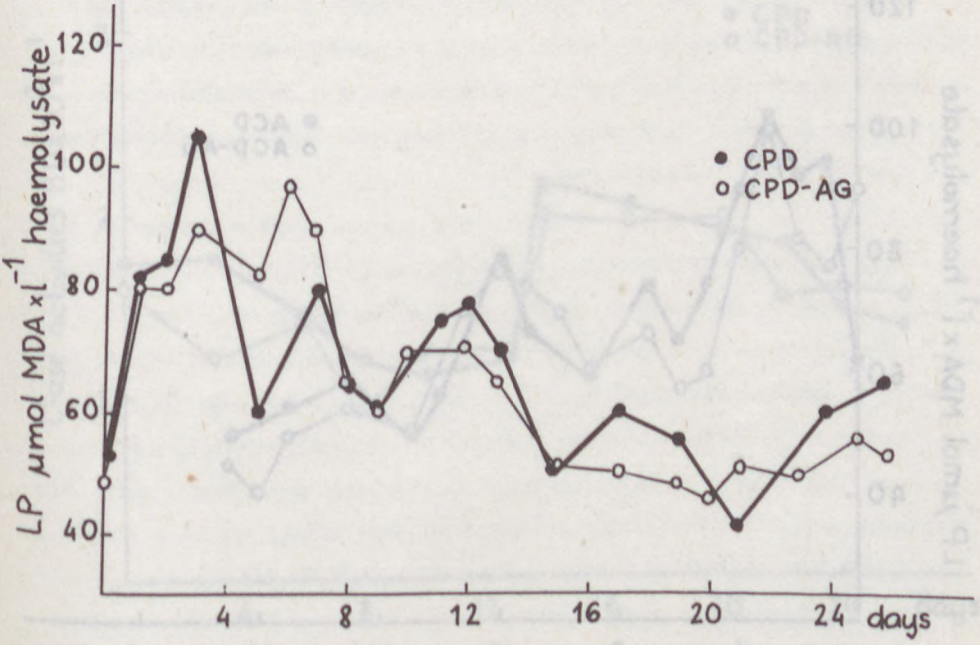

Figure 4. Variation of C-ase activity of RBC haemolysate preserved with CPD and CPD-A,G (BU/l haemolysate ± SEM)

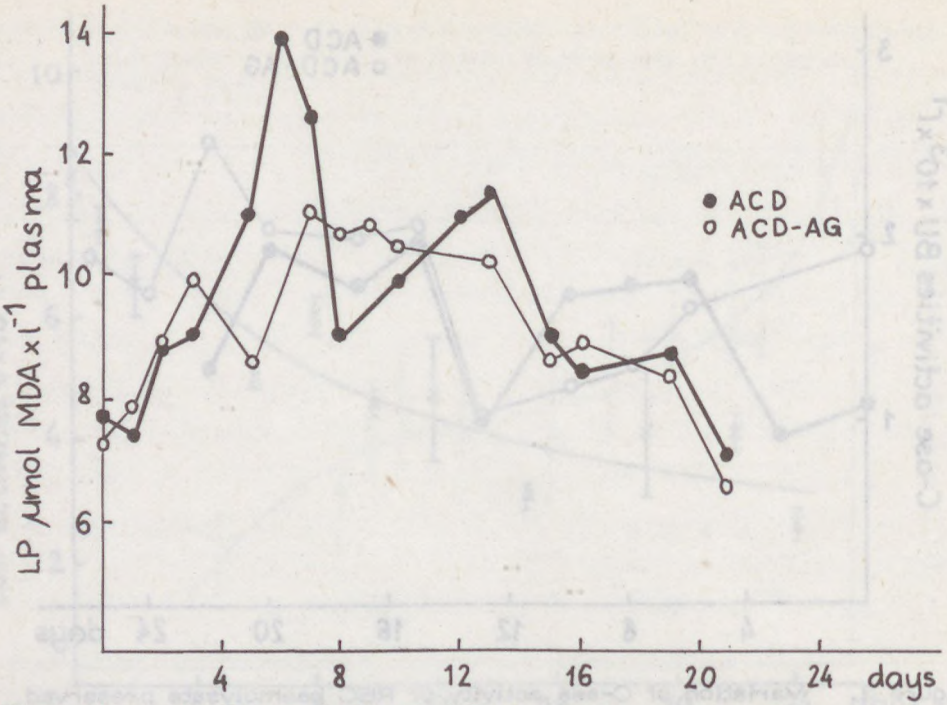

Figure 5. Variation of plasma LP of preserved with ACD and ACD-A, G (µmol MDA/l plasma)

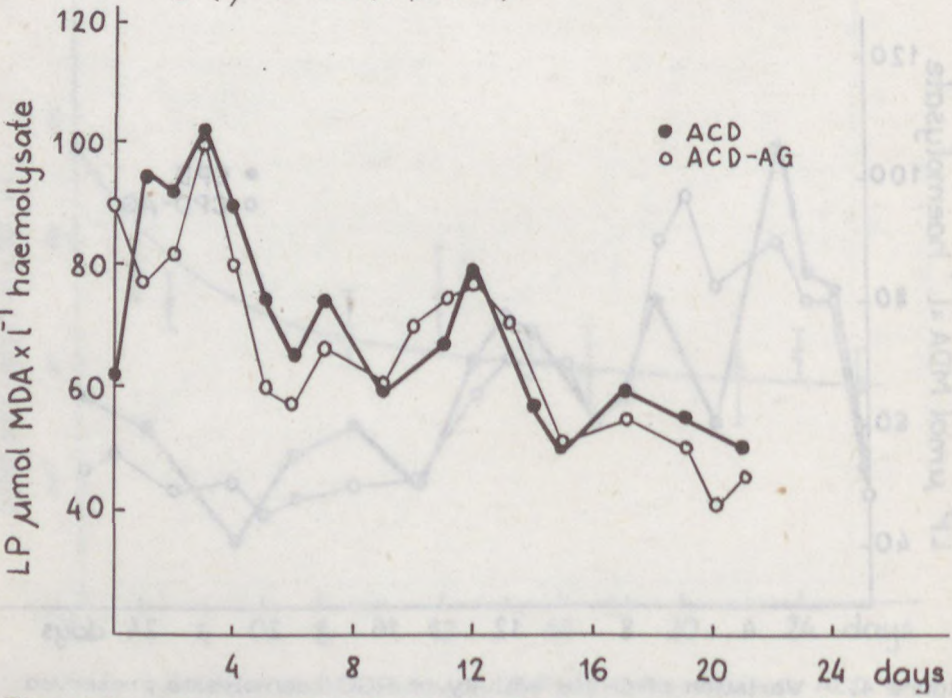

Figure 6. Variation of LP of haemolysate preserved with ACD and ACD-A,G (µmol MDA/l haemolysate)

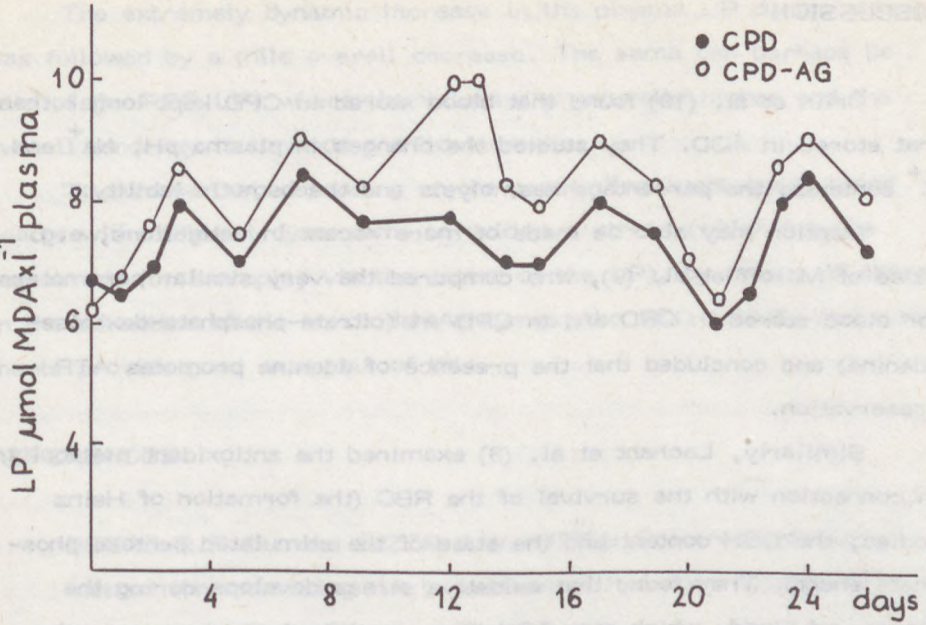

Figure 7. Variation of LP of plasma preserved with CPD and CPD-A,G (µmol MDA/l plasma)

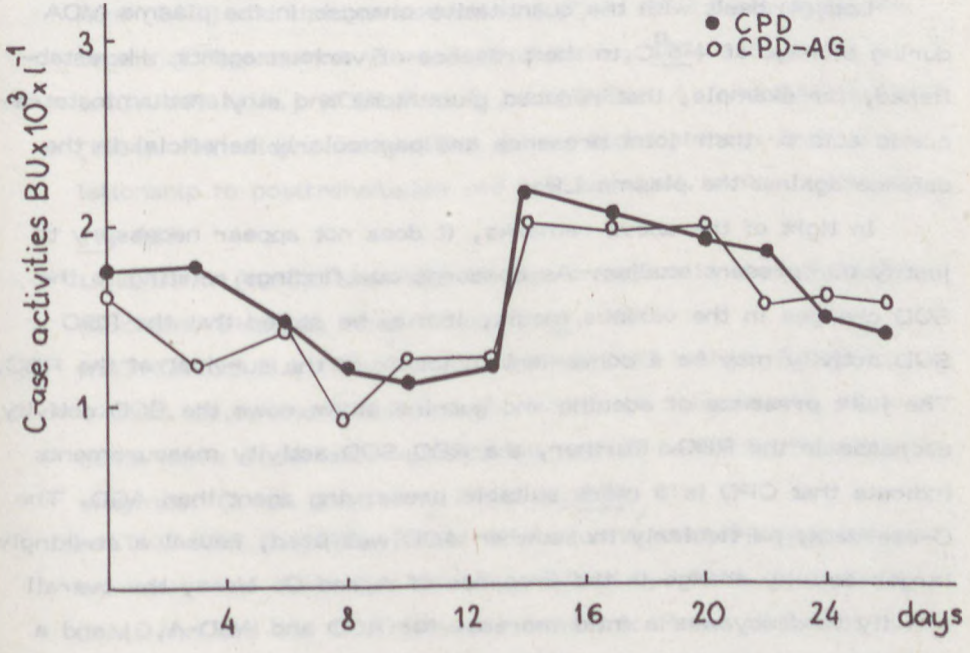

Figure 8. Variation of LP of haemolysate preserved with CPD and CPD-A,G (µmol MDA/l haemolysate)

Orlin et al. (10) found that blood stored in CPD kept longer than that stored in ACD. They studied the changes in plasma pH, Na^+ and K^+ contents, the percentage haemolysis and the osmotic lability.

Mention may also be made of more recent investigations, e.g. those of Moroff et al. (9), who compared the very similar parameters for blood stored in CPD and in CPDA-1 (citrate-phosphate-dextrose-adenine) and concluded that the presence of adenine promotes ATP preservation.

Similarly, Lachant et al. (3) examined the antioxidant metabolism in connection with the survival of the RBC (the formation of Heinz bodies, the GSH content and the state of the stimulated pentose phosphate shunt). They found that oxidative stress develops during the storage of blood, which may lead to a simultaneous decrease in the ATP content, and the defence against this oxidative stress governs the survival of the RBC in the peritransfusion period.

Lee (4) dealt with the quantitative changes in the plasma MDA during storage at 4-5°C in the presence of various agents. He established, for example, that reduced glutathione and ethylenediaminetetraacetic acid or their joint presence are particularly beneficial in the defence against the plasma LP.

In light of the above remarks, it does not appear necessary to justify our present studies. As concerns our findings relating to the SOD changes in the various media, it may be stated that the RBC SOD activity may be a convenient indicator of the survival of the RBC. The joint presence of adenine and guanine slows down the SOD activity decrease in the RBC. Further, the RBC SOD activity measurements indicate that CPD is a more suitable preserving agent than ACD. The C-ase data, particularly those when ACD was used, reveal a strikingly larger activity change in the presence of A and G. Here, the overall activity tendency was a mild increase for ACD and ACD-A,G, and a mild decrease for CPD and CPD-A,G.

The extremely dynamic increase in the plasma LP during storage was followed by a mild overall decrease. The same can perhaps be said of the RBC LP, where the values are naturally higher and the overall tendency is towards a mild increase.

To summarize, depending on the preserving agent used, blood reacts with different dynamisms to oxidative stress (which may be measured, for example, via the extent of the LP), by means of changes in the activities of the antioxidant enzymes; the SOD activity measurements provide good examples of this.

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Oxigen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

EFFECTS OF SEROTONIN RECEPTOR BLOCKERS ON THE LIPID PEROXIDATION AND ACTIVITIES OF SOME ANTIOXIDANT ENZYMES OF CHICKEN BRAIN HOMOGENATES

M. MÉZES

Department of Animal Physiology and Hygiene,
University of Agricultural Sciences,
Gödöllő, Hungary

SUMMARY

The effects of two serotonin antagonists, methysergide and pizotiphen, were investigated. Two different doses (1.0 and 1.5 mg/kg b.w. day^{-1} in the case of methysergide, and 1.0 and 2.0 mg/kg b.w. day^{-1} in the case of pizotiphen) were administered orally for a six-day period to immature female domestic fowls, and the activities of catalase and glutathione peroxidase in the whole brain homogenates. The lipid peroxidation value was measured as TBA-reactive substances. Significant differences were found in the catalase activity, but not the glutathione peroxidase activity. The effective dose was 1.0 mg for both drugs. The TBA-reactive substances measured for the 1.0 and 2.0 mg doses of pizotiphen differed significantly.

INTRODUCTION

Few data are available on the changes of lipid peroxidation and lipid peroxide metabolism enzyme activities in response to psychotropic agents (Szabó et al. 1983). It has been found that neuroleptics cause changes in the lipid peroxidation and in the activities of catalase and superoxide dismutase in brain homogenates. The oxygen free radicals can cause local neurocyte degeneration or enhance neuromelanin formation in the human brain (Das et al. 1978). Because of the relatively large quantity of unsaturated fatty acids present in the brain, the tissue is not very tolerant to peroxidative damage. The enzymes examined in this investigation protect the lipids against such damage (Matkovics et al. 1977a).

In the present work, the lipid peroxidation and the catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9) activities were measured in brain homogenates from control chickens and animals treated with the serotonin receptor blockers methysergide and pizotiphen.

MATERIALS AND METHODS

Immature female chickens aged four weeks and weighing 1020±120 g were used. They were fed with commercial food for chickens. Room temperature was 23±0.5°C and the light period was 12L:12D. The animals were sacrificed exactly 1 hour after the beginning of the light period on the day before treatment or on the 3rd or 6th day of treatment. After slaughtering, the full brain was immediately removed and frozen.

The whole brain was homogenized in ice-cold isotonic saline (0.65% w/v NaCl) in a ratio of 1:9. For the lipid peroxidation measurement, aliquots were taken from the total homogenates, while the enzyme and protein determinations were performed in the supernatants after centrifugation (10,000 g for 20 min at +4°C) of the homogenates. Protein content was measured according to Lowry et al. (1952). Catalase activity was determined by the spectrophotometric method of Beers and Sizer (1952). The enzyme activity was expressed in Bergmeyer units (BU) per gram protein content of the final supernatant. 1 BU is the amount of catalase which can decompose 1.0 g H_2O_2/min. The glutathione peroxidase activity was measured by the direct assay of Szabó (1983). The enzyme activity was expressed in units per gram protein content of the final supernatant. One unit is the amount of reduced glutathione (GSH) in nanomoles which is oxidized per minute in the system used. The lipid peroxidation was measured by the thiobarbituric acid method of Placer et al. (1966) and was expressed in terms of the malondialdehyde content, which served as standard material.

The treated chickens received methysergide (Deseril^{R}, Sandoz, Basel) orally for six days in a daily dose of 1.0 or 1.5 mg/kg b.w., or pizotiphen (Sandomigran^{R}, made under licence of Sandoz AG by Alkaloida, Tiszavasvári) in a daily dose of 1.0 or 2.0 mg/kg b.w. The control animals were treated at the same time, using a handling stress similar to that found effective in the metabolism of serotonin (Thierry et al. 1968).

RESULTS

The results are given in Tables 1 and 2. The data, which are the means and SD of five measurements in each group, led to the following findings:

- No significant change was found in the lipid peroxidation value as a result of methysergide treatment. Pizotiphen caused dose-dependent changes in the samples from day 6.

Table 1. Lipid peroxidation of chicken brain homogenates during serotonin receptor blocker treatments

		TBA-reactive substances (MDA μmol/g wet wt.)		
Day of treatment		0	3	6
Methysergide	0.0 mg	10.09±3.30	8.41±1.75	8.75±2.46
	1.0 mg	9.45±3.15	9.55±3.25	8.88±1.86
	1.5 mg	9.75±2.76	8.76±2.75	8.92±2.17
Pizotiphen	0.0 mg	8.65±2.28	8.70±3.11	11.14±3.25
	1.0 mg	8.70±2.46	9.72±3.52	13.15±2.88
	2.0 mg	8.17±2.46	7.78±1.21	8.89±1.69
Significance: Pizotiphen 1.0 mg vs 2.0 mg				$p < 0.05$

Table 2. Glutathione peroxidase and catalase activities of chicken brain homogenates

		Glutathione peroxidase (U/g protein)			Catalase (BU/g protein)		
Day of treatment		0	3	6	0	3	6
Methysergide	0.0 mg	23.44	26.20	28.42	133.40	138.40	176.80
		5.70	6.30	5.47	57.90	43.00	61.00
	1.0 mg	22.47	28.10	26.93	146.40	376.40	310.40
		3.46	2.15	6.64	58.00	41.00	41.00
	1.5 mg	22.43	25.75	26.17	142.40	176.40	85.40
		4.99	3.95	6.54	61.00	40.00	37.07
Pizotiphen	0.0 mg	36.48	46.94	30.73	142.43	153.60	159.50
		15.77	20.28	13.54	51.00	68.42	99.14
	1.0 mg	37.43	38.21	63.72	146.28	200.77	565.40
		15.48	22.03	37.54	51.09	62.13	135.40
	2.0 mg	30.17	49.23	50.15	144.18	186.03	194.19
		16.15	20.45	20.58	57.91	56.67	40.67
Significance:							
Methysergide	0.0 vs 1.0					$p < 0.01$	$p < 0.05$
	0.0 vs 1.5						$p < 0.05$
Pizotiphen	0.0 vs 1.0						$p < 0.05$

- The glutathione peroxidase activity did not change significantly in either the methysergide or the pizotiphen-treated groups as compared to the controls.

- The activity of catalase changed in the treated groups: increases were found at the dose of 1.0 mg/kg b.w. for both serotonin receptor blockers. Methysergide at the dose of 1.5 mg/kg b.w. caused a significant decrease on the sixth day of treatment.

DISCUSSION

Some measurements on glutathione peroxidase and catalase in various regions of the central nervous system have been reported in the case of rat (Brannan et al. 1980, 1981). No data are available on the enzyme activities in chicken brain. Concrete data relating to the lipid peroxidation value are not available either. As concerns the enzyme activities, some differences are to be found in the literature. Some authors suggest that the abilities of the enzymes to detoxify the peroxides in the brain appear to be insufficient (Marchena et al. 1974). Relatively high enzyme activities were found in the present investigation. A high glutathione peroxidase activity has also been found to be connected with a high MAO activity (Brannan et al. 1981). This means that the chicken brain may show a relatively high MAO activity, which was not detected during this experiment. This hypothesis was suggested by one of my previous findings, when MAO blocker treatment caused a marked decrease of brain glutathione peroxidase activity (Mézes, unpublished results). The catalase activity could change as a result of hydrogen peroxide production which was found earlier using hydrogen peroxide intake (Matkovics et al. 1977b). The changes observed this investigation suggest that the hydrogen peroxide content of the tissue changes in response to treatment. The period of six days seems to be effective for receptor blocking; some earlier observations suggest that the binding sites decrease within 48 hours when such

drugs are used (Blackshear et al. 1983). Both compounds competitively inhibit the serotonin receptors (Goodman and Gilman, 1975), and they have similar effects on the central nervous system; this similarity was found in the effects on the peroxidative metabolism as well.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

CHANGES IN LIPID PEROXIDATION AND ACTIVITIES OF SOME ANTIOXIDANT ENZYMES OF CHICKEN BRAIN HOMOGENATES DURING EARLY POSTNATAL DEVELOPMENT

M. MÉZES

Department of Animal Physiology and Hygiene,
University of Agricultural Sciences,
Gödöllő, Hungary

SUMMARY

The changes in the lipid peroxidation (TBA-reactive substances) and the activities of catalase and glutathione peroxidase in chicken brain homogenates were investigated during early postnatal development (1-35 days of age).

It was found that the rate of lipid peroxidation was nearly constant from hatching to 28 days of age and then increased significantly to the end of the investigated period. The catalase activity of brain homogenates was similar throughout the whole period, while the activity of glutathione peroxidase showed cyclical changes.

INTRODUCTION

It is a platitude for every physiologist that parameters of the internal milieu such as temperature, pH, ionic strength, etc., play very important modifying and regulatory roles in various physiological and pathophysiological processes. The tissue redox-state potential too has been found to be a regulator of such processes (Puppi and Dely, 1983). The importance of lipids and antioxidants from the aspects of the cell proliferation and cell cycle synchronization has also been studied theoretically (Polezhaev and Volkov, 1981) and experimentally (Gavino et al. 1981). The investigation of the nerve function in the central nervous system suggested a physiologically significant redox mechanism (Itokawa et al. 1972). For example, it was found that a

high amount of reduced glutathione destroys the opiate receptors in guinea-pig brain homogenates (Leslie et al. 1980).

The changes in the activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) have been studied in the skeletal muscles of chickens during early postnatal development (Mizuno, 1984).

The aim of the present study was to extend the previous results to the brain homogenates.

MATERIALS AND METHODS

Immature female chickens were used up to 35 days of age. They were fed with commercial food for chickens (starter diet). The room temperature changed in accordance with the generally applied technology, and constant illumination was used. The animals were sacrificed on the first day or at weekly intervals. After slaughtering, the full brain was immediately removed and frozen.

The whole brain was homogenized in ice-cold isotonic saline (0.65% w/v NaCl) in a ratio of 1:9. For the measurement of lipid peroxidation aliquots were taken from the total homogenates, while the enzyme and protein determinations were performed on the supernatants after centrifugation (10,000 g for 20 min at +4 ^{o}C) of the homogenates. Protein content was measured according to Lowry et al. (1951). Catalase activity was determined according to Beers and Sizer (1952). The enzyme activity was expressed in Bergmeyer units (BU) per gram protein content of the final supernatant. The glutathione peroxidase activity was measured using a direct assay, as modified by Szabó (1983). The enzyme activity was expressed in units (U) per gram protein content of the final supernatant. One unit was the amount of reduced glutathione (GSH) in nanomoles oxidized per minute in the system at 25^{o}C. The lipid peroxidation was measured by the thiobarbituric acid (TBA) method of Placer et al. (1966), and the quantity was expressed in malondialdehyde content per gram wet weight of tissue.

Significance values were calculated by Student's t test. The values were calculated between the groups as follows: day 1 vs day 7; day 7 vs day 14.

RESULTS

Table 1. Lipid peroxidation, catalase and glutathione peroxidase activities of chicken brain homogenates during early postnatal development

Age (days)	TBA-reactive substances (MDA µmol/g wet wt.)	Catalase (BU/g) 10,000 g supernatant protein	Glutathione peroxidase (U/g) protein
1	7.22±0.91	91.50±30.03	36.51±12.40
7	7.15±0.19	115.36±19.08	27.95± 9.94
14	7.87±1.18	140.91± 8.02*	43.60±22.50
21	6.02±1.29*	171.58±32.10	28.74±12.20
28	5.70±3.11	153.60±68.42	46.99±20.28
35	10.76±1.16**	122.57±40.50	21.97± 5.96*

Significance values:
* = $p < 0.05$
** = $p < 0.01$

The results are given in Table 1. The data, which are the means and SD of five determinations for each group, led to the following findings:

The lipid peroxidation decreased significantly from the 14th day of age and remained at a low level up to day 28, but then increased markedly to the end of the investigation (day 35).

The catalase activity of the chicken brain homogenates increased significantly up to day 14 and remained a relatively high level until the end of the investigation period.

The glutathione peroxidase activity displayed "cyclical" changes. It increased markedly but not significantly from the 7th to the 14th day of age, and decreased up to the next sampling time (day 21); this was followed by a non-significant increase, and then a significant decrease up to the end of the investigation.

DISCUSSION

Few data are available on the changes in the lipid peroxidation processes and the activity of the biological antioxidant defence system of chickens during early postnatal development (Marsh et al. 1981.; Mizuno, 1984). The results of the present study showed that the lipid peroxidation and the activities of the antioxidant enzymes change during postnatal development. The relatively large quantity of unsaturated fatty acids present in the brain means that the possibility of tissue lipid peroxidation becomes higher. The content of TBA-reactive substances increased up to the end of the investigation. The lipid composition may undergo changes during this period. Clarification of this problem necessitates further experiments.

The catalase activity increased gradually, which means that the brain could protect the neurocytes more effectively against free radical-initiated damage. The cyclical changes in the glutathione peroxidase activity of the brain homogenates suggested a different tissue distribution and absorption of selenium, which is a component of this metalloenzyme (Rotruck, 1973). Similar cyclical changes have been observed in the skeletal muscles of chickens (Mizuno, 1984).

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

TOTAL SOD ACTIVITIES OF ERYTHROCYTES AND LYMPHOCYTES IN CHRONIC ALCOHOLIC LIVER DISEASE BEFORE AND AFTER SILIBININ (LEGALON®) THERAPY

Gy. MÜZES, K. NÉKÁM, Gy. DEÁK, A. VERECKEI, A. ROMÁNY, I. LÁNG and J. FEHÉR

2nd Department of Medicine,
Semmelweis University of Medicine,
Budapest, Hungary

INTRODUCTION

The neutralization of free radical substances produced during the detoxification of xenobiotics and toxic materials requires a high antioxidant capacity (Hornsby and Crivello, 1983). In fact, the liver is one of the organs most protected against oxygen stress. Free radicals are presumed to play important roles in the pathogenesis of toxic and drug-induced liver diseases (Reynolds and Treinen-Moslen 1980; Fehér et al. 1982; Yoshikava and Kondo 1982; De Groot and Noll 1983; Ribiere et al. 1983; Shaw et al. 1983), and they can further increase the tissue damage seen in various forms of chronic liver diseases (Yoshikava and Kondo 1982). In order to confirm the role of oxygen stress in the pathogenesis of chronic liver diseases, we compared the total superoxide dismutase (SOD) activities of the erythrocytes and lymphocytes of patients with histologically proven fatty liver and micronodular cirrhosis with those of age- and sex-matched healthy subjects. We also evaluated the effect of in vivo treatment with the natural antioxidant flavonoid silibinin (Legalon®) on the total SOD activities of the erythrocytes and lymphocytes of the same patients following therapy for one month.

This work was supported by grants from the Hungarian Academy of Sciences and the Hungarian Ministry of Health.

PATIENTS AND METHODS

Twelve patients with chronic alcoholic liver disease were studied. The histological diagnosis was fatty degeneration or micronodular cirrhosis. Twelve age- and sex-matched healthy subjects served as controls. All patients received 210 mg of silibinin (Legalon ^{R}) daily for one month.

The SOD (E.C. 1.15.1.1) activities of the erythrocytes and lymphocytes were determined by the method of Misra and Fridovich (1972), based on the spontaneous autoxidation of epinephrine to adrenochrome in the presence of air at pH 10.2. The inhibition of this process depends on the amount of SOD. Results are given in units. One unit is the amount of enzyme causing a 50% inhibition of the autoxidation in one minute. Spectrophotometric measurements were performed at 37 ^{o}C with a Spectromom 204 instrument at 480 nm.

Erythrocyte lysates from patients and healthy individuals were prepared by adding distilled water to packed erythrocytes previously washed with isotonic saline solution. Haemoglobin was precipitated with a chloroform-ethanol mixture. The pale-yellow supernatant obtained after centrifugation was used for estimation of the specific activity of SOD (McCord and Fridovich 1969; Vanella et al. 1983).

Lymphocytes were separated from heparinized venous blood on a Ficoll-Uromiro gradient (Bøyum 1968). Phagocytic cells were removed by carbonyl-iron treatment. Viability, as judged by the trypan blue test, was higher than 98%. Monocyte contamination was less than 2%. Cells were suspended in PBS. Lymphocyte membranes were disrupted by sonication in ice-cold PBS. After centrigutation, enzyme analysis was performed on the supernatants. All data were expressed as units of total SOD per ml sample. Statistical analysis was performed by Student's t test.

RESULTS

The total SOD activities of the erythrocytes and lymphocytes of the patients and of the healthy individuals are listed in Table 1.

Table 1. Effect of treatment with silibinin on erythrocyte and lymphocyte SOD activities* of patients with chronic alcoholic liver disease.

	Before treatment		After treatment	
	ery	lym	ery	lym
Patients	61.8±15.4	38.6±6.7	109.2±22.4	78.9±14.5
Controls	153.8±21.4	105.7±21.5	-	-

*U/ml; ery: erythrocyte; lym: lymphocyte

The patients with chronic alcoholic liver disease had significantly lower erythrocyte and lymphocyte SOD activities initially than the mean values for the healthy control group. After treatment for one month with silibinin, the originally decreased SOD values of the patients were significantly increased both in the erythrocytes (Fig. 1) and in the lymphocytes (Fig. 2).

DISCUSSION

Previous findings concerning the erythrocyte SOD activity of patients with alcoholic liver disease are rather controversial. Emerit et al. (1984) found an increased erythrocyte Cu, Zn-SOD activity and macrocytosis in patients with chronic alcoholic hepatic lesions (fatty degeneration, alcoholic hepatitis and cirrhosis). They suggested that both hepatic lesions and macrocytosis might be caused by ethanol-induced free radical production, and proposed the measurement of erythrocyte Cu, Zn-SOD activity as a new marker to detect alcohol-

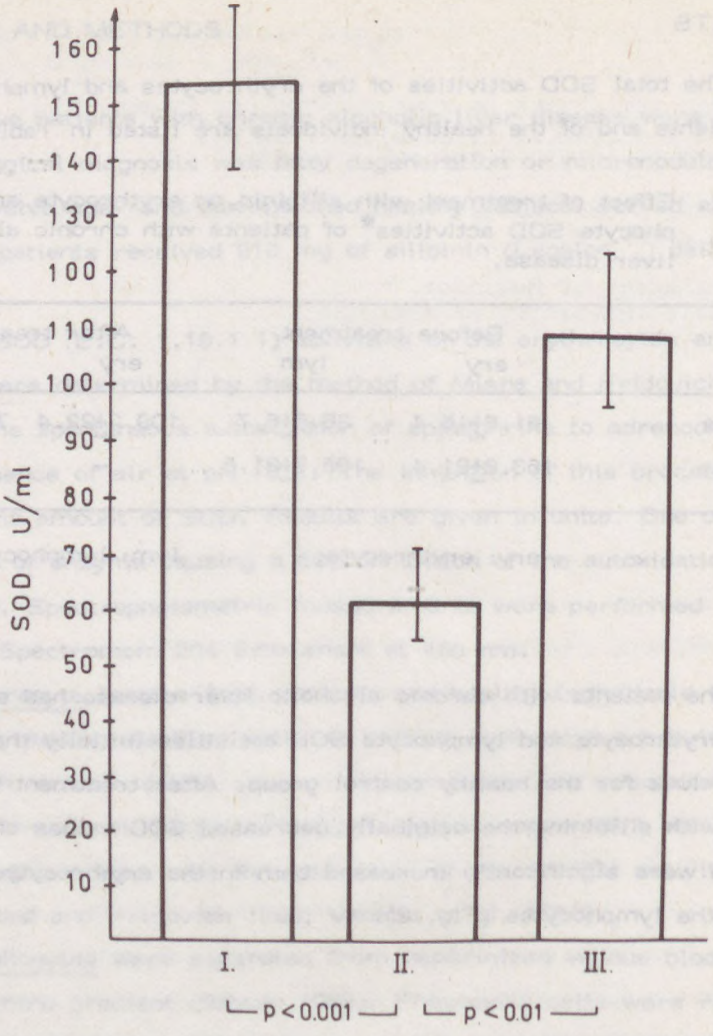

Fig. 1. Effect of silibinin on the total erythrocyte SOD activity of patients with chronic alcoholic liver disease

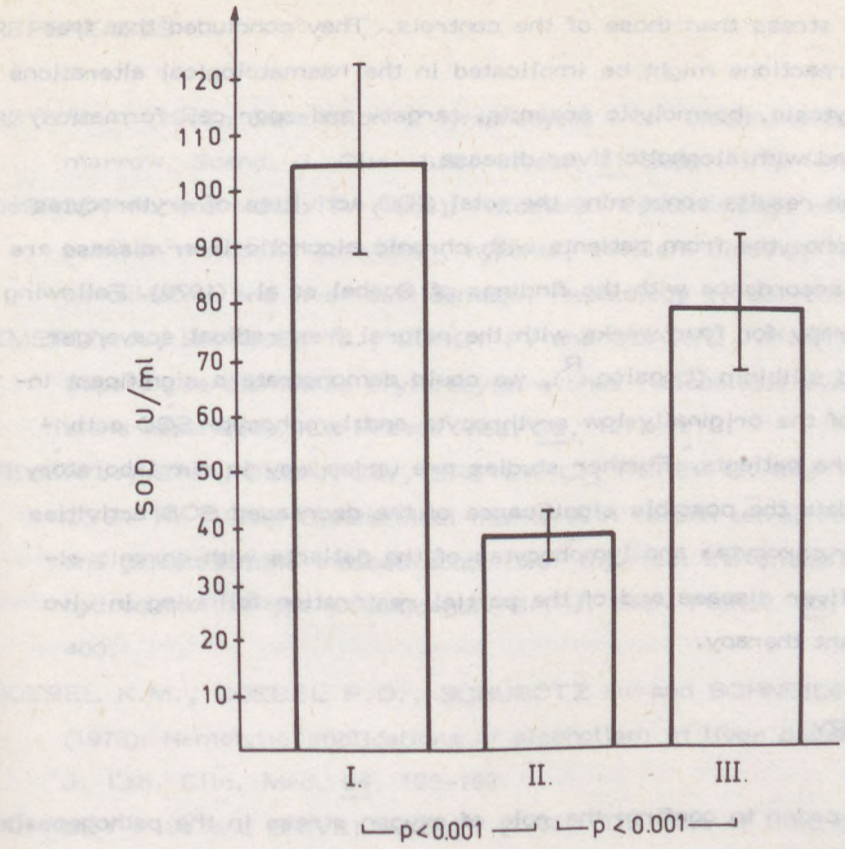

Fig. 2. Effect of silibinin on the total lymphocyte SOD activity of patients with chronic alcoholic liver disease

ism. However, Kocak-Toker et al. (1984) were unable to demonstrate any change in erythrocyte MDA production, GSH content or glutathione peroxidase activity in rats treated with ethanol in acute experiments. They suggested that the extremely efficient antioxidant defence system of the erythrocytes prevented ethanol-induced lipid peroxidation. Goebel et al. (1979) described reduced PUFA, GSH and vitamin E contents and a decreased pyruvate kinase activity in the erythrocytes of patients with Zieve syndrome. They also demonstrated a significantly lower vitamin E concentration in the sera of these patients. In vitro, they found that the erythrocytes of the patients were more susceptible

to H_2O_2 stress than those of the controls. They concluded that free radical reactions might be implicated in the haematological alterations (macrocytosis, haemolytic anaemia, target- and spur cell formation) associated with alcoholic liver disease.

Our results concerning the total SOD activities of erythrocytes and lymphocytes from patients with chronic alcoholic liver disease are in good accordance with the findings of Goebel et al. (1979). Following oral therapy for four weeks with the natural free radical scavenger flavonoid silibinin (Legalon ^{R}), we could demonstrate a significant increase of the originally low erythrocyte and lymphocyte SOD activities of the patients. Further studies are under way in our laboratory to elucidate the possible significance of the decreased SOD activities of the erythrocytes and lymphocytes of the patients with chronic alcoholic liver disease and of the partial restoration following in vivo antioxidant therapy.

SUMMARY

In order to confirm the role of oxygen stress in the pathogenesis of chronic liver diseases, the superoxide dismutase (SOD) activities of the erythrocytes and lymphocytes of patients with histologically proven alcoholic fatty liver and micronodular cirrhosis were compared with those of healthy control subjects. The effects of in vivo therapy for one month with the natural antioxidant flavonoid Legalon ^{R} on the erythrocyte and lymphocyte SOD activities of these patients were also evaluated. Patients with chronic alcoholic liver disease had significantly lower erythrocyte and lymphocyte SOD activities than the mean values for the healthy control group. After treatment for one month with silibinin, the originally decreased SOD values of the patients were significantly increased both in the erythrocytes and in the lymphocytes.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

EFFECTS OF ATROPINE AND CIMETIDINE ADMINISTERED IN CYTOPROTECTIVE AND ANTISECRETORY DOSES

Gy. MÓZSIK, B. CZEGLÉDI, G. SÜTŐ, Á. VINCZE and T. ZSOLDOS

First Department of Medicine
Department of Biology,
University Medical School,
H-7643 Pécs, Hungary

It is known that the superoxide anion is formed from molecular oxygen in the presence of some intracellular enzymes (cytochrome oxidase, NADPH-oxidizing enzyme, mixed function oxidase systems, etc.) (Tyler, 1975):

$$2\ O_2 + 2\ H^{\cdot} \xrightarrow{\text{oxidases}} 2\ O_2^{\cdot -} + 2\ H^{+}$$

The function of superoxide dismutase (SOD), a natural factor in living tissues, is to provide a defence against the potentially toxic effect of the superoxide anion generated by enzymes in cells metabolizing oxigen, as follows:

$$2\ O_2^{\cdot -} + 2\ H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2^{\cdot}$$

Following this, oxygen radical intermediates will also be produced ($^{\cdot}OH$, $^1O_2$) as a consequence of the reaction between the H_2O_2 formed and $O_2^{\cdot -}$ (Pryor, 1978):

Previously, it was shown that the development of gastric mucosal damage can be produced by the intragastric administration of 0.6 M HCl, 0.2 M NaOH, 25% NaCl or 96% ethanol (Mózsik et al. 1984a). The SOD activity significantly decreased in HCl-, NaOH- and hypertonic NaCl-induced gastric mucosal damage, while its value significantly increased in the ethanol-induced model. When atropine (in a

dose of 0.025 mg/kg), PGI_2 (5 µg/kg) or cimetidine (2.5 mg/kg) was given intragastrically 30 min before the administration of different necrotizing agents, the number and severity of the gastric mucosal lesions could be decreased significantly, in association with a decrease of the mucosal SOD activity in the ethanol-induced mucosal damage, and with increases of the mucosal SOD activity in the HCl^-, $NaOH^-$ and hypertonic NaCl-induced mucosal damage (Zsoldos et al. 1984). The changes in the membrane-bound ATP-dependent energy systems were studied at the time of development of the gastric mucosal damage produced by intragastric administration of 0.6 M HCl, 0.2 M NaOH, 25% NaCl or 96% ethanol, together with cytoprotection by atropine, PGI_2 or cimetidine (Mózsik et al. 1983a,b).

The aims of our study were:

1. to evaluate the changes in the gastric mucosal SOD activities in different models (HCl and ethanol-induced gastric mucosal damage) following cytoprotective and antisecretory doses of atropine and cimetidine;

2. to evaluate the changes in the membrane-bound ATP-dependent energy systems in these models;

3. to find some correlation(s) between the changes in the gastric mucosal SOD activity and the gastric mucosal ATP-dependent energy systems; and

4. to find some correlation(s) between the effects of atropine and cimetidine (given in cytoprotective and antisecretory doses) on the mucosal SOD activity and on the membrane-dependent energy systems.

MATERIALS AND METHODS

The observations were made on CFY rats of both sexes, weighing 180 to 210 g. The animals were fasted for 24 hours, but they received water and libitum.

Gastric mucosal damage was produced by the intragastric administration of 96% ethanol or 0.6 M HCl (1 ml). The animals were

killed one hour later, and the number and severity of gastric mucosal lesions (ulcers) were recorded.

The gastric mucosal SOD (EC.1.15.1.1) activity was measured by the method of Misra and Fridovich (1972), as modified by Matkovics et al. (1977). The protein content was measured by the method of Lowry et al. (1951). The gastric mucosal contents of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and lactate were measured enzymatically (Boehringer, Ingelheim, FRG), and tissue levels of cyclic AMP (cAMP) by RIA. The results were expressed in mmol/mg protein (lactate), nanomol/mg protein (ATP, ADP and AMP) or picomol/mg protein (cAMP). The results were expressed as percentages (means $\pm$ SEM) of the results obtained on the administration of 96% ethanol or 0.6 M HCl (= 100 per cent).

The unpaired Student's t test was used for statistical analysis of the results (between the groups relating to the presence of 96% ethanol or 0.6 M HCl and the atropine or cimetidine-treated groups).

RESULTS

The tissue levels of ATP, cAMP and AMP decreased significantly, while that of ADP increased at the time of development of HCl^-, $NaOH^-$ concentrated $NaCl^-$ or 96% ethanol-induced gastric mucosal lesions (Mózsik et al. 1983a). The biochemical changes in the gastric mucosal damage produced by the different necrotizing agents showed similarities in all models. The gastric mucosal SOD activity increased significantly during the development of ethanol-induced gastric mucosal damage, while its activity decreased significantly in the HCl^-, $NaOH^-$ or hypertonic NaCl-induced gastric mucosal lesions (Mózsik et al. 1984a, 1986b).

Tables 1 to 4 indicate that atropine and cimetidine, applied in cytoprotective and antisecretory doses, decrease the gastric mucosal SOD activity in ethanol-induced gastric damage, while they increase

Table 1. Correlations between the ulcer-preventive effect of atropine and the gastric mucosal superoxide dismutase (SOD) activity in ethanol-induced gastric mucosal damage in rats. Atropine was given intraperitoneally 30 min before the administration of ethanol. The results are presented as percentage (means + SEM) of the results for the ethanol-treated group (= 100 per cent). The statistical analysis was performed between the results for the ethanol-treated group versus the atropine-treated groups.

Groups of animals	n	Number of gastric mucosal lesions	Severity (sum)	SOD activity (IU/mg prot.)
Ethanol-treated only	10	100 ± 14	100 ± 7	100 ± 3
Ethanol + atropine (0.025 mg/kg)	10	57 ± 14^{+}	53 ± 4^{+++}	83 ± 4^{++}
Ethanol + atropine (0.2 mg/kg)	10	50 ± 7^{++}	37 ± 3^{+++}	18 ± 1^{+++}
Ethanol + atropine (1.0 mg/kg)	10	43 ± 7^{++}	35 ± 3^{+++}	20 ± 3^{+++}

+ = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

Table 2. Correlations between the ulcer-preventive effect of atropine and the gastric mucosal superoxide dismutase (SOD) activity in HCl-induced gastric mucosal damage in rats. Atropine was given intraperitoneally 30 min before the administration of 0.6 M HCl. The results are presented as percentages (means + SEM) of the results HCl-treated group (= 100 per cent). The statistical analysis was performed between the results for the HCl-treated group vs. the atropine-treated groups.

Groups of animals	n	Number of gastric mucosal lesions	Severity (sum)	SOD activity (IU/mg prot.)
HCl-treated only	10	100 ± 13	100 ± 7	100 ± 8
HCl + atropine (0.025 mg/kg)	10	20 ± 7^{+++}	21 + 2^{+++}	1015 ±84^{+++}
HCl + atropine (0.2 mg/kg)	10	53 ± 7^{+++}	32 ± 4^{+++}	1169 ±77^{+++}
HCl + atropine (1.0 mg/kg)	10	53 ± 7^{+++}	16 ± 4^{+++}	1400 ±150^{+++}

+ = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

Table 3. Correlations between the ulcer-preventive effect of cimetidine and the gastric mucosal superoxide dismutase (SOD) activity in ethanol-induced gastric mucosal damage in rats. Cimetidine was given intragastrically 30 min before the administration of ethanol. The results are presented as percentages (means + SEM) of the results for the ethanol-treated group (= 100 per cent). The statistical analysis was performed between the results for the ethanol-treated group versus the cimetidine-treated groups.

Groups of animals	n	Number of gastric mucosal lesions	Severity (sum)	SOD activity (IU/mg prot.)
Ethanol-treated only	10	100 ± 13	100 ± 7	100 ± 3
Ethanol + cimetidine (2.5 mg/kg)	10	43 ± 7^{+++}	58 ± 3^{+++}	44 ± 5^{+++}
Ethanol + cimetidine (10 mg/kg)	10	35 ± 7^{+++}	30 ± 2^{+++}	38 ± 3^{+++}
Ethanol + cimetidine (50 mg/kg)	10	20 ± 7^{+++}	33 ± 8^{+++}	35 ± 3^{+++}

$^{+++}$ = $p < 0.001$

Table 4. Correlations between the ulcer-preventive effect of cimetidine and the gastric mucosal superoxide dismutase (SOD) in HCl-induced gastric mucosal damage in rats. Cimetidine was given intraperitoneally 30 min before the administration of 0.6 M HCl. The results are presented as percentages (means + SEM) of the results for the HCl-treated group (= 100 per cent). The statistical analysis was performed between the results for the HCl-treated group versus the cimetidine-treated groups.

Groups of animals	n	Number of gastric mucosal lesions	Severity (sum)	SOD activity (IU/mg prot.)
HCl-treated only	10	100 ± 13	100 ± 7	100 ± 8
HCl + cimetidine (2.5 mg/kg)	10	33 ± 7^{+++}	28 ± 3^{+++}	1230 ±77^{+++}
HCl + cimetidine (10 mg/kg)	10	27 ± 7^{+++}	24 ± 2^{+++}	1307 ±77^{+++}
HCl+ cimetidine (50 mg/kg)	10	20 ± 7^{+++}	17 ± 3^{+++}	1385 ±77^{+++}

$^{+++}$ = $p < 0.001$

Table 5. Atropine-induced biochemical changes in the gastric mucosa in HCl-induced gastric mucosal damage. The results are presented as percentages (means + SEM) of the values for the HCl-treated group (= 100 per cent). Atropine was given intraperitoneally 30 min before the administration of 0.6 M HCl. The statistical analysis was performed between the results for the HCl-treated group versus the group versus the atropine-treated groups.

Examined biochemical parameters	Groups of animals			
	treated with 0.6 M HCl only (n=10)	treated with atropine 0.025 mg/kg (n = 10)	0.2 mg/kg (n = 10)	1.0 mg/kg (n = 10)
ATP	100 ± 12	132 ± 12^{++}	132 ± 6^{++}	132 ± 19
ADP	100 ± 7	45 ± 10^{+++}	47 ± 9^{+++}	48 ± 9^{+++}
AMP	100 ± 11	96 ± 8	146 ±13^{+}	169 ± 19^{++}
cAMP	100 ± 2	280 ± 5^{+++}	335 ±35^{+++}	450 ± 60^{+++}
Lactate	100 ± 10	62 ± 16^{+}	74 ± 8	68 ± 9

+ = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$

the gastric mucosal SOD activity in the 0.6 M HCl-induced gastric mucosal lesions. It was surprising that atropine and cimetidine act in similar ways in a given model, but in opposite ways in the two models examined. On the other hand, no significant difference was observed in the atropine- and cimetidine-induced gastric mucosal SOD activities.

Table 5 shows the atropine-induced biochemical changes in the membrane-bound ATP-dependent energy systems and in the lactate of the gastric mucosa in the HCl model. It is to be observed that the ATP-cAMP transformation is stimulated, while the ATP-ADP transformation is inhibited, by the administration of atropine. These changes showed a close dose-response correlation. No significant change was found in the lactate level.

After the administration of cimetidine, the tissue level of cAMP was unchanged, the AMP level was decreased, and the ATP-ADP transformation was increased (Moron et al. 1984).

DISCUSSION

In previous studies, a similarity in the biochemical alternations was found during the development of gastric mucosal lesions produced by different necrotizing agents (Mózsik et al. 1983a), while the gastric mucosal SOD activity changed in significantly different ways in these models (Mózsik et al. 1984a): its activity decreased in HCl-, NaOH- and hypertonic NaCl-induced gastric mucosal damage and increased in the ethanol-induced model. Atropine, cimetidine and PGI_2 (applied in cytoprotective doses) decreased the ethanol-induced increase of gastric mucosal SOD activity, and increased its activity produced by the application of 0.6 M HCl (Zsoldos et al. 1984), in association with the development of a gastric cytoprotective effect. Vitamin A and β-carotene prevented the ethanol and 0.6 M HCl-induced gastric mucosal damage, but the SOD activity was unchanges in the HCl-induced model (Mózsik et al. 1984b).

The results presented here indicate a slight dose-response curve for the gastric mucosal SOD activity when atropine and cimetidine were given in cytoprotective and antisecretory doses. The cytoprotective dose is 0.025 mg/kg for atropine (Mózsik et al. 1983b), and 2.5 mg/kg for cimetidine (Moron et al. 1983).

The results in Table 5 and other observations (Moron et al. 1981 , 1984; Mózsik et al. 1983b) indicated that the biochemical backgrounds of the development of atropine and cimetidine-induced cytoprotection differ significantly (Mózsik et al. 1983b), while that of PGI_2 is similar in the different models (Mózsik et al. 1983a). The ulcer prevention induced by atropine and cimetidine in cytoprotective and antisecretory doses showed close dose-response correlations, as did the membrane-bound ATP-dependent energy systems (Morón et al. 1984; Mózsik et al. 1986a). Additionally, atropine and cimetidine produced opposite changes in the gastric mucosal SOD activities in ethanol and HCl-induced mucosal damage.

It has been concluded that:

1. no close correlation exists between the changes in gastric mucosal SOD activity and in the membrane-bound ATP-dependent energy systems, as concerns either the development of gastric mucosal damage produced by different necrotizing agents, or its prevention with atropine, cimetidine, PGI_2, vitamin A or β-carotene;

2. the free radical mechanisms represent only one type of the different cellular parameters involved the development of gastric mucosal lesions and their prevention; and

3. the changes in the membrane-bound ATP-dependent energy systems are more sensitive experimental parameters than those in the free radical mechanisms.

These results are in good agreement with other observations (Mózsik et al. 1985, 1986b).

The lack of correlation between the changes in the membrane-bound ATP-dependent energy systems and in the free radical mechanisms can be explained in different ways:

1. the time of our observations (1 hour after the administration of necrotizing agents) is not the optimal time to study this correlation;

2. there is really no close correlation between the membrane-bound ATP-dependent energy systems and the free radical mechanisms relating to the development of gastric mucosal damage and its prevention.

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Oxygen Free Radicals and the Tissue Ijury
B. Matkovics, D. Boda and H. Kalász (Eds)

CLINICAL SIGNIFICANCE OF OXIDATIVE PROCESSES IN HUMAN GRANULOCYTES

J.T. NAGY, T. FÜLÖP Jr., G. PARAGH and G. FÓRIS

First Department of Medicine,
University Medical School,
Debrecen, Hungary

SUMMARY

The reactive oxygen species (ROS) production by human resting granulocytes and the "respiratory burst" (RB) after stimulation were studied in healthy subjects as well as in patients with different disorders. It was found that the O_2 consumption, and O_2^- and H_2O_2 generation by the resting cells were increased with aging, in different autoimmune disorders and in type 2 diabetes mellitus (DM). After stimulation through the Fc receptors, the RB was lower in all of the studied diseases as well as in the aged subjects, whereas granulocytes obtained from patients with autoimmune disorders did not change in reactivity after stimulation through the cholinergic receptors or directly by elevation of the intracellular free Ca^{2+} and protein kinase C (PKC) activity. These results suggest an impaired function of the Fc receptors only in autoimmune disorders (immune thyroiditis, glomerulonephritis and autoimmune DM). After stimulation through the cholinergic receptors by carbachol, the ROS generation was decreased in both the aged subjects and the patients with type 2 DM, suggesting a disturbance of post-receptorial coupling of some receptors which are negatively coupled to adenylate cyclase. Both A23187 and phorbol myristate acetate (PMA), which affect the granulocytes directly, were able to produce RB, except for cells obtained from patients with type 2 DM. Therefore, it was concluded that in granulocytes of type 2 DM, impairment of both Ca/calmodulin and PKC may play some role in the disturbed functions of the phagocytic cells.

INTRODUCTION

The ROS-generating system of granulocytes plays an important role in the host resistance against invading microorganisms, but it has a dangerous effect in autoimmune diseases and inflammatory pro-

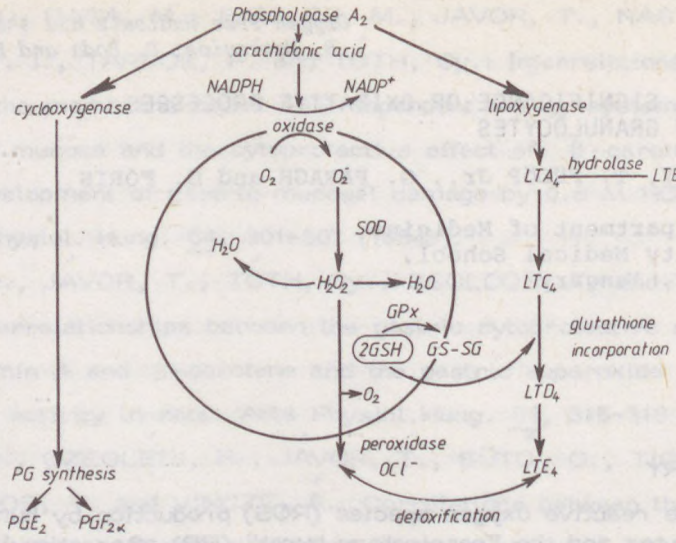

Fig. 1. The mechanism of oxidative processes in phagocytic cells. The relationship between ROS generation and lipid peroxidation

cesses, damaging different organs (1-4). The key enzyme of the ROS is the HMP shunt-dependent NADPH oxidase.

A product of this complicated enzyme system is superoxide (O_2^-), which is converted to H_2O_2 by superoxide dismutase (SOD). Further detoxification of H_2O_2 in granulocytes is carried out by glutathione peroxidase (GPx) with a concomitant oxidation of the reduced glutathione (GSH). At the end of this cycle, glutathione reductase (GR) reduces the oxidized form of glutathione (GS-SG). Intracellular H_2O_2 is able to penetrate the membrane and in the extracellular space OCl^- is generated by myeloperoxidases. The ROS are responsible for two important effector functions of granulocytes: intracellular killing activity and extracellular cytotoxicity. Therefore, in the past ten years, growing interest in the oxidative processes of phagocytic cells has resulted in a better understanding of the non-specific immunological processes. Despite the intensive investigations there is no direct evidence that, except for some enzyme defects (CGD, G6-P deficiency and myeloperoxidase deficiency), a correlation exists between the

measurable decrease of the ROS products and the incidence of infectious illnesses.

The activity of NADPH oxidase is triggered by the intracellular levels of cGMP and arachidonic acid (5). It is therefore easy to understand that an elevation of phospholipase A_2 (which is an integral part of the Fc receptor) during Fc receptor-mediated phagocytosis has a stimulatory effect on the NADPH oxidase (6). All the ligands processing their post-receptor effects through the cleavage of inositol triphosphate (chemotactic peptides, leukotriene B_4, lymphokines, cholinergic agonists and neuropeptides) are also able to initiate the ROS (7-10). On the other hand, all the ligands activating adenylate cyclase have an inhibitory effect on the RB (11).

An interesting possibility is provided by ligands which act through the cleavage of phosphatidyl inositol to activate not only the ROS-generating system, but also the arachidonic acid cascade (12). While the extremely toxic leukotriene C_4 and D_4 are detoxified almost solely by the ROS product OCl^-, it may be presumed that the effect of each system could be neutralized by the other (13). Although there is no evidence of a direct correlation between one or more measurable parameters of the ROS and the susceptibility to infectious diseases, there is no doubt that in certain pathological conditions not affecting the non-specific immune system directly, the resistance of the organism is decreased.

In our laboratory the O_2 consumption, the O_2^- and H_2O_2 production, the GPx and GR activities and the intracellular levels of GSH and GS-SG were determined in granulocytes obtained from young and aged healthy subjects, and from patients suffering from autoimmune thyroiditis, glomerulonephritis, type 1 DM or type 2 DM. The determinations were carried out in both resting and stimulated cells. For stimulation, we applied ligands which can stimulate cells through specific receptors, or drugs which directly stimulate the calmodulin system (14) or PKC (15). In this way we were able to elucidate the receptor activity and the post-receptorial impairment of signal transmission in different disorders.

METHODS

Patients: As part of a large screening program involving the inhabitants of a retirement home, 20 healthy elderly males (aged 60-87 years) and 20 healthy elderly females (aged 60-90 years) were selected on the basis of very careful physical and mental examinations, confirmed by some routine biochemical, biological and radiological tests. Ten young males (aged 20-25 years) and 10 young females (aged 20-25 years), all medical students, served as voluntary controls.

Patients with autoimmune thyroiditis (17 females, aged 17-37 years) in an active state were diagnosed on the basis of the clinical signs and the fine needle biopsy findings.

Patients with type 1 DM (8 females, aged 24-35 years) were selected on the basis of the elevated serum glucose concentration, no signs of secondary diabetes, and insulin dependence.

Patients with type 2 DM (7 subjects, aged 58-65 years) averaged 128% of the ideal body weight. They had a mean fasting blood glucose of 202 mg/dl. None of the subjects were taking any regular medication, including oral hypoglycaemic drugs and insulin.

Granulocytes were separated from the blood by the method of Böyum (16). O_2 consumption was measured with a Clark electrode by the method of Tanabe (17). O_2^- generation was determined by the method of Cohen and Chovaniek (18). H_2O_2 production by granulocytes was detected as described by Pick and Keisari (19). Glutathione peroxidase activity was measured by the method of Paglia and Valentine (20). Glutathione reductase activity was determined according to the method of Beutler (21). The reduced and oxidized forms of intracellular glutathione were determined by the method of Hissin and Hilf (22).

Experimental conditions: Suspensions of granulocytes were made in RPMI 1640 medium (Gibco) in Nunc tubes of different sizes. All incubations were carried out in an ASSAB CO_2 Incubator (CO_2 5%, air 95%, humidity 95%) at 37°C. For phagocytosis, heat-killed and pre-

opsonized yeast cells were applied at a 1:20 effector:target ratio. Carbachol was used at a concentration of 10^{-6} M, A 23187 (Calbiochem) at 10^{-6} M, and PMA (Sigma) at 10^{-6} M.

RESULTS AND DISCUSSION

Parameters of the oxidative processes in resting granulocytes obtained from patients with different disorders are demonstrated in Fig. 2. It seems to be important that, not only in the different illnesses, but also in the healthy aged subjects, a trend to a virtual activation of ROS generation was detected. Significant increases appeared in the oxygen consumption, the O_2^- and H_2O_2 production, and the luminol-dependent chemiluminescence of the resting granulocytes in aged subjects, in different autoimmune disorders and in cases of insulin-resistant type 2 DM. Marked decrements of GPx activity were measured in

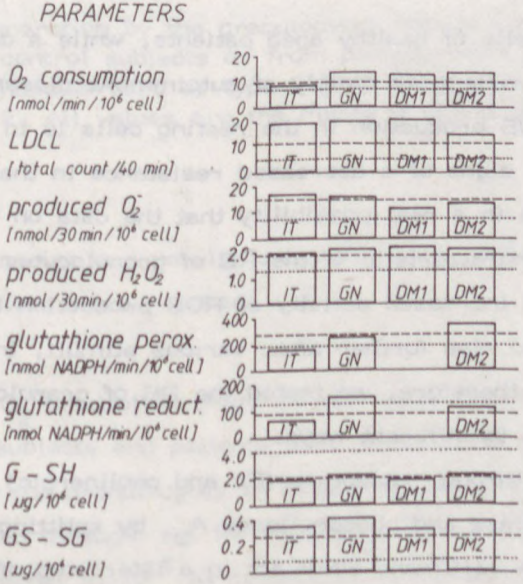

Fig. 2. Parameters of oxidative processes in granulocytes. ..... control healthy young subjects; ----- control healthy aged subjects. IT = immune thyroiditis, GN = glomerulonephritis, DM 1 = type 1 DM, DM 2 = type 2 DM

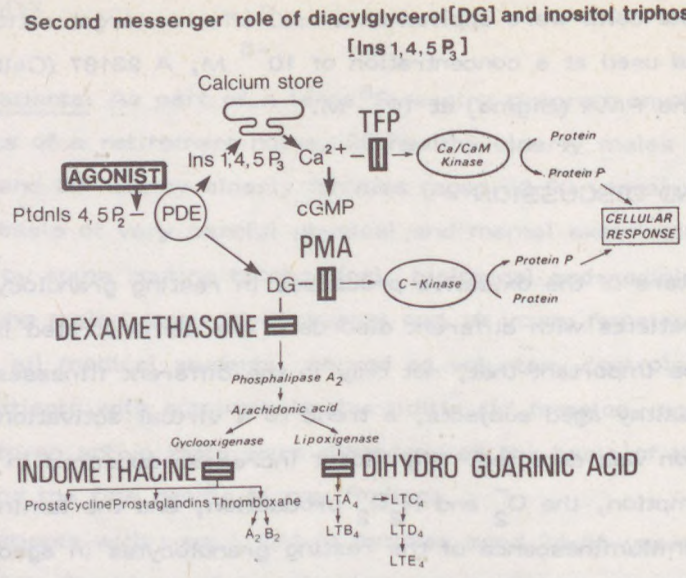

Fig. 3. Crucial points useful according to the Berridge scheme for the inhibition of activation

the phagocytic cells of healthy aged patients, while a decreased GR activity was characteristic mainly of autoimmune diseases. This enhancement of ROS production in the resting cells is in violent contrast with the clinical signs of a decreased resistance in these illnesses. Therefore, there is a real possibility that the data on the resting cells are not really characteristic of the RB of granulocytes. It is also presumable that the increased activity of ROS production in the resting cells is unable to rise further after various stimuli. In the next series of experiments, therefore, we tested the RB of granulocytes by stimulating them in different ways.

Activation through receptors (Fc and cholinergic) is carried out by guanylate cyclase and phospholipase A_2, by splitting phosphatidyl inositol, while A 23187 and PMA act in a later step of the enzyme cascade (Fig. 3). The Ca ionophore A 23187 activates the Ca/calmodulin system, while PMA, as a stable derivative of diacylglycerol, has a direct effect on PKC.

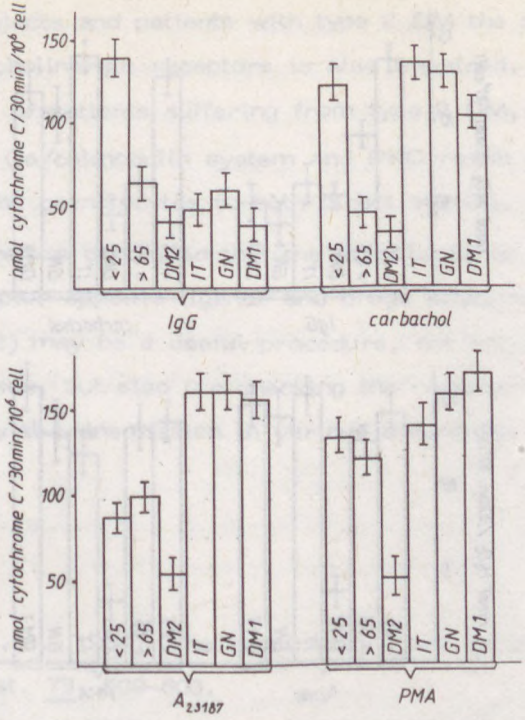

Fig. 4. O_2^- generation by the granulocytes obtained from young and aged control subjects or from patients with different autoimmune disorders or type 2 DM. For abbreviations, see Fig. 2. All values are the means ± SD of the determinations

To summarize our results using this stimulatory system, the following conclusions were reached:

1. The O_2^- production (Fig. 4) was lower by the granulocytes of healthy aged subjects and patients with autoimmune disorders or type 2 DM than by the granulocytes of healthy young controls, if the cells were stimulated through the Fc receptors during yeast cell phagocytosis.

2. Carbachol in 10^{-6} M concentration markedly stimulated the granulocytes obtained from patients with autoimmune diseases, whereas the reaction of granulocytes obtained from healthy aged subjects with or without DM was markedly diminished.

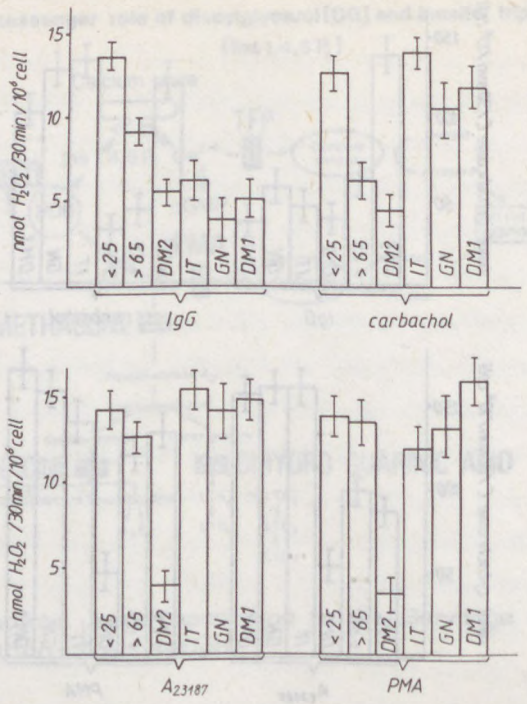

Fig. 5. H_2O_2 generation by the granulocytes obtained from young and aged control subjects or from patients with different autoimmune disorders or type 2 DM. For abbreviations, see Fig. 2. For footnotes, see Fig. 4

3. The Ca ionophore A 23187, which elevates the intracellular free Ca^{2+} level in the granulocytes, and PMA in 10^{-6} M concentration, which stimulates PKC directly, triggered the O_2^- generation of the granulocytes, if the cells were obtained from healthy young or aged subjects or from patients with autoimmune disorders. It is noteworthy that the granulocytes of patients with type 2 DM were unresponsive to both drugs.

In an identical system, the H_2O_2 generation was similar to the O_2^- production after various stimuli (Fig. 5) in different patients.

Our results suggest that the RB of granulocytes induced through the Fc receptors during yeast cell phagocytosis is markedly impaired in various autoimmune disorders, whereas in the granulocytes of

healthy aged subjects and patients with type 2 DM the post-receptorial coupling of the cholinergic receptors is also impaired. In addition, in the granulocytes of patients suffering from type 2 DM, the disturbed activities of the Ca/calmodulin system and PKC result in an unresponsiveness of the granulocytes to both direct stimuli.

It was therefore concluded that the stimulation of ROS generation by applying receptor-specific ligands and drugs affecting the phagocytic cells directly (12) may be a useful procedure, not only for studying oxidative processes, but also for checking the receptor coupling and the disturbed signal transmission in various disorders.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

THE ROLE OF OXYGEN FREE RADICALS IN CELLULAR AGING: A REVIEW

I. Zs.-NAGY

F. Verzár International Laboratory for Experimental Gerontology (VILEG),
Hungarian Section, University Medical School,
H-4012 Debrecen, Hungary

ABSTRACT

The main characteristics of the oxygen free radicals and the mechanisms of their formation in vivo are summarized. Natural defense mechanisms like the superoxide dismutase, catalase and glutathione-peroxidase help the living systems to avoid the damaging effects of those radicals. Emphasis will be given to the role of $OH^{\cdot}$ free radicals which react so fast with the organic compounds that no enzymatic protective mechanism can really be efficient against them, therefore, the living systems are forced to replace the damaged macromolecules continuously. It is shown that due to their extremely high reactivity, the $OH^{\cdot}$ free radicals damage amino acids, proteins and lipids and the consequences of this damage depend on the actual composition of the molecular environment: in the relatively compact membrane structures the damage manifests itself more frequently as newly formed intermolecular bonds, whereas in the highly diluted cytosol it results in only intramolecular changes. This is the reason why the cell membrane damage represents the rate limiting factor in the aging process as predicted by the membrane hypothesis of aging. As a matter of fact, the cell membrane proteins display the highest turnover rate among the cell components. The free-radical induced damage of the cell membrane components results in an increasing loss of the membrane permeability for potassium (and probably for water). These permeability losses lead to an age-dependent condensation of the intracellular and intranuclear colloids reducing this way all the enzyme activities including those performing the RNA and protein synthesis. The membrane hypothesis of aging explains the effect of the oxygen free radicals in the living cells and outlines a reasonable physiological mechanism of general validity for the experimental gerontology. This concept has been checked also by some dietary (Vitamin E deficient diet in rats) and pharmacological interventions (treatment with centrophenoxine being a $OH^{\cdot}$ radical scavenger) and a full support was found in those experimental models for the brain cells.

INTRODUCTION

The goal of any gerontological research should be to find the explanation for the generally recognized main characteristics of the aging process, i.e., the deleteriousness, the progressiveness, the intrinsicalness and the universalness (Strehler, 1959, 1982). At the same time one has to explain the differences in the longevity of the species, which is obviously genetically determined.

To find the explanations for biosenescence has always been a great challenge for the scientists in all historical periods. However, it is almost impossible to make any clear image or valid generalizations from the currently available literature, since there is an extreme divergency of theories, hypotheses and experimental approaches to the problem of aging of biological systems. The main reason for such a confusing situation is that gerontology is still lacking a solid theoretical basis (Comfort, 1979). In other words, we have to realize that most of the actual gerontological research is performed only at a phenomenological level without asking the basic questions of gerontology. Due to the universal character of aging (Strehler, 1959, 1982) each biological individual displays some structural and functional alterations after having reached the maturation. Therefore, wherever we touch the living systems, we can obtain descriptive age-dependent data from all of the organs, cell types, etc., however, it is extremely difficult to find really causal interrelationships of general validity. Henceforth, one has to emphasize that some conceptual approaches should be developed first, and only in possession of some clear ideas may it become possible to concentrate the experimental efforts to the theoretically feasible ways.

It is impossible to give even a very superficial overview of the existing ideas on aging in the present paper. However, some recent reviews are available (Zs.-Nagy, 1985, 1986a) explaining the actual state of art from a theoretical point of view based on the concept of Esposito (1983), and

describing in detail the logic I intend to follow in this paper.

There is no doubt that the main task of experimental gerontology is to explain the age-dependent decrease of the protein turnover of all living systems (Zs.-Nagy, 1985, 1986a). For achieving this goal one has to (i) identify the damaging factors in physical, chemical and physicochemical terms, which force the cells to replace their components continuously; and (ii) identify the physiological mechanisms being responsible for the fact that the rate of replacement of the damaged components is not maintained at the same level for ever as it is observable in the young or adult age.

This paper will outline the actual knowledge regarding these 2 main tasks.

ON THE NATURE OF THE DAMAGING FACTORS

1. Oxygen free radicals

Much evidence has been accumulated during the recent decade showing that oxygen free radicals occur in biological systems (Fridovich, 1978; Nohl and Hegner, 1978) and are involved in cellular aging (Harman, 1956, 1981; Totter,1980; Cutler, 1984; Sohal et al., 1984, etc.). Although due to some methodological problems, the importance of free radicals in aging is based largely on circumstantial evidence, some data seem to be of great importance: For example, Tolmasoff et al. (1980) have demonstrated that the superoxide dismutase (SOD) activity, if related to the specific metabolic rate of the given tissue, displays a linear correlation with the maximum life span potential of the species. Furthermore, Cutler (1985) showed that the peroxide-producing potential of brain and kidney is inversely correlated with the longevity of mammalian species.These data gain an especially high weight in the light of some molecular biological observations, according to which just a small oxidative damage on one single histidine moiety of the glutamine synthetase is sufficient to destroy the enzyme function and

activate the proteolytic degradation of the damaged molecule (Levine, 1983a, 1983b).

The actual knowledge on the oxygen free radicals is summarized in Fig.1. It is obvious that the monovalent reduction of the molecular oxygen leads to the formation of superoxide anion radicals, which are dismutated very actively by the SOD (McCord and Fridovich, 1969a, 1969b). The product of this reaction is hydrogen peroxide which is much less harmful for the cells, nevertheless, it should also be eliminated. Two main systems perform this job: (i) the catalases (Fridovich, 1976) (wherever present) are sensitive only for relatively high hydrogen peroxide concentrations, whereas the glutathione peroxidase (GPO) reacts also with the low concentrations of H_2O_2. Nevertheless, GPO requires a substrate (glutathione) for this reaction which may not be available always in the cells. As a consequence of this situation, one can assume that some freely diffusing H_2O_2 may always be present in the living systems and may participate in reactions with transient metals like Fe^{2+} (Fenton-reaction: Fenton, 1894). This type of reaction generates $OH^{\cdot}$ free radicals which are extremely reactive. It picks up an electron from any of the neighboring molecules within about 2 molecular collisions, i.e., will form covalent cross-links in or between the organic molecules. Due to the extremely high reactivity of the $OH^{\cdot}$ radicals, practically no enzymatic or any other protection is possible against them except the one which has been realized by the evolution of the life: the continuous replacement of the damaged components by resynthesis and degradation of the damaged molecules. It has been shown that even in vivo a sufficient amount of Fe^{2+} may be available (Floyd and Lewis, 1983) for a Fenton reaction, and the $OH^{\cdot}$ radicals deriving from this reaction are able to attack the amino acids and proteins very efficiently (Floyd and Zs.-Nagy, 1984; Zs.-Nagy and Floyd, 1984a).

At this point one has to make a special consideration. Namely, the $OH^{\cdot}$ radical events are obviously realized in a statistical manner, i.e., practically all the organic molecules can be targets of this reaction, and the consequences

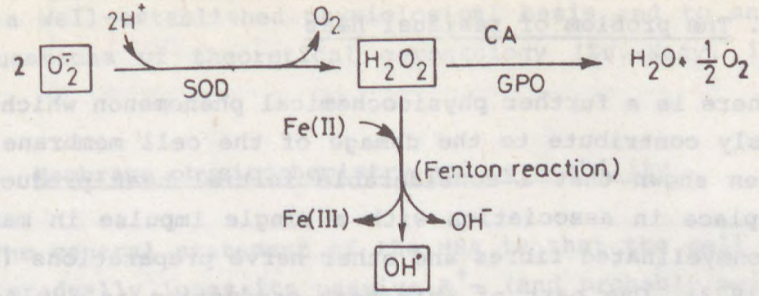

Figure 1. The summary of the pathways generating various types of oxyradicals in biological systems, indicating the possible defense mechanisms against them. Other explanations can be found in the text.

of such a radical attack may be different according to the molecular environment: if the organic molecule is in the cytosol where the nearest other molecules are relatively far away, the probability is high that the OH• radical attack results in only some intramolecular damage, i.e., one radical event can destroy only one single macromolecule. On the other hand, in the more compact biological structures, like the membranes, the molecules are so near to each other that the probability of intermolecular cross-link formation increases considerably, i.e., one radical event may destroy more than one macromolecule. In other words, OH• radical attacks may destroy the membrane components to a higher extent than the cytosolic components. This consideration agrees with the fact that the highest turnover of the components has always been observed in the membrane proteins. For example,some protein fractions of the hepatocyte membrane displayed a half-life of only 4 h in normal adult rats, meanwhile other proteins had 10-fold longer half-lives (Tauber and Reutter,1981). Therefore, one has to consider the possibility very seriously that the cell membrane damage may be of considerably higher extent than that of any other cellular component, therefore, membrane alterations may represent the leading factor in cellular aging.

2. The problem of residual heat

There is a further physicochemical phenomenon which may seriously contribute to the damage of the cell membrane. It has been shown that a considerable initial heat production takes place in association with a single impulse in mammalian nonmyelinated fibres and other nerve preparations (Ritchie, 1973). One part of this heat production is due to to free energy changes when the membrane electric capacity is discharged, however, most of it is explained by an entropy decrease of the dielectric when the electric field across it is removed. The main trouble is, however, that about 10 % of this heat is not reabsorbed, i.e., a residual heat remains in the membrane after each discharge (Ritchie, 1973). It has been shown in theoretical calculations (Zs.-Nagy, 1979) that the residual heat may be considerable in quantitative terms, and since it may remain in the membrane only in form of chemical bonds, one has to assume that the elaboration of impulses by the cell membrane (which is especially frequent in case of the nerve cells) represents a further factor of damage for the cell membrane in addition to the effect of the free-radicals.

These data demonstrate that even among the various types of membrane structures, the cell membrane may be the main target of the possible damaging factors. A research activity of about 10 years have demonstrated that this is really the case.

THE PHYSIOLOGICAL MECHANISM OF CELLULAR AGING ON THE BASIS OF THE MEMBRANE HYPOTHESIS OF AGING

Below an attempt will be made to explain the general age-dependent decrease of protein turnover in terms of a working hypothesis outlined first in 1978 as the membrane hypothesis of aging (MHA) (Zs.-Nagy, 1978). This concept is summarized in Figure 2 and the following text shall list the evidence and give explanations why we consider this concept as a valid aging hypothesis being completely suitable to

offer a well-established physiological basis and to answer the questions of theoretical gerontology (Zs.-Nagy, 1985; 1986a).

1. Membrane physicochemistry and permeability

The central statement of the MHA is that the cell membrane gradually loses its passive K^+- (and probably water-) permeability during aging due to the free-radical induced and other damages of its components (Fig. 2). Let us see now the evidences supporting this statement:

(i) Lipid fluidity. It has been demonstrated by fluorescence anisotropy measurements that the lipid layer of the synaptosomal membrane of rats displays an age-dependent decrease of fluidity (i.e., an increase of its "microviscosity") (Nagy et al., 1983a). The very same conclusion was reached using spin label methods in electron spin resonance spectroscopy of the same type of membrane preparations (Nagy et al., 1983b). The possible role of the alterations in the membrane lipid asymmetry in aging (through some alterations of the transmembrane processes) has recently been discussed in detail (Schroeder, 1984). The observed alterations in the lipid fluidity and the asymmetry of the lipid layer may well contribute to an overall loss of permeability of the membrane for K^+ (and for water) with advancing age.

(ii) Molecular weight distribution of proteins in the synaptosomal membranes. It has been shown that this distribution is shifted toward the higher molecular weights in the adult and old rats. This observation is consistent with an increased cross-linking of proteins in the membrane and with a loss of permeability (Nagy and Zs.-Nagy, 1984).

(iii) Lateral diffusion of membrane proteins. By using the fluorescence recovery after photobleaching (FRAP) technique on hepatocytes, it was observed that the average lateral diffusion constant of proteins in the cell membrane is inversely correlated with the age of rats (Zs.-Nagy et al., 1984, 1986). This negative linear correlation is so strong that rather precise age-estimations of the animals can be

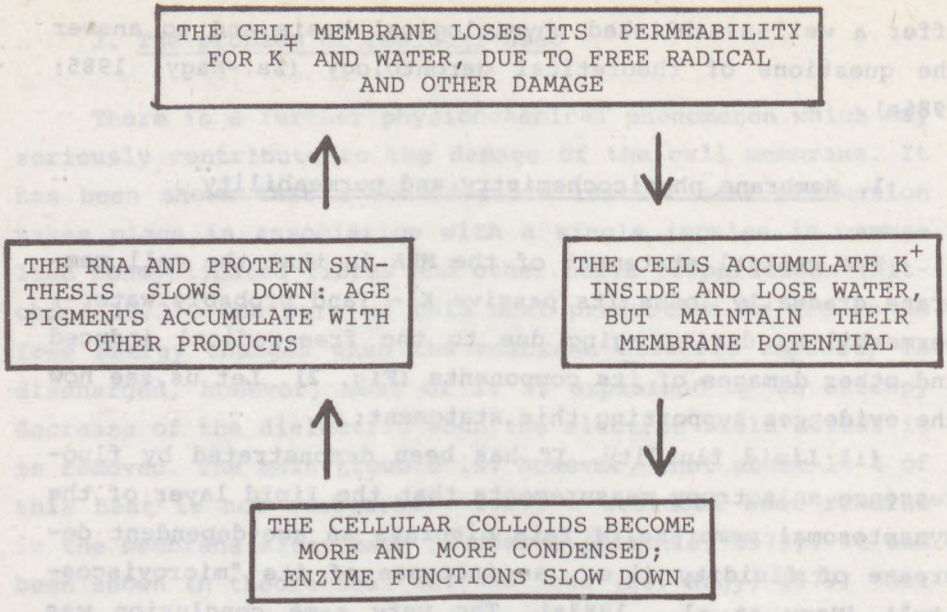

Figure 2. A schematic outline of the membrane hypothesis of aging (Zs.-Nagy, 1978, 1979, 1985, 1986a). The arrows indicate causal interrelationships between the phenomena described briefly in the figure. One can see that this process is a circulus vitiosus leading to a complete functional failure of the cell.

performed simply by measuring the lateral diffusion constant of proteins in the hepatocyte membrane (Kitani et al.,1986). It has also been shown in a theoretical model that the observed rate of decrease of the lateral diffusion constant of membrane proteins corresponds to the rate of loss of passive K^+ permeability described below (Zs.-Nagy, 1986b). Experimental evidence shows that chemical cross-linking of the membrane proteins decreases rather strongly their lateral mobility (Lustyik et al., 1986).

(iv) Measurements on the K^+ permeability.This parameter has been measured by two independent methods and animal models. First,it could be predicted theoretically that an increase of the so-called rubidium discrimination ratio will indicate a decrease of the passive Rb^+ and K^+ permeability of the cell membrane. As a matter of fact, both the liver and the brain cells of rats displayed a more than 100 % in-

crease of this discrimination ratio in Rb-substitution experiments in vivo, resulting in a calculated loss of K^+ permeability of about 40 % for the brain cells between 2 and 24 months of age (Zs.-Nagy et al., 1982a; Gyenes et al., 1984). The second system used for this purpose was an identified giant neuron of a snail where the intracellular electrophysiology and the X-ray microanalytic determination of the intracellular water and ion contents could be combined on the very same cells. These experiments revealed that the relative passive K^+ and Cl^- permeabilities of the neuronal cell membrane decreased by about 50 % between the young and old age (Zs.-Nagy et al., 1985). It should be noted that the age-dependent water loss of the snail neurons was even of higher extent than observed in rat brain cortical cells.

These data regarding the K^+ permeability of the nerve cell membrane support the MHA, since these results offer a logical explanation for the age-dependent increase of the intracellular potassium concentration in the same way as predicted originally (Zs.-Nagy, 1978, 1979).

2. Intracellular ion and water contents

As a straight consequence of the loss of passive permeability of the membrane for K^+ (and for water), the intracellular ionic strength (mainly K^+) increases and the water content decreases with advancing age (Fig. 2).

In order to measure these parameters, considerable methodical developments were needed in the field of X-ray microanalysis (Zs.-Nagy et al., 1977, 1982b, Zs.-Nagy, 1983; Lustyik and Zs.-Nagy, 1985). The results obtained in various cellular models demonstrate two main tendencies as follows.

(i) The intracellular K^+ content of the nerve cells increases about 30-40 % (in rats) or more (in snails) during the life-span (Pieri et al., 1977; Zs.-Nagy et al., 1979, 1985; Lustyik and Zs.-Nagy, 1985). Such an increase exerts a strong condensing effect on the cytoplasmic and nuclear colloids as it has been demonstrated many years ago by X-ray diffraction methods (Bernal and Fankuchen, 1941).

(ii) The intracellular water content of the nerve cells decreases from about 80 % by weight of the young animals to about 70-73 % in the old ones (Zs.-Nagy et al., 1982b, 1985; Lustyik and Zs.-Nagy, 1985).

3. Intracellular density

The above results indicate unanimously that the intracellular density of the cell colloids increases with age. An increase of the dry mass content from 20 to 27-30 % by weight is quite a considerable change. An increase of the intracellular microviscosity was demonstrated also in isolated hepatocytes by using the measurement of fluorescence polarization of fluorescein emission incorporated as a non-fluorescing compound and split by the intracellular esterases (Lustyik and Tatár-Kiss, 1985).This increase of the density causes an overall decrease of the enzyme activities in the sense of a theoretical enzyme kinetic model (Damjanovich and Somogyi, 1973, Somogyi and Damjanovich, 1975; Somogyi et al., 1978).It is well known that the chromatin is especially sensitive toward the ionic strength of the medium (see for ref.: Zs.-Nagy, 1979), therefore, one can expect that the rate of transcription and translation will be reduced simply because of the increased intracellular density (and viscosity).

Obviously, one has to ask the question what happens with the osmotic balance under such conditions. Apparently it is maintained also in the old cells, and one has to explain why the increased K^+ content does not cause osmotic problems. One has to consider several possibilities as follows.

(i) Intracellular osmotic pressure may be generated only by ions or molecules for which the cell membrane is practically impermeable, whereas the solvent (water) can freely pass the membrane. The potassium ions do not contribute seriously to the osmotic pressure of the cytoplasm, since the membrane of young animals is highly permeable for this ion, and even in the old age, the rest of the passive permeability for this ion is still high enough to avoid osmotic dysbalance.

(ii) The intracellular colloid osmotic pressure is very high in the young brain (Tomita et al., 1979), which is due obviously to the high water-solubility of the components. However, the intracellular colloid osmotic pressure may decrease during aging, since the water solubility of the proteins decreases considerably (Zs.-Nagy et al., 1981), i.e.,the loss of the colloid osmotic pressure may compensate for the increased intracellular ionic strength.

(iii) The water permeability of the cell membrane may decrease during aging, therefore, the effect of the higher ion concentrations may be altered on the total osmotic pressure.

It should be stressed that all these possibilities are realistic in the sense of the actual knowledge, and further experimental studies are needed to reveal their proportional contribution to the maintenance of the osmotic equilibrium.

4. The rates of RNA and protein synthesis

Our radioisotope experiments in vivo revealed that both the total, and the mRNA synthesis rate decreases more than 50 % between 2 and 26 months of age in rat brain cortex (Semsei et al., 1982). These results together with the other available data (See for ref.: Zs.-Nagy, 1985, 1986a) strongly indicate that the rate of protein synthesis decreases with age in almost all experimental models studied so far. It should be stressed again that this fact finds its satisfactory explanation in the effect of increased intracellular viscosity on all enzyme functions, including the accumulation of the so called age pigments (lipofuscin). Lipofuscin is accumulated apparently just because the protein synthesis is slowed down and the lysosomal enzymes necessary for the decomposition are missing. This assumption is supported by the experimental fact that inhibition of lysosomal proteases by leupeptin results in lipofuscin accumulation in the neurons of young rats within a couple of days (Ivy et al., 1984). In other words, one can assume that lipofuscin is not a special product of the aging cells, but the old cells dis-

play a lower rate of elimination of the damaged components than the young ones, and therefore, the waste products accumulate in the postmitotic cells.

Another point should be made clear: The nature of the molecular damage is not different in the young and old systems, on the contrary, due to the higher metabolic rate of the young systems, even more harmful radicals are formed in them per unit of time per cell. However, due to the highly diluted character of the young systems, the rate of protein synthesis can be maintained to such a high extent that the individual is not only able to reproduce its damaging components but it is even growing. On the other hand, during the maturation process the living mass reaches a state of density in which the rates of damage and replacement are just in equilibrium, i.e., the growth is not possible any more. At last, in spite of a decreased metabolic rate observed in the old age, due to the increase of the intracellular density, the free radical induced damage will be more and more efficient in the sense that fewer radicals produce relatively more macromolecular damage, therefore, the aging process will progrediate. It may also be of importance that e.g.the SOD displays a salt-dependence which may render less efficient the defense mechanism against the superoxide radicals in the old cells (Semsei and Zs.-Nagy, 1984, 1986).

SOME FURTHER EVIDENCES IN FAVOR OF THE MHA

It seems to be obvious that the MHA can be tested from various points of view by using some pharmacological or dietary interventions in vivo. Here I list such experimental results without being complete.

1. Acceleration of the aging rate

(i) On the basis of the assumption that one of the most important protective factor against lipid peroxidation (see for ref.: Donato and Sohal, 1981), is vitamin E, experiments were carried out with vitamin E deficient diet in rats. It

has been observed that this diet causes an accelerated macroscopic aging of the rats which is accompanied with the same type of changes in the intracellular ion and water content of the brain cells as observed during the normal aging process (Bertoni-Freddari et al., 1981).

(ii) Starting from the known fact that the blood brain barrier is extremely well closed for iron, Fe^{2+} salts were injected in the cerebrospinal fluid of young rats. This intervention is extremely toxic for the rats, however,if still tolerable doses were applied repeatedly for 3 days, the synaptosomal fractions of the brain cortex showed similar alterations as observed during the normal aging process (Nagy et al., 1985).

2. Deceleration of the aging rate

Possibilities of this type of intervention will be demonstrated on the example of centrophenoxine (CPH) treatment. CPH consists of p-chlorophenoxy-acetic acid (PCPA) and dimethylamino-ethanol; it is a brain metabolic stimulant (or nootropic drug). DMAE is incorporated into the cell membrane of neurons (see for ref.: Zs.-Nagy et al., 1979; Zs.-Nagy and Semsei, 1984). It has been shown that its DMAE moiety is an efficient $OH^{\cdot}$ radical scavenger (Zs.-Nagy and Nagy, 1980; Zs.-Nagy and Floyd, 1984b), and as such may represent some radical protection in the old nerve cells. Treatments of old rats for 40-60 days with CPH resulted in a series of beneficial effects listed below.

(i) Membrane lipid fluidity of synaptosomes as measured by fluorescence anisotropy method increased and returned almost to the adult level (Nagy et al., 1983a).

(ii) Synaptosomal membranes were protected considerably by CPH against the effect of iron overload in the CSF (Nagy et al., 1985).

(iii) The age-dependent shift of the molecular weight distribution of synaptosomal proteins to higher values was reversed by CPH (Nagy and Zs.-Nagy, 1984).

(iv) CPH increases the passive potassium permeability of the neuronal cell membrane, i.e., it decreases the intracellular potassium content and rehydrates the cytoplasm considerably (Zs.-Nagy et al.,1979; Lustyik and Zs.-Nagy,1985).

(v) CPH treatment improves the age-dependent deterioration of some synaptic parameters measured by electron microscopic morphometry in the cerebellar glomerulus of rats (Bertoni-Freddari et al., 1982).

(vi) CPH increases the rates of total and mRNA synthesis in the brain cortex of old rats (Zs.-Nagy and Semsei, 1984).

(vii) CPH treatment reversed the age-dependent decrease of water solubility of proteins and the increase of the total protein content in the brain of rats (Zs.-Nagy et al., 1981).

(viii) CPH treatment is able to prolong the medium life-span and to improve the learning ability of several experimental animal species (see for ref.: Zs.-Nagy et al., 1979; Zs.-Nagy and Semsei, 1984).

CONCLUDING REMARKS

This paper contains a view of the problem of aging which is certainly unusual for most of the gerontologists. Nevertheless, it seems to be sure that no experimental approach can be valid, if it does not have a solid theoretical basis in agreement with the actual cell biological knowledge. It should be stressed that aging can be studied only by using multidisciplinary methods, and this requirement renders extremely difficult all the experimental work.

It should be stressed that the membrane hypothesis of aging (Zs.-Nagy, 1978) has developed independently from the theoretical concepts of Esposito (1983) and the fit of these two independent lines to each other proved to be very impressive (Zs.-Nagy, 1986a). This coincidence indicates in itself that it is worth while to follow this strategy because elements of the truth can be present in it.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

OXIDIZED AND REDUCED GLUTATHIONE LEVELS IN BLOOD SAMPLES FROM PREMATURE INFANTS WITH RDS AND CRITICALLY ILL CHILDREN

I. NÉMETH and D. BODA

Paediatric Department, University School of Medicine, Szeged, Hungary

SUMMARY

Both the oxidized and the total glutathione concentrations of the plasma and the whole blood were determined with a sensitive method, using glutathione reductase. No consequent change in either the total or the oxidized glutathione level of the plasma was found in patients under intensive care or in hypoxic premature infants. Pronounced increases in the concentration of oxidized glutathione and in the ratio of oxidized/reduced glutathione in the whole blood were measured during oxygen therapy in the course of perinatal hypoxia and in intensive care patients suffering from acute metabolic disorders. It is suggested that the concentration of oxidized glutathione or the ratio of oxidized/reduced glutathione for the whole blood is an appropriate index of oxidative stress.

INTRODUCTION

The presence of glutathione tripeptide in all cells and its essential protective role against intracellular reactive oxygen radicals (mainly through the glutathione peroxidase system) are well established. The ratio of the reduced and oxidized forms of glutathione (GSH, GSSG) in the cells is an indicator of oxidative stress. In rats treated with oxidative compounds Adams et al. (1) proved that blood plasma samples can be used to determine the ratio of the reduced and oxidized forms of glutathione in the hepatic cells. They found that plasma GSSG measurements were especially informative on oxidative stress. This suggested to us that the ratio of GSSG and GSH in blood plasma may be an appropriate index of the severity and outcome of pathological

states where damage through the activity of free oxygen radicals is highly possible, such as during oxygen therapy in the course of perinatal hypoxia, or in intensive care patients suffering from acute metabolic disorders.

METHODS

Highly sensitive determinations of glutathione concentration and of the ratio of GSSG and GSH were carried out using the method of Tietze (7). The combined actions of DTNB (5,5′-dithio-bis-(2 nitrobenzoic acid)) and NADPH in the presence of glutathione reductase result in a reaction cycle, the rate of which depends on the concentration of glutathione; it is recorded at 412 nm during the first 6 minutes of the reaction. The assay responds to both GSH and GSSG, so that GSSG must be determined separately after alkylation of the GSH with NEM (N-ethylmaleimide) according to Akerboom and Sies (2). Separation of GSSG and NEM was achieved by gel filtration with Sephadex G-10 (4).

Both total and oxidized glutathione concentrations were determined in plasma and whole blood samples from children, newborn infants, premature infants with respiratory distress syndrome (RDS), with or without adjuvant allopurinol therapy, and critically ill infants and children. An animal model for the investigation of oxidant injury according to Adams et al. (1) was also used. Wistar rats treated with paraquat (Gramoxon®) in a dose of 0.29 mmol/kg i.p. were sacrificed 1, 2 or 4 hours following paraquat administration, and arterial blood samples were taken by aortic puncture. Plasma samples anticoagulated with EDTA and deproteinized with metaphosphoric acid were used, as well as whole blood samples as hemolysates.

RESULTS AND DISCUSSION

The plasma glutathione concentrations were first determined. The average value for GSSG in the control patient group was 0.51±0.22 µmol/l. The total glutathione level was 1.06±0.35 µmol/l (mean ± S.D., n=10). No consequent change in either total or oxidized glutathione levels was found in patients under intensive care or in severe hypoxic premature infants. This observation confirms the conclusion drawn by Beutler and Gelbart (3) from their investigations on total glutathione in human plasma: the plasma glutathione level has no specific significance in man.

Accordingly, whole blood was taken to measure the oxidized and total glutathione concentrations. The values were calculated with reference to hemoglobin (Hb) as they are derived from erythrocytes. Since the total glutathione is expressed in µmol/g Hb and the GSSG in nmol/g Hb, the total glutathione will be referred as to GSH.

Figure 1 shows the percentage of GSSG/GSH in whole blood in groups of patients with different illnesses. Control children, infants and prematures exhibited an average ratio of about 0.15%, while prematures with adaptation problems or symptoms of RDS sometimes displayed far higher values. The ratio in children under intensive care ranged from the average for the control group to rather high levels.

Figure 2 depicts the whole blood GSH concentrations in the same patients. The average levels were found to be higher in control prematures and infants than in control children. As already indicated, newborn infants have a higher erythrocyte GSH content than adults (5). Even premature newborn infants with adaptation problems and symptoms of RDS displayed normal GSH values. Higher levels were measured in children under intensive care. A strikingly high value was found in one patient suffering from bronchopulmonary dysplasia (28.2 µmol/g Hb). This extremely high value fell when the respiratory treatment and oxygen therapy were terminated.

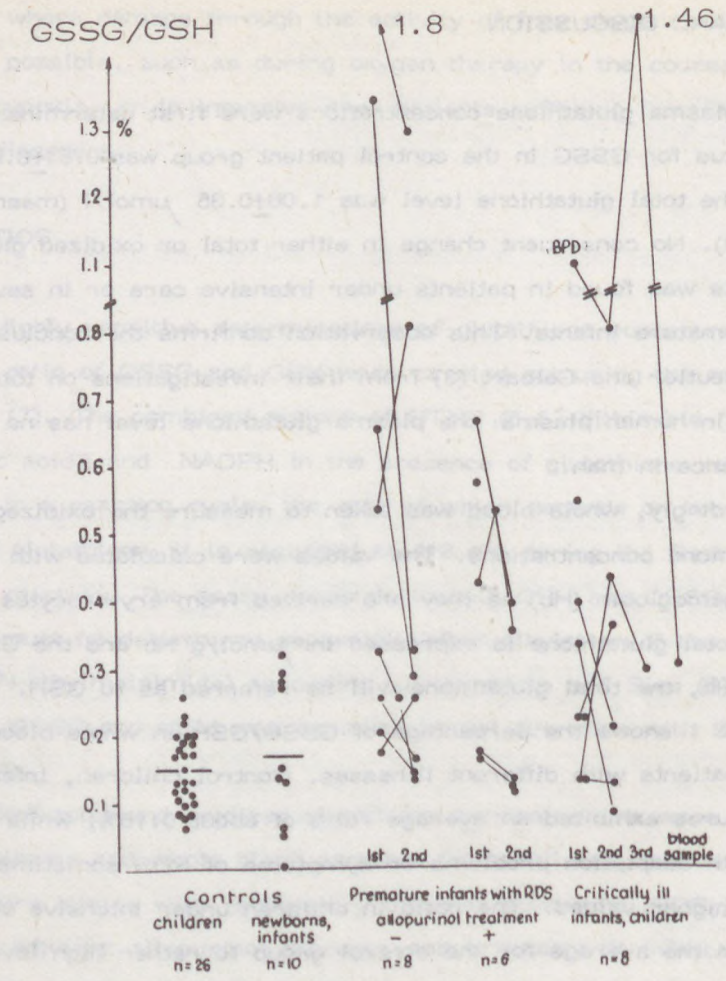

Fig. 1. Oxidized glutathione level as a percentage of the reduced glutathione level in the whole blood of controls and patients with various illnesses

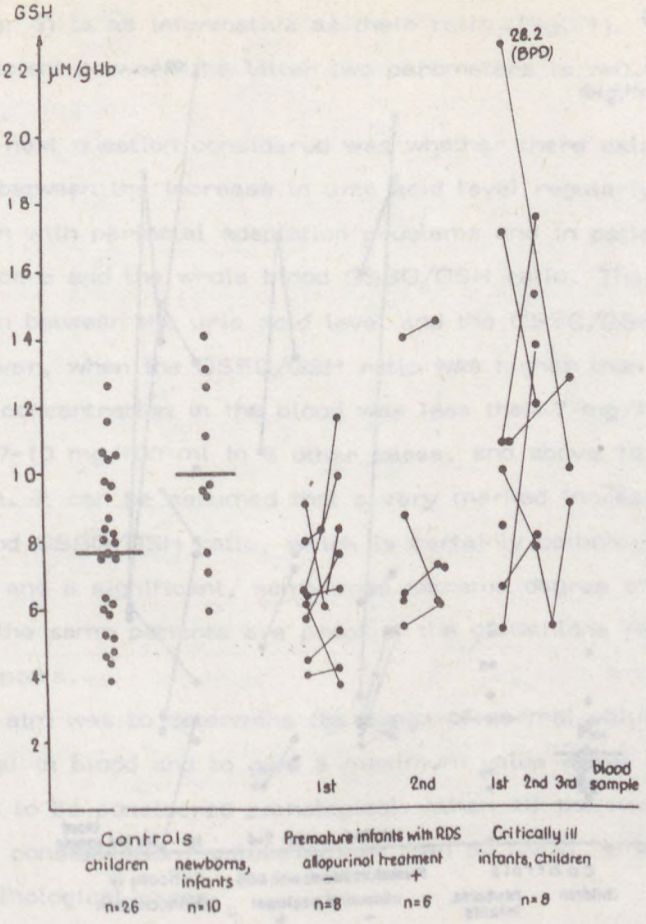

Fig. 2. Concentration of reduced glutathione in the whole blood of controls and patients with various illnesses

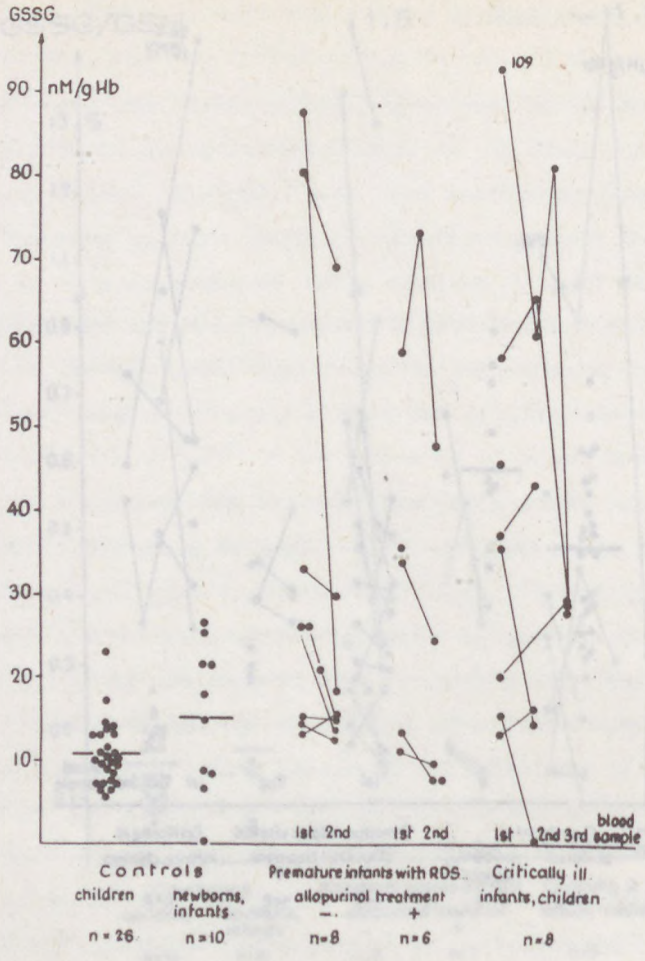

Fig. 3. Concentration of oxidized glutathione in the whole blood of controls and patients with various illnesses

The GSSG levels in whole blood are to be seen in Fig. 3. The GSSG values from the first samples examined markedly exceeded the levels for the controls in half of the prematures with adaptation problems and children under intensive care. These high values, however, decreased as the state of the patient improved. Consequently, as the GSH level is relatively constant (Fig. 2), the concentration of GSSG

alone (Fig. 3) is as informative as their ratio (Fig. 1). The correlation coefficient between the latter two parameters is $r=0.831$; $n = 82$; $p < 0.001$.

The next question considered was whether there exists a correspondence between the increase in uric acid level regularly observed in association with perinatal adaptation problems and in patients under intensive care and the whole blood GSSG/GSH ratio. There was no correlation between the uric acid level and the GSSG/GSH ratio overall. However, when the GSSG/GSH ratio was higher than 0.5%(n=12), the uric acid concentration in the blood was less than 7 mg/100 ml in only 3 cases, 7-10 mg/100 ml in 3 other cases, and above 10 mg/100 ml in 6 cases. It can be assumed that a very marked increase in the whole blood GSSG/GSH ratio, which is certainly pathological above 0.5% (6), and a significant, sometimes extreme degree of hyperuricemia in the same patients are proof of the glutathione ratio being a sign of hypoxia.

Our aim was to determine the range of normal values for the GSSG level in blood and to give a maximum value above which the levels are to be considered pathological. When all the results were taken into consideration, values higher than 50 nM/g Hb were observed only in pathological cases.

The question we have to answer when evaluating our results is exactly what the whole blood GSSG concentration is an indicator of. Adams et al. (1) considered that a rise in the plasma GSSG level is a sensitive sign of oxidative stress of the whole body in rats. Thus, their experiments on rats were repeated and the GSSG levels and the GSSG/GSH ratios for the plasma and the whole blood were compared 1, 2 and 4 hours following paraquat treatment. As demonstrated in Fig. 4, the changes both in the GSSG level and in the ratio of the glutathiones are parallel in rat plasma and whole blood. This result confirms our assumption that in man, where the plasma glutathione level cannot be measured, determination of the GSSG level of the whole blood is an appropriate way of getting information on the oxidative stress not only of the erythrocytes, but of the whole body as well.

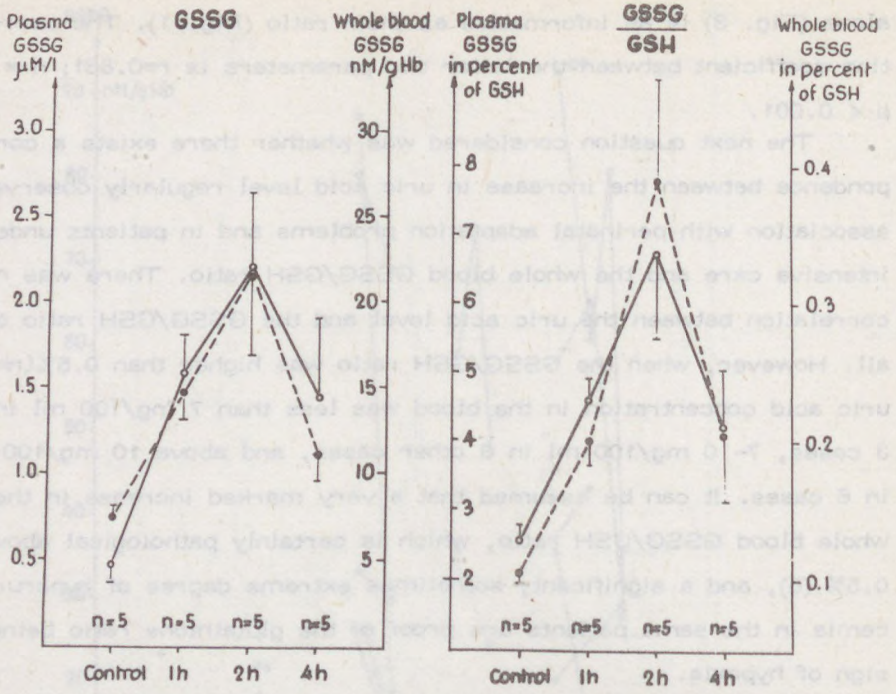

Fig. 4. Concentration of oxidized glutathione and the ratio of oxidized/reduced glutathione in the plasma and the whole blood of rats treated with paraquat (0.29 mmol/kg i.p.)
Plasma samples: ○—○—○
Whole blood samples: ●—●—●

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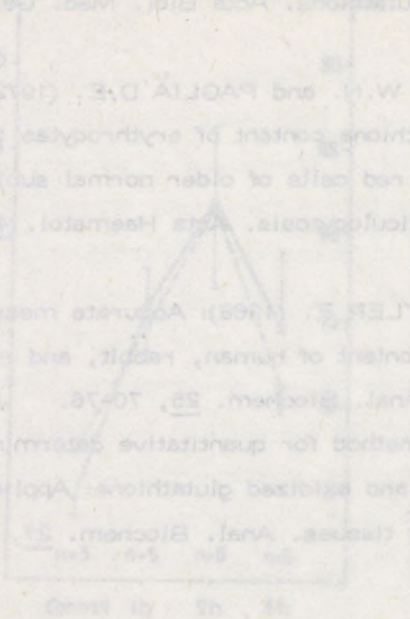

Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

FREE RADICAL-INDUCED DISEASES IN THE NEONATE

G. OROSZLÁN, L. LAKATOS and L. KARMAZSIN

Department of Paediatrics, University Medical School, Debrecen, Hungary

SUMMARY

Over the past decade, a large body of literature has accumulated indicating that free radical-induced peroxidation of membrane lipids may lead to an irreversible insult that ultimately results in cell death. Clinical and experimental evidence has suggested that oxygen toxicity is a major factor in the etiology of bronchopulmonary dysplasia, retinopathy of prematurity, hemolytic diseases, hyaline membrane disease and post-ischemic brain damage. The role of active radicals developing from molecular oxygen and the free radical-induced diseases in the neonate are discussed.

INTRODUCTION

Oxygen free radicals are known to cause the peroxidation of polyunsaturated fatty acids in biological membranes. Antioxidant defense mechanisms are essential against the destructive potential of oxidizing free radicals.

Three types of defense mechanisms have been described. The first results from the various physiological barriers confronting oxygen as it travels from the inspired air in the alveolar airspace into the pulmonary capillary blood and is carried by hemoglobin via the arterial and capillary circulations to the cells. The second type of antioxidant defense includes enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, transferrin, ceruloplasmin and NADPH-generating systems, which demonstrate increased activity with adaptation to hyperoxia. The third type of endogenous antioxidant pro-

tection is provided by adequate tissue levels of antioxidant compounds such as vitamin E, selenium, sulfur amino acids and vitamin C.

Oxygen toxicity is the resultant of two biological processes acting in opposite directions. On the one hand, the production of free radicals and the chain reaction initiated by them tend to lead to disintegration of the cell and the cellular organelles. On the other hand, there exists a sophisticated defense system acting at several levels that prevents or even repairs the tissue damage. Under physiological conditions there is an equilibrium between the two systems: the identical rates of production and elimination ensure only the "normal" function of the lipid peroxidation (LP). Newborns with pathological condition and preterm infants, however, are in a peculiar situation. In these infants and to some extent in normal-term babies as well, a disequilibrium resulting in an increased vulnerability to oxygen toxicity may develop during adaptation to extrauterine life. In addition, defense mechanisms against the oxygen radicals are also immature in them. There are two main reasons for this increased vulnerability: an increased exposure to situations of prolonged oxidative stress and an inefficient antioxidant defense mechanism.

INCREASED EXPOSURE TO SITUATIONS OF PROLONGED OXIDATIVE STRESS

1. Before and during birth the newborn may be exposed to maternal and fetal injuries resulting in an increased rate of LP: EPH-gestosis, intrauterine infections, various maternal diseases, placenta previa, premature rupture of the membranes, exposure of the mother to drugs and radiation, especially tocolytics, procedures during labor such as oxygen inhalation, anesthesia, analgesia, oxytocin, etc. (32).

2. The greatest risk is oxygen therapy some time after birth, frequently applied as a life-saving measure necessary for the prevention of sequelae of hypoxia (16). The most vulnerable individuals in this respect are babies of very low birth weight and newborns in whom the partial tension of arterial oxygen continuously exceeds the tolerable

level. For this reason, in neonatal units all over the world attempts at measuring arterial oxygen tension are made to avoid prolonged hyperoxemic periods (35). Unfortunately, only partial success has been achieved, and even in intensive care units not all the grave sequelae of oxygen toxicity can be avoided.

3. During the hypoxic state the activities of the antioxidant enzymes are decreased. In this situation, even a small amount of free radicals may destroy the cellular integrity. After the hypoxic state, during the reperfusion phase a great load of oxygen free radicals occurs, causing the destruction of the biological membranes. In fact, both hypoxia and hyperoxia may lead to the generation of free radicals, i.e. to LP.

4. A frequent damaging factor is hyperbilirubinemia. Apart from its unfavorable effect on the central nervous system (23), bilirubin has been shown to be a photodynamic agent, i.e. it produces singlet oxygen in the presence of oxygen and light, thereby enhancing the peroxidation of unsaturated fatty acids (12).

5. Certain therapeutic measures destined to surmount neonatal hyperbilirubinemia, such as phototherapy and exchange transfusion, may also be potentially harmful in this respect. Phototherapy enhances the isomerization of bilirubin into rapidly eliminated and less toxic compounds. This process is accompanied by an increased production of singlet oxygen. This radical plays a minor role in bilirubin oxidation, but it may be responsible for the potential hazards of phototherapy; damage to DNA, mutagenesis, carcinogenesis, sexual dysfunction, etc. (7, 33). Similarly, exchange transfusion and transfusion of adult blood may increase the oxygen toxicity by the lower oxygen affinity of adult hemoglobin compared to that of fetal hemoglobin (6).

6. Septic and hypotensive conditions, including the idiopathic respiratory distress syndrome (IRDS) or hyaline membrane disease (HMD), resuscitation and reparation after resuscitation increase the risk of oxygen damage. In chronic and acute inflammations the polymorphonuclear leukocytes, macrophages and monocytes produce a con-

siderable amount of superoxide radical; this radical has a direct bactericidal effect and promotes the formation of the superoxide-dependent chemotactic factor and the production of hydroxyl radicals. Both compounds have a favorable effect in the killing process directed against bacteria and other invasive agents (2). In the immature organism, however, with its deficient antioxidant defense, the aggressive radicals may damage the cellular integrity.

7. Certain drugs may increase the proneness to oxygen toxicity. Some have a direct sensitizing effect in favor of oxygen toxicity, e.g. riboflavin, methylene blue, salicylates, etc. Others enhance the albumin-bilirubin dissociation or weaken the activity of glucuronyl transferase, thereby promoting bilirubin and oxygen toxicity at the same time (23); sulfonamides and chloramphenicol are in this group. Finally, artificial ventilation and X-ray exposure, not infrequent in modern intensive care, lead directly to the overproduction of harmful oxygen radicals (3).

INEFFICIENT ANTIOXIDANT DEFENSE MECHANISM

1. In newborn babies the physiological barriers lining the internal route of oxygen transport are immature. Immaturity and gestational age show a direct correlation. The size, the weight, the alveolarity, the collagen content, the concentration of the pulmonary surfactant, the proportion of interstitial elements, the capillary microcirculation, the number of alveolar epithelial cells, the respiratory capacity and the compliance of the lung all differ from those prevailing in the baby born at term (15).

2. The activities of specific antioxidant enzymes gradually increase during gestation and in the early postnatal period. The most potent inductor of this activity is oxygen itself, and hyperoxia speeds up the enzyme adaptation process (39). This may explain the finding that under prolonged oxygen exposure newborn animals survive much longer than adult animals; this does not imply that they do not suffer

permanent tissue damage. Similarly, pretreatment with antioxidants abolishes the usual biochemical reaction to hyperoxia. Our own experiments with superoxide dismutase (SOD) on the effects of D-penicillamine resulted in similar findings (17). The neonatal insufficiency of the enzymatic defense is markedly promoted by heavy metals, and especially by iron and copper ions. These latter accumulate to a considerable degree in the fetal tissues during the last weeks of gestation (30). After birth an additional unfavorable factor is the increased hemolysis of the fetal erythrocytes. The metal ions directly influence the oxidative processes and, in addition, inhibit the activity of delta-amino-levulinic acid synthetase, an enzyme governing the production of heme-containing proteins such as catalase, peroxidase and cytochrome P-450. They also stimulate heme oxygenase, an enzyme initiating heme catabolism (22, 26).

3. Newborn babies, especially very low birth weight preterm babies, are insufficiently supplied with endogenous antioxidants such as vitamin E, selenium and reduced glutathione.

FREE RADICAL-INDUCED DISEASES IN THE NEONATE

These disorders are specific to the neonatal period. They have a multicausal etiology, their common feature being the chain reaction leading to tissue damage initiated by free oxygen radicals.

Hemolytic diseases

Auto-oxidation of the newborn's erythrocytes proceeds at a rate three times higher than in adults. An especially unfavorable situation is encountered in babies affected by a congenital deficiency of glucose-6-phosphate dehydrogenase, pyruvate kinase or glutathione reductase (34).

Retinopathy of prematurity (ROP)

Terry gave the first description of retrolental fibroplasia (RLF) of premature babies in 1942 (36). With the increased survival rate of premature infants, especially those of very low birth weight, there has been a concomitant increase in RLF, now known as ROP. Although the duration of exposure to an elevated oxygen tension and the immaturity of the retina are regarded as the principal factors associated with the disease, other factors too contribute. The risk factors for ROP are: respiratory distress syndrome, multiple episodes of bradycardia and apnoea, exchange transfusion, HMD, anemia of prematurity, hyperbilirubinemia, avitaminosis E, cardiovascular defects, infectious diseases, etc. Nevertheless, the two main and most important factors in the pathogenesis of ROP are oxygen administration and the immaturity of the newborn's retinal vessels. The susceptibility of the eye, and especially the retina, to oxidative damage has been known for a number of years. The premature human infant retina is especially vulnerable to high levels of oxygen (5, 10, 27, 28). That this effect may be mediated via the LP is suggested by recent studies which showed the antioxidant vitamin E to be efficacious in moderating the severity of ROP (14). Lakatos et al. (18, 20) reported that prophylactic treatment with D-penicillamine was successful in reducing the frequency of ROP. More direct evidence of the involvement of the LP in photoreceptor degeneration has came from several laboratories.

The environment and the composition of the retina clearly make it an ideal "substrate" for LP reactions. Photoreceptor ROS (rod outer segment) membranes contain the highest levels of long-chain polyunsaturated fatty acids of any tissue in the body. These membranes are constantly bombarded by photons, which may act directly or indirectly through photosensitizers to produce damaging oxygen free radicals.

The retina has several defense mechanisms which protect it from oxidative damage. The photoreceptor ROS contains high levels of vit-

amin E and SOD. Although defense mechanisms are present in the retina, under certain conditions the oxidative stress may be sufficient to exceed the ability of this tissue to protect itself.

Bronchopulmonary dysplasia (BPD)

A better understanding of the pathomechanism of IRDS has led to the introduction of continuous positive airway pressure (CPAP) ventilation, bringing about a dramatic improvement in the prognosis of the disease (11). However, BPD, a new clinical entity, has appeared on the scene (24). BPD is a complex chronic lung disorder, usually but not exclusively found in infants who have been ventilated for RDS. While all ventilated infants are vulnerable to this condition, the frequency of BPD appears highest in the smallest infants undergoing the longest periods of assisted ventilation, and 25-30% of very low birth weight infants on ventilators may develop some degree of BPD.

The pathogenesis of BPD seems to represent a combined result of various insults to the lung, including overdistension, prolonged exposure to high atmospheric oxygen concentrations, persistent overperfusion of the pulmonary capillary bed by a patent ductus arteriosus, and interstitial and intra-alveolar edema as a final common pathway preceding the development of atelectasia and fibrosis.

The clinical features of BPD are well known, but its pathophysiology remains obscure. Indirect evidence suggests that free radicals, especially the superoxide anion and its by-products such as hydroxyl radical, contribute to the destruction of lung tissue in the neonate (8, 13). At normal oxygen tension the superoxide anion is removed in microseconds by dismutation mediated by SOD. When tissues are subjected to the increased oxygen tension typically used in the treatment of neonatal pulmonary disease, this enzyme system may be overwhelmed. Superoxide anions accumulate, leading to cell membrane and cytosol destruction and damage to the pulmonary alveolar macrophages, with the release of chemoattractant substances (9). The chemoattract-

ants cause an outpouring of polymorphonuclear leukocytes, which carry collagenases and elastases that may cause further connective tissue damage to the lung and initiate an exaggerated, abnormal repair process (dysplasia).

Hyaline membrane disease (HMD) or IRDS

This condition is specific to preterm babies and pathological newborns and leads to alveolar hyaline membrane production. The process is related to oxygen toxicity in several respects. The increased catabolism due to the severe condition of the newborn may increase the production of free radicals, and the therapeutic efforts may directly enhance LP. Recently, it has been shown that the peroxidative chain reaction triggered by oxygen radicals may play a primary role, since the hyaline membrane is basically a LP product (4); it can be induced experimentally by ionizing radiation, and the type II pneumocytes producing the surfactant are damaged by oxygen radicals (1).

Post-ischemic brain damage

Asphyxia is a condition in which the brain is subjected not only to hypoxia, but also to ischemia and hypercarbia. The biochemical and physiologic changes attending acute asphyxia have been studied extensively. Alterations in cerebral blood flow induced by asphyxia are important in the understanding of the genesis of brain injuries (37). Initially, there is a redistribution of the cardiac output, so that a large proportion enters the brain. This results in a 30 to 175% increase in the cerebral blood flow. At the same time, there is a loss of cerebral vascular autoregulation (21). As a consequence, the cerebral arterioles fail to respond to changes in perfusion pressure and carbon dioxide concentrations, resulting in a pressure-passive cerebral blood flow. As asphyxia persists, the cardiac output drops and hypotension follows. Since the cerebral autoregulation is no longer func-

tional, the arteriolar system is unable to respond to the decreased perfusion pressure with vasodilatation, and the result is a striking reduction of the cerebral blood flow (21). The cerebral blood flow may be further compromised by the development of localized or generalized brain edema.

It is still not clear which components of the complex postischemic pathophysiology are the most important in causing neuronal destruction and death. The importance of calcium ions in addition to prostaglandins and other metabolites of arachidonic acid is beyond doubt. Another possibly important component is an increased concentration of free radicals during or after ischemia (29, 31). Free radicals have the capacity to initiate destructive reactions in biological membranes and other cellular structures, and may be of central importance in causing further damage during or after ischemia.

D-PENICILLAMINE FOR THE PREVENTION OF OXYGEN TOXICITY

D-Penicillamine (DPA), which is used in our clinic to prevent oxygen toxicity, has a beneficial influence on all the three types of antioxidant defense mechanisms:

- it stabilizes the biological membranes;
- it increases the activities of antioxidant enzymes such as catalase and peroxidases; and
- as a sulfur amino acid, it provides higher levels of antioxidant compounds.

It seems that in preventing hyperbilirubinemia and the retinopathy of prematurity the mechanism of action of DPA is the protection of biomembranes against LP (19, 20, 25).

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IN VIVO ANTIOXIDANT AND IMMUNOSTIMULATORY EFFECTS OF A HEPATOPROTECTIVE FLAVONOID DRUG, CYANIDANOL, IN PATIENTS WITH CHRONIC LIVER DISEASE

A. PÁR, M. MÉZES*, P. NÉMETH, Julia SZEKERES-BARTHO, T. MAGYARLAKI, Mária PAÁL, E. KEREKES and T. JÁVOR

First Department of Medicine, Institute of Pathology and Microbiology, University Medical School, Pécs
*Department of Animal Physiology and Hygiene, University of Agricultural Sciences, Gödöllő, County Blood Transfusion Centre, Pécs, Hungary

SUMMARY

Cyanidanol, a flavonoid with the composition 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7 triol, has been shown to have hepatoprotective, radical scavenger and immunomodulatory properties. The present study was designed to assess its in vivo actions on the blood lipid peroxide status and antioxidant enzymes, as well as on certain immune functions in patients with chronic hepatitis.

Changes in the serum malondialdehyde (thiobarbituric acid-reactive substance) and superoxide dismutase (Cu,Zn-SOD) levels were measured in parallel with determination of the activities of blood glutathione peroxidase and catalase. Additionally, the serum concentration of the natural antioxidant vitamin E was followed. Studies were also made of the immunoregulatory cell subsets (OKT4 and OKT8 cell counts and the helper/suppressor ratio), the mitogen-induced lymphoproliferative response and the natural killer (NK) cell activity of patients with chronic hepatitis, before and after cyanidanol treatment.

Twenty-nine (18 male and 11 female) patients (aged 24-80 years, mean 50 years) with biopsy-proven chronic liver disease (20 hepatitis B virus-positive, 4 HBV-negative and 5 alcoholic hepatitis) were investigated. Cyanidanol was given orally (Catergen tablets, Zyma licence, Biogal, Debrecen) at a daily dose of 1.5-3.0 g; the duration of treat-

ment was from 1 week to three months, depending on the different protocols. This cyanidanol therapy resulted in normalization of the elevated serum malondialdehyde concentration after a 3-month treatment period. At the same time, after a transient increase, the high serum SOD content decreased significantly, while the blood activities of glutathione peroxidase and catalase increased. The serum vitamin E level had also risen by the end of the 3-month therapy. After a one-month course of cyanidanol treatment, significant increases in the mitogen-induced lymphoproliferative response and in the helper/suppressor ratio were seen in patients with chronic HBV-hepatitis. The NK cell activity of patients with HBV-infection was also enhanced by cyanidanol, even after a one-week treatment period. The results presented here suggest that the observed effects on the antioxidant and immunologic defence mechanisms may play a role in the efficacy of the drug in chronic hepatitis.

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B. Matkovics, D. Boda and H. Kalász (Eds)

BEFLAVIN® (B_2 VITAMIN) TREATMENT IN PATHOLOGICAL JAUNDICE OF THE NEWBORN DUE TO ABO INCOMPATIBILITY

L. PATAKI, B. MATKOVICS*, A. MOLNÁR, Z. NOVÁK and Sz.I. VARGA*

Children's Hospital and Outpatient Department,
Szeged, Hungary
*Biological Isotope Laboratory,
József Attila University of Szeged,
Szeged, Hungary

ABSTRACT

Comparative studies of antioxidant enzymes and lipid peroxidation were carried out in pathological neonatal jaundice caused by ABO incompatibility. The bilirubin level fell significantly during irradiation with blue light supplemented with vitamin B_2. Of the antioxidant enzymes, only catalase displayed a significant change in the same group.

INTRODUCTION

Vitamin B_2 has been observed to make bilirubin sensitive to light and to enhance its photocatabolism (7, 23). It has therefore been suggested that the compound should be tried clinically (5, 16, 19, 28). Vitamin B_2 does not influence the albuminbilirubin complex, and its therapeutic use involves no danger of bilirubin displacement. The advantages of preventive vitamin B_2 treatment were reported in an earlier study (20). The aim of the present examinations was to investigate the use of vitamin B_2 combined with phototherapy, which has proved effective in the prevention and rapid decrease of high bile pigment levels.

MATERIAL AND METHODS

28 mature full-term newborn infants with ABO incompatibility were examined. All were symptom-free except for jaundice, and the bile pigment levels measured on admission were so high that exchange blood transfusion was necessary in all cases. The examinations were performed within a 3-hour period during preparation for blood transfusion. 14 patients were treated for 3 hours with phototherapy only. The other 14 received vitamin B_2 combined with phototherapy. Vitamin B_2 was given in the form of Beflavin (Hoffman La-Roche). It was administered very slowly, intravenously, in a single dose of 10 mg/kg dissolved in physiological NaCl (1:3, w/v). The bilirubin levels measured on adminission, i.e. before the treatment, will be referred to as the 0-hour values. Three hours later the bilirubin determination was repeated (3-hour values) and the results for the two treatment groups were compared statistically.

The changes in activity of some of the antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (C-ase), and in the lipid peroxidation during treatment were examined by means of biochemical methods (2, 12, 18, 21), simultaneously with the serum bilirubin determinations.

RESULTS

Data on patients treated with phototherapy only or with vitamin B_2 combined with phototherapy are shown in Table 1. There were no essential differences between the two groups (0-hour) as concerns birth weight, age and 0-hour bilirubin level. The 3-hour bilirubin levels were found to be slightly higher in the phototherapy group. The increase was not statistically significant compared to the 0-hour value. The vitamin B_2 group exhibited a decreased 3-hour bilirubin level in each case. The decrease here was significant compared to the 0-hour value. Analysis of the 3-hour bilirubin values for the two

Table 1. Comparative data of newborn infants treated with phototherapy only or riboflavin-combined phototherapy

Group of patients	$\bar{X} \pm$ S.D.				Statistical significance
	Birth weight (g)	Age (h)	Serum bilirubin (micromol/l)		
			0 hour	3 hours	
Phototherapy treated patients (n=14)	3405±467	49.7±28.8	349.2±76.6	381.0±92.4	n.s.
Riboflavin-combined phototherapy treated patients (n=14)	3271±588	50.8±26.7	366.8±66.2	279.5±51.0	$p < 0.01$
Statistical significance	n.s.	n.s.	n.s.	$p < 0.01$	

groups again gave a significant difference. In 8 of the 14 vitamin B_2-treated patients the bilirubin levels had diminished to such an extent that exchange blood transfusion was no longer needed. In the other group, blood transfusion had to be performed in all the cases.

The examinations of the antioxidant enzymes gave the following results: No significant difference could be detected between the SOD levels in the two groups (Fig. 1). The catalase activity was significantly higher before treatment in the vitamin B_2 group (Fig. 2). The LP values in the two groups were not significantly different (Fig. 3).

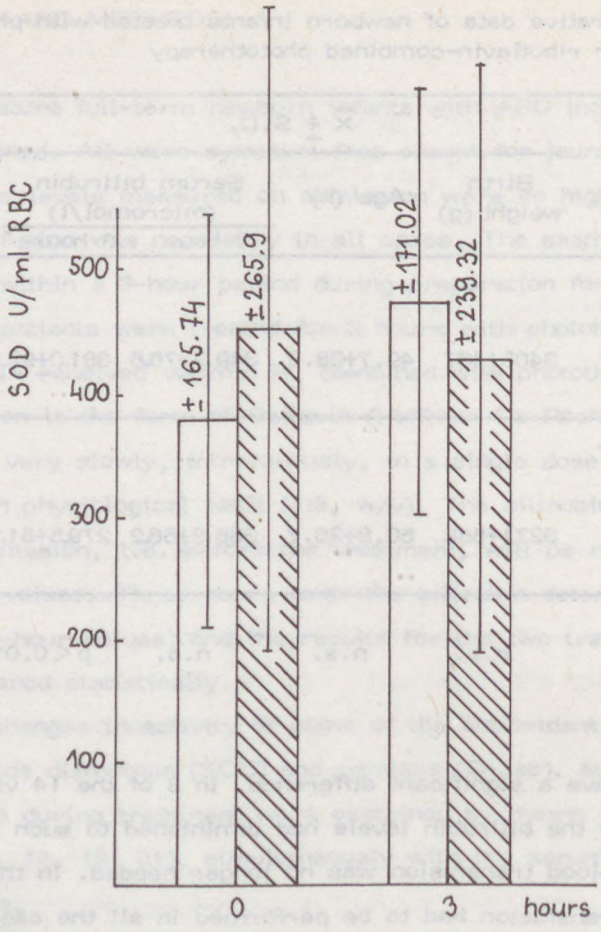

Fig. 1. Superoxide dismutase activity in patients treated with phototherapy only and with riboflavin-combined phototherapy before (0 hour) and 3 hours after treatment

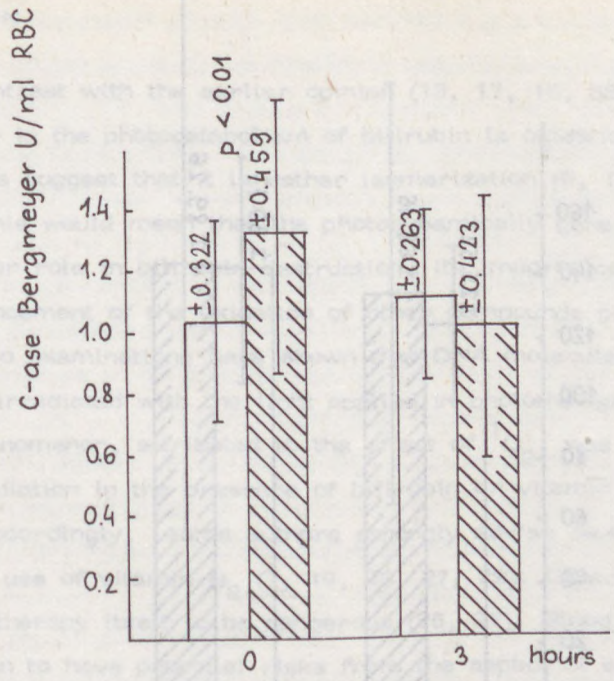

Fig. 2. Catalase activity in patients treated with phototherapy only or riboflavin-combined phototherapy before (0 hour) and 3 hours after treatment

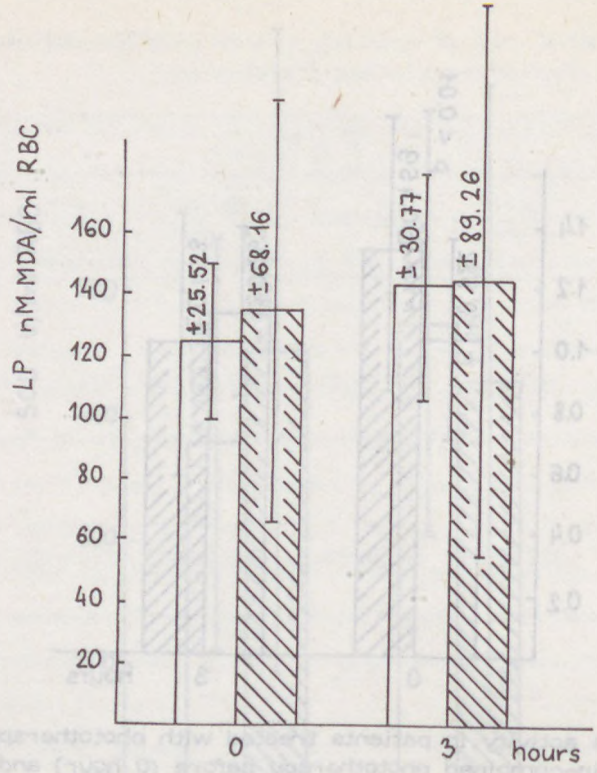

Fig. 3. Lipid peroxidation in patients treated with phototherapy only or riboflavin-combined phototherapy before (0 hour) and 3 hours after treatment

DISCUSSION

In contrast with the earlier opinion (13, 17, 19, 33) that the main factor in the photocatabolism of bilirubin is oxidation, recent observations suggest that it is rather isomerization (9, 10, 11, 14, 15, 24). This would mean that the photodynamically generated $^1O_2$ has only a minor role in bilirubin destruction, its importance rather lying in its enhancement of the oxidation of other compounds present (4, 8, 23). In vitro examinations have shown that DNA molecules may be changed if irradiated with the light applied in phototherapy (27). A similar phenomenon, attributed to the effect of $^1O_2$, was observed during irradiation in the presence of bilirubin or vitamin B_2 (6, 22, 25, 26). Accordingly, some authors strongly advise caution with the therapeutic use of vitamin B_2 (7, 19, 23, 27, 29). Others even consider phototherapy itself to be dangerous (25, 27). Blood transfusion too is known to have potential risks from the aspect of oxygen toxicity (1). However, statistical analysis of our results did not suggest that the 3-hour treatment combined with phototherapy may be more harmful towards the vitamin B_2 SOD and C-ase activities and the lipid peroxidation than the usual phototherapy (Figs 1-3). On the other hand, this combination of vitamin B_2 with phototherapy during preparation for blood transfusion led to such a strong decrease of the high bile pigment levels that exchange blood transfusion was no longer needed in the majority of the cases.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

ROLE OF FREE RADICAL REACTIONS AND RECENTLY DEVELOPED POSSIBILITIES OF PHARMACOTHERAPY IN ADULT RESPIRATORY DISTRESS SYNDROME

I. PÉNZES, V. BÄR and J. ILLEI

Korányi National Institute for Tuberculosis and Pulmonology,
National Institute for Neurosurgery,
Budapest, Hungary

Adult respiratory distress syndrome (ARDS) is a catastrophic illness currently displaying an increasing incidence. A substantial body of evidence has accumulated concerning its pathomechanism, but its mortality ranges from 40 to 100 per cent, regardless of the provoking factors. The disease is characterized by an increased permeability of the pulmonary capillaries, pulmonary edema and acute respiratory failure. Several hypotheses have been considered as potential causes of ARDS: endothelial injury due to alveolar hypoxemia (Hyman and Kadowitz 1975), pathologic changes in the pulmonary microvasculature, resulting in a "low output syndrome" (Pénzes et al. 1977, 1979), the aggregation of platelets and leukocytes (activation of complement and coagulation factors), other types of mechanical vessel occlusion, and the release of various humoral mediators, including endogenous vasoactive substances. The pathogenetic role of lysosomal proteases released from aggregated thrombocytes, granulocytes and mastocytes has also been suggested. Histamine, serotonin and their derivatives and/or metabolites, catecholamines, prostaglandins, bradykinin, ADP, lysosomal elastases and collagenases too may be important.

It appears that oxygen-derived free radicals released from polymorphonuclear leukocytes play a central role in some or all forms of ARDS. The accumulation of polymorphonuclear leukocytes is a common feature of inflammatory reactions. Leukocytes rapidly invade the lung in laboratory animals and human beings. Leukostasis in the lung has

already been demonstrated in traumatic and hemorrhagic shock (Schlag and Redl 1981, Wilson 1982), after burn trauma (Till et al. 1983), in sepsis (Powe et al. 1982, Wong et al. 1984), in hyperoxic lung (Fox et al. 1981, Jenkonson 1982), after microembolization (Flick et al. 1981) and during extracorporal circulation (Craddock et al. 1977, Zimmermann and Amory 1982).

Stimulated leukocytes release humoral and inflammatory mediators, which enhance the inflammatory response. The activated inflammatory cells produce large amounts of oxygen free radicals and release them into the extracellular space.

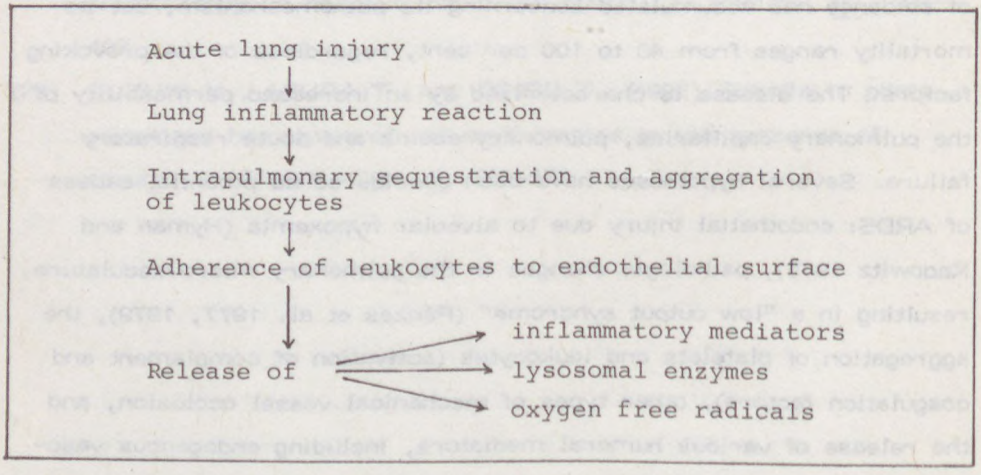

Fig. 1. Inflammatory reactions in the lung (Bertrand 1985)

Oxygen free radicals are cytotoxic to the endothelial cells (Del Maestro 1981, Weiss et al. 1981). The polymorphonuclear leukocyte membrane adheres to the endothelial surface (Shasby et al. 1983, Bowman et al. 1983).

It is common knowledge that superoxide anion radicals are formed not only in the mitochondrial space, but also in the other surfaces of the membranes of the leukocytes (Salin and McCord 1977).

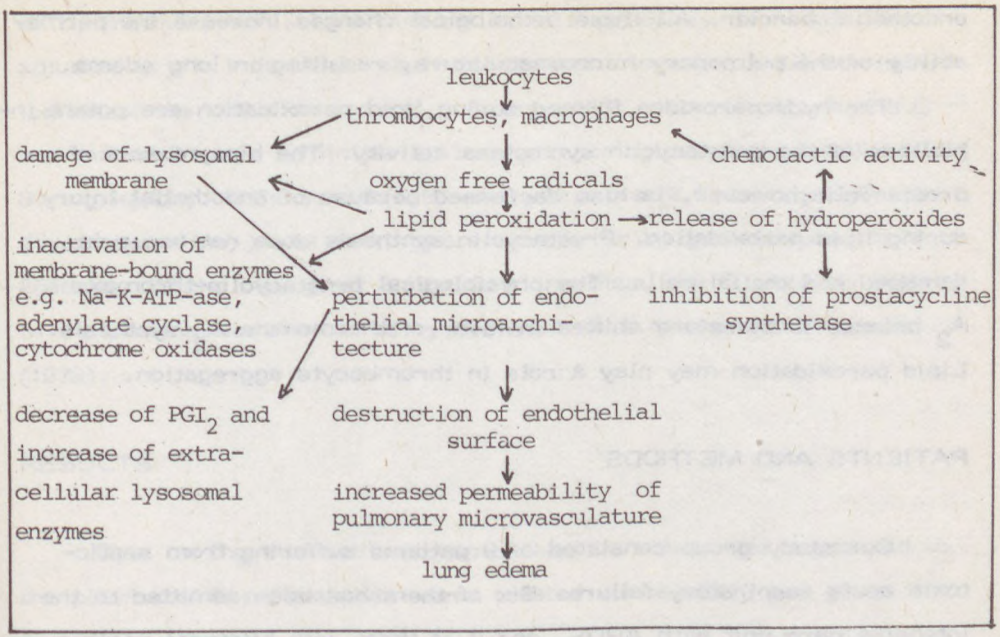

Fig. 2. Proposed mechanism of lipid peroxidation in acute respiratory failure

The role of these oxygen free radicals has already been recognized in the pathogenesis of experimentally-induced acute pulmonary edema in rabbits (Shasby et al. 1981). Experimental evidence suggests that these reactive species may also play a pathogenic role in acute lung injury.

All oxygen free radicals attack the side-chain of polyunsaturated fatty acids of lipid membranes while inducing a peroxidative chain reaction. Consequently, lipid peroxidation destroys the membrane integrity, resulting in the relase of several cellular components, e.g. lysosomal enzymes into the extracellular space. The release of hydroperoxides, endoperoxides and prostaglandins is likewise to be observed.

Free radicals destroy the endothelial barrier; they degrade hyaluronic acid (Halliwell 1978) and extracellular fibronectin (Aukburg et al. 1981, Fischer 1982), the latter being essential components of the

endothelial barrier. All these pathological changes increase the permeability of the pulmonary microvasculature, resulting in lung edema.

The hydroperoxides formed during lipid peroxidation are potent inhibitors of the prostacyclin synthetase activity. The biosynthesis of prostacyclin,however, is also decreased because of endothelial injury during lipid peroxidation. Prostacyclin synthesis does not occur in damaged endothelial cells. The physiological prostacyclin-thromoxane A_2 balance is therefore shifted in favor of thromoxane A_2 synthesis. Lipid peroxidation may play a role in thrombocyte aggregation.

PATIENTS AND METHODS

Our study group consisted of 9 patients suffering from septic-toxic acute respiratory failure. Six of them had been admitted to the intensive care unit with ARDS, and 3 of them with bilateral pneumonia. All these patients recovered, and the clinical course of their diseases, as well as the laboratory findings and the measured parameters, will therefore be interpreted together. Three additional patients had been referred to our unit in terminal stage ARDS. Their disease led to a fatal outcome. The observations and findings during their treatment will be discussed separately. Quantitative estimations of lipid peroxidation products were performed by measuring the amount of the thiobarbituric acid-reactive substances in the plasma. For this purpose the method of Stuart et al. (1975) was applied. Measurements were performed on the day of admission, on days 1, 2, 3 and 5 of treatment, and when the patient had become symptom- and complaint-free. The course of the disease was monitored by means of 3-6 blood gas analyses daily, X-ray pictures of the thorax in order to follow X-ray morphological changes in the lung, careful clinical observation and routinely performed laboratory examinations. In 4 patients, hemodynamic monitoring was carried out after Swan-Ganz catheterization. All patients received the conventional treatment for ARDS, consisting of methyl-prednisolone medication (30 mg/kg/day) for 3 days, follow-

ed by methyl-prednisolone medication in a gradually decresed dose adjusted to the individual requirements and tolerance, until normalization of the diffusion capacity. Amino acids, vitamins E, A and C and mannitol were administered in pharmacological doses. Additonally, 30 mg/kg/day of Sensorad (6,6´-methylene-bis-(2,2,4-trimethyl-1,2-dihydroquinoline)) was administered orally or by gastric tube for 4 to 26 days. Of the biological antioxidant enzymes, the CuZn-SOD activity in the plasma was measured by the method of Misra and Fridovich (1972).

RESULTS

Data on the thiobarbituric acid-reactive substances measured in the plasma of 9 patients who were treated successfully are given in Fig. 3.

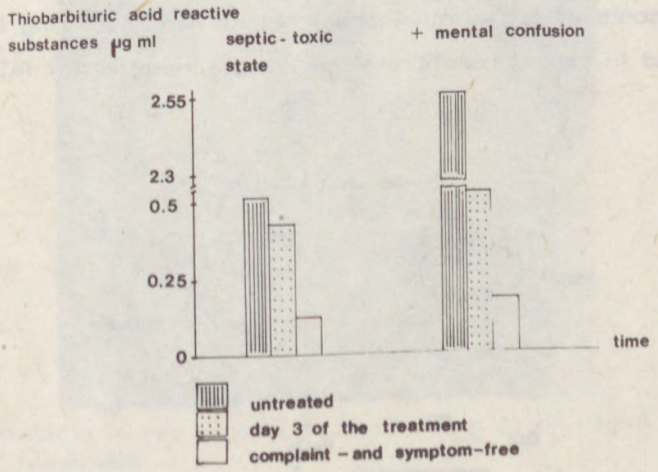

Fig. 3. Plasma concentration of thiobarbituric acid-reactive substances in patients with acute respiratory failure (n = 9)

The results suggest that lipid peroxidation is involved in the pathogenesis of acute respiratory failure. In patients with septic-toxic ARDS accompanied by mental confusion, the concentration of the thiobarbituric acid-reactive substances was found to be one order of magnitude higher than in those without mental confusion. The concentration of thiobarbituric acid-reactive substances decreased as the patients recovered. SOD activities were found to vary in acute respiratory failure. However, they were stabilized by the time of the recovery.

The superoxide anion is the main course of OH radical formation (Del Maestro et al. 1980), as shown in Fig. 4.

It is of special interest that the human organism does not possess defense mechanisms to inactivate OH radicals. However, on the action of Fe^{3+} ions, OH radicals are formed from O_2^-, and by this pathway the superoxide anion radicals may "bypass" the natural defense mechanisms (superoxide dismutase, catalase). It should be emphasized that we consider the results of SOD activity measurements in the plasma as preliminary results of indicative importance. Below, we report on some of our clinical observations demonstrating the effects of Sensorad in patients suffering from acute respiratory failure.

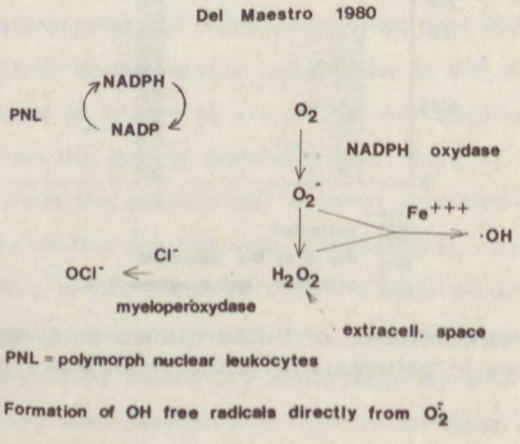

Fig. 4. Formation of OH free radicals from the superoxide anion radical (Del Maestro 1980)

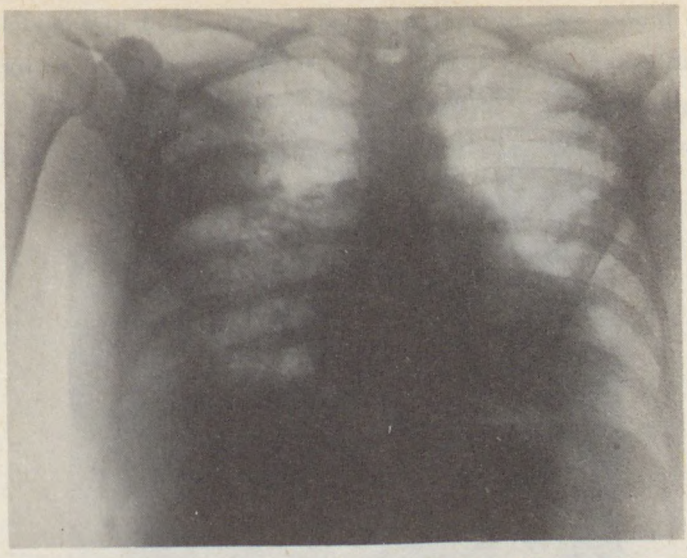

Fig. 5. Thoracic X-ray picture of H.J. (case 1) on admission

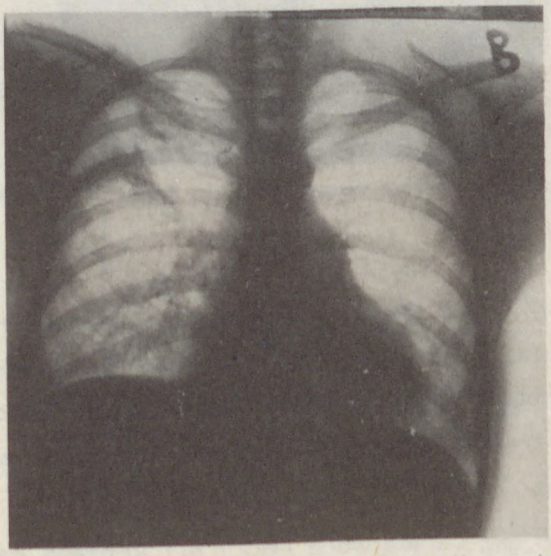

Fig. 6. Thoracic X-ray picture of H.J. (case 1) on day 4 of treatment

Case 1. H.J., a 34-year-old woman with closed and open heart surgery in the history, was admitted to the intensive care unit with cyanosis, tachypnea, tachycardia and mental confusion. The thoracic X-ray picture showed extensive infiltration in the right lung and less extensive infiltration in the left lung, as shown in Fig. 5.

The blood gas parameters were: PaO_2: 7.3 kPa and $PaCO_2$: 5.7 kPa in room air. On day 4 of treatment, the infiltrations had almost completely disappeared, as revealed by the thoracic X-ray picture (Fig. 6), and the blood gas values were: PaO_2: 11.3 kPa and $PaCO_2$: 4.4 kPa.

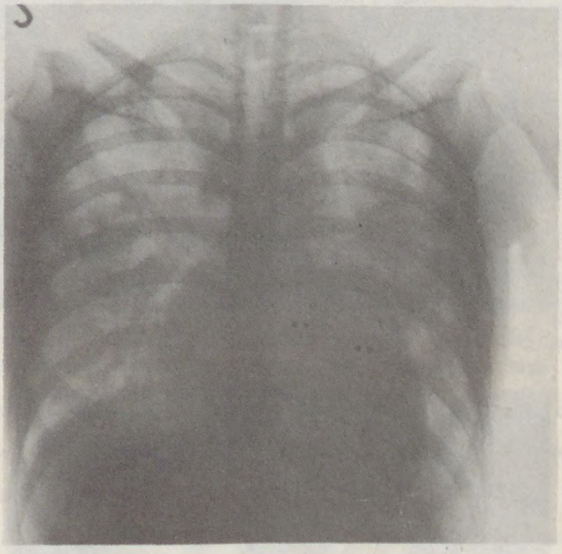

Fig. 7. Thoracic X-ray picture of M.J. (case 2) on admission

Case 2. M.J., a 19-year-old girl, suffering from grave anemia (hematocrit: 0.14), was referred to the intensive care unit because of a "homologous blood syndrome"-induced ARDS, which developed after repeated blood transfusions. The thoracic X-ray picture on admission is shown in Fig. 7.

The blood gas values were PaO_2: 8.1 kPa and $PaCO_2$: 2.2 kPa in room air. On day 4 of treatment the values were PaO_2: 10.9 kPa and $PaCO_2$: 3.8 kPa. On day 5 no changes were found in the thoracic X-ray picture, as shown in Fig. 8.

Radical scavenger medication was administered for 21 days, after which the spirometric parameters and diffusion capacity had normalized, as revealed by Hewlett-Packard spirometry (Fig. 9).

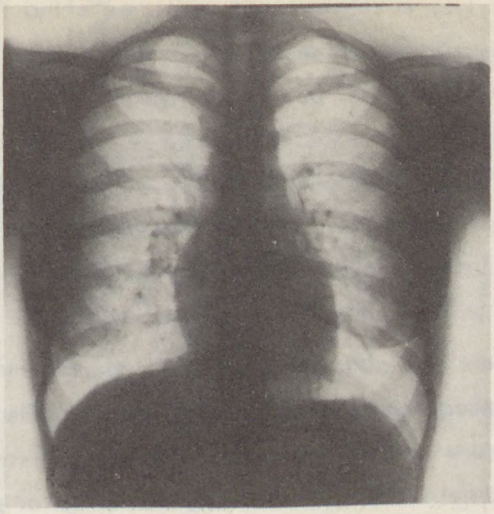

Fig. 8. Thoracic X-ray picture of M.J. (case 2) on day 5 of treatment

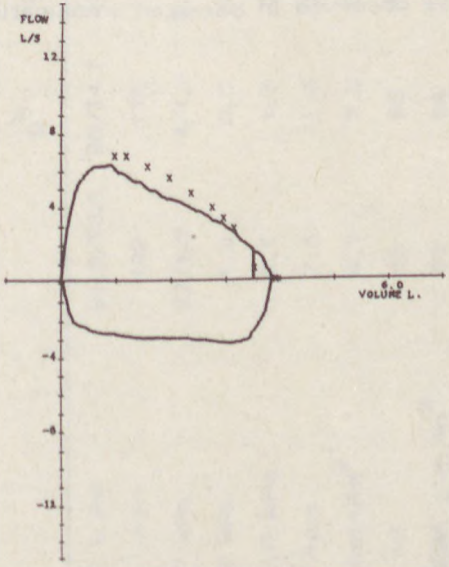

Fig. 9. Hewlett-Packard spirometry on M.J. (case 2) on day 1 of treatment

Case 3. Sz.L., a 22-year-old man, suffering from paraquat intoxication which occurred 20 days after admission. The PaO_2 value was 8-12 kPa (breathing 100 per cent oxygen using a Siemens-Servo mechanical ventilator), and the calculated Q_s/Q_t varied between 55.6 and 36%. The clinical state on admission was assessed as the terminal stage of paraquat intoxication. Radical scavenger (Sensorad)medication was started, but after this the pulmonary arterial pressure increase usually observed in hypoxia did not occur, as shown in Table 1.

In this patient the concentration of thiobarbituric acid-reactive substances decreased transiently in response to Sensorad, but it increased again before death. This observation confirms the statement published in the Environmental Health Criteria 39 of WHO, to which paraquat intoxication is a prototype of the pathologic free radical reactions. In the other two cases with a fatal outcome, the underlying disease was hemorrhagic pancreatitis with associated ARDS. The lipid peroxidation parameters and clinical course of the disease, however, were similar to those observed in paraquat intoxication.

Table 1. Pulmonary artery pressure in Sz.L. (case 3)

	13.IX.	14.			15.			16.
		9^h	14^h	19^h	9^h	11^h	14^h	9^h
SBP k Pa	21.3/13.3	20/14.7	17.3/12	17.3/10.6	20/13.3	25.3/14.7	18.7/12	24/14.7
HR ^{1}/min	122	118	112	100	114	139	118	104
PAP kPa	2.7/1.3	4/1.6	2.9/1.6	2.7/1.6	5.6/2.7	6.4/4	4/2.4	4/2.1
CVP kPa	1.2	0.5	0.3	0	0.5	0.4	0.5	0.7
PCWP kPa	0.7	1.3	0.8	0.7	1.1	1.9	1.3	1.3
CO^I/min	7.5	11.6	5.9	5.7	7.3	9.6	10	8.8
CI^I/min/m^2	4.7	7.2	3.7	3.5	4.5	5.9	6.2	5.5
SV ml	62	96	53	57	64	69	85	85
LVSWI g·m/m^2	60	94	43	44	59	72	69	88
RVSW g·m/m^2	3.1	15.9	6.7	7.8	12	21.1	14.5	12.2
PVR dyn cm^{-5} sec	107	90	149	154	197	208	112	109
SVR dyn cm^{-5} sec	1184	855	1315	1347	1227	1108	816	1162

CONCLUSIONS

After administration of the dihydroquinoline-type radical scavanger Sensorad, an unexpected dramatic improvement and/or disappearance of the X-ray morphological changes in the lung were observed, together with a substantial clinical improvement.

The blood gas values also improved. The mean values of PaO_2 and $PaCO_2$ of this group of patients in room air wereas follows: on the admission day: PaO_2: 7.1,(6.5 - 8.7) kPa; $PaCO_2$: 5.2 (2.7 - 6.7) kPa; on day 4: PaO_2: 10.8 (9.1 - 12.6), $PaCO_2$: 4.4 (3.8 - 5.4); on day 5: PaO_2: 11.8 (11.1 - 12.6), $PaCO_2$: 4.4 (4.1 - 4.8); after recovery: PaO_2: 12.2 (11.7 - 12.9), $PaCO_2$: 4.4 (4.2 - 4.9).

The usually observed hypoxia-induced pulmonary arterial pressure increase did not occur in patients treated with Sensorad.

On the basis of the above experience, we suggest that the administration of dihydroquinoline-type radical scavengers in addition to the "conventional complex treatment" promises an advance in the treatment of ARDS and other forms of acute respiratory failure.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

PATHOLOGICAL FREE RADICAL REACTIONS IN ADOLESCENCE; MODEL EXPERIMENTS IN YOUNG RATS

Zs. POLLÁK, S. SULYOK*, I. KEMENES and J. FEHÉR**

Health Department of the Social Insurance Centre, Budapest
*Hospital of the Hungarian State Railways, Budapest
**2nd Department of Medicine, Semmelweis University of Medicine,
Budapest, Hungary

Hormonal contraception, frequently beginning in adolescence, may induce thromboembolic complications and myocardial infarction. Liver damage has also been recognized as an important risk factor in the reproductive age (3, 6, 7, 8, 16, 24, 25). The role of free radical reactions in clot formation has been verified (14). Xenobiotics, including hormonal contraceptive agents, induce lipid peroxidation in several mammalian organs, including the liver. Long-acting risk factors are of special importance from the point of view of prevention. The mechanisms of these free radical reactions have been studied by our team (10).

Dihydroquinoline-type radical scavengers have been developed. 6,6-Methylene-bis(2,2,4-trimethyl-1,2-dihydroquinoline) (trade name Sensorad, code name MTDQ) is a lipid-soluble radical scavenger. 6,6-Methylene-bis(2,2-dimethyl-4-methanesulfonic acid sodium-1,2-dihydroquinoline) (code name MTDQ-DA) is a water-soluble radical scavenger (1, 2). These compounds were found to be non-toxic in several mammalian species, including humans (4, 5, 17, 18). Earlier studies revealed their decreasing action on the carbontetrachloride - and/or galactosamine - enhanced malondialdehyde serum concentration, as well as on enzyme activity changes caused by organic liver damage (9). Malondialdehyde is an indicator of prostaglandin formation in platelets (19).

The aim of the present studies was to investigate whether MTDQ and MTDQ-DA affect an increased malondialdehyde concentration in

platelets, as well as liver damage induced by high doses of combined norgestrel ethinylestradiol, both contained in the frequently-used Hungarian contraceptive, Ovidon.

MATERIALS AND METHODS

First series

Young, sexually mature female Balb-C rats weighing 120 g each were used. Four groups were formed, each containing 5 animals. Group 1 received 1 tbl. Ovidon containing 0.25 mg d-norgestrel and 0.05 mg ethinylestradiol, pulverized and suspended in 1 ml milk, through a gastric tube daily on 5 consecutive days. Group 2 received 1 tbl. Ovidon + 30 mg MTDQ-DA suspended in 1 ml milk through a gastric tube, daily for 5 days. Group 3 was given 1 tbl. Ovidon + 35 mg MTDQ suspended in 1 ml milk, daily for 5 days. Group 4 received only 1 ml milk, daily for 5 days. On day 5, blood was taken under Nembutal anesthesia by means of an abdominal section, 6 hours after the last treatment.

Second series

Adult, sexually mature female Balb-C rats weighing 220 g each were used. Four groups consisting of 10 animals each were formed. The treatment of the individual groups was identical to that used in the first series. The aim was to establish whether the results showed age- and weight-dependent differences.

The malondialdehyde concentration in the platelets was determined by the method of Stuart et al. (19, 22). The liver function was estimated by measuring GOT, gamma-GT, and alkaline phosphatase activities with an F-B automatic analyzer, using Boehringer's test. Statistical analysis was performed with Student's test.

RESULTS

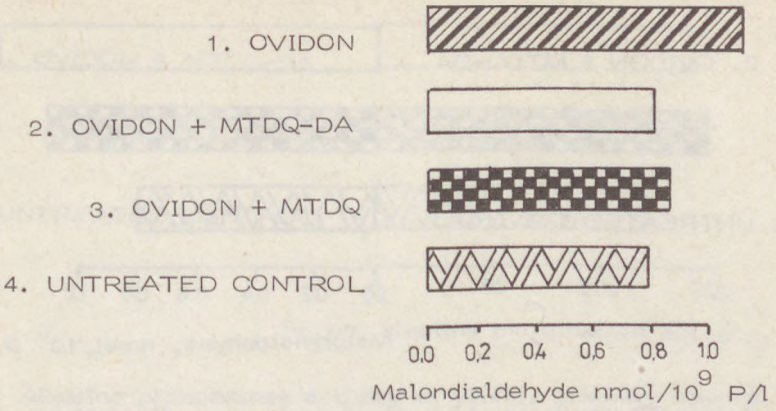

Fig. 1. Malondialdehyde in platelets of young, sexually mature (120 g) rats

Figure 1 shows the malondialdehyde concentrations in the platelets of the animals of groups 1 to 4 of the first series, expressed in nmol/10^9 P/l.

As compared to the untreated controls, the combined hormone administration induced a highly significant increase of the malondialdehyde concentration. The simultaneous administration of Ovidon and MTDQ-DA led to a significantly lower malondialdehyde concentration than that induced with Ovidon. The simultaneous administration of MTDQ also significantly moderated the malondialdehyde concentration increase produced with Ovidon alone. The malondialdehyde concentrations after Ovidon + MTDQ-DA or Ovidon + MTDQ administration were not significantly different from that for the untreated controls.

Figure 2 shows the average malondialdehyde concentrations in the platelets of the animals belonging to groups 1 to 4 of the second series, expressed in nmol/10^9 P/l.

It can be seen that Ovidon induced a highly significant increase of the malondialdehyde concentration in the thrombocytes as compared to the untreated controls. The Ovidon-induced malondialdehyde concentration increase was significantly lower if either MTDQ-DA or

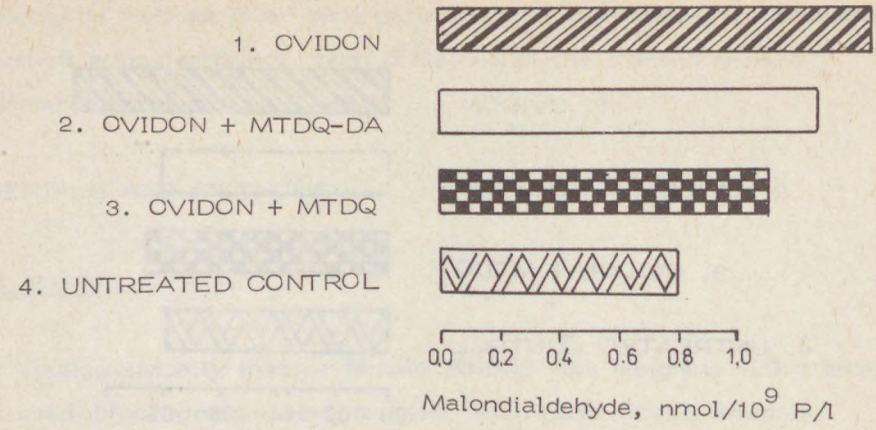

Fig. 2. Malondialdehyde in platelets of adult, sexually mature (220 g) rats

MTDQ was administered simultaneously with the Ovidon. As compared to the untreated control group, the malondialdehyde concentration increase was found to be highly significant after Ovidon + MTDQ-DA or Ovidon + MTDQ.

Figure 3 shows the alkaline phosphatase activity levels, expressed in IU/l, observed in the animals belonging to groups 1 to 4 of the first series.

It may be seen that Ovidon administration caused a significant increase of the enzyme activity compared to the level in the untreated controls. The simultaneous administration of Ovidon + MTDQ-DA or Ovidon + MTDQ resulted in significantly lower increases. As compared to the untreated controls, the differences in enzyme activity were found to be significant.

Figure 4 shows the alkaline phosphatase activity levels, expressed in IU/l, in the animals belonging to groups 1 to 4 of the second series.

Ovidon administration induced a highly significant increase of the alkaline phosphatase activity as compared to the level in the untreated animals. The simultaneous administration of MTDQ-DA or MTDQ

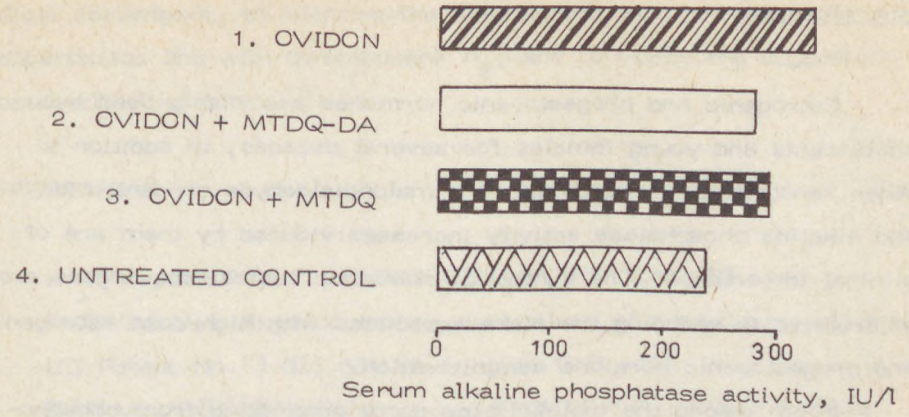

Fig. 3. Alkaline phosphatase activity in young, sexually mature (120 g) rats

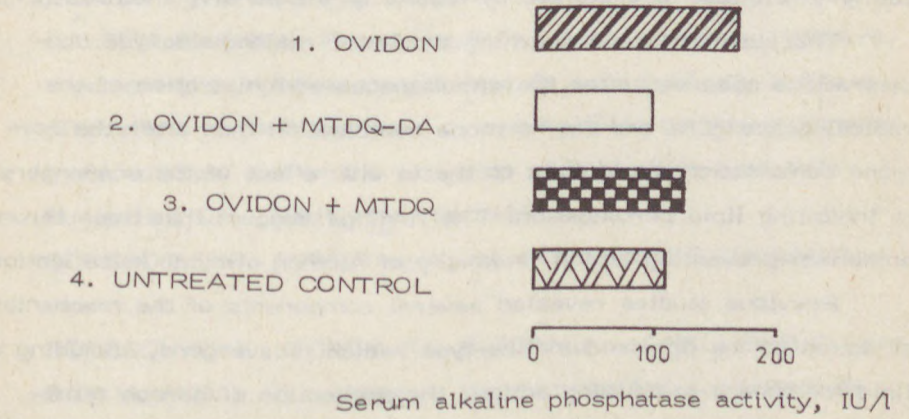

Fig. 4. Alkaline phosphatase activity in adult, sexually mature (220 g) rats

significantly inhibited this activity increase. None of the mentioned treatment modalities induced significant changes in GOT or gamma-GT activities. Detailed results therefore have been omitted.

DISCUSSION

Estrogenic and progestogenic hormones are widely used by adolescents and young females for several decades, in addition to other xenobiotics. Accordingly, the malondialdehyde concentration and alkaline phosphatase activity increases induced by them are of clinical importance. The synthetic, non-toxic radical scavengers are of promise in reducing the risks associated with high-dose estrogenic and progestogenic hormone administration.

From among the metabolic products originating from phospholipids that increase or are involved in thrombocyte aggregation, malondialdehyde was chosen because its measurement can be carried out easily in clinical laboratories by means of inexpensive methods (21).

The observations concerning the lower malondialdehyde concentrations observed after the simultaneous administration of the radical scavengers and the hormone combination than after the hormone combination alone point to the in vivo effect of the scavengers in inhibiting lipid peroxidation. The findings suggest that their thromboembolism-preventing action is worthy of further clinical investigation.

Previous studies revealed several components of the mechanisms of action of the dihydroquinoline-type radical scavengers, including a lipid membrane-stabilizing action, the prevention of carbon tetrachloride- and galactosamine-induced liver damage, hepatocyte protection, and the inhibition of xenobiotic-induced lipid peroxidation. Among the subcellular biochemical effects, the inhibition of microsomal enzymes, the protection of lysosomal membranes, the inhibition of lipid peroxide formation and the formation of oxygen-derived free radicals should be mentioned (9, 11). Further, it has been observed that these radical scavengers do not induce immunosuppression (13, 15). One of them, mixed MTDQ, has been widely used as a radiation-sensitizing agent in several hundred cancer patients (18).

Further studies are planned to clarify whether the contraceptive action of Ovidon is influenced by the simultaneous administration of

radical scavengers, to examine the interactions with prostaglandin endoperoxides and with thromboxane A_2, and to verify the suggested prostacyclin-like action.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

ETHANOL INTOXICATION AND SUPEROXIDE DISMUTASE ACTIVITY LEVELS

DO QUY HAI and B. MATKOVICS

Biological Isotope Laboratory,
József Attila University,
Szeged, Hungary

SUMMARY

Pathways not involving alcohol dehydrogenase and aldehyde dehydrogenase or the intermediate in the elimination of acetaldehyde in the ethanol metabolism are surveyed. Mainly catalase, superoxide dismutase, glutathione peroxidase and cytochrome P-450 are considered, together with the HO˙ radical and the reactions in which this radical is involved as the transforming agent in the ethanol metabolism. Certain other parameters too are discussed.

INTRODUCTION

As a consequence of the huge problem of alcoholism, an increasing number of articles deal with the mechanism of ethanol (EtOH) addiction and dependence, the regulation of the metabolism, and the mechanism of action of drugs used to help the individual break away from his or her addiction.

Accordingly, virtually all metabolic studies to date are being reconsidered, in an attempt to compartmentalize the metabolic pathways.

We should like to present an account of pathways that are not the major ones in the EtOH metabolism, e.g. Fig. 1 demonstrates that, besides alcohol dehydrogenase (ADH; EC 1.1.1.1) (16), the mixed function oxidases (MFOs), here cytochrome P-450 (c. P-450) and catalase (C-ase; EC 1.11.1.6) take part in the breakdown of EtOH. In all of these cases, the intermediate in the metabolism is acetal-

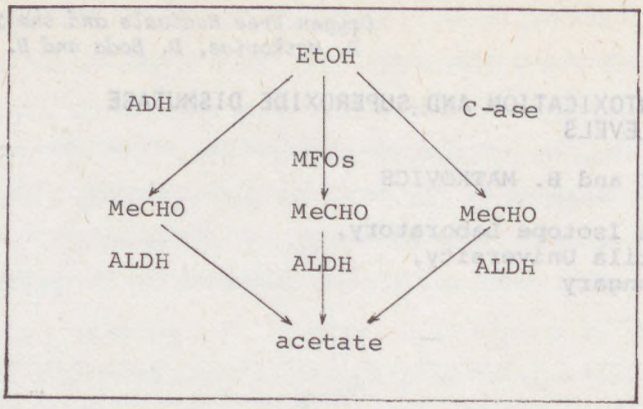


Fig. 1

dehyde (MeCHO), which is converted to acetate by aldehyde dehydrogenase (ALDH; EC 1.2.1.3) (8).

Hydrogen peroxide is formed in the following reaction, which is catalysed by superoxide dismutase (SOD; EC 1.15.1.1):

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Since H_2O_2 is decomposed by C-ase, we considered it necessary to measure not only the C-ase activity, but also the SOD activity. Glutathione peroxidase (GP-ase; EC 1.11.1.7) too plays an important role in H_2O_2 decomposition, and hence the GP-ase activity was also determined.

According to Oei et al. (1982) (10), the C-ase and GP-ase activities increase in rat heart and liver in response to chronic EtOH. Kocak-Tocker et al. (1985) (4) reported that the GP-ase activity is unchanged after acute EtOH treatment.

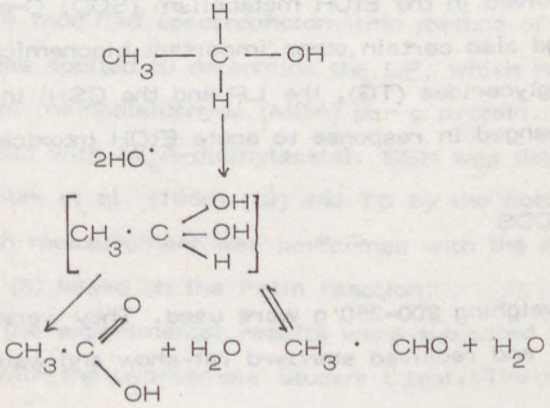
Not much is known of the participation of C-ase and c. P-450 in the EtOH metabolism.

Marklund et al. (1983) (6) found that the activity of Cu,Zn-SOD is slightly decreased, while that of Mn-SOD is slightly increased, in the various brain parts of alcoholics.

It must be mentioned that if the activities of SOD and C-ase decrease, then the quantities of O_2^- and H_2O_2 rise, and the cells undergo damage from the HO$^\cdot$ radical released in the Haber-Weiss reaction:

$$O_2^- + H_2O_2 + H^+ \longrightarrow HO^\cdot + H_2O + O_2$$

The HO$^\cdot$ radical also reacts with EtOH, to yield first MeCHO and then acetate:



This reaction leads to an increased level of MeCHO in the organism.

Speisky et al. (1985) (14) recently reported that MeCHO reacts with reduced glutathione (GSH) to form a complex:

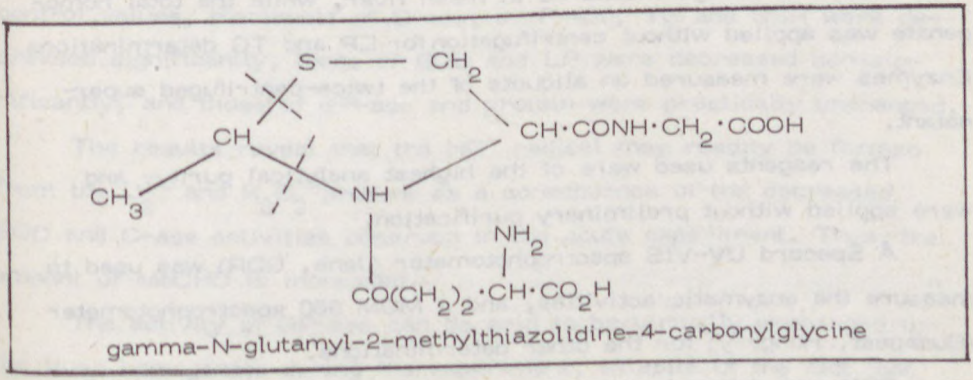


Fig. 2

They also found that the GSH level in the liver is decreased by about one-third 5 h after acute EtOH intoxication, thus MeCHO may block GSH synthesis.

It must be added that O_2^- and HO$^\cdot$ radical formation may lead to an increased lipid peroxidation (LP). Rosenblum et al. (1985) (12) ascribed the EtOH-induced liver and testis lesions in rats to the LP enhancement, as the primary membrane damage.

We have investigated how the activities of various specific and non-specific enzymes involved in the EtOH metabolism (SOD, C-ase, GP-ase and c. P-450) and also certain other important biochemical parameters (the liver triglycerides (TG), the LP and the GSH) in rat liver homogenates are changed in response to acute EtOH intoxication.

MATERIALS AND METHODS

Female CFY rats weighing 200-250 g were used. They were kept under identical conditions and received standard rat-show and water ad libitum.

Before treatment, the rats were starved for 24 h. They were then injected i.p. with 5 g EtOH/kg, and 1 h later were decapitated under Nembutal (40 mg/kg) anaesthesia. The liver was excised and homogenized under cooling in a Potter teflon homogenizer (4 ml 0.9% NaCl solution/g wet liver).

GSH was always measured in fresh liver, while the total homogenate was applied without centrifugation for LP and TG determinations. Enzymes were measured on aliquots of the twice-centrifuged supernatant.

The reagents used were of the highest analytical purity, and were applied without preliminary purification.

A Specord UV-VIS spectrophotometer (Jena, GDR) was used to measure the enzymatic activities, and a MOM 360 spectrophotometer (Budapest, Hungary) for the other determinations.

SOD was determined by the methods of Misra et al. (1972) (9) and Matkovics et al. (1977) (7);. both are based on inhibition of the

epinephrine - adrenochrome transformation, which depends on the quantity of SOD. C-ase activity was measured by the ultraviolet method of Beers et al. (1952) (1). GP-ase was measured by a combination of the methods of Chiu et al. (1976) (2) and Sedlak et al. (1968) (13) with cumene hydroperoxide as substrate. The determinations of ADH and ALDH were performed by following the reduction of NAD to NADH at 340 nm. c. P-450 was determined by the method of Greim et al. (1970) (3).

A modified spectrophotometric method of Placer et al. (1966) (11) was applied to determine the LP, which was expressed in units of nmol malondialdehyde (MDA) per g protein. The calibration plot was produced with MDA-diethylacetal. GSH was determined by the method of Sedlak et al. (1968) (13) and TG by the acetylacetone procedure (15). Protein measurement was performed with the method of Lowry et al. (1951) (5) based on the Folin reaction.

The experimental results were subjected to mathematical evaluation with the two-sample Student t test. The data are given as the means ($\bar{x}$) and standard errors ($\pm$ SE) of several parallel measurements, varying between 5 and 10.

The period of 1 h after the administration of 5 g EtOH/kg was selected as the most suitable for our investigations on the basis of a number of preliminary experiments and the literature data.

The results are presented in Tables I and II. Relative to the control values, the levels of C-ase, c. P-450, TG and GSH were decreased significantly, those of SOD and LP were decreased non-significantly, and those of GP-ase and protein were practically unchanged.

The results reveal that the HO$^{\cdot}$ radical may readily be formed from the O_2^- and H_2O_2 present as a consequence of the decreased SOD and C-ase activities observed in the acute experiment. Thus, the amount of MeCHO is increased.

The activity of GP-ase can be said to be virtually unchanged in the liver homogenate during the experiment, in spite of the fact that the amount of GSH decreased. The GSH presumably reacts directly with the MeCHO as mentioned above.

Table I.

SOD U/g protein		Mn-SOD U/g protein		Cu, Zn-SOD U/g protein	
Control n=6	EtOH n=6	Control n=6	EtOH n=6	Control n=6	EtOH n=6
53,000 ±8,900	49,000 ±8,700	2,100 ± 400	2,200 ± 200	50,900 ±9,000	46,900 ±8,600

C-ase BU/g protein		GP-ase U/g protein		c. P-450 nM/mg protein	
Control n=9	EtOH n=9	Control n=3	EtOH n=3	Control n=6	EtOH n=6
7.3 ±1.4	6.1 ±0.9	920 ±130	1.030 ± 180	0.9664 ±0.0796	0.7742 ±0.1972

$p < 0.05$ $p < 0.05$

Table II.

LP nM MDA/g protein		TG µmol/g protein	
Control n=9	EtOH n=9	Control n=6	EtOH n=6
321.2 ±105.6	296.1 ±66.9	64.8 ±16.4	52.4 ±17.2

$p < 0.2$

GSH µM/g protein		Protein g/100 g wet wt.	
Control n=9	EtOH n=9	Control n=18	EtOH n=19
38.5 ± 5.3	23.5 ± 5.7	11.6 ±1.1	11.3 ±1.6

$p < 0.01$

We assume that the decrease of c. P-450 (MFO) can be explained by the MeCHO sensitivity of the system.

The LP decrease was not significant but was general, and may be explained by the scavenging effects of EtOH on oxygen radicals.

The decrease of TG in acute EtOH intoxication may occur through the TG entering the blood.

Thus, our measurements appear to support the supposition that the antioxidant enzyme system, like all of the other parameters measured, plays a role in the elimination of EtOH. The extents to which the parameters in question are involved in EtOH elimination will form the subject of future studies.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

ANTIOXIDANT PROTECTION AGAINST FREE RADICAL-MEDIATED MYOCARDIAL INJURY

E. RÖTH, B. TÖRÖK, V. BÄR* and Gy. TEMES

Department of Experimental Surgery,
University of Medicine, Pécs,
*Material Chemical Works,
Budapest, Hungary

INTRODUCTION

Previous studies have indicated (4, 5, 9) that oxygen-derived free radicals may contribute to the myocardial damage induced by various pathologic states, including ischemia and reflow. The major cytotoxic effect of oxygenerated free radicals(2)may be the peroxidation of lipid compounds of the membrane bilayer, together with a reduced cellular defense mechanism. In the event of the exhaustion of the natural scavengers, the synthetic antioxidants can play a substantial role (1, 7). For this purpose we have investigated the effect of a new Hungarian antioxidant compound, MTDQ-DA, i.e. 6,6 -methylene-bis-2,2-dimethyl-4-methanesulfonic acid sodium, 1,2-dihydroquinoline (trade name KONTRAD), which exerts radical scavenger action in various biological systems (3, 10). Our earlier investigations confirmed that this antioxidant may substitue endogenous scavengers and blocks propagation of the Haber-Weiss reaction in myocardial ischemia (11, 12).

The objective of the present work was to study the lipid peroxidation of myocardial tissue and scavenger compound after transient coronary ligation followed by reperfusion in untreated animals and in animals with antioxidant protection.

MATERIAL AND METHODS

45 mongrel dogs (15–20 kg) were premedicated, then anesthetized with sodium barbital (20 mg/kg) and ventilated artificialiy with oxygen–nitrous oxide in the ratio 1:3.

Group I (15 dogs): control animals without antioxidant treatment. By means of left thoracotomy, LAD (left anterior descending coronary artery) ligation was performed just distal to the first major diagonal branch. After ischemia for 30 min, or 1 or 2 hours, reperfusion was maintained for 1 hour.

Group II (15 dogs): the surgical procedure was the same as in the first group, but a simultaneous intravenous drip infusion of 150 mg/kg MTDQ–DA was given during the ligation and reperfusion.

Group III (15 dogs): 150 mg/kg MTDQ–DA was administered to the animals for 2 weeks before the surgical procedure. LAD ligation was performed on day 14 by means of the above technique.

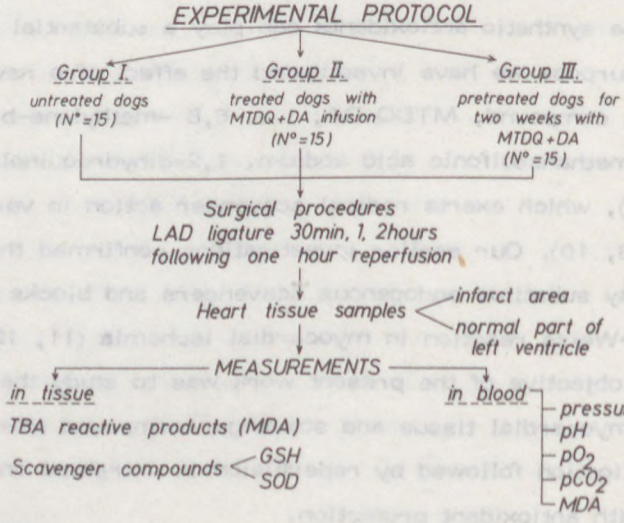

Fig. 1. Scheme of experimental protocol

In all three groups, at the end of reperfusion the hearts were excised and tissue samples were taken from the infarct and intact parts of the left ventricular wall for biochemical measurements.

During the experiments, a standard ECG was recorded on a 6-channel polygraph and a catheter was inserted into the femoral artery and vein to measure the systemic blood pressure and to take venous blood samples for biochemical investigations.

Biochemical assays

a) The lipid peroxidation end-product, mainly malondialdehyde (MDA), gives a reaction with thiobarbituric acid which can be measured photometrically at 532 nm (11).

b) The reduced glutathione (GSH) content was measured by a modified method with a colorimetric reaction at 412 nm (10, 12).

c) The superoxide dismutase (SOD) activity was determined according to Misra and Fridovich (8), measuring the transformation of adrenaline to adrenochrome.

Values of MDA and GSH were expressed as percentages while SOD activity was calculated in Unit/g wet tissue. The Student t-test was used to evaluate the differences between the control and experimental groups.

RESULTS

Figure 2 depicts the alternations in the thiobarbituric acid-reactive products. In the control group, the MDA was already elevated after the one-hour ischemia, but the elevation was significant after the 2-hour ischemia and reperfusion. In the second and third groups, where antioxidant treatment was administered, the end-product of lipid peroxidation remained near the normal level, independently of the former ischemic period.

The level of GSH did not show any changes after the 30-min ischemia and reperfusion in any of the three groups. In Group I,

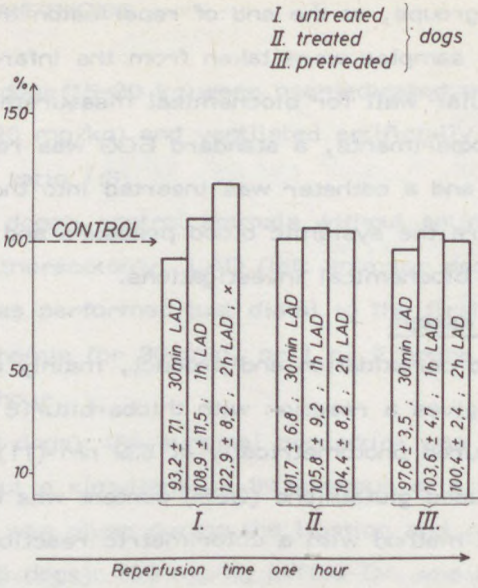

Fig. 2. Alterations in thiobarbituric acid-reactive products (MDA) in infarct area

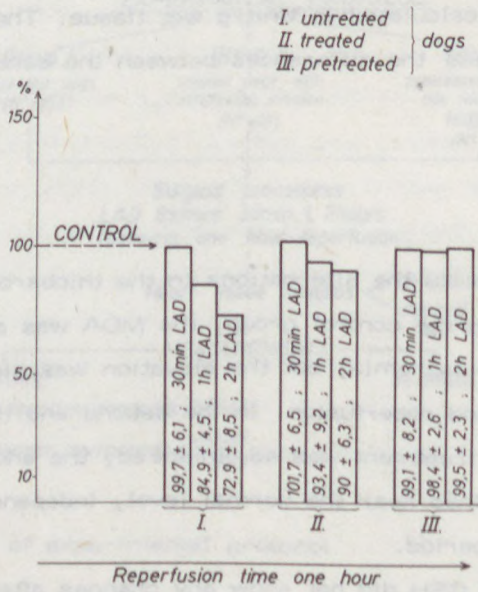

Fig. 3. Alterations in reduced glutathione (GSH) in infarct area

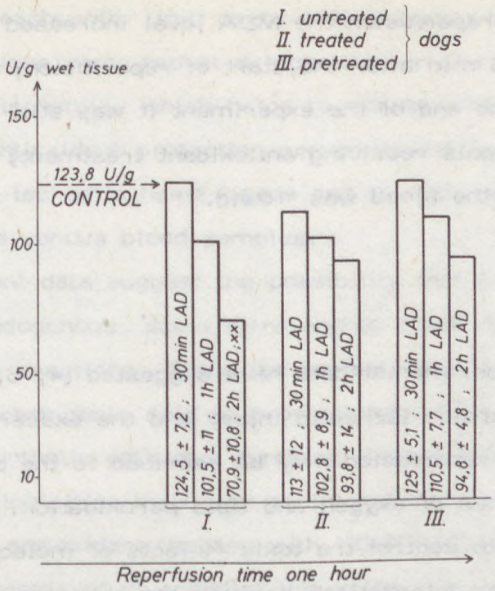

Fig. 4. Superoxide dismutase (SOD) in infarct area

after the 1 and 2-hour ischemia this endogenous thiol compound decreased sharply in the infarct area of the left ventricle. In Group II, where the animals received the KONTRAD infusion, this depletion was only moderate. In Group III, after pretreatment with MTDQ-DA, the GSH remained at the normal level, even after the 2-hour ischemia and reperfusion.

The activity of SOD in normal heart tissue in dogs in about 123 U/g wet tissue. The SOD activity remained unchanged after the 30-min ischemia. In the untreated animals, the enzyme activity decreased gradually and after the 2-hour ischemia its value was significantly lower than in the normal tissue. In Groups II and III, we observed slight decreases of the enzyme content.

We measured the thiobarbituric acid-reactive compounds in the venous blood samples at the beginning of anesthesia, at the end of ligation and 5, 10, 15, 30 and 45 min after reperfusion. In the control

group and during reperfusion the MDA level increased continuously. Its peak was found 15 min after the start of reperfusion; thereafter it decreased, but at the end of the experiment it was still above the normal level. In animals receiving antioxidant treatment, no elevation of the MDA level in the blood was found.

DISCUSSION

Different experimental data have suggested (4, 6, 7, 12) that the pathogenesis of cardiac ischemic injury and the exacerbation of hypoxia-induced injury on reperfusion may be ascribed to the production of activated metabolites of oxygen and lipid peroxidation. The ability of the heart muscle to control the toxic effects of molecular oxygen depends upon complex interactions between the abnormal production of dangerous free radicals and the endogenous scavenger content of the myocardial tissue (4). Ischemia followed by reperfusion may cause serious tissue injury because of the more abundant production of free radicals and the damage to the protective enzyme systems. A burst of O_2^- production is associated with the conversion of hypoxanthine to xanthine by xanthine oxidase with the reintroduction of oxygen. Other sources of free radicals may be the autoxidation of catecholamines, the activation of neutrophils and alterations in the redox state of mitochondria, resulting in O_2^- release.

Our present work showed that transient myocardial ischemia followed by reperfusion casues different serious biochemical alterations in the heart tissue. We could not find differences after a 30-min ischemia and a one-hour reperfusion between control and antioxidant-treated animals. After one-hour LAD ligation, in the control group the scavenger compounds showed alterations indicating that the natural defense mechanism had been damaged. After a longer ischemic time, this event appeared in more serious form.

The administration of MTDQ-DA may prevent or decrease the biochemical consequences of myocardial ischemia and reflow. We as-

sumed that it reacts with lipid peroxides, trapping the free radicals. Reduced glutathione remained at a high level in the infarct area even after a 2-hour ischemia, which helps to maintain the normal cellular function. Our antioxidant protection prevented the increase of lipid peroxidation in the ischemic heart tissue and in this way MDA release did not occur in the venous blood samples.

The present data suggest the possibility that MTDQ-DA helps to preserve the endogenous scavengers and to block the propagation of dangerous chain reactions. Since the oxygen radicals stimulate the conversion of arachidonic acid to prostaglandin G_2 and activate the prostaglandin synthetic pathway, synthetic free radical scavengers with cyclooxygenase inhibition may play an important role.

Since the antioxidant therapy with KONTRAD had favourable effects on the outcome of myocardial infarction (12), in this context it should be assumed that this non-toxic radical scavenger might open new perspectives for increase of the survival of tissues exposed to free radicals produced during ischemia and reperfusion.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

EXAMINATION OF OXYGEN-DERIVED FREE RADICALS IN OSTEOARTHROTIC PATIENTS TREATED WITH A GLYOSAMINOGLYCAN POLYSULPHATE

A.M. SÁRKÖZI, M. MÉZES* and G. BARTOSIEWICZ

Dept. of Rheumatology, Central Hospital of Hungarian State Railways, Budapest
*Dept. of Animal Physiology and Hygiene, University of Agricultural Sciences, Gödöllő, Hungary

SUMMARY

The synovial fluid level of malonyldialdehyde and the activities of catalase and glutathione peroxidase were repeatedly examined during intraarticular treatment with glycosaminoglycan polysulphate in osteoarthrosis. The changes in the peroxide level (represented by malonyldialdehyde) and the activity of glutathione peroxidase were inverse. A temporary brief increase was found in the catalase activity, with one of longer duration in the glutathione peroxidase activity, followed by a continuously decreasing catalase activity. These biochemical changes were compared with the clinical state of the patients.

INTRODUCTION

Osteoarthrosis (OA) is characterized by a net loss of proteoglycan and collagen, with a resultant loss of cartilage. Inflammation is not a prominent feature in OA, although it may be said that the disease starts as a degenerative process, but progresses with secondary inflammatory components. There are signs of limited inflammation within the synovium, and immune complexes are also often present in the articular cartilage of OA patients (8, 12).

There is increasing evidence from in vitro studies that free radicals are involved in the degradation of collagen and proteoglycan. These radicals can be generated by macrophages and polymorphonuclear leukocytes during inflammation in response to chemotactic stimuli or endocytosis of immune complexes (4, 7, 14, 15).

Treatment with Arteparon (Luitpold-Werk, Munich), an analogous compound to the cartilage matrix, represents therapy at a biochemical level in OA. Biochemically, this is a glycosaminoglycan polysulphate (GAGPS). An inhibitory effect of GAGPS was found on glycosaminoglycan-cleaving enzymes, elastase and cathepsin B1 and G, the latter being of importance in the degradation both of collagens and of proteoglycans (3, 11, 13, 17).

In the present work we examine whether GAGPS has an influence on the oxygen-derived free radical-controlling system.

METHODS

GAGPS (50 mg) was administered intraarticularly twice a week to 17 patients suffering from OA of the knee joint. The total dose was 500 mg. 12 patients were male and 5 were female. The average age was 45 years. The complaints had started 1-6 years previously, 10 patients had a primary, and 7 a secondary type of the disease. Prior to the treatment a slight joint inflammation could be detected in each case, without a marked joint effusion. GAGPS treatment was repeated at an interval of 72 hours, and thus the synovial fluid reflected the state 72 hours after the drug administration. In this period, the GAGPS was accumulated in the cartilage (9).

The changes in malonyldialdehyde level (thiobarbituric acid-reactive compounds) and the activities of catalase and glutathione peroxidase were repeatedly examined in the synovial fluid. Malonyldialdehyde, being an end-product of the lipid peroxidation, permits conclusions concerning the peroxide content; it was examined via the thiobarbituric acid reaction (16, 18). Malonyldialdehyde tetraacetate (Fluka AG., Basel) was used as a standard. Catalase activity was measured in Bergmeyer units by spectrophotometry (1 U = cleavage of 1 g hydrogen peroxide/minute at 25°C). Catalase activity, referred to 1 g protein, was determined by the biuret method. It is well known that this enzyme inactivates hydrogen peroxides. Glutathione peroxidase (im-

portant in the linking of hydroperoxides) was examined by the method of Szabó (19).

RESULTS

No synovial fluid could be obtained following the 7th injection, i.e. after a total dose of 350 mg, which itself is a sign of the lack of inflammation. Previously, only 0.5–2 ml synovial fluid could be aspirated during each occasion.

The catalase activity displayed a decreasing tendency in the synovial fluid, but without any significant changes. The transient increase following the first injection seems to be correlated with the slight transitory joint inflammation experienced in one-third of the patients. The decrease was more marked in the second part of the treatment, which was in accordance with the definite clinical improvement (Fig. 1).

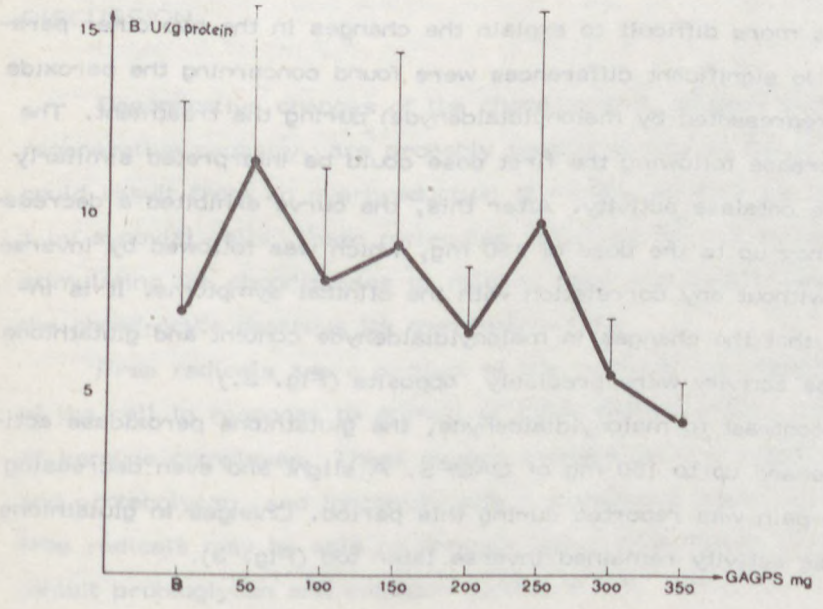

Figure 1. Catalase activity ($\bar{X}$ + S.D.) in the synovial fluid during treatment with GAGPS (B = before)

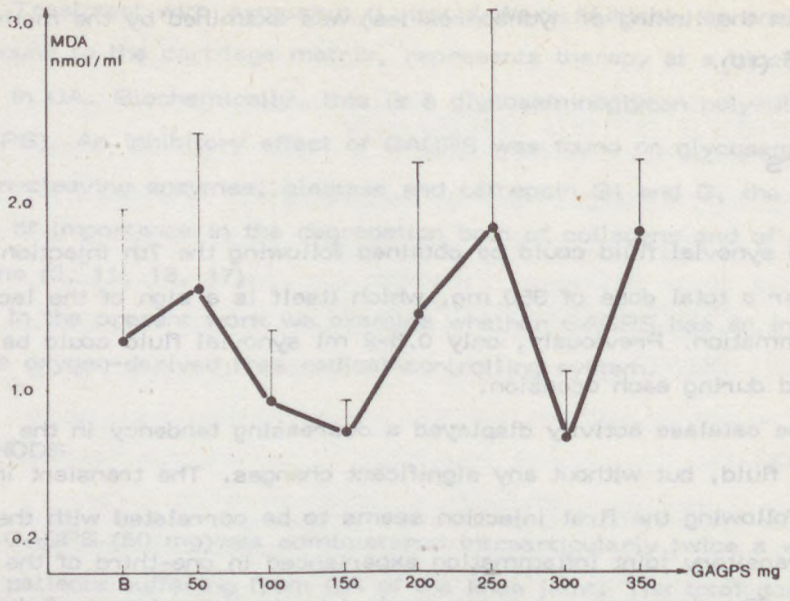

Figure 2. Changes in TBA-reactive compounds (malonyldialdehyde) ($\bar{X} \pm$ S.D.) in the synovial fluid of OA patients treated intraarticularly with GAGPS

It is more difficult to explain the changes in the two other parameters. No significant differences were found concerning the peroxide content (represented by malonyldialdehyde) during the treatment. The slight increase following the first dose could be interpreted similarly as for the catalase activity. After this, the curve exhibited a decreasing tendency up to the dose of 150 mg, which was followed by inverse phases, without any correlation with the clinical symptoms. It is interesting that the changes in malonyldialdehyde content and glutathione peroxidase activity were precisely opposite (Fig. 2.)

In contrast to malonyldialdehyde, the glutathione peroxidase activity increased up to 150 mg of GAGPS. A slight and even decreasing articular pain was reported during this period. Changes in glutathione peroxidase activity remained inverse later too (Fig. 3).

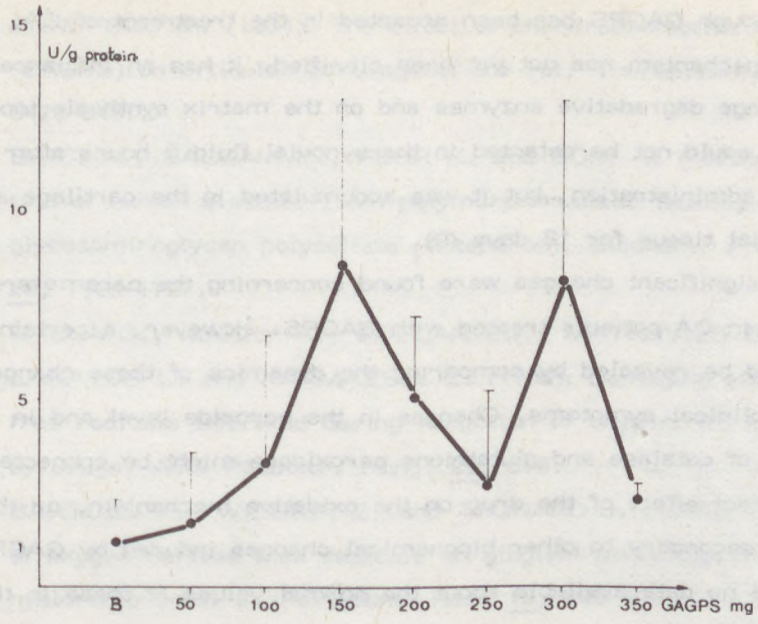

Figure 3. Glutathione peroxidase activity ($\bar{X} \pm$ S.D.) in the synovial fluid during treatment with GAGPS

DISCUSSION

Degenerative changes of the chondrocytes, coupled with a lack of regenerative capacity, are probably central events in OA (1). This could result from an overproduction of molecules such as interleukyn 1 by synovial cells, these molecules inhibiting normal synthesis and stimulating the chondrocytes to release degenerative proteinases. Thus, the chondrocyte destroys its own matrix (11).

Free radicals are a product of the primary "respiratory burst" of the cell in response to stimuli of inflammation and/or endocytosis of immune complexes. These oxygen metabolites can attack collagen and proteoglycan, and inactivate alfa 1-proteinase inhibitor. Further, free radicals may be able to activate latent proteinases and even to inhibit proteoglycan and collagen synthesis (5, 10).

Although GAGPS has been accepted in the treatment of OA, its working mechanism has not yet been clarified. It has an influence on the cartilage degradative enzymes and on the matrix synthesis too (2). The drug could not be detected in the synovial fluid 3 hours after intra-articular administration, but it was accumulated in the cartilage and in the synovial tissue for 12 days (9).

No significant changes were found concerning the parameters we examined in OA patients treated with GAGPS. However, a certain tendency could be revealed by comparing the dynamics of these changes with the clinical symptoms. Changes in the peroxide level and in the activities of catalase and glutathione peroxidase might be connected with a direct effect of the drug on the oxidative mechanism, or they might be secondary to other biochemical changes induced by GAGPS. There are no data available about the normal values of these in the synovial fluid.

Changes in the peroxide level (represented by malonyldialdehyde) and in the activity of glutathione peroxidase were inverse, showing that these parameters formed a system in this case. A temporary brief increase was found in catalase activity, and one of longer duration in the case of glutathione peroxidase, followed by a continuously decreasing catalase activity; this was matched by the clinical picture. Following the 9th day of GAGPS treatment, an inverse peroxide level and glutathione peroxidase activity fluctuations were found; these were not in accordance with the clinical state, which showed a permanent improvement and a lack of inflammation. These inverse phases might be connected with biochemical changes different from the former ones, which makes further investigations necessary.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

CORRELATION BETWEEN FREE RADICALS AND THE DEVELOPMENT OF ETHANOL-INDUCED GASTRIC MUCOSAL DAMAGE

G. SÜTŐ, Á. VINCZE, T. ZSOLDOS* and Gy. MÓZSIK

First Department of Medicine and
*Department of Biology,
University Medical School,
H-7643 Pécs, Hungary

Free radicals have been implicated in the pathogenesis of tissue damage caused by physical agents (e.g. ionizing radiation), chemicals (e.g. carbon tetrachloride or paraquat) or ischemia/reperfusion. Numerous compounds (with and without sulfhydryl groups) scavenge free radicals, decrease lipid peroxidation and prevent organ injury.

Many structurally unrelated chemicals damage the gastric mucosa in experimental animals and humans. The most videly used chemicals in animal models are 0.6 M HCl, 0.2 M NaOH, 96% ethanol, hypertonic NaCl and drugs (aspirin, indomethacin, reserpine and epinephrine). We have postulated that free radicals may be common biochemical mediators of the gastric mucosal injury induced by chemicals. Furthermore, free radicals and their scavengers might play a role in the prevention of gastric mucosal lesions by different compounds (Czeglédi et al.,1984, 1985; Jávor et al.,1983, 1985; Mózsik et al., 1984a,b; 1986,b).

Previously, the gastric mucosal superoxide dismutase (SOD) was measured under different experimental circumstances (Czeglédi et al., 1984, 1985; Mózsik et al., 1984a,b, 1985, 1986a,b,; Zsoldos et al., 1984) in order to evaluate its role in the development of gastric mucosal lesions and in the prevention of mucosal damage. These results did not reveal a close correlation between the mentioned parameters, which would have provided a better understanding of the suggested mechanisms in these processes.

Szabó (1984) reported that early and late biochemical and vascular reactions were found in the development of ethanol-induced gastric mucosal damage.

In this paper, we analyse the changes in the gastric mucosal catalase (CAT), glutathione peroxidase (GSH-Px), reduced glutathione (GSH), SOD and malondialdehyde (MDA) at different times (0, 1, 5, 15, 30 and 60 min) after the administration of 96% ethanol (1 ml intragastrically), in relation to the development of gastric mucosal damage. The aim was to evaluate possible correlations betwen the free radical mechanisms and ulcer development in the ethanol model.

MATERIALS AND METHODS

The observations were made on both male and female rats of the CFY strain, weighing 180 to 210 g. The animals were fasted for 24 hours before the experiments, but they received water ad libitum. Examinations were carried out in the morning. The gastric mucosal damage was produced by the intragastric administration of 1 ml 96% ethanol. The animals were killed 0, 1, 5, 15, 30 or 60 min after the administration of ethanol.

The following parameters were measured: the number and severity of gastric mucosal lesions (ulcers); the CAT activity (by the method of Beers and Sizer, 1952); the GSH activity (by the method of Ellman, 1979); GSH-Px activity (by the method of Sedlak and Lindsay, 1968); the SOD activity (by the method of Misra and Fridovich, 1972, as modified by Matkovics et al., 1977); the MDA content (by the method of Fong et al., 1972, as modified by Zsoldos et al., 1983); and the protein content (by the method of Lowry et al., 1951).

The results were calculated per mg protein (means ± SEM).

RESULTS

The CAT activity was increased at 1 min, the GSH-Px activity from 1 to 60 min, and the SOD activity from 15 to 60 min after etha-

MECHANISMS OF FREE RADICALS AND DEVELOPMENT OF ETHANOL-INDUCED GASTRIC MUCOSAL LESIONS IN RATS

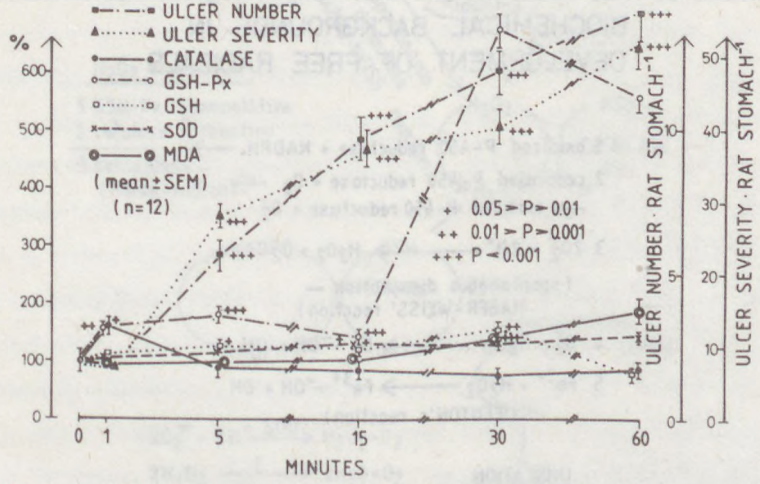

Fig. 1. Changes in the activities of gastric mucosal CAT, GSH-Px, SOD, GSH and MDA in connection with ulcer development, at different times after the administration of ethanol. The results were expressed as percentages (means ± SEM) of the results obtained at 0 min. The unpaired Student's t test was used for statistical analysis of the results

MECHANISMS OF FREE RADICALS AND DEVELOPMENT OF ETHANOL-INDUCED GASTRIC MUCOSAL LESIONS IN RATS

TIME PERIODS

	1 min	5 min	15 min	30 min	60 min
ULCER NUMBER	—	↑↑↑	↑↑↑	↑↑↑	↑↑↑
ULCER SEVERITY	—	↑↑↑	↑↑↑	↑↑↑	↑↑↑
CATALASE	↑	NS	NS	NS	NS
GSH-Px	↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
GSH	NS	↑	↑	↑↑	NS
SOD	NS	NS	↑	↑↑↑	↑↑↑
MDA	NS	NS	NS	NS	↑↑↑

NS = NOT SIGNIFICANT ↑ = 0.05 > P > 0.01 ↑↑ = 0.01 > P > 0.001 ↑↑↑ = P < 0.001
↑ = INCREASE

Fig. 2. Summary of correlation between free radical mechanisms and development of ethanol-induced gastric mucosal damage (see also Fig. 1)

nol administration. The content of GSH was increased from 1 to 30 min, and that of MDA at 60 min. The number of gastric lesions rose progressively after ethanol administration (Figs 1 and 2).

DISCUSSION

Figures 3 and 4 indicate the possible main mechanisms of the development of free radicals and their physiological "detoxication".

BIOCHEMICAL BACKGROUNDS IN DEVELOPMENT OF FREE RADICALS

1. oxidized P-450 reductase + NADPH $\longrightarrow$
2. reducated P-450 reductase + O_2 $\longrightarrow$
   $\longrightarrow$ oxidized P-450 reductase + O_2^-
3. $2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$
   (spontaneous dismutation — HABER-WEISS' reaction)
4. $O_2^- + H_2O_2 \longrightarrow O_2 + {}^-OH + {}^{\cdot}OH$
5. $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^-OH + {}^{\cdot}OH$
   (FENTON's reaction)

INDICATION:
MEASUREMENT OF H_2O_2

Fig. 3. Schematic presentation of the development of free radicals in the different tissues

The changes in the activities of GSH-Px and CAT occur in the first step of the breakdown of H_2O_2, and that of horseradish peroxidase in the second step. $O^{\cdot-}$, originating from O_2, is transformed to H_2O_2. The CAT and GSH-Px indicate the "quick" reactions, while the SOD changes appear in the late phase only. MDA changes are obtained as consequence of lipid peroxidation.

Starting out from these results, the changes in tissue biochemism can be followed via the increased activities of CAT and GSH-Px, and via the increased SOD activity and MDA content, in the early and late periods of development of mucosal damage. The increased activites of CAT and GSH-Px preceded the macroscopic appearance of gastric mucosal damage. The etiological role of these enzymes is suggested by results obtained from other observations: the prostacyclin methyl ester-β-cyclodextrin comples (PCCD) prevented the development of ethanol-induced gastric mucosal damage in association with decreased CAT, GSH-Px and SOD activities (Vincze et al., 1986). The content of MDA

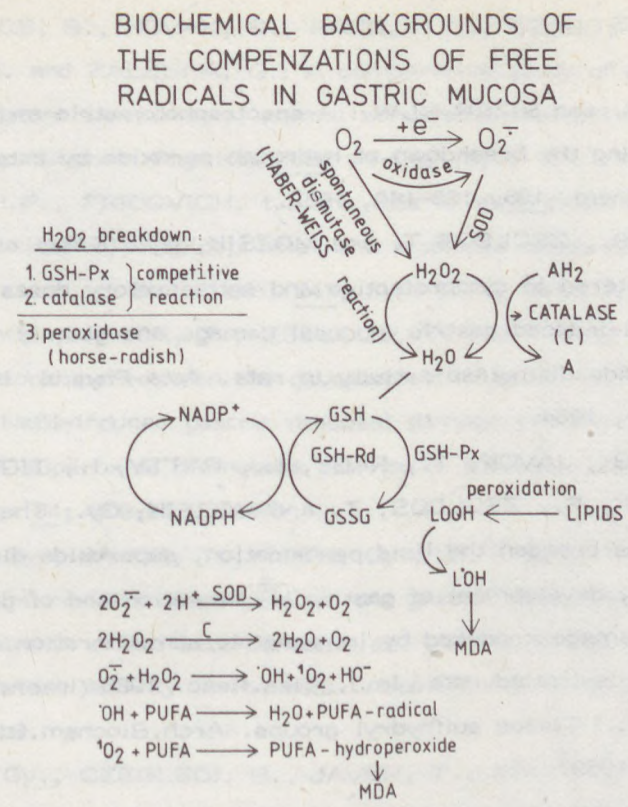

Fig. 4. Main steps of physiological "detoxication" of free radicals. Abbreviations: superoxide dismutase, SOD; reduced glutathione, GSH; glutathione peroxidase, GSH-Px; glutathione reductase, GSH-Rd; polyunsaturated fatty acids, PUFA; malondialdehyde, MDA

increased as a consequency of the development of ethanol-induced gastric mucosal damage, while its value naturally decreased in association with a decreased extent of ethanol-induced mucosal damage on the application of PCCD.

Independently of these results, no close correlation can be found between the free radical mechanisms and the development of ethanol-induced gastric mucosal damage.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

PROTECTIVE MECHANISMS IN PLANTS AGAINST PHOTODESTRUCTION OF CHLOROPHYLL

Z. SZIGETI and D. VÁGUJFALVI

Department of Plant Physiology,
Eötvös Loránd University,
H-1445, Budapest, Hungary

SUMMARY

The chlorosis of herbicide-treated leaves is a consequence of the irreversible photodestruction of chlorophylls. This photodestruction needs molecular O_2, or activated oxygen species such as O_2^- or $^1O_2$. Some related mechanisms exist in the chloroplasts for the protection against oxidative destruction. These are: 1. the quenching of $^1O_2$ or triplet chlorophyll by different compounds, 2. the functioning of superoxide dismutase and other O_2^- eliminating systems, 3. the Foyer - Halliwell cycle, 4. the xanthophyll-epoxide cycle.

INTRODUCTION

In the course of our laboratory experiments with herbicides, we have found chlorophyll destruction in different in vivo and in vitro systems (13). This led us to a detailed investigation of this process and of the possible protective mechanisms (14, 15). The experimental data showed that the most effective protectors against the photooxidative bleaching of the chlorophylls in a chloroplast suspension were ascorbate isoascorbate and L-dihydroxyphenylalanine. NADH was only moderately effective, while glutathione and cysteine were not able to protect the chlorophyll from destruction, especially when electron transport inhibitors were present (15).

Here, we present a further discussion of our ideas about a general scheme proposed earlier, summarizing the possible interactions of excited chlorophylls, activated oxygen species and reductants (15) from another aspect.

RESULTS AND DISCUSSION

The scheme summarizing the processes connected with chlorophyll photooxidation and the possible protective mechanisms is shown in Fig. 1. The Figure consists of six functionally different parts.

1. The excitation of chlorophyll by a light quantum $h\nu_1$, its spontaneous quenching by the emission of fluorescence $h\nu_2$ or phosphorescence $h\nu_3$, and the participation of chlorophyll in the photosynthesis under physiological conditions. $^1CHL_0$ and $^1CHL_1$: singlet chlorophyll in the ground and in the excited state, $^3CHL_1$: triplet chlorophyll. The proportion of $^3CHL_1$ molecules unable to dissipate their absorbed energy is 3-5% (9).
2. The reaction of $^3CHL_1$ with molecular O_2 yields $^1O_2$, a very reactive oxygen species. The $^3CHL_1$ molecules are the main sources of $^1O_2$ in chloroplasts. This $^1O_2$ oxidizes $^1CHL_0$ (2). Another possible reaction between $^3CHL_1$ and $^3O_2$ yields a complex $[CHL^+...O_2^-]$, resembling an ion-pair.
3. There are four possible ways for the functioning of naturally-occurring carotenoids (CAR) as protectors against photooxidative damage in the chloroplasts: a) the quenching of $^3CHL_1$ by ^1CAR or b) by ^3CAR (20), c) the quenching of $^1O_2$ by ^1CAR, and d) the reaction of a special carotenoid, zeaxanthine, with $^1O_2$ results in antheraxanthine, a xanthophyll-epoxide (11). The reduction of antheraxanthine to zeaxanthine needs light, ascorbate, glutathione and NADPH (5, 11). The functioning of this "xanthophyll cycle" in vivo is to some extent questionable (6, 7), because the rate of oxygen uptake attributable to this cycle is rather low (12).

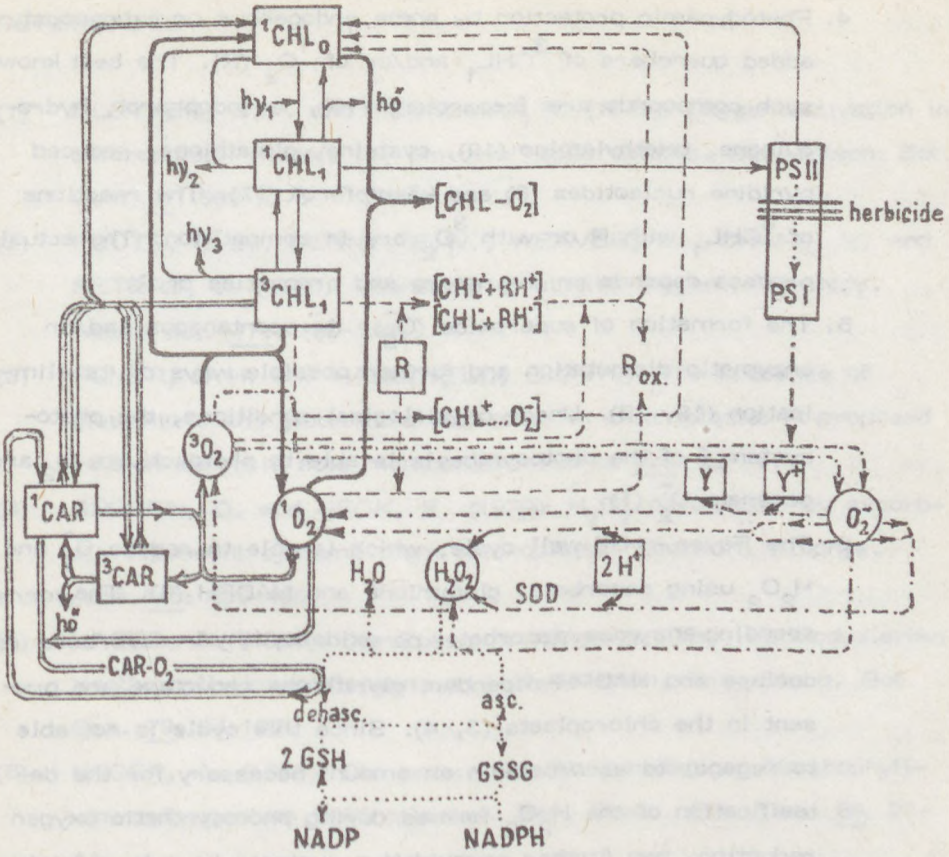

Fig. 1 Scheme of chlorophyll photooxidation and of possible protective mechanisms. 1. Excitation of chlorophyll, its spontaneous quenching, and the participation of chlorophyll in photosynthesis (——). 2. Reaction of triplet chlorophyll with molecular oxygen, yielding singlet oxygen (▬▬). 3. Role of carotenoids in chlorophyll protection (═══). 4. Protection by photodynamic means (---). 5. Reactions connected with generation and elimination of superoxide (-· -·-). 6. The Foyer - Halliwell cycle (.....)

4. Photodynamic protection by some endogenous or exogenously added quenchers of $^3CHL_1$ and/or of $^1O_2$ (R). The best known such compounds are β-carotene (16), α-tocopherol, hydroquinone, triethylamine (10), cysteine, glutathione, reduced pyridine nucleotides (8) and kaempferol (17). The reactions of $^3CHL_1$ with R or with $^3O_2$ are in competition. The actual process depends on the nature and properties of R.
5. The formation of superoxide (O_2^-), its spontaneous and/or enzymatic dismutation and further possible ways of its elimination (18, 19). Under physiological conditions, the photosystem I of the photosynthesis is able to photoactivate O_2 and generate O_2^- (1).
6. The Foyer - Halliwell cycle, which is able to reduce O_2^- and H_2O_2 using ascorbate, glutathione and NADPH (3). The corresponding enzymes ascorbate peroxidase, dehydroascorbate reductase and NADPH-dependent glutathione reductase are present in the chloroplasts (3, 4). Since this cycle is not able to regenerate ascorbate in an amount necessary for the detoxification of the H_2O_2 formed during photosynthetic oxygen reduction, two further peroxidative systems have been postulated and verified (4).

CONCLUSIONS

As has been shown there are several possibilities for the generation of different reactive oxygen forms, i.e. strong damaging agents, in chloroplasts. Different activated chlorophyll forms and oxygen species can be eliminated by the same reductant in several ways. The mechanism actually functioning depends on the nature of the system investigated (intact leaves, isolated chloroplasts or extracted pigments), on the lifetimes of the excited or activated molecules, and on the concentrations, localizations and mobilities of the interacting molecules under the given conditions.

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Oxygen Free Radicals and the Tissue Injury
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TISSUE INJURIES INDUCED BY FREE RADICALS, AND THEIR INTERPRETATION

B. TÖRÖK, E. RÖTH and B. MATKOVICS*

Department of Experimental Surgery,
University of Medicine, Pécs,
*Biological Isotope Laboratory, University of
Szeged, Hungary

The biological changes occurring during regional myocardial ischaemia produced by a sudden occlusion of a major coronary artery evoke a chain of pathological events. The decrease in arterial flow causes ischaemia and/or an anaerobic metabolism, which is followed by a quick decrease in cellular energy level with cessation of specialized functions and in the case of irreversibility cell death occurs (5). The mass of injured cells may give rise to the potentially ingravescent deterioration of electrical and mechanical activity.

Lipid peroxidation is known to be involved in many biological damage processes (1, 2). As we proposed earlier, lipid peroxidation not only causes the destruction of the myocardial membranes, but also gives rise to a chemical product that can propagate the damage to distant sites (7, 8).

To avoid confusion, the fundamental questions have to be answered as to what intracellular changes cause the death of ischaemic myocytes, and why. It is known both from the literature and from own investigations that the onset of the irreversible phase is associated with the depletion of high-energy phosphates; the ATP content will be very low (8% of the control or less) and signs of extensive membrane damage appear (6). These ultrastructural events involve direct and indirect changes (membrane expansion with an increase of the membrane permeability, and consequently with pathologic ion and water shifts, intracellular, myofibrillar and mitochondrial oedema, disintegration, rupture

and lysis of different membrane structures with dislocation of Ca^{2+} ions from the extracellular space into the myocytes, with resultant irreversible actomyosin coupling and disruption of the regulated machinery of the cell, the crucial loss of membrane-bound enzymes with their leakage to the extracellular space, etc.).

The growing recognition that oxygen radicals are ubiquitous in aerobic living organisms has prompted a flood of publications dealing with the impact of these radicals on cellular integrity and the promotion of different diseases, including myocardial infarct.

There is no longer doubt that free radicals are produced in myocardial cells under physiological and pathological conditions. It appears probable that these free radicals imply a severe threat to the myocytes, although natural defensive systems have also evolved.

Under physiological conditions, the very reactive end-product of the Haber-Weiss reaction, the hydroxyl radical ($OH^{\cdot}$), is not or only hardly formed, since the required precursors, the superoxide radical (O_2^-) and H_2O_2, are immediately scavenged by the ubiquitous superoxide dismutase (SOD) and by the catalase/peroxidase system, respectively. Under pathological conditions, such as in acute myocardial infarction, tissue destruction will be extensive owing to the rapid decrease of natural scavenging. In an ischaemic state, the aggressive hydroxyl radical attacks the membrane polyunsaturated fatty acids by abstracting a hydrogen and leaving a lipid radical (Lipid $C^{\cdot}$), which induces a chain reaction forming alkoxy (Lipid $O^{\cdot}$) and peroxy (Lipid $OO^{\cdot}$) radicals. The increasing rate of lipid peroxidation will result in an increase of the already outlined membrane destruction.

It must be mentioned that the activation of lipid peroxidation may also occur under hyperoxic conditions; the amount of molecular oxygen in the membrane matrix is then sharply elevated and the possibility of oxygen interaction with the reduced electron carriers in the mitochondrial and microsomal electron transporting chain is increased. The same process could potentially be expected in reperfusion or reoxygenation states; in this case a considerable accumulation of reduced

carriers is followed by an excessive supply of oxygen. Reperfusion therapy of a myocardial infarct will result not in an improvement, but in contracture, cellular necrosis and intracellular calcium overload. This phenomenon has been termed oxygen paradox and/or calcium paradox, "two facets of the same problem" (3, 4).

On the basis of an experimental myocardial infarct model, we illustrate a summarizing scheme for cellular sources and targets of free radicals and display the major stages of protective systems (Fig. 1). The aim of the scheme is to signalize the severity of the pathological processes in the myocardial infarct area, border zone and intact region. Our findings suggest that the most endangered process arises at the border zone where on the inner side a chain of ischaemic events appears with increase of the free radical reactions and decrease of the scavenging activity, while on the outer side a normal oxygen supply may be present, with an increased possibility of oxygen interaction with reduced electron carriers in the marginal diffusion zone. On the outer side there is also an unchanged blood supply with O_2 transport; moreover, the contraction activity of the intact myocardial fibres exerts tugging and strain on the already non-contracting ischaemic fibres of the central infarct areas.

From a consideration of the experimental findings, certain conclusions may be drawn:

1. There is a strong relation between the structural disintegration and functional disorders of membranes in ischaemic states.

2. The membrane damage is a primary consequence of severe ATP depletion, but the potential mechanisms for irreversibility may be interpreted in various ways.

3. The increase of aggressive free radicals and the depletion of endogenous scavenger compounds indicate an essential hazard to membrane disintegration.

4. The calcium-paradox and/or oxygen paradox are "two facets of the same problem", since the highly reactive oxygen intermediates play a significant role in the initiation of both paradoxes.

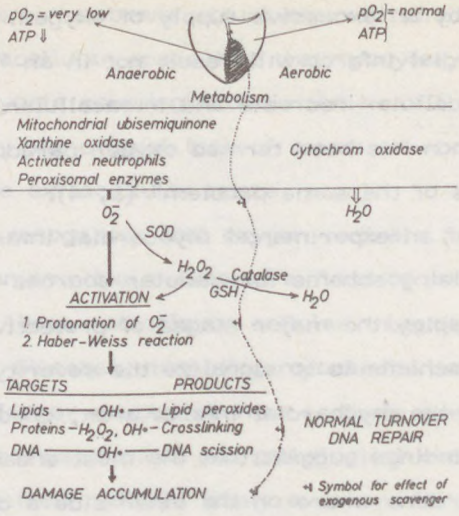

Fig. 1. Some biochemical events in the myocardial infarct area, border zone and intact region

5. Exogenous antioxidant therapy may substitute the endogenous scavengers and allows blockage of the propagation of the Haber-Weiss reaction, reducing the progress of irreversible changes.

6. MTDQ proves to be a potent exogenous scavenger.

The concepts that oxygen free radical production is an important mediator in the pathology of myocardial ischaemic injury, and that the free radicals are significant promoters in cardiac muscle disintegration, seem valid.

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Oxygen Free Radicals and the Tissue Injury
B. Matkovics, D. Boda and H. Kalász (Eds)

CORRELATION BETWEEN THE ACTIVITY OF RED BLOOD CELL ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION DURING THE BEFLAVIN® CATALYSED PHOTOSENSIBILIZATION

SZ.I. VARGA, B. MATKOVICS, L. PATAKI*, Z. NOVÁK* and A. MOLNÁR*

Biological Isotope Laboratory,
József Attila University, Szeged, Hungary
*Municipal Children's Hospital,
Szeged, Hungary

SUMMARY

The antioxidant enzyme activities and lipid peroxidation (LP) of the blood of mature hyperbilirubinaemic neonates born at full term were determined and compared with the corresponding values for normal non-hyperbilirubinaemic neonates. A number of procedures aimed at eliminating the hyperbilirubinaemia were tested.

Our results on the antioxidant enzyme activities and lipid peroxidation were as follows: In the blood of hyperbilirubinaemic neonates (requiring "combined" treatment), the superoxide dismutase (SOD) and catalase (C-ase) activities and LP are significantly lower than in the blood of normal neonates.

The results support the conception that hyperbilirubinaemia requiring "combined" treatment develops only in neonates with a low antioxidant enzyme activity and in whom the oxygen radicals necessary for bilirubin elimination are formed slowly.

INTRODUCTION

Following numerous investigations and their criticism (4), we set out to study the role played in the elimination of a large quantity of bilirubin by molecular oxygen (MO) and the radicals formed from MO under biological conditions. We also wished to establish how hyperbilirubinaemia and efforts to eliminate it this influence the antioxidant enzyme system and lipid peroxidation. As the substance most easily examined, blood was used in our determinations. The activities of the following antioxidant enzymes were determined in red blood cell (RBC) haemolysates: superoxide dismutase (SOD or Cu,Zn-SOD: EC 1.16.1.6),

catalase (EC 1.11.1.6) and glutathione peroxidase (GP-ase; EC 1.11.1.9). Measurements and comparisons were also made of the total haemolysate LP, i.e. the amount of thiobarbituric acid (TBA)-active substances. These LP measurements were designed to obtain total amount of oxygen radicals featuring in the oxidative membrane damage.

SUBJECTS AND METHODS

In the examined groups, blood was taken from some readily accessible vein except umbilical vein. Clotting was inhibited with a few drops of heparin solution (G. Richter Pharmaceutical Works, Budapest, Hungary). The RBCs were separated from the plasma by centrifugation. The upper layer, containing the plasma, was removed by suction. The RBC layer was next washed twice with isotonic NaCl solution (pH 7.0), and the supernatants after centrifugation were again removed by suction. A 10-fold volume of distilled water was added to the concentrated RBC layer, and the haemolysis was made complete by freezing and thawing. Aliquots of the fresh haemolysate were used for enzyme activity and protein quantitative measurements.

Before SOD estimation, the haemolysate was diluted with a 2:1 mixture of ethanol-chloroform (0.75 ml mixture was added to 1 ml haemolysate, and the precipitate was separated from the supernatant by centrifugation, aliquots of the supernatant being used for measurements). For quantitative determination of SOD activity, use was made of the enzyme-dependent inhibition of the epinephrine-adrenochrome transformation, as in other cases (6, 7).

C-ase activity was determined at 240 nm via the consumption of H_2O_2 in unit time (1, 6), and was expressed in Bergmeyer units (BU).

For measurements of GP-ase activity, aliquots were mixed with cumene hydroperoxide, as GP-ase substrate, after a given time the reaction being stopped and the GSH residue measured with the Ellman reagent (3, 13). The quantity of TBA-reactive substances was measured with TBA reagent, and expressed in nmol malondialdehyde (MDA)/g

protein. The calibration plot was prepared with MDA-diaethyl acetal (Merck, Darmstadt, FRG) (9).

The total quantity of proteins was determined with the Folin reagent, human serum albumin being used to prepare the calibration curve (5).

Examinations were carried out on normal, full-term neonates (i) and on neonates with hyperbilirubinaemia. It should be stressed that the latter were also born at full term and had normal weight, i.e. were not premature.

The hyperbilirubinaemic neonates received different types of treatment: (ii) treatment with blue light: (iii) exchange transfusion, preceded and continued by blue light treatment; (iv) a combination of riboflavin and blue light treatment. Thus, the "combined treatment" was begun when no change occurred in the high bilirubin value of the blood in response to 24-hour blue light treatment. The number of cases (n) in the study groups was 12-15.

Every group contained neonates of both sexes. The average weights in the various groups were as follows:

(i) control group: 3565 $\pm$ 584.9,

(ii) group treated with blue light: 3720 $\pm$ 431.4 g,

(iii) group undergoing replacement transfusion: 2877.7 $\pm$ 820.5 g,

(iv) group treated with riboflavin + blue light: 3409.0 $\pm$ 533.5 g.

Average weight of treated groups: 3355.5 $\pm$ 593.8 g.

A KLA-21 20 W Baby Blue instrument (Medicor, Budapest, Hungary) was used for blue light treatment.

The reagents used for the measurements were always of the highest analytical purity. They were commercial products of Sigma (USA) or Reanal (Budapest, Hungary).

Beflavin^{R} (Hoffman-La Roche, Switzerland) was used as riboflavin preparation.

The results were evaluated statistically. All data are given as means of $\underline{n}$ measurements $\pm$ SD.

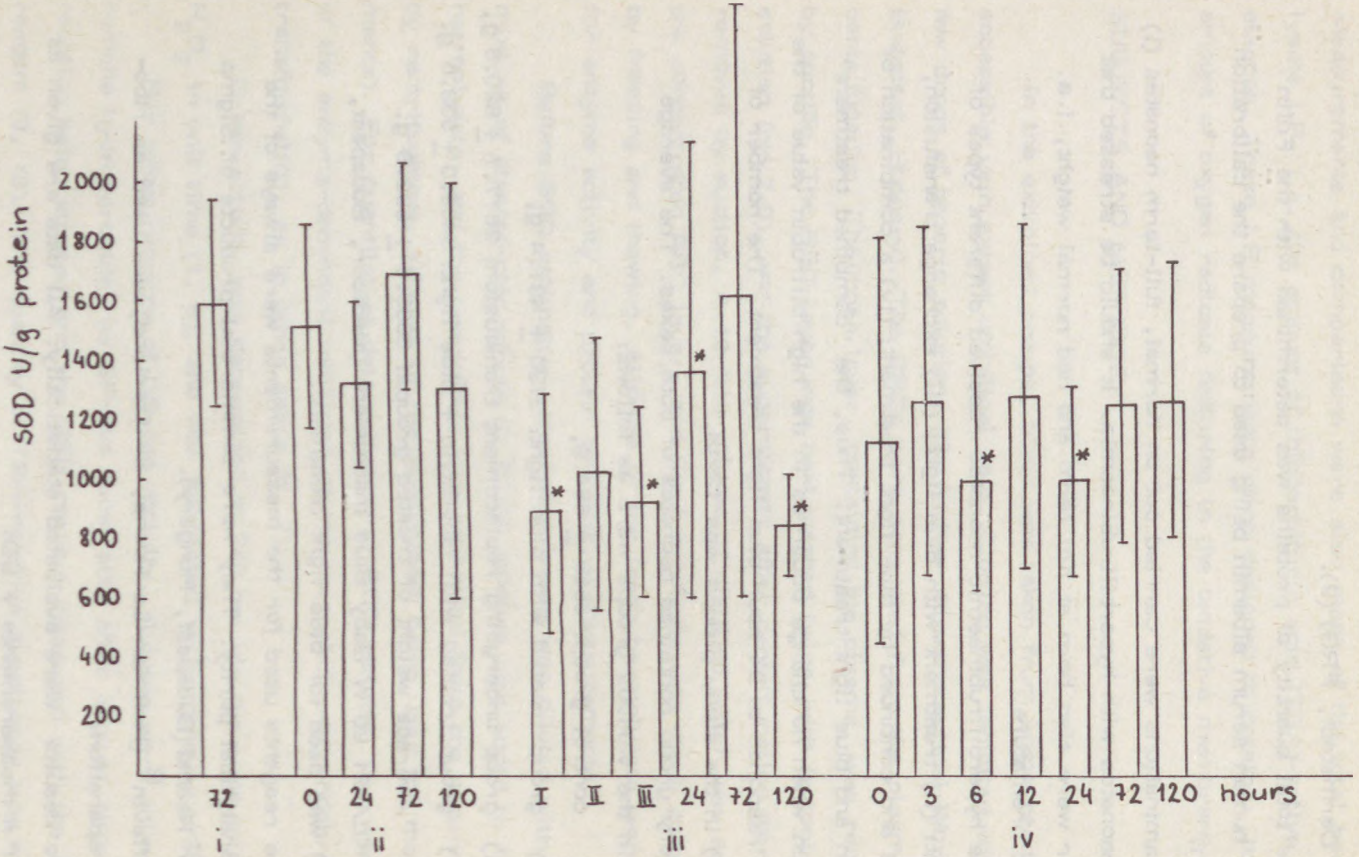

Fig. 1. Cu, Zn-SOD activity of haemolysates. Sequence: (i) normal neonates; (ii) hyperbilirubinaemic neonates treated with blue light; (iii) combined blue light + replacement transfusion (24-h blue light treatment preceding replacement transfusion) and (iv) blue light + Beflavin^{R} (24-h blue light treatment preceding Beflavin^{R}). Numbers under columns are examination times post partum

in hours. In group (iii), activities adn LP measured before (I), during (II) and after (III) replacement transfusion. 24, 72 and 120-h volumes relate to times following completion of replacement transfusion. In group (iv), determinations made after 3, 6, 12, 24 hours etc.
Data are means ± S.E.M.: $p < 0.05$–0.001

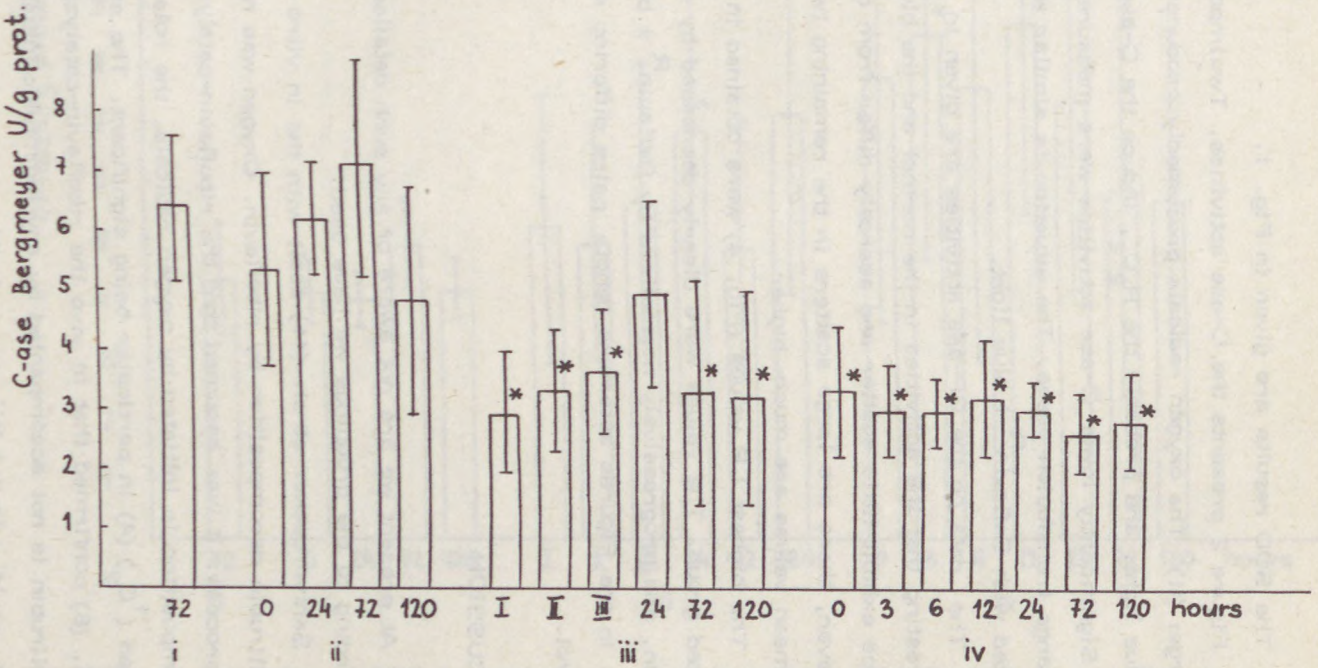

Fig. 2. C-ase activities. For details, see Fig. 1 and text

RESULTS

The SOD results are given in Fig. 1.

Figure 2 presents the C-ase activities. Two important findings emerge: (ii) The oxygen radicals presumedly produced on the action of blue light, and probably the H_2O_2, induce the C-ase activity (12). (iii) Significantly lower C-ase activities were measured in blue light + exchange transfusion cases. The situation is similar to that in cases treated with Beflavin^R + blue light.

The data on the GP-ase activities are given in Fig. 3. It is interesting that the activities in the normal and the blue light-treated groups exhibit little scatter and scarcely differ from one another. However, there are large scatters in the remaining two groups, where the mean values are much higher.

The highest LP values (Fig. 4) were obtained in the blue light-treated group. The values were clearly decreased by exchange transfusion, and progressively normalized by Beflavin^R + blue light.

In the Figures asterisks denote pairs differing significantly from control.

DISCUSSION

At present we are not aware of any such detailed examinations according to the groupings we have used.

Santvordecker et al. (11) dealt with the in vitro enhancement of bilirubin decomposition by riboflavin. Oxygen was necessary for the process. It was assumed that the riboflavin-catalyzed bilirubin decomposition is initiated by oxygen radicals, the role of singlet oxygen ($^1O_2$) (4) in particular being significant. The studies by Pataki et al. (8) confirmed that in vivo the riboflavin-catalyzed decomposition of bilirubin is not accompanied by any harmful clinical consequence which would rule out the very valuable catalytic use of riboflavin in the therapy of hyperbilirubinaemias. In the present work we set out

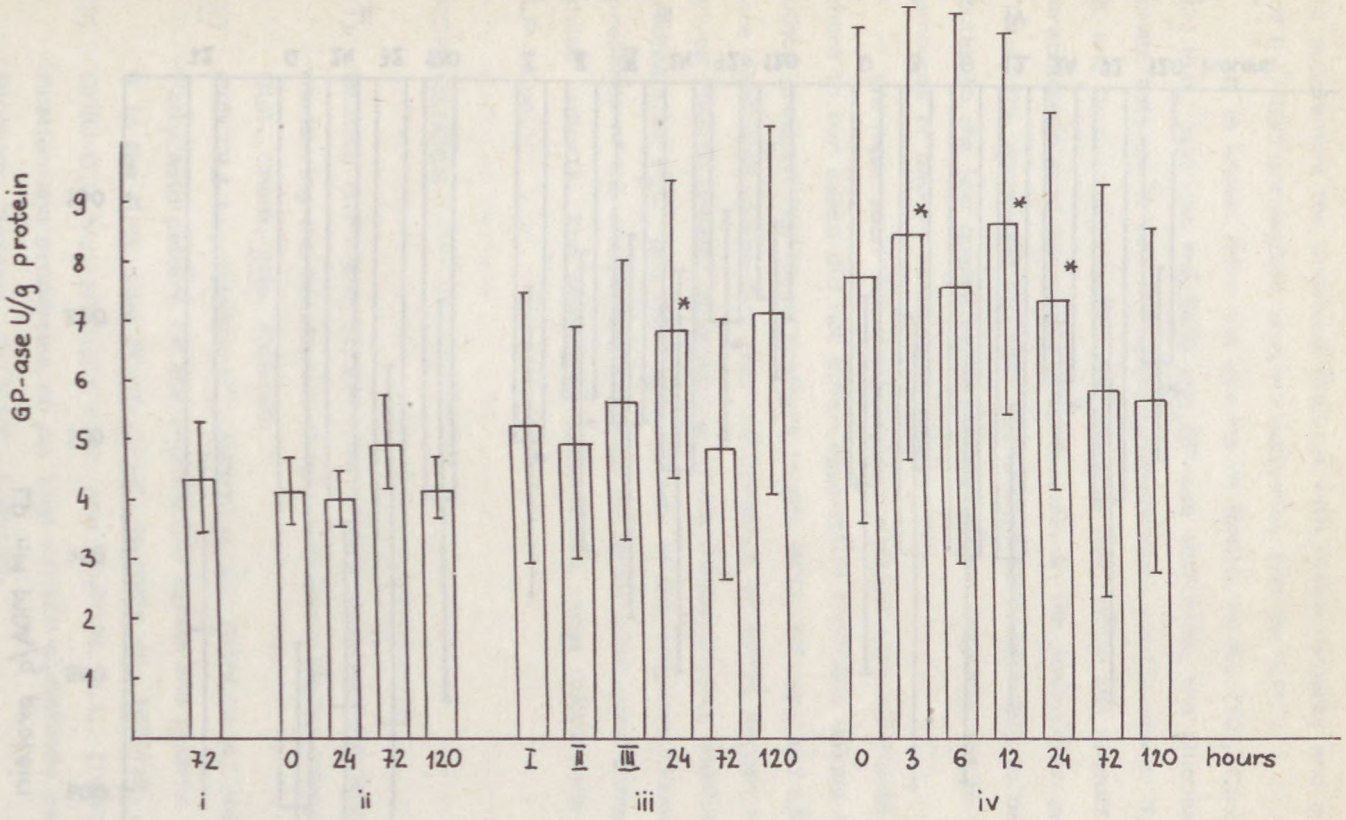

Fig. 3. GP-ase activities of RBC haemolysates, in U/g protein. Details in text and Fig. 1

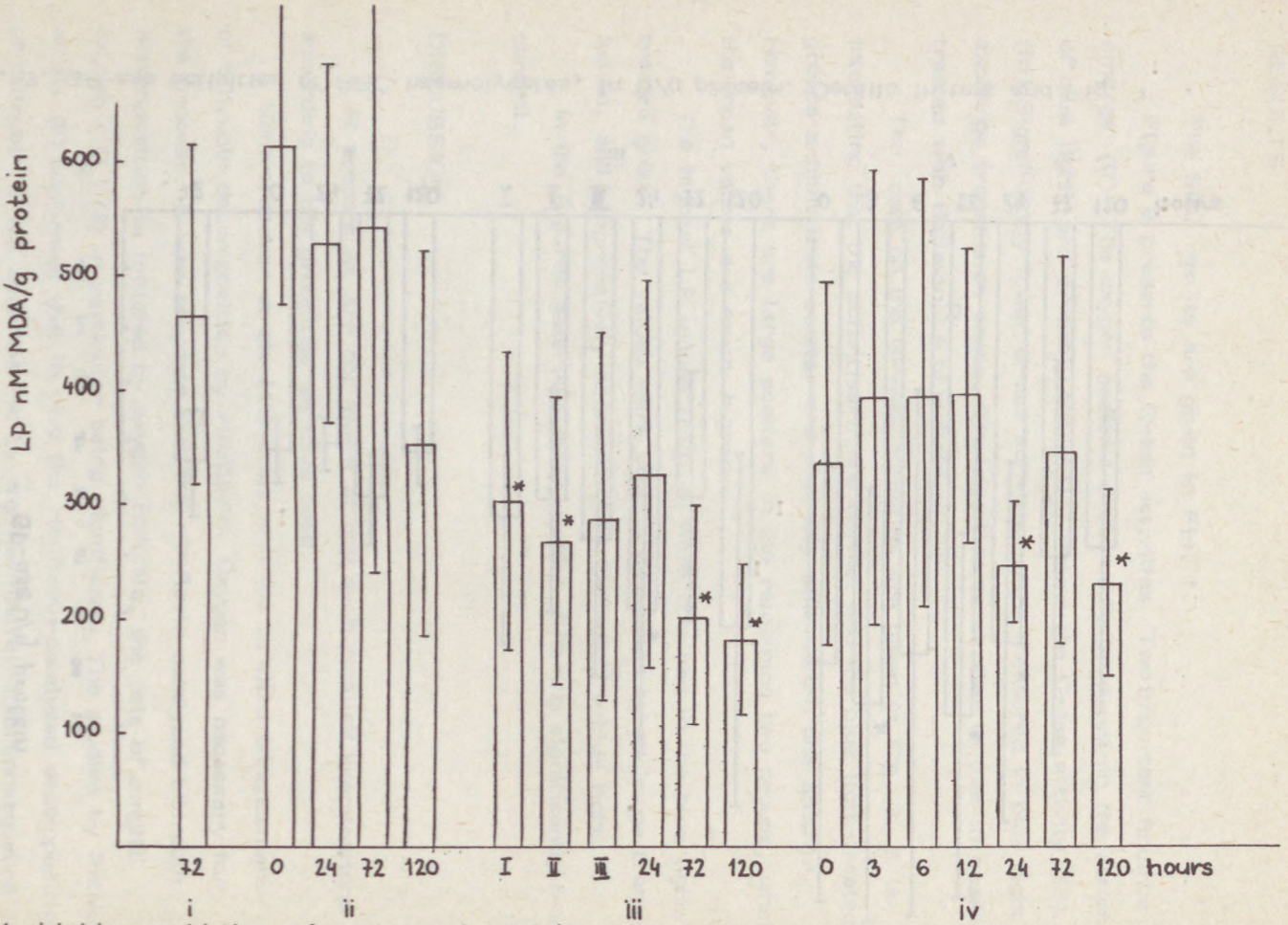

Fig. 4. Lipid peroxidation values in nmol MDA/g protein haemolysate. Details in text and Fig. 1

to supplement the previous findings with measurements and comparisons of the RBC antioxidant enzyme activities and the LP.

It is known from the results of Rotilio et al. (10), Ciccoli et al. (2) that, just like the SOD and GP-ase activities, the glucose-6-phosphate activity is low in hyperbilirubinaemic neonatal blood. The vitamin E and linoleic acid levels are likewise low, while the amount of arachidonic acid decreases progressively in the postpartum period. Thus, the decreased bilirubin elimination is presumably to be attributed to the low quantities of the antioxidant enzymes and to the small amounts of oxygen radicals formed.

We have seen that the activities attained with riboflavin treatment in our cases did not differ essentially from the values in the other groups. The large scatters in the SOD, GP-ase and LP data are attributed to the fact that the presence of active oxygen radicals gives rise to greater fluctuations in the measurements involving radicals or H_2O_2 and GSH as substrate of the enzymes. However, the presence of the antioxidant enzymes or other substrates compensates these radicals. The same may refer to the large fluctuations in the LP value.

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Oxygen Free Radicals and the Tissue Injury
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CORRELATIONS BETWEEN FREE RADICALS AND THE PROSTACYCLIN METHYL ESTER-β-CYCLODEXTRIN COMPLEX (PCCD)-INDUCED GASTRIC CYTOPROTECTIVE EFFECT IN EXPERIMENTAL ULCER

Á. VINCZE, G. SÜTŐ, T. ZSOLDOS* and Gy. MÓZSIK

First Department of Medicine and
*Department of Biology,
University Medical School,
H-7643 Pécs, Hungary

Prostaglandins (PGs) and prostacyclin I_2 (PGI_2) have been proven to be protective compounds against the development of gastric mucosal damage produced by different necrotizing agents, such as 0.6 M HCl, 0.2 M NaOH, 25% NaCl, 96% ethanol, or thermal injury (Morón et al., 1981, 1983; Mózsik et al., 1982, 1983; Robert, 1979; Robert et al., 1979), without any significant inhibition of gastric acid secretion. This phenomenon was termed "gastric cytoprotection" by Robert (1979). The cytoprotective activity of PGI_2 is attenuated by its instability.

The prostacyclin methyl ester- β-cyclodextrin complex (PCCD) was prepared by Kovács et al. (1984), and this was followed by extensive chemical and biological research (Székely et al., 1984). PCCD is an inclusion complex of PGI_2 methyl ester in β-cyclodextrin (3% active ingredient) with increased stability. In biological observations, PCCD was found to be a powerful cytoprotective agent (Mózsik et al., 1985).

The aim of this examination was to find some correlation between the PCCD-induced gastric cytoprotective effect and the changes in gastric mucosal catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities, and in reduced glutathione (GSH) and malondialdehyde (MDA) contents.

MATERIALS AND METHODS

The observations were made on CFY rats of both sexes, weighing 180 to 210 g. The animals were fasted for 24 hours before the experiments, but they received water ad libitum.

The gastric mucosal lesions were produced by the intragastric administration of 96% ethanol. In other series of observations, PCCD was freshly dissolved and given intragastrically in doses of 5 or 50 μg/kg. The animals were killed 0, 1, 5, 15, 30 or 60 min after the administration of ethanol. PCCD was given 30 min before the administration of ethanol.

The following experimental parameters were measured: the number and severity of gastric mucosal lesions (ulcers); the CAT activity in the gastric mucosa, by the method of Beers and Sizer (1952); the GSH content in the gastric mucosa, by the method of Ellman (1959); the GSH-Px activity in the gastric mucosa, by the method of Sedlak and Lindsay (1968); the gastric mucosal SOD activity, by the method of Misra and Fridovich (1972), as modified by Matkovics et al. (1977); the MDA content, by the method of Fong et al. (1972), as modified by Zsoldos et al. (1983); and the protein content, by the method of Lowry et al. (1951).

The number and severity of the lesions were calculated per rat (means ± SEM), while the biochemical values were calculated per mg protein (means ± SEM). The results in Tables 1 and 2 are given as percentages (means ± SEM). The number and severity of the gastric mucosal lesions are expressed as percentages of the values obtained 1 hour after ethanol administration, while the biochemical results are expressed as percentage of the values obtained 0 min after the administration of ethanol. The unpaired Student's t test was used for the statistical analysis of the results.

RESULTS

The results are given in Tables 1 and 2.

Table 1. Prostacyclin methyl ester–β-cyclodextrin complex (PCCD)-induced changes in the number and severity of ethanol-induced gastric mucosal lesions. The results are presented as percentages (means ± SEM) of the results obtained 1 hour after ethanol administration. Each group contained 10 animals

Groups of animals	Time after administration of ethanol (min) 1	5	15	30	60
	Number of gastric mucosal lesions				
A	0 ± 0	45 ± 5	75 ± 7	85 ± 7	100 ± 7
B	0 ± 0	21 ± 7^{+}	69 ± 7	58 ± 7^{+}	54 ± 7^{++}
C	0 ± 0	11 ± 4^{+++}	15 ± 7^{+++}	39 ± 7^{+++}	46 ± 7^{+++}
	Severity of gastric mucosal lesions				
A	0 ± 0	54 ± 3	71 ± 6	77 ± 6	100 ± 6
B	0 ± 0	17 ± 3^{+++}	50 ± 7^{+}	48 ±10^{+}	67 ± 7^{+}
C	0 ± 0	11 ± 1^{+++}	33 ± 7^{+++}	33 ± 7^{+++}	42 ±10^{+++}

Abbreviations:

A = animals treated with ethanol only; B = animals treated with ethanol + 5 µg/kg PCCD (given ip. 30 min before ethanol administration); C = animals treated with ethanol + 50 µg/kg PCCD.

+ = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$ (A vs B and C)

The results in this Table show that the gastric mucosal lesions first appear macroscopically 5 min after ethanol administration. Their occurrence is inhibited dose-dependently by the application of PCCD in doses of 5 or 50 µg/kg.

Table 2. PCCD-induced changes in the gastric mucosal CAT, GSH-Px, SOD, GSH and MDA. The results are presented as percentages of the values for the untreated controls (= 100 per cent) (means ± SEM)

Groups of animals	Time after administration of ethanol (min) 1	5	15	30	60
			CAT activity		
A	207±4	100±4	89±4	55±4	86±4
B	43±2^{+++}	41±3^{+++}	50±3^{+++}	153±8^{+++}	114±8^{++}
C	76±8^{+++}	56±5^{+++}	90±13	101±6^{+++}	61±8
			GSH-Px activity		
A	145±9	178±9	144±6	640±9	563±25
B	55±4^{+++}	59±3^{+++}	46±3^{+++}	36±3^{+++}	39±4^{+++}
C	30±3^{+++}	24±3^{+++}	32±6^{+++}	44±6^{+++}	30±4^{+++}
			GSH content		
A	119±7	124±6	124±6	156±9	87±6
B	79±6^{++}	73±4^{+++}	69±3^{+++}	66±4^{+++}	63±4^{+}
C	47±6^{+++}	34±4^{+++}	45±6^{+++}	58±7^{+++}	46±4^{+++}
			SOD activity		
A	110±5	117±8	124±7	139±7	145±5
B	122±6	153±12^{+}	208±16^{+++}	108±6^{++}	122±15
C	87±12	45±9^{+++}	35±3^{+++}	43±9^{+++}	39±7^{+++}
			MDA content		
A	102±3	105±2	97±3	134±4	145±7
B	125±8	98±2	85±3^{+}	105±4^{+++}	85±4^{+++}
C	82±2^{+++}	72±3^{+++}	78±4^{++}	94±4^{+++}	86±4^{+++}

Abbreviations:

A = animals treated with ethanol only; B = animals treated with ethanol + 5 µg/kg PCCD; C = animals treated with ethanol + 50 µg/kg PCCD.
+ = $p < 0.05$ ++ = $p < 0.01$ +++ = $p < 0.001$ (A vs B and C)

DISCUSSION

Our results indicate that the development of ethanol-induced gastric mucosal damage can be inhibited dose-dependently by the application of PCCD. When the details of the free radical mechanisms were studied during the PCCD effect, we could not find a good correlation between the PCCD-induced changes in the mentioned parameters: No close correlation was observed in the PCCD-induced changes in the gastric mucosal SOD and CAT activities. The other parameters, such as GSH-Px activity and GSH and MDA content, were decreased by the application of PCCD. It was interesting that the increased CAT activity appeared 30 and 60 min after the administration of ethanol.

After a careful review of our results, it must be concluded that no close correlation exists between the free radical mechanisms and the PCCD-induced gastric cytoprotective effect. When we analyzed the correlation between the development of ethanol-induced gastric mucosal damage and the free radical mechanisms, a similar conclusion was reached (Sütő et al., 1986). Other similar findings have been published (Mózsik et al., 1986).

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Oxygen Free Radicals and the Tissue Injury
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GLUTATHIONE AND PEROXIDE METABOLISM IN MALARIA PARASITIZED ERYHROCYTES

B. FRITSCH, A. DIECKMANN, B. MENZ, A. WENDEL,
E. HEMPELMANN*, K.G. FRITSCH*, W. STAHL**, G. EISENBRAND**,
D.T. SPIRA***, H. SCHIRMER*, G. FRITSCH and A. JUNG

Physiologisch-chemisches Institut der Universität (Director: Prof. Dr. D. Mecke) Hoppe-Seyler-Str. 1, D-7400 Tübingen, FRG,
*Institut für Biochemie II., Universität Heidelberg, FRG,
**Institut für Lebensmittelchemie, Universität Kaiserslautern, FRG
***The Hebrew University, Jerusalem, Israel

The protection of the intracellular environment against oxidative damage is of general importance in cellular metabolism.
At a conference like this, where the role of free oxygen radicals in tissue injury is discussed, it seems necessary to mention that oxygen radicals play a useful and important role in the defense-mechanism against all sorts of parasites. At present, these radicals are thought to belong to the main mediators in the immune reactions against intracellular protozoan parasites (1). The susceptibility of parasites to this processe however, depends on their antioxidant capacity.

In most cells, the main antioxidant capacity is provided by the glutathione-redox-cycle. The decrease of the activity of one or more of the enzymes which are involved in the maintenance of the glutathione status of a cell leads to an increased susceptibility for peroxidative damage (Fig. 1). The relation of Oxidative Stress Versus Antioxidant Capacity -OSVAC-(2) is decisive for the survival or non-survival of cells under various conditions like exposition to alkylating agents, peroxides-generating drugs or even macrophage-mediated cell lysis.
It is generally accepted that malaria infected erythrocytes are susceptible to oxidative damage; the biochemical basis for this phenomenon, however, is not clear. Because of the intracellular parasitism of Plasmodia it is widely assumed that these parasites are more or less dependent on the intermediary metabolism of the host cell, e.g. on the erythrocytic redox cycle (3, 12). The parasitized red blood cell,

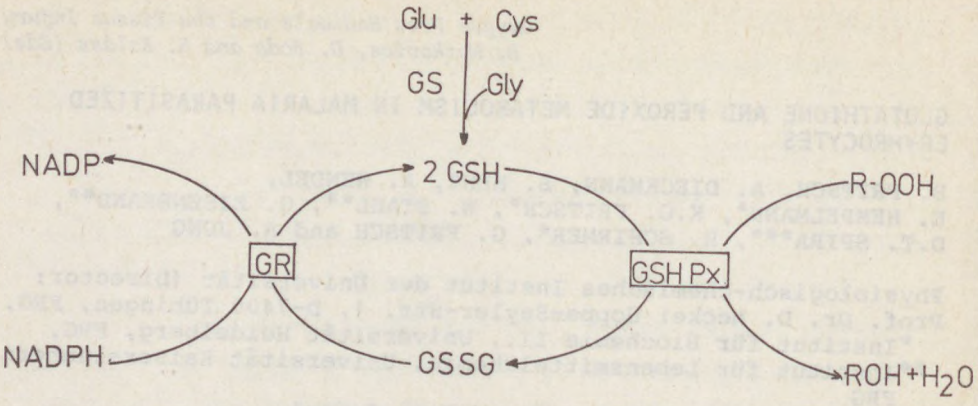


Fig. 1. Schematical drawing of glutathione-metabolism
GS glutathione-synthetase
GR glutathione-reductase
GSH-Px glutathione-peroxidase

however, is a multicompartment system and it is very difficult to distinguish between the metabolic activities of host and guest cell, e.g., between the antioxidant capacity of both cells.

In order to differentiate between the glutathione-cycle of the parasite and host erythrocyte, we have investigated this pathway in P.vinckei and P.berghei infected mouse erythrocytes and in P.falciparum parasitized human red blood cells.

We tried to answer the following questions:

1. do malaria parasites have an own glutathione-redox-cycle or are they dependent on the metabolic activities of the host cell cycle and
2. if there is a parasitic glutathione-cycle, are there qualitative and/or quantitative differences between host and guest cell?

In earlier investigations (4, 5, 6) we found in P.vinckei infected mouse erythrocytes in correlation to the growth rate of the parasite, a steep increase in glutathione (GSH) content and increasing activities of glutathione-synthetase (EC 6.3.2.3), glutathione-reductase (EC 1.6.4.2) and, in parallel to the degradation of hemoglobin, a decreasing activity of catalase. The results gave strong indications that

these parasites have at least two of the three enzymes of the glutathione-cycle but seem to lack catalase activity. The experiments on the glutathione-peroxidase (EC 1.11.1.9), however, were not equally conclusive.

In order to investigate the existence or absence of a parasitic glutathione-peroxidase and to elucidate the influence of the host cell enzyme on the development of the malaria parasite, we "produced" glutathione-peroxidase deficient erythrocytes by feeding mice for several months with a selenium deficient diet.

Normal (Se^+) and selenium deficient (Se^-) mice, the latter having an erythrocytic glutathione-peroxidase activity of less than 10% of control mice, were infected with P.vinckei and P.berghei, respectively. We found no significant differences in the development of parasitemia for both strains in Se^+ and Se^- mice. The only difference was a slightly increased malonyl-dialdehyde content in parasitized Se^- red blood cells. The development of the parasite therefore does not seem to depend on glutathione-peroxidase of the host erythrocyte.

To assess the enzymes of the glutathione-cycle in P.vinckei infected Se^+ and Se^- erythrocytes in correlation to the intraerythrocytic multiplication of the parasite, the cells were separated into the different stages of blood schizogony by density gradient centrifugation (4, 7).

In correlation to the different stages of blood schizogony and to the parasitic marker enzyme (NADP-dependent glutamate-dehydrogenase (4)) we found:

1. in Se^+ as well as in Se^- host cells, an increase of glutathione-synthetase and glutathione-reductase activities (figs. 2, 3);

2. in Se^+ erythrocytes, decreasing glutathione-peroxidase activity with hydroperoxide (H_2O_2) as a substrate, slightly increasing activity with t-butyl-hydroperoxide and steeply increasing activity with cumene-hydroperoxide as the substrate (fig. 4a);

3. in Se^- erythrocytes, low glutathione-peroxidase activity in all stages with the substrate H_2O_2 and increasing activities with the or-

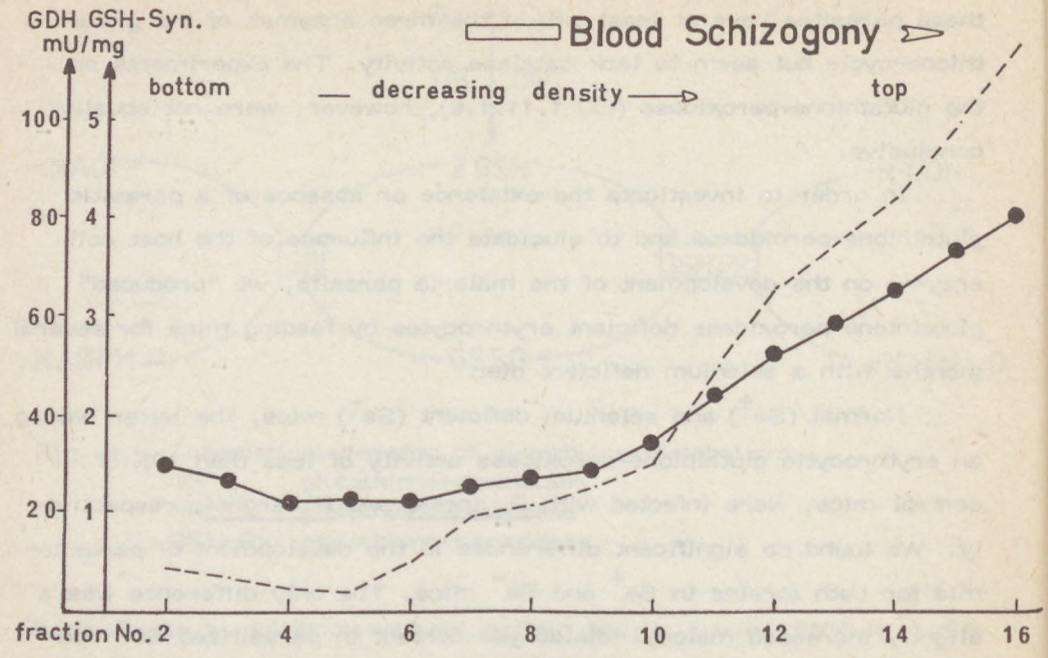
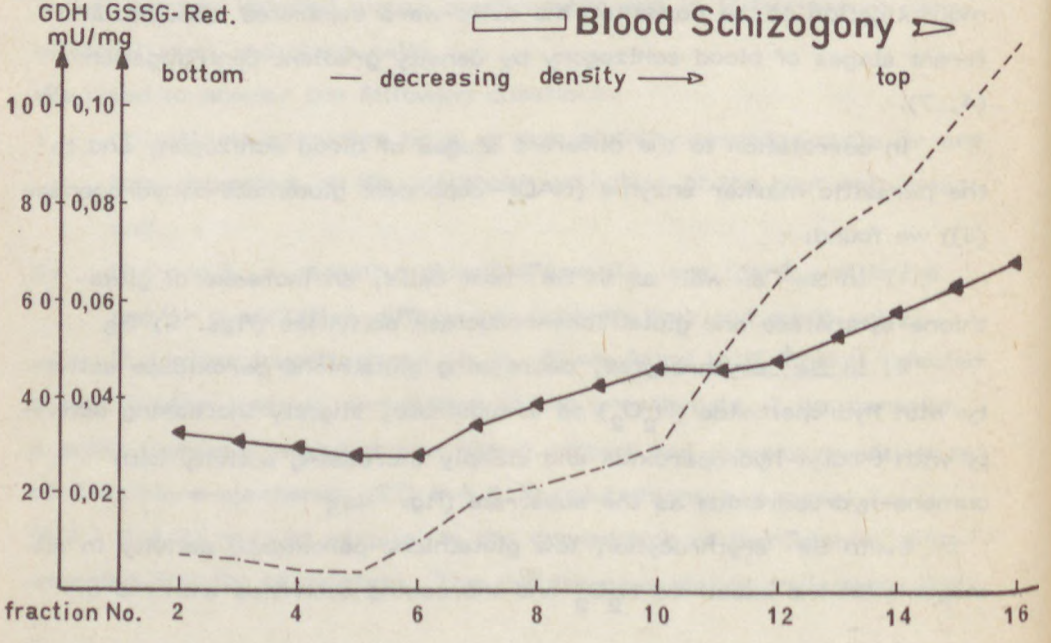


Fig. 2. Glutathione-synthetase in P.vinckei parasitized erythrocytes separated into the different stages of blood schizogony by percoll density gradient centrifugation.
●——● glutathione-synthetase (GSH-Syn.)
------ glutamate-dehydrogenase (GDH), parasitic marker enzyme



ganic hydroperoxides as substrates, especially with cumene-hydroperoxide (fig. 4b).

In P.vinckei and in chloroquine resistant P.berghei (K 65), isolated from Se^+ and Se^- host erythrocytes, the enzyme activity of glutathione-peroxidase was very low with H_2O_2, but high with cumene-hydroperoxide (fig. 5); this activity pattern is typical for a selenium independent enzyme (8).

The enzymes of the glutathione-redox-cycle are also measurable in isolated P.falciparum, the human malaria parasite. We found that the substrate specificity of the glutathione-peroxidase of P.falciparum is comparable to that of rodent malaria parasites. Like in rodent malaria, we found that glutathione (GSH) is also remarkably increased in P.falciparum infected erythrocytes.

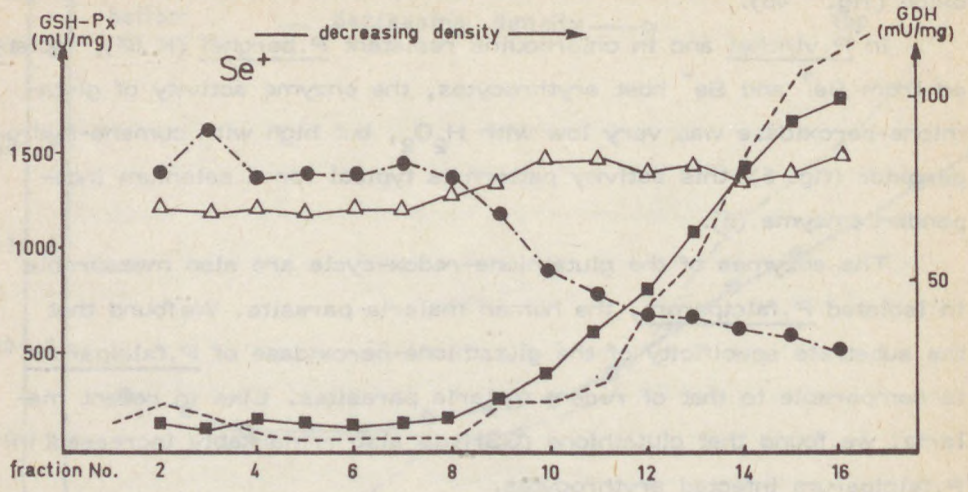
P.falciparum is inhibited in vitro by D,L/R,S-buthionine sulfoximin (BSO) and by 1,3-bis-(2chloro-ethyl)-1-nitrosourea (BCNU), inhibitors of glutathione-synthetase and glutathione-reductase; the concentrations that result in a 50% inhibition are 50 μM and 70 μM, respectively.

Pretreatment of uninfected human red blood cells for 24 hours with 1 mM BSO or 1 mM BCNU, resulted in a more than 95% inhibition of glutathione-synthetase and a 90% inhibition of glutathione-reductase, respectively. No inhibition of the development of P.falciparum could be observed in such host erythrocytes with chemically induced enzyme deficiencies. These results demonstrate very clearly that the parasites possess own enzymes of the glutathione-redox-cycle and that they are independent of the redox-metabolism of their host cell. This holds true as long as the erythrocyte is not lysed by oxidative damage and the parasite has to join this lethal event.

The substrate specificity of the parasitic glutathione-peroxidase and an apparent lack of a parasitic catalase activity indicate that

Fig. 3. Glutathione-reductase in P.vinckei parasitized erythrocytes separated into the different stages of blood schizogony by Percoll density gradient centrifugation.
←——→ glutathione-reductase (GSSG-Red.)
-------- glutamate-dehydrogenase (GDH), parasitic marker enzyme

a



b

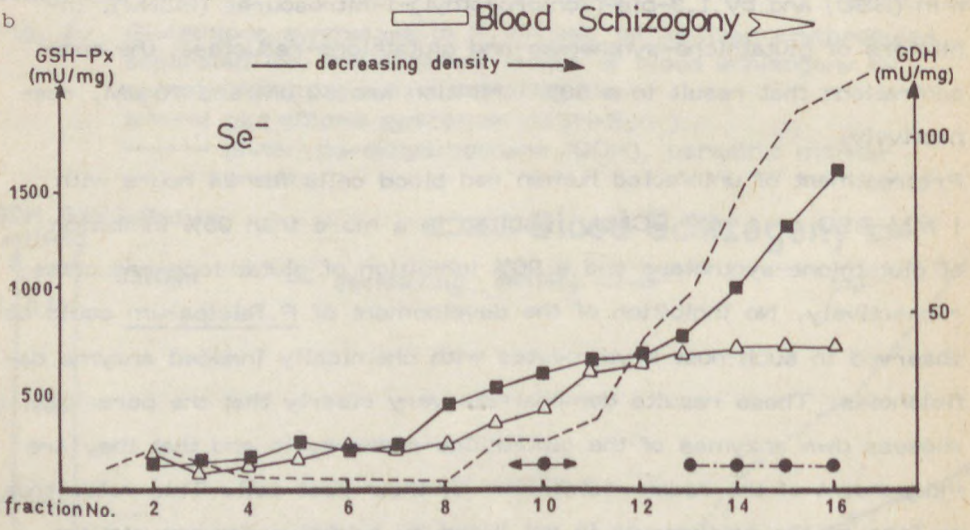


Fig. 4a,4b Glutathione peroxidase activities with different substrates in P.vinckei parasitized erythrocytes from selenium-deficient (Se^-) and supplemented (Se^+) NMRI-mice. The cells were separated into the different stages of blood schizogony by Percoll density gradient centrifugation.
The substrates were: o—o H_2O_2
■—■ cumene-hydroperoxide
Δ—Δ t-butyl-hydroperoxide
Glutamate dehydrogenase (GDH)-----

OSVAC of the parasite and its host cell must be seen independently of each other. In contrast to the red blood cell, the malaria parasite is sensitive to H_2O_2-generators or t-butyl-hydroperoxide because of its low capacity to eliminate these peroxides; as demonstrated in vivo by other authors (9). On the other hand, the antioxidant capacity of the host erythrocyte decreases continuously in the course of the intracellular development of the parasite because of the degradation of host cell stroma (hemoglobin and the stroma enzymes which are involved in the protection of the red cell membrane). Erythrocytes, infected with mature parasites are therefore more sensitive for radical-generators like Isouramil than younger stages (10); the same should be true for a macrophage-mediated cell lysis. The question, whether malaria parasites possess an own superoxide-dismutase is not answered finally. Independently of the origin of a superoxide-dismutase (synthesized by the parasite, as suggested by us or adopted from the erythrocyte, as suggested by other authors (12)), this enzyme activity would detoxify superoxide radicals but thereby increase the steady-state concentration of H_2O_2.

In general we conclude: malaria parasites possess an own glutathione-redox-cycle and are therefore independent of the correspondent cycle of the host cell. This conclusion is supported by the observation that under appropriate conditions even erythrocytes with specific enzyme defects regarding their redox-system, are suitable host cells for these parasites; e.g. genetically determined or "artificially" made enzyme defects like glucose-6-phosphate dehydrogenase deficiency (11) or erythrocytes with experimentally inhibited glutathione enzymes (see above). Because of the high GSH concentration and the enzyme equipment one can conclude that the antioxidant capacity of malaria parasites is remarkably high. The differences in the quality of the antioxidant capacity of host and guest cell, however, are reflecting differences in the susceptibility to oxidative damage of both cells (depending on the nature of the peroxide). The differences in the capability of host cell and parasite to detoxify peroxides, are a good

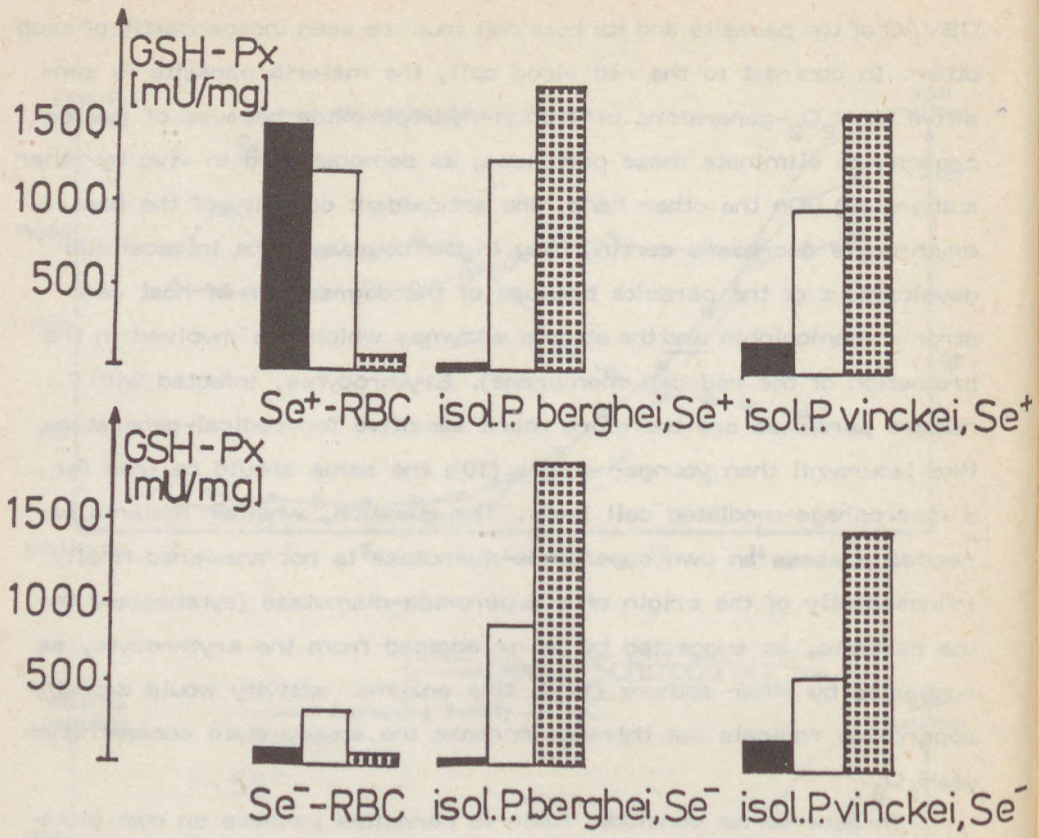


Fig. 5. Glutathione-peroxidase activities with different substrates in uninfected erythrocytes from selenium-deficient (Se^-) and supplemented (Se^+) NMRI-mice and in isolated plasmodia. The substrates were: H_2O_2, cumene hydroperoxide, t-butyl-hydroperoxide

prospect for a selective destroying of the parasite by suitable compounds. Qinghaosu, an endoperoxide, isolated from the medical plant Artemisia anua, might be such a drug - probably as an inhibitor of the parasitic glutathione-peroxidase.

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This work was supported by the WORLD HEALTH ORGANIZATION and by the MÜNCHNER APPARATEBAU, Kimmel & Ko, KG, FRG

SUBJECT INDEX

The material of a two-day Symposium held in Szeged concerning recent research on the oxygen-free radicals and the tissue injury has been compiled in this book.

Essentially topics closely related to radiation biology, radiation protection, gerontology, cell protection with antioxidants, oxidative cell damage and its protection in the newborn and paediatric clinics, the oxidative metabolism of human red blood cells and white blood cells are treated. Results on cell damages caused by oxidative processes, such as the oxidative origin of the rheumatism in the human organism, as well as toxic procedures of oxygen-free radicals in plants caused and generated by herbicides are also presented.

The possible relationships between the presence of oxygen-free radicals and cataracts of old age, hyperbiliruninaemia of newborns, ethanol intoxications, toxic effect of Gramoxone® in humans, and the oxidative damage caused by the cardiac infarct are explored.

This book is intended for medical practitioners, biologists, chemists involved either in practical or in theoretical research of radiation protection, gerontology or pathophysiological processes caused by the oxygen-free radicals and will be a valuable asset of libraries of medical clinics, research institutes and universities.

ISBN 963 05 4951 4

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