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THESIS

EFFECT OF ALLOPURINOL TREATMENT OF PREECLAMPTIC MOTHERS ON THE ANTIOXIDANT CAPACITY OF THE NEWBORN (A HYPOTHESIS AND METHODOLOGICAL APPROACH)

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CONTENTS

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ABBREVIATIONS

CPO, cumene hydroperoxide
EDRF, endothelium derived relaxing factor
GSH, reduced form of glutathione
GSSG, oxidized form of glutathione
NTBI, non-transferrin bound iron
$OH^{\cdot}$, hydroxyl radical
PG I_2, prostacyclin
TRAP, total free radical trapping antioxidant parameter
TX A_2, thromboxane A_2

INTRODUCTION

Preeclampsia, a frequent disorder of human pregnancy, characterized by persistent arterial hypertension and proteinuria appearing after the 20th week of gestation, is one of the most important causes of premature birth. Although the syndrome is common and well known, its pathogenesis is still unclear. So far there have beeen two major hypotheses offered to explain the generalized vasoconstriction found in preeclampsia (Fig.1).

The first one suggests that there is an imbalance in the production of vasoactive prostaglandines caused by circulating lipid peroxidation products (Fig.2). Prostacyclin (PG I_2) is a vasodilator and inhibitor of platelet aggregation, whereas thromboxane A_2 (TX A_2) is a vasoconstrictor and a promoter of platelet aggregation. Circulating products of the excessive lipid peroxidation in preeclampsia inhibit the synthesis of prostacyclin via inhibition of prostacyclin syntethase enzyme. Thromboxane A_2 synthesis, however, is not negatively

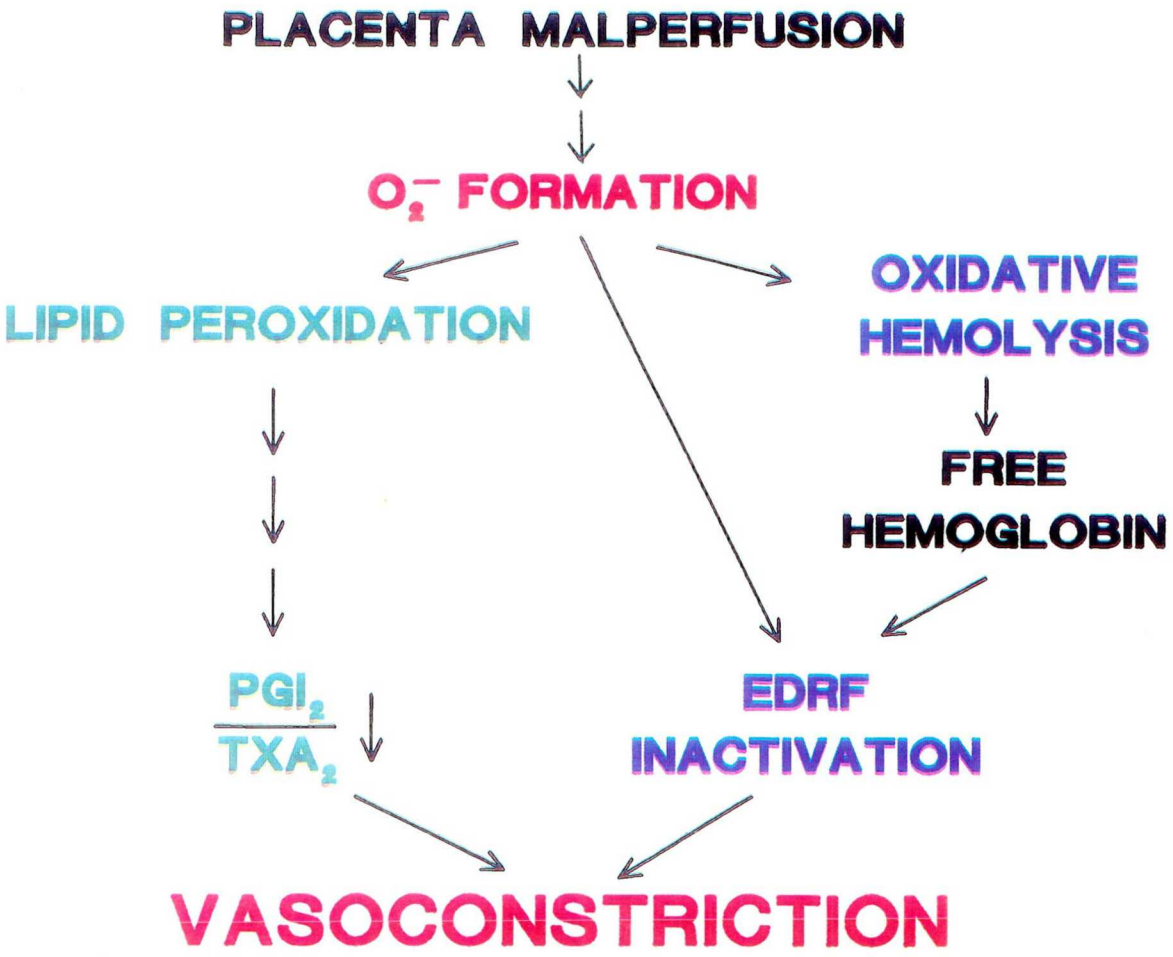


Figure 1.

Hypotheses to the pathomechanism of preeclampsia

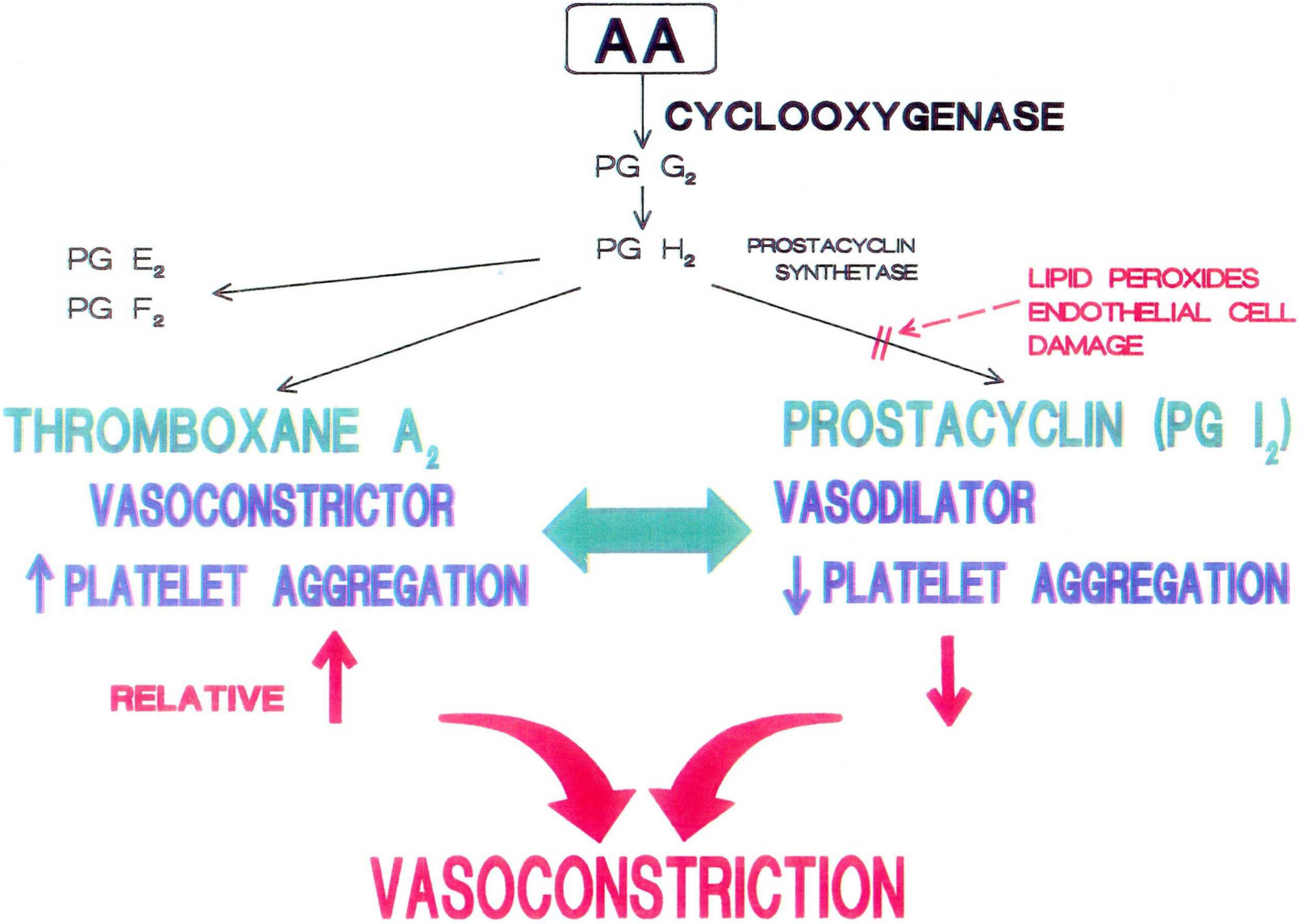


Figure 2. **The role of vasoactive prostaglandines in the pathomechanism of preeclampsia**

influenced by lipid peroxides. This results in an imbalance between the vasoconstrictor TX A_2 and vasodilator PG I_2, in which the former dominates resulting in a generalized vasoconstriction (2,29,30).

According to the other hypothesis free hemoglobin as well as superoxide free radicals inactivate the endothelium derived relaxing factor (EDRF) produced by vascular endothelial cells (2,3,31). EDRF, identified as either nitric oxide or a related compound, plays an important role in the control of tissue blood flow. In preeclampsia the increased amount of free hemoglobin (coming probably from the increased rate of oxidative hemolysis) binds to, and inhibits the effects of EDRF, which results in vasoconstriction (Fig 1.).

We offer the hypothesis that in the background of both mechanisms there may be a free radical mediated oxidative stress.

The primary defect in preeclampsia has been shown to be a severely depressed uteroplacental blood flow (1) with a consecutive tissue hypoxia. It is well known that in hypoxic tissues hypoxanthine is accumulated as a result of the inreased ATP breakdown. At the same time xanthine dehydrogenase enzyme, normally present in endothelial cells, is transformed into xanthine oxidase (Fig 3.). In the presence of this enzyme superoxide free radicals are formed. These products can be toxic in themselves but their toxicity increases greatly in the presence of iron, as iron catalyzes the formation of the extremely reactive hydroxyl radicals ($OH^{\cdot}$) in the iron catalyzed Haber-Weiss reaction:

$$O_2^- + H_2O_2 \xrightarrow{\text{IRON}} O_2 + OH^- + OH^{\cdot}$$

The severity of the damage caused by free radicals depends on the actual antioxidant status of the body. One of the most important superoxide scavengers in erythrocytes is the glutathione redox system. During oxidative stress GSH, the reduced form, is oxidized and GSSG is formed, therefore there is a rapid decrease in the amount of GSH (Fig.3.). GSSG is then reduced by the NADPH-dependent glutathione reductase enzyme. Thus, it is not only the total amount of GSH that is important in the antioxidant defence system, but also the capacity of erythrocytes to regenerate GSH after an oxidative stress.

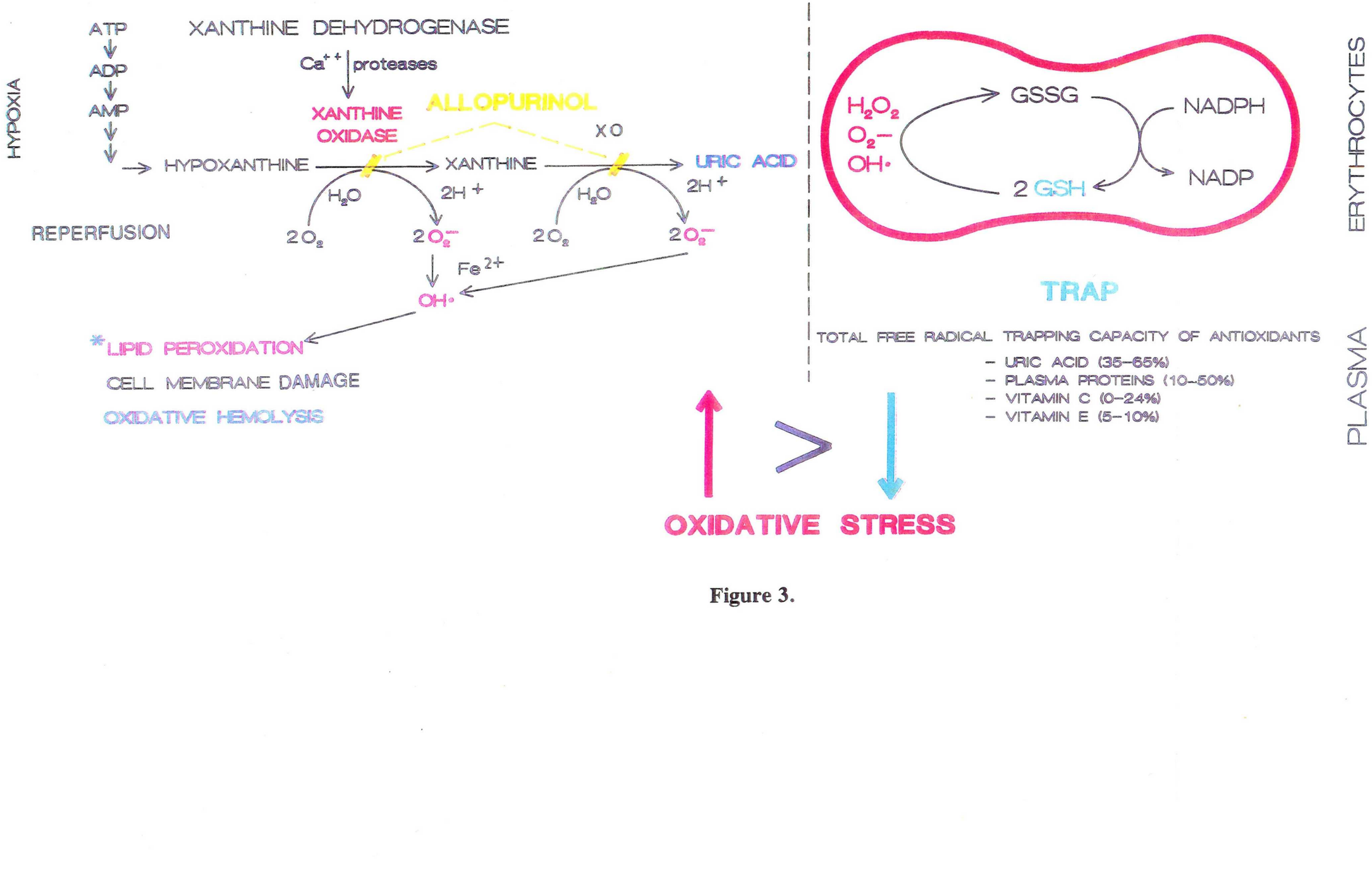

THE XANTHINE OXIDASE ENZYME SYSTEM IN HYPOXIC TISSUES
ANTI OXIDANTS
HYPOXIA
ATP
ADP
AMP
XANTHINE DEHYDROGENASE
Ca++
proteases
XANTHINE OXIDASE
ALLOPURINOL
XO
HYPOXANTHINE
XANTHINE
URIC ACID
H2O
2H+
2O2
2O2−
REPERFUSION
Fe2+
OH·
*LIPID PEROXIDATION
CELL MEMBRANE DAMAGE
OXIDATIVE HEMOLYSIS
H2O2
O2−
OH·
GSSG
NADPH
2 GSH
NADP
ERYTHROCYTES
TRAP
TOTAL FREE RADICAL TRAPPING CAPACITY OF ANTIOXIDANTS
- URIC ACID (35–65%)
- PLASMA PROTEINS (10–50%)
- VITAMIN C (0–24%)
- VITAMIN E (5–10%)
PLASMA
OXIDATIVE STRESS

Figure 3.

There are several antioxidants in the plasma as well, e.g. uric acid, SH groups of plasma proteins, vitamin E and vitamin C. TRAP, the total free radical trapping capacity of plasma antioxidants is the result of the synergistic action of these antioxidants (4,5).

Although a variety of the above mentioned antioxidants serve to control the harmful effect of free radicals, under certain conditions the protective mechanisms can be overwhelmed. This imbalance between oxidant and antioxidant forces in which the former dominates is defined as oxidative stress, resulting in uncontrolled lipid peroxidation and cell membrane damage (Fig.3.).

Our hypothesis about the possible pathogenic role of an increased xanthine oxidase enzyme activity generating superoxide free radicals seems to be supported by the fact that in many preeclamptic pregnancies not only hyperuricaemia, but also an elevated serum level of lipid peroxidation products and an increased rate of hemolysis has been found which can all indicate an oxidative stress.

If, indeed, a free radical induced oxidative stress is in the background of preeclampsia, then allopurinol, a specific xanthine oxidase enzyme inhibitor must have a beneficial effect on the newborn. By reducing superoxide generation allopurinol decreases the oxidative stress on the infant.

As in Holland allopurinol is given as an adjuvant treatment to some preeclamptic mothers with hyperuricaemia, it has given us the chance to evaluate its effect on the newborn.

PATIENTS AND METHODS

Our patients fall into 3 groups (Fig.4). All of them are prematures, their gestational ages are between 30 and 35 weeks. In the first 2 groups there are prematures born from preeclamptic pregnancies, mothers of babies in group II received allopurinol treatment during pregnancy. (The usual dosage of allopurinol: if serum uric acid level is above 0.36 mmol/l then 300-900 mg daily.) Babies in the control group are born from normal pregnancies, but prematures.

PATIENT GROUPS

I. PREMATURES PREECLAMPSIA

II. PREMATURES PREECLAMPSIA ALLOPURINOL

III. (30-35 wks) CONTROL

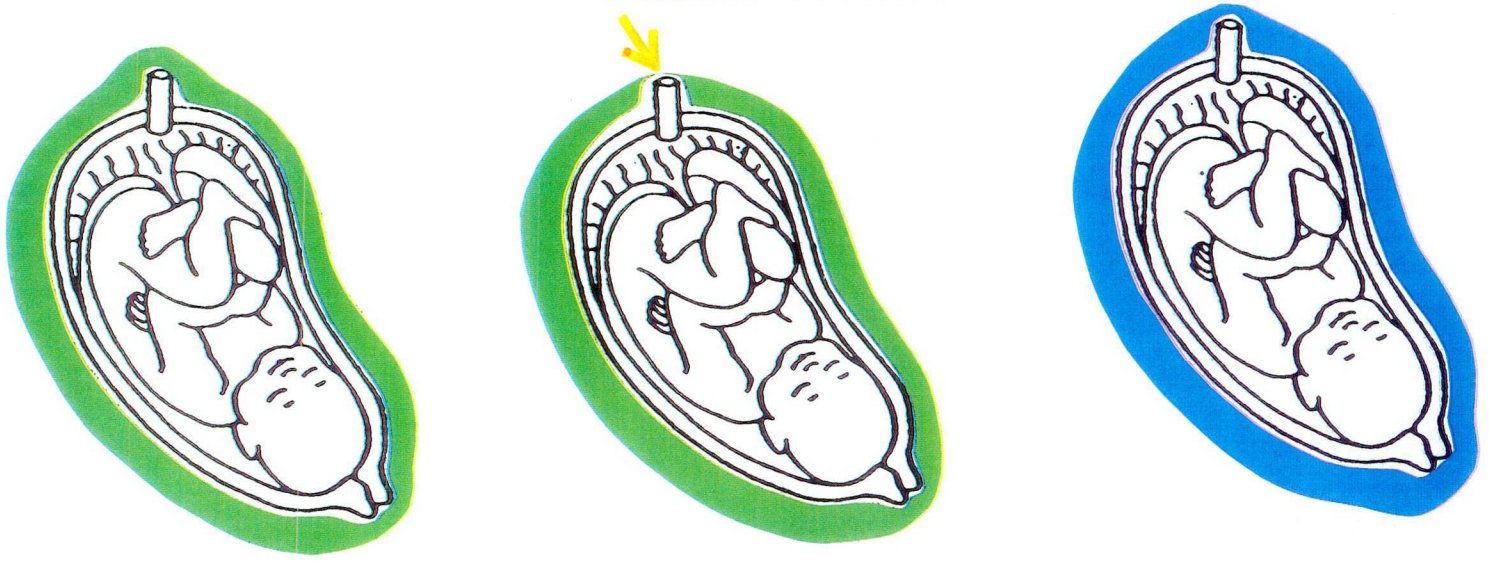


Figure 4.

Venous cord blood samples are taken from the separated placenta within 15 minutes after birth. The blood is collected over Na_2EDTA and immediatelly centrifuged (800xg for 10 min). The supernatant (plasma) is stored at -70°C for later determination of TRAP and uric acid concentrations ($d_)$,$e_)$, see below), whereas measurements from the packed cells ($a_)$,$b_)$,$c_)$, see below) are carried out immediatelly.

In order to obtain information about the actual antioxidant status of our patients we have planned to determine the following parameters (Fig.5.):

a) total GSH concentration of erythrocytes indicating the 'in vivo' oxidative stress;

b) the capacity of erythrocytes to regenerate GSH after an 'in vitro' oxidative stress;

c) the proportion of hemolysis after an 'in vitro' oxidative stress as an indicator of the oxidative susceptibility of erythrocytes;

d) TRAP, as a parameter of the total free radical trapping capacity of plasma antioxidants;

e) serum uric acid concentrations indicating the xanthine oxidase enzyme activity.

The total GSH concentration of erythrocytes and the capacity of eythrocytes to regenerate GSH after an 'in vitro' peroxidative stress are both determined by the glutathione stability test according to Koster et al (7) (Fig.6.). Separated packed cells are washed 3 times with a 0.1 M phosphate buffer containing 10 mM glucose (pH=7.4) and diluted with the same buffer solution. The cells are then exposed to cumene hydroperoxide (CPO) at concentrations of 0.75 mM and 1.5 mM. They are incubated at 37°C in shaking waterbath for 60 minutes. Parallel control studies are performed by means of incubation of cells in the buffer solution alone. Samples are taken at 0, 5, 10, 15, 20, 25, 30, 40, 50, 60 minutes and reduced glutathione (GSH) concentrations are determined from them with the GSH assay according to Beutler et al (39) based on the reduction of dithiobis-nitrobenzoic acid (DTNB). GSH concentrations at the indicated time points are calculated and expressed as a percentage of the total GSH concentration of erythrocytes.

During the control studies GSH concentrations remained constant, giving the original total GSH concentrations of erythrocytes, expressed in nmol/ml packed cells.

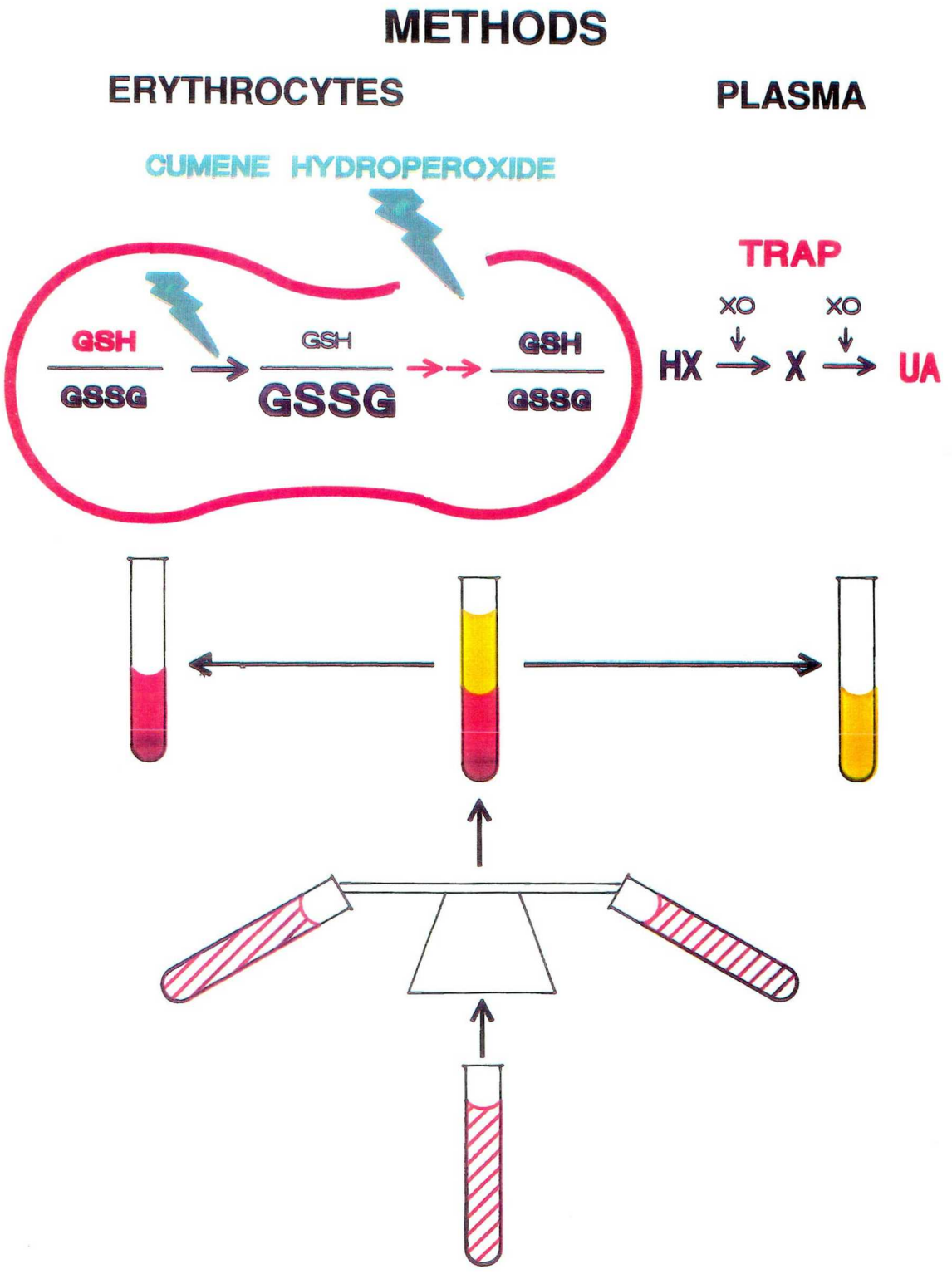

METHODS
ERYTHROCYTES
PLASMA
CUMENE HYDROPEROXIDE
TRAP
GSH
GSSG
GSH
GSSG
GSH
GSSG
XO
XO
HX
X
UA

Figure 5.

GSH ASSAY (Koster et al.)

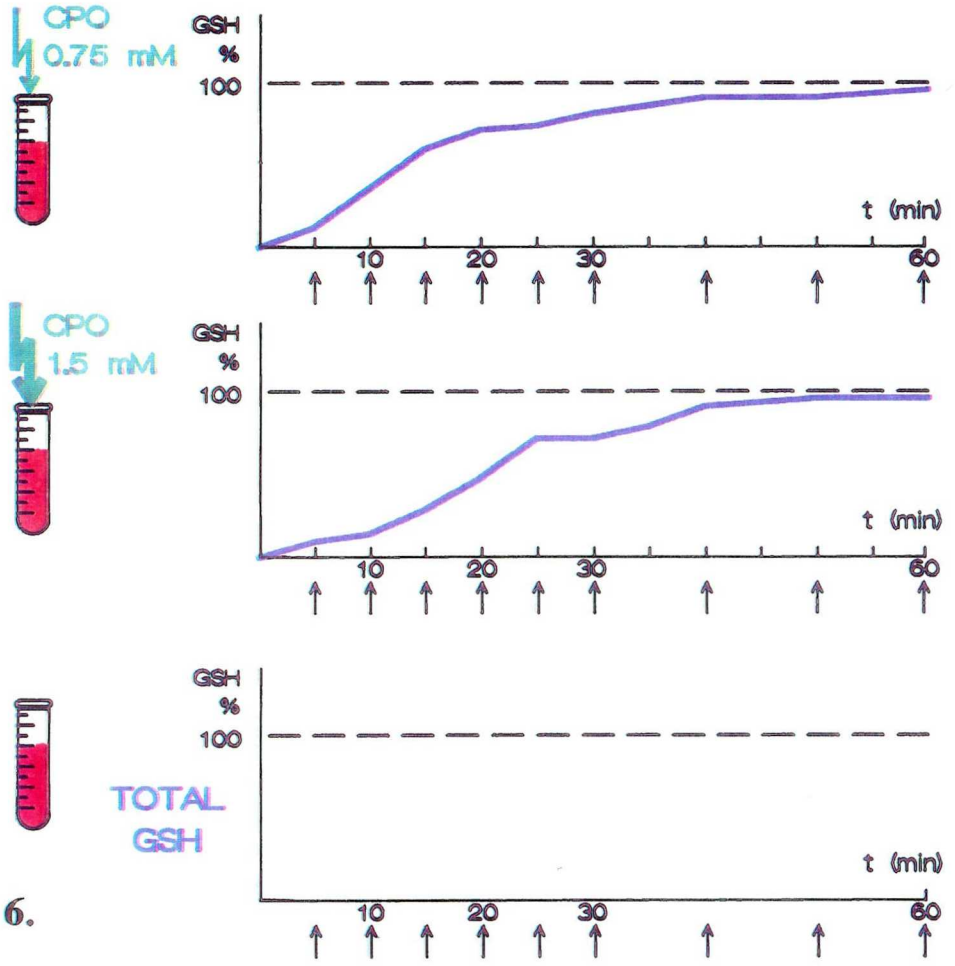


Figure 6.

The oxidative susceptibility of erythrocytes, indicated by the rate of hemolysis after an 'in vitro' peroxidative stress is determined as follows (Fig.7.): Washed, diluted packed cells are given a peroxidative stress with CPO (1.5 mM). After 60 minutes' incubation at 37°C the sample is centrifuged and hemoglobin concentration of the supernatant is determined with a hemoglobin reagent (containing 100 mg NaCN + 300 mg $K_3Fe(CN)_6$/L 10 mM phosphate buffer, pH=7.4), absorptions are read at 546 nm. Total hemoglobin concentration of the packed cells is determined with the same method after they are hemolyzed in a hypotonic solution. The rate of hemolysis due to the 'in vitro' peroxidative stress is calculated by comparing the supernatant's hemoglobin concentration to the total hemoglobin concentration of the packed cells.

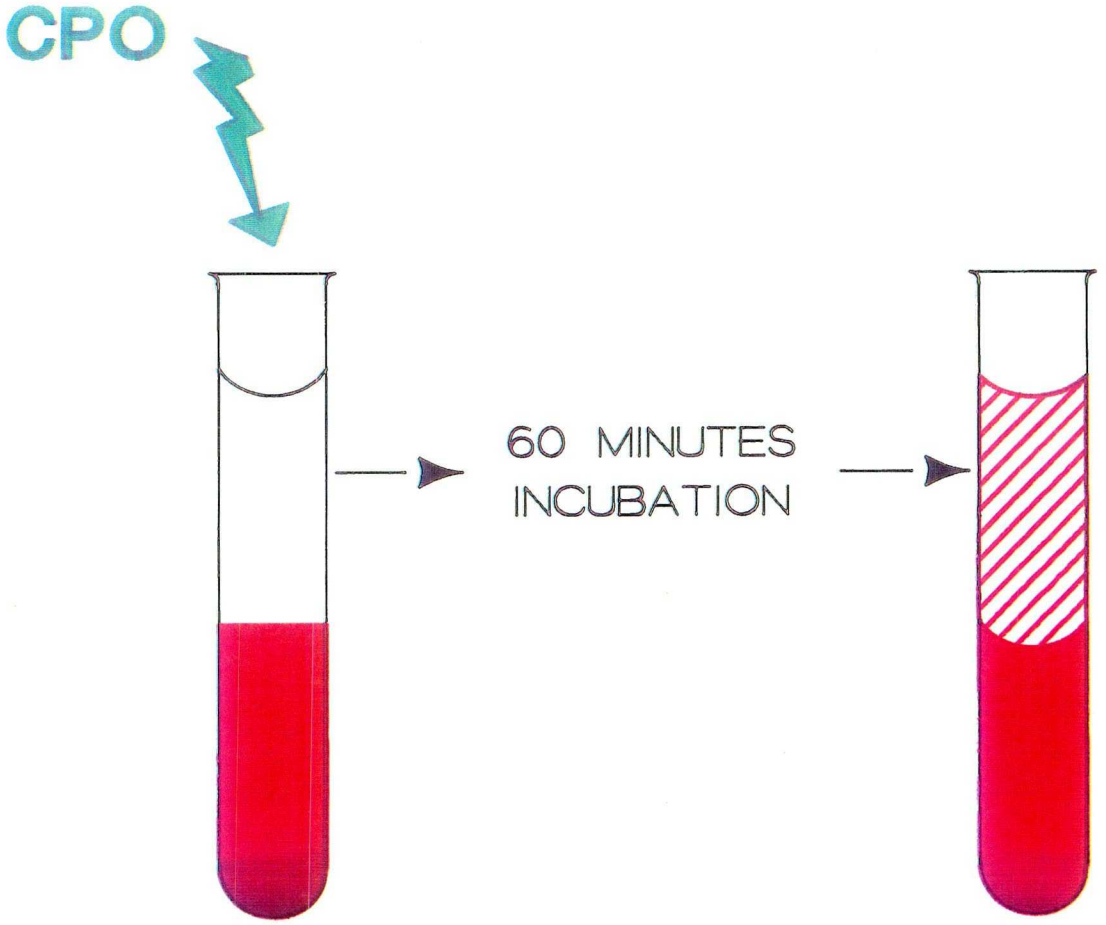
TRAP, as a parameter of the total free radical trapping capacity of plasma antioxidants was measured with the TRAP assay (4,5,6,8). The principle of the measurement is as follows (Fig.8.):

A buffer solution contains linoleic acid, as the substrate of controlled lipid peroxidation, ABAP (2,2'-azo-bis(amidopropane)), a thermal labile free radical initiator and the plasma sample containing a certain amount of antioxidants. The system is closed, oxygen concentration is continuously measured with a Clarke-type oxygen electrode.

Without the antioxidants free radicals would rapidly be consumed for lipid peroxidation, and thus the total oxygen concentration of the system would fall steeply. But as long as the added plasma has a high trapping capacity lipid peroxidation is inhibited - therefore the fall in the total oxygen concentration of the system is not so steep. The higher its antioxidant capacity the longer the plasma can inhibit lipid peroxidation. Antioxidant activity of the serum is therefore measured by the time taken to prevent maximum oxygen uptake. The system is calibrated by the addition of a known amount of water soluble vitamin E analogue (Trolox=6 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with a known trapping capacity.

The serum uric acid concentration is determined as an indicator of the xanthine oxidase enzyme activity.

OXIDATIVE SUSCEPTIBILITY OF ERYTHROCYTES



$$\% \text{ OF HEMOLYSIS} = \frac{\text{HAEMOGLOBIN CONCENTRATION IN SUPERNATANT}}{\text{TOTAL HAEMOGLOBIN CONCENTRATION}} \times 100$$

Figure 7.

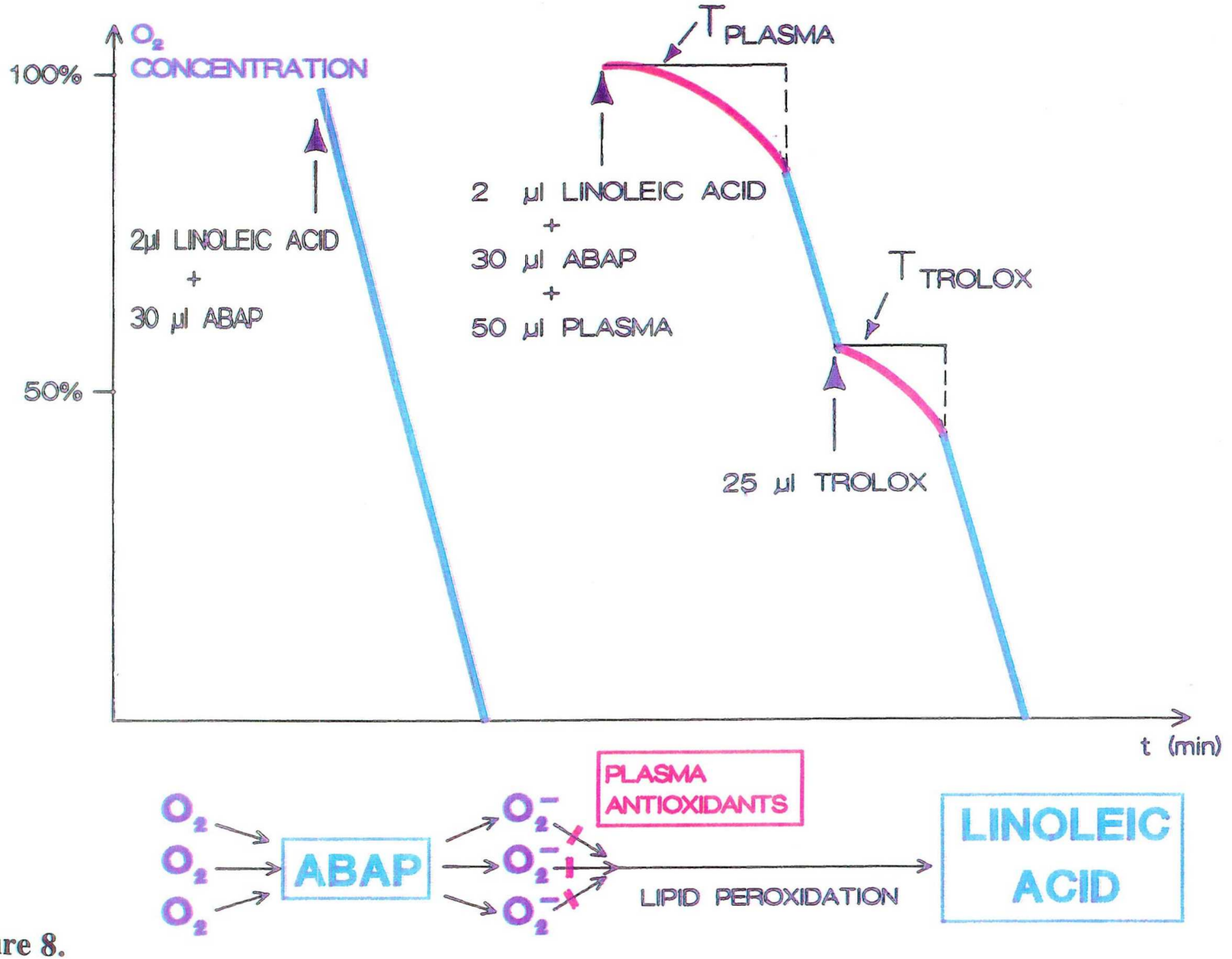

O_2 CONCENTRATION
100%
50%
2µl LINOLEIC ACID
+
30 µl ABAP
T_{PLASMA}
2 µl LINOLEIC ACID
+
30 µl ABAP
+
50 µl PLASMA
T_{TROLOX}
25 µl TROLOX
t (min)
O_2
O_2
O_2
ABAP
O_2^-
O_2^-
O_2^-
PLASMA ANTIOXIDANTS
LIPID PEROXIDATION
LINOLEIC ACID

Figure 8.

RESULTS

The aim of this study is to introduce the idea of the possible beneficial effect of allopurinol treatment in preeclampsia on the basis of various publications, and to present the methods, already elaborated, which will enable us to find proof to this hypothesis. However, some initial results obtained with methods a)-c) are shown in Table 1.

In all 3 patient groups the initial GSH concentration of the erythrocytes was restored within 60 minutes after the 'in vitro' peroxidative stress, no matter which concentration of CPO was used. There tends to be some difference, however, in the erythrocytes' GSH concentrations indicating a stronger 'in vivo' oxidative stress in the non-treated preeclamptic patient group, which seems to have been prevented by allopurinol treatment. The rate of hemolysis after the 'in vitro' peroxidative stress shows some difference as well in patient groups I and II, suggesting a higher fragility, a higher oxidative susceptibility of erythrocytes in the non-treated patient group.

DISCUSSION

In the present study it is suggested that adjuvant allopurinol treatment of preeclamptic mothers may result in a decrease in the oxidative stress on the baby, a decrease in the oxidative susceptibility of its eythrocytes and, consequently, an improvement of the newborn's clinical condition.

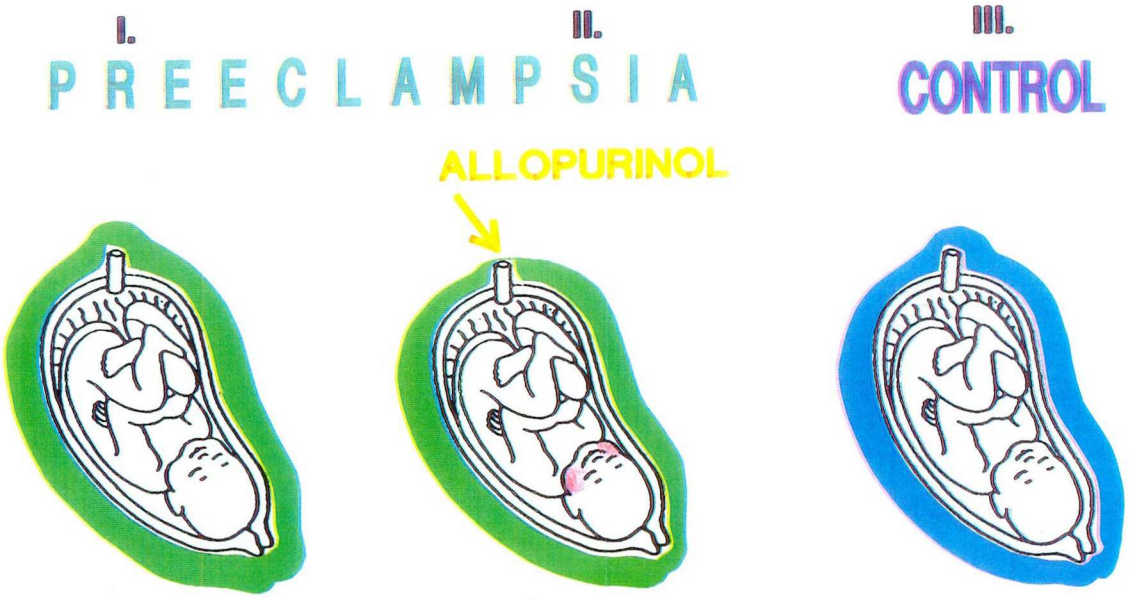
It has been suggested by several authors that the most severe oxidative damage is caused not only directly by superoxide anions, but also by the even more reactive hydroxyl radicals ($OH^{\cdot}$) (10,11,12,13,14,15,17,18,19). They are generated in the presence of non-transferrin-bound iron (NTBI) (9) in the Fenton reaction:

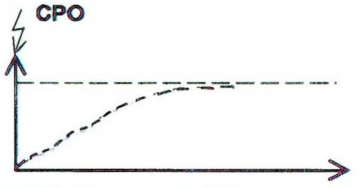
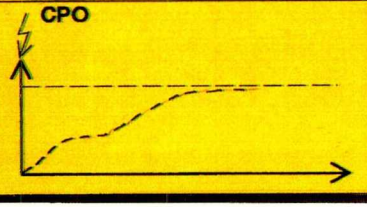
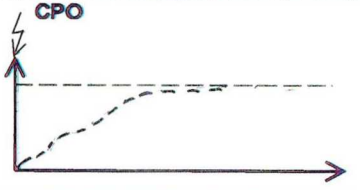
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Iron that is not bound to the usual transport protein but to different extracellular low molecular weight compounds is referred to as NTBI. It becomes elevated when transferrin saturation is high and transferrin is no longer able to bind iron. (Excessive parenteral iron administration during pregnancy may also be

Table 1.

RESULTS



	I. PREECLAMPSIA	II. PREECLAMPSIA + ALLOPURINOL	III. CONTROL
TOTAL GSH CONCENTRATION	1875.0 nmol/ml packed cells 2543.8 nmol/ml packed cells	3089.7 nmol/ml packed cells 3223.2 nmol/ml packed cells	3308.1 nmol/ml packed cells 3467.4 nmol/ml packed cells 2629.9 nmol/ml packed cells
% OF HEMOLYSIS	22.8 % 71.0 %	1.9 % 5.9 %	0.4 % 9.1 % 9.6 %
GSH STABILITY			

considered as a possible factor leading to iron overload.)

Considering the fact that superoxide (generated by the xanthine oxidase enzyme system) and other free radicals can release iron from ferritin (10,15,16,18), it is possible that the oxidative damage in the newborn is, at least partly, caused by hydroxyl radicals.

It has also been proved that the erythrocytes of the newborn are more susceptible to oxidative stress and there is a high tendency of hemoglobin oxidation in neonates (20,21,22). The oxidative derivates of hemoglobin: oxihemoglobin and methemoglobin are able to generate $OH^{\cdot}$ and another, also highly damaging 'reactive species'(13) - they may account for the most severe oxidative damage in neonates.

By reducing the superoxide radical generation with allopurinol treatment we might reduce the iron release from ferritin and the production of oxidized hemoglobin derivates, thus preventing the formation of the highly reactive $OH^{\cdot}$ radicals.

Allopurinol treatment has already been found to be beneficial both in human (26,38) and in experimental animal (27) subjects endangered by hypoxia and oxidative stress. What we suggest in this study is practically an allopurinol treatment started before birth. The question must be raised whether allopurinol passes through the placental barrier and can in fact have a therapeutic effect on the baby 'in utero'. No data concerning this particular problem have been found in literature. A relatively simple micromethod, however, suitable for measuring blood levels of allopurinol and its xanthine oxidase inhibitory activity has been developed and published (28) and with this method allopurinol was detected in the blood of infants following allopurinol treatment during pregnancy (Boda,D., unpublished data).

In spite of the fact that xanthine oxidase enzyme activity can be determined directly with the caffeine test (25), we suggest serum uric acid determination as an indicator of xanthine oxidase enzyme activity. Considering the fact that we use cord blood for our measurements, it is obvious that the caffeine test cannot be performed. On the other hand, serum uric acid levels have been shown to correlate well with the results of the caffeine test (25), therefore we consider serum

uric acid level to be a parameter sufficiently informative on xanthine oxidase enzyme activity.

Many investigators reported increased serum uric acid levels in women with preeclampsia (33), and also a positive correlation was demonstrated between the magnitude of serum uric acid levels, clinical severity of preeclampsia and perinatal outcome (34-37). It was also found that serum uric acid levels may begin to rise before the appearance of hypertension and proteinuria (32), meaning that the rise in the serum level of uric acid is probably not caused by renal impairment but by the increased xanthine oxidase activity.

Although in the present study GSH concentration of erythrocytes is used as an indicator of the 'in vivo' oxidative stress, it has been suggested (23,24) that the ratio of oxidized/reduced glutathione (GSSG/GSH) is a much more sensitive and reliable indicator. However, the determination of the latter is much more complicated, therefore it was purely for technical reasons that GSH determination was chosen.

The defences against oxigen toxicity include enzymes (e.g. superoxide dismutase, glutathione peroxidase), metal chelators (transferrin, ceruloplasmin) and various plasma antioxidants e.g. vitamin E (VE), vitamin C (VC), sulfhydryl groups (SH), uric acid (UA), etc. (5). Synergistic interactions occur between these various antioxidants and therefore complete understanding of the total antioxidant defence system in the baby requires measurement of the combined effect of the antioxidants and not just the measurement of their individual concentrations (4). There are two major types of antioxidants. Those that reduce the rate of production of new radicals, e.g. transferrin, allopurinol, are referred to as primary or preventive antioxidants. Antioxidants that trap radicals and thereby reduce the chain length of oxidation are referred to as secondary or chain-breaking antioxidants, e.g. vitamin E and C (5). The TRAP provides information on the total capacity of the chain-breaking (secondary) antioxidants in the plasma. TRAP can either be measured directly with the TRAP assay ($TRAP_{meas}$), or calculated from the measured concentrations of the individual plasma antioxidants by using their experimentally determined efficiency values (8) as follows:

$$TRAP_{calc}(\mu mol/l) = 1.3[UA] + 1.7[VC] + 2.0[VE] + 0.2[SH]$$

It has been found however, that the values of $TRAP_{calc}$ were considerably lower than the $TRAP_{meas}$ (5). The reason for this can be on one hand the fact, that not all of the plasma antioxidants have been identified yet. On the other hand, the $TRAP_{calc}$ does not take into account the very important interactions that occur between antioxidants when they work in unision (4). For example, the recycling of vitamin E by vitamin C is believed to be an important mechanism in defending against lipid peroxidation (4). These interactions do occur when the $TRAP_{meas}$ assay is performed and may explain the greater than predicted free radical trapping capacity in plasma (5). Determination of $TRAP_{meas}$ therefore gives reliable information on the actual antioxidant capacity of the plasma taking all (even the unidentified) antioxidants as well as their synergistic actions into account.

Our initial results, as well as data from publications suggest that allopurinol treatment of preeclamptic mothers may have a beneficial effect on their babies probably due to the specific inhibition of xanthine oxidase enzyme and thus the reduced generation of reactive superoxide radicals. Further investigations in order to find out more about the pathogenesis of preeclampsia and the possible role of oxygen toxicity in it are in progress.

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