THE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF RETINOPATHY OF PREMATURITY

Ph.D. thesis

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1. LIST OF PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. **Papp, A.**, Nemeth, I., Karg, E., Papp, E.: Glutathione status in retinopathy of prematurity. Free Rad. Biol. Med., Vol 27, Nos .7/8, pp.738-743, 1999.

II. **Papp, A.**, Nemeth, I., Pelle, Zs.: A retrospective ophthalmological and biochemical study on the antioxidant defense capacity of patients suffering from retinopathy of prematurity. In: Mozsik Gy., Emerit I., Feher J., Matkovics B., Vincze A. (Eds.): Oxygen Free Radicals and Scavengers in the Natural Sciences, Akademiai Kiado, Budapest, pp. 203, 1993.

III. **Papp, A.**, Nemeth, I., Pelle, Zs., Tekulics, P.: A one-year ophthalmical and biochemical follow-up study on prematures with high risk of retinopathy. In: Mozsik Gy., Emerit I., Feher J., Matkovics B., Vincze A. (Eds.): Oxygen Free Radicals and Scavengers in the Natural Sciences, Akademiai Kiado, Budapest, pp. 199, 1993.

IV. **Papp, A.,** Nemeth, I., Pelle, Zs.: A retrospective, biochemical study on the antioxidant defense capacity of patients suffering from Retinopathy of Prematurity. Orv. Hetil., 134/19: 1021-1026, 1993.

V. **Papp, A.**, Nemeth, I., Pelle, Zs., Tekulits, P.: A prospective, biochemical study on the antioxidant defense capacity of patients suffering from Retinopathy of Prematurity. Orv. Hetil. 201: 201-205, 1997.

VI. **Papp, A.**, Nemeth, I., Pelle, Zs., Tekulics, P.: Antioxidant vitamins and iron-pool in preterm infants suffering from Retinopathy of Prematurity. Opht. Hung. (Szemeszet), 132/1:51-55, 1995.

2. ABBREVIATIONS

APH – acetylphenylhydrazine

G6PD – glucose-6-phosphate-dehydrogenase

GSH – reduced glutathione

GSSG – oxidized glutathione

GSSG/GSH – glutathione redox ratio

GSH-Px – gluathione peroxidase

GSH-Red – glutathione reductase

Hb – hemoglobin

NEM – n-ethylmaleimide

RBCs – red blood cells

ROP – retinopathy of prematurity

Se – Selenium

SOD – superoxide dismutase

Cat – catalase

IRBP – interstitial retinol binding protein

– SH – free sulphydryl groups

EPO – erythropoietin

3. SUMMARY

Background: Retinopathy of Prematurity (ROP) is one of the leading causes of childhood blindness. As a result of the improved and careful management in the neonatal intensive care units the survival rate of very small premature infants dramatically increased during the last two decades. A new ROP epidemic started among the very low birth weight and high-risk preterm infants. The etiology ROP is multifactorial and the pathomechanism is still not clear. Better understanding of the environmental and individual factors that cause retinal neovascularisation and lead to this disabling condition requires further investigation. One of the proposed pathomechanisms of ROP is oxygen radical injury. It occurs when the body's natural antioxidant capacity is overwhelmed by the free oxygen radicals produced during the perinatal period.

Objective: On the hypothesis that a special susceptibility of prematures with ROP does exist (e.g. some kind of trace element or vitamin deficiency causing decreased activity of antioxidant enzymes), retrospective and prospective studies were carried out. The similarity between the erythrocyte (RBC) membrane and the immature retina both in their structure and antioxidant defense systems led us to study RBC as indicator of the oxidative status of patients.

Study design and patients

Retrospective study

Supposing that premature infants may have defects in their antioxidant systems which can be detected later, even in childhood, a retrospective study of 50 ROP patients of different ages (between 6 weeks and 6 years), who had been born prematurely (gestational age: 28.7±1.3 weeks; birth

weight: 1210±313 g; mean±SD) and were suffering from different stages of ROP (stage 2-5) was carried out.

Prospective study

After confirming the presence of the diminished antioxidant defense capacity in our retrospective study, a prospective study was planned. Our one-year study was carried out on 60 premature infants (gestational age: 32.8±3.1 weeks, range: 26-35 weeks); birth weights under 2000 g (mean: 1529±302 gs, range: 980-1840) admitted immediately after birth to the Perinatal Intensive Care Unit of the Department of Pediatrics for oxygen therapy (FIO_2 >0.6 longer than 24 hrs). In our prospective study a close ophthalmological follow-up was provided in order to monitor the presence and the outcome of ROP. Both the ROP screening and the biochemical tests were started at the age of 6 weeks. We examined the biochemical parameters of available mothers as well, supposing the mother's responsibility in determining the infant's antioxidant defense capacity, via her nutritional status (e.g. vitamin and trace element supply) and lifestyle (e.g. smoking) during pregnancy.

Methods: The ratio of oxidized/reduced glutathione (GSSG/GSH) was used as a parameter of *in vivo* oxidative stress. The degree of GSH stability after an *in vitro* oxidative insult and the presence and amount of hemoglobin oxidation products (methemoglobin, hemichrome) reflected the antioxidant protective capacity of RBCs. Blood selenium levels were measured by fluorometry. Plasma concentrations of free sulphydryl groups were determined by a spectrophotometric method.

Plasma concentrations of vitamin E and vitamin A were measured simultaneously by a HPLC method. Radioimmunassay was performed to determine ferritin concentrations.

Results: 1) A significant increase in the GSSG/GSH as a specific sign of acute oxidative stress was only seen in the youngest patients (<3 months). 2) The compromised antioxidant defense capacity could be detected in all ROP patients even in childhood following an in vitro oxidative stress. 3) The very low selenium levels in all ROP patients both in the retrospective and prospective studies indicated a reduced GSH-peroxidase activity. The same tendency was seen in the mothers. 4) The concentrations of free sulphydryl groups in the plasma were significantly lower in ROP patients; maternal levels showed the same relationship. 5) Marked decrease of plasma vitamin E concentrations were determined both in our ROP patients and in their mothers. 6) Vitamin E supplementation seems to have positive effect on prevention of ROP progression: both the ophthalmological and selenium status improved after per os vitamin E administration. 7) Plasma vitamin A concentrations were lower in those premature infants suffering from ROP 8) Ferritin concentration, which is an indicator of the body's iron load, was much higher in ROP patients than in their mothers.

Conclusions: 1) The RBC glutathione redox ratio is an adequate parameter to reflect the oxidative stress in the acute phase of ROP. 2) ROP may develop in those patients who have reduced antioxidant defense capacity (genetic or acquired). 3) Selenium depletion in Hungary might be an important predisposing factor to ROP. 4) The fact that during our screening for ROP we could only diagnose 5 cases of mild ROP from 60 high-risk premature infants, leads us to the conclusion that the management of preterm infants at the Perinatal Intensive Care Unit of our University and the secondary treatment at the Children's Hospital is very careful. 5) The close correlation found between the low free sulphydryl group and selenium levels of mothers and babies suggest that supplementation with sulfur containing aminoacids (e.g. methionin,

cystein) and selenium during pregnancy would improve the antioxidant capacity of premature infants. 6) The very low vitamin E levels measured both in our ROP patients and in their mothers suggest that an 'antioxidant cocktail' described in literature (containing Se and vitamin E) given to the high-risk mothers before delivery might be useful in the prevention of ROP. 7) According to our results, per os vitamin E supplementation seems to have a positive effect on the prevention of ROP progression. Because of the potential harmful side effects, however, vitamin E treatment must be closely monitored by repeated measurements of plasma vitamin E level. 8) The fact that in spite of the mothers' iron deficiency, fully saturated iron pools were detected in their preterm infants, leads us to the conclusion that during the first 6 weeks of life iron overload occurs and might be one of the main factors leading to Retinopathy of Prematurity.

4. INTRODUCTION

4.1. Retinopathy of prematurity

4.1.1 History

Retinopathy of prematurity (ROP) is a disease of the incompletely vascularized immature retina, characterized by retinovitreal neovascularization and retinal scarring. In the most severe cases retinal detachment can occur, leading to serious visual disability and even complete blindness [26, 76]. The disease was first reported in 1942 by *Terry* [113]. The cause of the first ROP epidemic appeared to be the excess oxygen, administered in high concentrations to premature infants in order to keep them alive. ROP was the first disease entity, at least in neonatology, which was referred to the effect of hyperoxia, thus, the original classical manifestation of oxygen toxicity due to excessive oxygen administration in premature infants [14].

Although oxygen therapy has subsequently been restricted and is now precisely controlled in modern neonatal intensive care units, we are in the midst of a second epidemic of a blinding disease which - many anticipated - would have been disappeared by now. *Phelps* estimated the number of ROP-blinded infants and found it comparable to the number of annual cases recorded at the peak of the first epidemic [79]. Despite the use careful oxygen monitoring, other investigators have noted the continued occurrence of new cases of ROP [29,37, 68,84]. Thus, ROP still remains one of the major causes of childhood blindness.

The increased survival rate of the very low birth weight infants and the special characteristics of retinal development can provide a possible explanation to the question why ROP never completely vanished. The inner retinal vasculature develops from spindle cells, which are the

precursor cells of the retinal endothelial cells. Spindle cells are largely in place by the 16th week of gestation [50]. The central retinal artery grows through the optic nerve, branching into arterioles that advance centrifugally across the retinal surface. By 32 weeks of gestational age, peripheral retinal vascularisation is taken place in most infants but may not be complete until 40 weeks of gestation [33]. As a result of the improved obstetric techniques and modern technologies of the perinatal intensive care, even extremely immature infants are able to survive, requiring more of their retinal vasculature to develop ex utero, often in the environment of a neonatal intensive care unit [23,29-31].

4.1.2. Risk Factors

Apart from immaturity and oxygen exposure approximately 50 more recognized risk factors have been reported [95]. Numerous studies have attempted to identify and give relative significance to different risk factors that are associated with the occurrence of ROP. However, in spite of the long list of the recognized ethiological factors, such as hyperoxia, hypoxia, immaturity (low birth weight and gestational age), low APGAR scores, apnoe, anemia, blood transfusions, sepsis, light exposition, lactic acidosis [44], administration of xanthine derivates, vitamin E deficiency, multiple gestation, advanced maternal age, diabetes, preeclampsia, toxemia, amnionitis, premature rupture of membranes, bleeding, administration of β_2 blockers, antihistamines in late pregnancy etc., the pathogenesis of this potentially blinding disease is still unclear. [6,7,8,12,33,37,47,67,81,95,121] ROP has even been documented among infants with various congenital malformation syndromes, such as anencephaly, trisomy 18, cri-du-chat syndrome, supporting the theory of intrauterine causation [27,37]. The possible role of genetics has been

supported by molecular genetic studies: some infants with advanced retinopathy have missens mutations in the gene for Norrie's disease [98]. The occurrence of ROP in prematures who received no oxygen treatment at all [27] or in mature newborns with cyanotic congenital heart disease [48] suggested the presence of further, as yet unknown risk factors in the pathogenesis. Logistic regression analysis has demonstrated that the gestational age and the frequency of blood transfusions are the only parameters independently associated both with the occurrence of ROP and with its severity [20,37,45,89].

4.2. Oxygen Radical Disease of Prematurity

4.2.1. Free oxygen radicals

Free radicals are compounds with one or more unpaired electrons. They are highly reactive and can initiate chain reactions, which form new free radicals. Oxygen free radicals like the superoxide radical (O_2^-), the hydroxyl radical (OH^-) and the singlet oxygen can damage membranes by lipid peroxidation, inactivating enzymes, and damaging disulfide bonds of proteins. They can even cause DNS injury [28,90].

Free radicals are created in the human body even in the course of the normal metabolism but also by activated leukocytes as a part of the inflammatory defense mechanism against microorganisms. During oxygen treatment, phototherapy, blood transfusions, etc. they are generated in increased quantity.

4.2.2. The defense system against oxygen radicals

The antioxidant defense mechanism of the body includes cellular and extracellular enzymes such as superoxide dismutase (SOD), catalase (Cat), glutathione reductase (GSH-Red) and glutathione peroxidase

(GSH-Px). GSH-Px is selenium (Se) dependent and therefore selenium plays an important role in the natural defense against free radicals. The other important free radical scavengers are: glutathione, free sulfhydril groups, vitamins E and C, caroteinoids in addition to serum albumin and metabolic products such as uric acid and bilirubin.

4.2.3. Oxidative stress and the preterm newborn

Oxidative stress occurs when the production of free radicals overwhelms the body's natural antioxidant defence systems, resulting in oxidative damage of the cells [101]. All infants, regardless of gestational age, have shown evidence of oxidative damage during the first few days after birth [90].

In humans and most of the animals studied, antioxidant enzymes are expressed in high concentration only at the end of gestation in preparation of the relatively hyperoxic extrauterine environment [86] Thus, preterm infants with their immature enzymes are pariculary vulnerable to oxidative stress. Genetic variability, maternal oxidative stress, lifestyle (e.g. smoking, nutrition) and maternal antioxidant capacity are all contributing factors which determine how the infant will withstand the stress of being born into a relatively hyperoxic environment (compared to the intrauterine experience). After birth, once the antioxidant defence capacity of the premature infant is overwhelmed, they become susceptible to several neonatal diseases. The pathogenic role of oxygen toxicity has been recognized in retinopathy of prematurity (ROP), respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), subependymal and intraventricular hemorrhage (IVH) and persistent ductus arteriosus Botalli (PDA). Saugstad has proposed the term 'Oxygen Radical Disease in Neonatology' to make it clear that these conditions may be different manifestations of the same disease [90]. Because of the greater incidence

of these disorders in preterm infants with deficient antioxidant systems, a better name for this disorder, according to Sullivan, is 'Oxygen Radical Disease of Prematurity' [106].

4.3. Similarities between the human retina and red blood cells

4.3.1. Susceptibility to oxidative stress

The retina is one of the organs most directly exposed to high oxygen tension, and the endothelial cells of its microvessels as well as the cells of the retinal epithelium are highly susceptible to oxidative injury. Red blood cells (RBC) are frequently exposed to oxidative stress as well. They take up oxygen in the lungs at high oxygen tension, contain the greatest part of the body's iron in hemoglobin (Hb), and during the permanent autooxidation of Hb, active oxygen species are generated in them. RBCs handle the permanent intracellular oxidative stress through the combined activities of SOD, Cat the glutathione-dependent defence system. The most important intracellular antioxidant, glutathione, is present mainly in a reduced form (GSH), and at a far lower concentration in an oxidized form (GSSG) [103]. The efficient recycling of GSSG to GSH through the interrelated enzyme systems: glutathione reductase (E.C. 1.6.4.2) and glucose-6-phosphate-dehydrogenase (G6PD; E.C. 1.1.1.49) is a requirement for adequate antioxidant protection [103].

4.3.2. Similarities in structure, metabolism and antioxidant defence

There are several similarities between the human retina and the red blood cells (RBCs): their membrane structures (very long chain unsaturated fatty acids with a marked susceptibility to oxidative insult), their metabolism (high demands for glucose and oxygen) and their antioxidant defense mechanisms (glutathione-dependent defense systems,

Gsh-Px, SOD...etc.) show marked similarities [22,82].

Therefore, the oxidative status of the RBCs can be used as a biological index of retinal oxidative injury [80,116]. On the other hand, there is increasing evidence that the efficient glutathione recycling of the RBCs is involved in the protection against oxidative damage not only of the RBCs themselves, but also of other tissues (e.g. lung) in premature infants [19,63-65,96].

4.4. Aims of the thesis

4.4.1. Retrospective study

Based on the overview of the relevant literature we supposed that a special susceptibility of prematures with ROP does exist (e.g. some kind of trace element or vitamin deficiency causing decreased activity of antioxidant enzymes), which can be detected later, even in childhood. Based on this hypothesis a retrospective study of patients of different ages (born prematurely) suffering from different stages of ROP was carried out [71-73].

4.4.2. Prospective study

After confirming the presence of a diminished antioxidant defense capacity in our retrospective study, a prospective study was planned. We carried out a one-year study on previously oxygen-treated, low birth weight premature infants, following them from the age of 6 weeks until their first birthday. In our prospective study a close ophthalmological follow-up was provided in order to monitor the presence and the final outcome of ROP [71, 74-75].

4.4.3. Maternal examinations

Maternal oxidative stress, lifestyle (e.g. smoking, nutrition) and maternal antioxidant defense capacity are all important factors which determine how the infant will withstand the oxidative stress after birth. Therefore, our third aim was to find the maternal factors contributing to the special susceptibility of ROP patients to free radicals. This study was carried out to assess the relationship between maternal and fetal levels of different antioxidants, vitamins and trace elements [71, 74-75].

5. PATIENTS AND METHODS

5.1 Patients

5.1.1. Retrospective study

We studied 50 ROP patients of different ages (between 6 weeks and 6 years), who had been born prematurely (gestational age: 28.7±1.3 weeks; birth weight: 1210±313 g; mean±SD). 12 patients were studied at the time of their ROP screening at the Perinatal Intensive Care Unit. 14 infants and 24 children suffering from a visual handicap due to preceding ROP were studied at the ophthalmological follow-up clinic. Their biochemical data were compared with those of controls of similar age (n=56), who had also been born prematurely (gestational age: 29.2±1.5 weeks; birth weight: 1305±283g), and treated with the same therapeutic principles, including oxygen therapy, but who had recovered without bronchopulmonary dysplasia or ROP [71-73].

The first ophthalmological examinations were carried out at the postconceptional age of 34-36 weeks or at 6 weeks postnatal age, whichever came first. Indirect ophthalmoscopy was used after pupillary

dilatation to evaluate the severity of the retinal manifestation of ROP. Staging was based on the International Classification of ROP [114].

5.1.2. Prospective study

Our one-year study was carried out in 60 premature infants (gestational age: 32.8±3.1 weeks, range: 26-35 weeks); birth weights under 2000 g (mean: 1529±302 gs, range: 980-1840), admitted immediately after birth to our Perinatal Intensive Care Unit for oxygen therapy (FIO_2 >0.6 longer than 24 hrs) and intensive care. Patients in the control group were, gestational age (33.1±1.1 weeks) and weight-matched (1609±102 gs) premature infants (n= 20) who required only minimal oxygen therapy (FIO2< 0.3) [70,74-75].

Ophthalmological examination was first carried out at 34-36 weeks of postconceptional age (postnatal 6.2±1.2 weeks). The ocular investigation was repeated every 1 or 2 weeks thereafter until our patients' first birthday. Direct and indirect ophthalmoscopy was performed, following maximal pupillary dilatation in order to monitor the retinal vascularisation. In some cases, ultrasound was used as well to evaluate the severity of retinal manifestation. ROP staging was based on the International Classification of ROP [114] as follows. Prethreshold ROP stage 1: demarcation line; stage 2: ridge; stage 3: ridge and extraretinal fibrovascular proliferation. The indication for cryotherapy was threshold ROP (stage 3 ROP with 'plus disease') [76,114].

According to their ophthalmological status, patients were divided into 3 groups: 1) ROP free group (n=27; gestational age 33.7± 2.5 weeks; birth weight: 1707±306 g; mean±SD): the fundus was normal. 2) ROP suspect group (n=28, gestational age: 32.3±3.2 weeks; birth weight: 1418±273 g): because of the abnormal tortuosity of retinal vessels and the pale, avascular temporal retinal periphery, this group required special attention, they were given vitamin E (30 mg oral doses twice a week). 3)

Pretreshold ROP group (n=5; gestational age: 29.8±3.2 weeks; birth weight: 1310±330 g; $p<0.05$): stage 1-2 and 2-3 ROP, different types of junctions were seen between the vascular and avascular part of the retina from a demarcation line to a highly elevated ridge. None of our patients suffered from severe, stage 4-5 ROP.

5.1.3. Maternal examinations

At the time of the first ROP-screening (34-36 weeks postconceptional age or 6 weeks postanatal age, whichever came first), the first biochemical examination was carried out on the infants as well as on 44 mothers [70,74-75].

5.1.4. Ethical Consideration

All of our studies were approved and monitored by the Scientific Committee and the Ethical Council of the University. The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the parents. 1.5 ml whole blood was collected over EDTA from a peripheral vein from each subject.

5.2. Methods

5.2.1 Biochemical assays

Highly sensitive and specific separate determinations of GSSG and GSH+GSSG concentrations were carried out using the method described by Nemeth and Boda [65]. This is a combination of standard methods [1,34,103,117] used after validation for accurate determination, especially of GSSG values in the presence of much higher concentrations of GSH (max. 50 times), in the presence of Hb and its oxidation products, and after sample storage.

In order to avoid artifactual oxidation of GSH in blood or in assay conditions, samples for GSSG measurement (25 µl of whole blood collected over EDTA) were hemolyzed within 15 min after venipuncture with cold buffer in the presence of N-ethylmaleimide (NEM; final concentration 0.02 M in 0.01 M phosphate buffer containing 0.005 M EDTA) [117]. The reaction of NEM with GSH results in the formation of a stable complex, which prevents its possible oxidation to GSSG. After incubation with NEM for 60 min at 25 °C metaphosphoric acid (final concentration 3%, w/v) was used to precipitate proteins [103]. Supernatant was stored at -20 °C (max. 6 days) until spectrophotometric determination of GSSG. As NEM is an inhibitor of GSH-Red, it was separated from the supernatant by gel filtration with Sephadex G-10 and controlled by measuring the absorbance of NEM at 315nm [34] immediately before GSSG measurement.

For measuring the total concentration of GSH+GSSG according to Tietze [117], 25 µl of whole blood anticoagulated with EDTA was immediately hemolyzed in 2.5 ml of cold 0.01 M sodium phosphate buffer containing 5 mM EDTA, pH=7.5, and stored at -20 °C until spectrophotometric analysis (max. 6 days). The possible oxidation of GSH to GSSG catalyzed by heme compounds during storage has no influence on the total amount of GSH+GSSG.

The same standard glutathione assay mixture was used for the analysis of GSSG and GSSG+GSH concentrations. Reagents were dissolved in 0.1M sodium phosphate/0.005M EDTA buffer, pH=7.5; the final volumes were 1.0ml. Components were added in the following order: 5,5`-dithio-bis-2-nitrobenzoic acid (DTNB) (0.6 µM), GSSG (0.4 ml of supernatant after NEM removal) or GSH+GSSG (25 µl of hemolysate), glutathione reductase (10µg) and NADPH (0.2 µM). The combined actions of DTNB and NADPH in the presence of GSH-Red result in a reaction cycle the rate of which depends on the concentration of

GSH+GSSG recorded at 412 nm during the first 6 minutes.

GSH (μmol) and GSSG (nmol) concentrations were calculated by using 5-100 nmol GSSG standards and expressed with reference to Hb determined simultaneously by the cyanmethemoglobin method [65]. As the main role of GSH is the protection of Hb, it is necessary to correct the measured GSH for the actual Hb value as described by the authors of standard methods [1,103,117].

The `GSH stability test` according to Beutler [10] was described to measure the capacity of the glutathione redox system to protect Hb after an in vitro oxidative stress. Acetylphenylhydrazine (APH) (0.33 mM) was added to the whole blood sample, together with sufficient glucose. Following incubation at 37 °C for 60 min with APH, RBCs deficient in G6PD, but not the normal ones, suffer a marked fall in GSH level, accompanied by a pronounced Hb oxidation [beutler]. The proportion of oxidized derivatives of Hb (metHb and hemichrome) along with the concentration of GSH before and after APH incubation was determined by the method of Szebeni and Winterbourn [110, 124] and Tietze [117] respectively.

Selenium concentrations in erythrocytes were determined by the diaminonaphthalene fluorometric method described by the Lalonde [53]. After destroying the cell membranes of the washed erythrocytes, the Se content of the RBCs was converted into pieso-selenol complex by adding 2.3 diamino-napthalene (DAN, SIGMA). Fluorescence was measured at 360nm excitation and 520nm emission wavelengths using Hitachi spectroflourometer. Se concentrations were expressed with reference to Hb.

The concentration of free sulfhydryl groups (-SH) in the plasma was determined by a spectrophotometric method described by Koster [49]. The Ellmann`s reagent's color change was detected at 412nm.

Concentrations of vitamin E (alpha-tocopherol) and vitamin A (retinol) were measured simultaneously from 100µl plasma by HPLC method according to Catignani [15].

Ferritin concentration, as an indicator of the body's iron pool, was measured from 50µl plasma by a radioimmunassay method, using the reagent kits made by the National Institute of Isotopes.

5.2.2. Statistical analysis

Clinical data on the patients are reported as means ± standard deviations (x ± SD). Statistical analyses included both parametric (variance analysis, Tukey test and Student`s t test) and nonparametric tests (Wilcoxon rank test). When the variances between pairs of groups differed significantly from each other ($p < 0.05$ in the F-test), we used the Welch test (d probe) instead of a t test to compare the mean values. Correlations between parameters were characterized by calculation of the linear regression and correlation coefficients. The significance level for all tests was taken as $p=0.05$.

6. RESULTS

6.1. Retrospective study

6.1.1 GSH redox system

A significant increase in the GSSG/GSH as a specific sign of acute, *in vivo* oxidative stress was only seen in the patients younger than 3 months old with simultaneous active ROP, as compared both with the controls of the same age and the ROP patients of any older age [71-73].
There was a significant negative correlation between the GSH oxidation (measured either as GSSG concentration or as the ratio GSSG/GSH) and

the total Hb concentration in the ROP patients less than 3 months old. Thus, the extent of *in vivo* oxidative stress showed a correlation with the extent of anemia in these prematures with simultaneous active ROP [72].

6.1.2 APH challenge and response: GSH stability test and hemoglobin oxidation

Following an *in vitro* oxidative stress (incubation with APH) compromised antioxidant defense capacity could be detected in all ROP patients. Greater fall in GSH was seen in all the ROP patient groups as compared with the controls. The proportions of oxidized derivatives of Hb (metHb+hemichrome) were higher in each ROP group, as well. There was a highly significant negative correlation between the GSH recycling and the Hb oxidation caused by APH both in the controls and in the patients with ROP. Greater GSH depletion was accompanied by a more extensive oxidation of Hb after a calibrated *in vitro* oxidative stress [71-73].

6.1.3 Blood selenium concentration

Compared to the controls very low blood Se levels were measured in all ROP patient groups reflecting reduced GSH-Px activity in each age group [71,73].

6.2 Prospective study

6.2.1.Individual parameters: gestational age and birth weight

The gestational age and the birth weight of patients diagnosed with stage 1-3 ROP were significantly lower than those of prematures in the other two groups (ROP free and ROP suspects) [70,74-75].

6.2.2. GSH redox system

A significant increase in the GSSG/GSH ratio as a specific sign of acute oxidative stress could be seen in all of our premature patient groups. We did not find significant differences either in concentrations and ratios or in stability of GSH between the patient groups according to their ROP status. A significant negative correlation between the GSH oxidation (measured either as GSSG concentration or as the ratio GSSG/GSH) and the total Hb concentration was detectable in all 3 patient groups [70,74].

6.2.3. Concentrations of free sulfhydryl groups in the prematures and their mothers

Concentrations of plasma -SH groups measured in the ROP patient group were significantly lower than in the other prematures without ROP. By measuring the -SH groups in their mothers' plasma, the same tendency was seen: the lowest -SH group concentrations were detected in the mothers of those prematures suffering from ROP [70,74].

6.2.4. Selenium concentrations in the prematures and their mothers

Extremely low RBC Se levels were detected in the ROP patients compared to the other two premature groups without ROP. Se concentrations in their mothers showed the same relationship: the lowest levels were seen at those mothers whose babies suffered from ROP [70,74].

6.2.5. The effect of vitamin E administration on the concentrations of free sulfhydryl groups and RBC selenium levels

In the ROP-suspect group of prematures vitamin E treatment was started. 30 mg was administered orally, twice a week. The treatment was closely monitored by repeated plasma vitamin E level determinations. At the age of 6 months we could already see the positive effect of vitamin E

on the ophthalmological status, i.e. the avascular, pale periphery began to be vascularised and slow regression was seen. At this time a second biochemical investigation was carried out in our vitamin E treated ROP suspect patients. Both the concentrations of free sulfhydryl groups and Se were increased after per os vitamin E treatment [70,74].

6.2.6. Plasma concentrations of antioxidant vitamins: E and A

There was a marked decrease in plasma vitamin E concentrations in the patient group diagnosed with ROP, as compared both with the other two premature groups and the vitamin E range. However, plasma vitamin E levels in the ROP free premature groups were also slightly below the lowest level of the physiological range, reflecting a general vitamin E deficiency of the preterm neonate.

Maternal vitamin E concentrations showed a very similar tendency: the lowest vitamin E levels were detected in those mothers whose preterm babies suffered from active ROP, concentrations of plasma vitamin E were slightly higher in the other two mother groups. On the other hand, plasma vitamin A concentrations were only lower in our ROP patients, as compared to the other, ROP-free groups and the physiological values. Plasma vitamin A levels of the ROP-free prematures and all of the mothers, regardless of the ophthalmolgical status of their babies, were in the physiological range [75].

6.2.7. Plasma ferritin levels

Plasma ferritin concentration, which is an indicator of the body's iron pool was significantly higher in the ROP patient group, as compared with the other ROP free prematures. Surprisingly, maternal ferritin levels showed the opposite tendency: the lowest plasma ferritin levels were detected in the mothers of the ROP patient group, but ferritin levels in the other two mother groups were still lower than that of their babies [75].

7. DISCUSSION

ROP is a disease of immature retinal vessels, an ocular manifestation of 'Oxygen Radical Disease of prematurity'[106]. The retina is one of the organs most directly exposed to high oxygen tension and the endothelial cells of its microvessels are highly susceptible to free oxygen radical injury. There is evidence that light exposure -even standard levels of nursery light- may also play a role in the pathogenesis of ROP by photosensitization.[30-31]. High levels of a hematogenous photosensitizer, protopophyrin IX, a photoactive compound that produces reactive oxygen species when exposed to light, was detected in preterm neonates as well [13]. The unique sensitivity of the retina to oxidative injury is probably due to its high content of highly unsaturated fatty acids, which are susceptible to the reactive oxygen species generated in the simultaneous presence of light and a high concentration of oxygen. Retinal hemorrhage is a frequent finding in all newborn infants shortly after birth [26]. Prematures, however, are even more prone to develop retinal oxidative injury: besides undergoing a hyperoxic insult on moving from the relatively hypoxic intrauterine environment to room air, they usually require special therapeutic interventions such as administration of high concentrations of oxygen, phototherapy, blood transfusions etc.

The similarity between the human retina and the RBCs concerning their membrane structure (very long chain unsaturated fatty acids with a marked susceptibility to oxidative insult), their metabolism (high demands for glucose and oxygen) and their antioxidant defence mechanisms [22,82] lead us to study the RBC as a model to demonstrate and follow the oxidative status of the RBCs as a biological index of retinal oxidative injury [80,116] in our ROP patients. Furthermore, there is increasing evidence that the efficient glutathione recycling of the RBCs is involved in the protection against oxidative damage not only of the

RBCs themselves, but also of other tissues (e.g. lung) in premature infants [19, 63-65,96].

Based on the fact, that low gestational age and the frequency of blood transfusions are the only parameters independently associated both with the occurrence of ROP and with its severity [20,37,45,89] we hypothetized that, as immaturity and anemia and are common pathological signs of the disease, the RBCs of ROP patients might display a special susceptibility to free oxygen radicals. On the hypothesis of an imbalance between the production of free oxygen radicals and the antioxidant protective capacity of the RBCs, a retrospective study of ROP patients of different ages was carried out first. The aim of our pilot study was to acquire information on the glutathione antioxidant status of the RBCs in ROP patients in different stages of the illness, and to assess the GSH recycling capacity of the RBCs providing the antioxidant protection of intracellular Hb after an *in vitro o*xidative insult. Intracellular Hb is protected against oxidative injury by the antioxidant mechanisms of the RBCs`, mainly by the glutathione redox system. Intracellular GSH plays a key role in the protection of the RBCs not only by protecting Hb from oxidation [124], but also as a scavenger of free hemin [100]. Hemin with its hydrophobic nature, is a more potent catalyst of lipid peroxidation in RBC membrane than nonheme irons, and plays a significant role in the acceleration of RBC destruction [18].

In the control patients of our first, retrospective study, the GSH concentration was highest in the youngest patient group in accordance with the results of previous studies, where the RBCs of healthy newborn babies (both term and preterm) displayed a higher GSH content [19,96] and a more efficient GSSG recycling after an in vitro oxidative insult compared with adults [19].

However, reduced GSH levels together with increased GSSG concentrations, resulting in high GSSG/GSH ratio, were present only in

prematures less than 3 months old simultaneously with the clinical signs of the acute phase of ROP. This finding suggested that the GSSG/GSH was a reliable parameter of an oxidative injury present in the most immature and most susceptible babies with active ROP.

We found a significant negative correlation between the GSH oxidation and the Hb concentration in the prematures with active ROP. This fact supported our hypothesis that the permanent oxidative stress contributed to the development of anemia due to oxidative hemolysis through a GSH-depleting mechanism. The possibility of a defective GSH synthesis as a cause of the reduced GSH concentration in the newborn period has recently been excluded [56].

In another recent study on prematures with chronic lung disease and 31% ROP incidence whose gestational age was similar to that in our group, a significantly reduced GSH concentration was similarly measured in the RBCs on day 28 as compared with the controls [60]. The prolonged oxidative stress, together with the suspected loss of riboflavin, a coenzyme for glutathione reductase, were suggested to result in the impaired GSH recycling [60]. Evidence from previous studies indicated that the GSH recycling capacity was more important in resisting oxygen toxicity than the GSH concentration itself [19,60,108].

A method of calibrated *in vitro* oxidative challenge with APH was evolved by *Beutler* [10] in order to recognize patients with defective G6PD activity resulting in insufficient GSH recycling. Subjects with reduced recycling capacity are in danger of oxidative hemolysis after the administration of oxidant drugs or during infections [5,9,125]. However, in our retrospective study we proved the presence of defective GSH recycling not only in active ROP patients but also in infants and children with preceding ROP as compared with that of the controls. On the basis our findings, we suggest that a defective GSH recycling capacity of RBCs could be a factor predisposing to oxidative hemolysis and ROP.

Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (Cat) are the most important enzymes in the defence system against free oxygen radicals. SOD, of which several different types have been described [58], protects against the superoxide radical and catalyses its transformation from superoxide to hydrogen peroxide. Cat and GSH-Px are necessary for rapid conversion of hyrogen peroxide to molecular oxygen and water. Assessing the operation of the GSH-Px system *in vivo*, an increased pathway activity has been observed upon exposing isolated perfused rat lung, ox retina and human RBCs to elevated O_2 [35]. GSH-Px (E.C. 1.11.1.9) consists of four protein subunits, each of which contains one atom of the element selenium (Se) at its active site. Thus, Se is an integral part of GSH-Px and therefore has a key role in the GSH protection against oxidative damage such as hemoglobin oxidation and the RBC hemolysis [87,90]. Linear relationship has been reported between the RBC's GSH-Px activity and Se concentrations in several studies [83,87]. It has been suggested later by another research group that RBCs of Se deficient individuals not only lack GSH-Px activity but also lack GSH-Px protein, thus Se controls the synthesis of the GSH-Px. [111-112]. This strong association led us to study Se status in order to assess the GSH-Px activity in our patient groups. According to the literature, to determine a long-term index of Se status, the RBC's Se concentration is a more reliable measurement since plasma Se concentration is influenced more readily by changes in the actual Se intake [83]. Therefore we determined the RBC Se concentrations in our retrospective study. The very low blood Se levels measured by us in all ROP patient groups reflected reduced GSH-Px activity in each age group [71,73], as compared to the age matched controls. Surprisingly, Se status of our healthy controls was low as well in comparison to the Western-European normal Se values, which were determined with the same method [115]. This finding

suggested that Se depletion in Hungary might be an important predisposing factor to ROP.

In order to further investigate the possible association between low selenium status and development of ROP, a planned, prospective study was warranted, which provides stronger evidence for causation than a retrospective observation. According to our results in the prospective study, extremely low RBC Se levels were detected in the ROP patients compared to the other two premature groups without ROP. Se concentrations in their mothers showed the same relationship: the lowest levels were seen at those mothers whose babies suffered from ROP. The maternal Se deficiency observed may lead to diminished retinal GSH-Px activity in the fetal retina. GSH-Px, the enzyme which provides the only antioxidant protection of human retinal precursor ('spindle') cells before the 28th gestational week, is already detectable in retinas of human embryos weighing more than 160g. [39,41,50,62]. For the proper GSH-Px synthesis and enzymatic activity Se is required. To obtain and maintain an adequate fetal Se level is extremely important because before the 28th gestational week retinal photoreceptors are not secreting the lipid soluble interstitial retinol binding protein (IRBP), a carrier protein for vitamin E, and therefore spindle cells cannot receive any protection from vitamin E supplementation [40, 42-43]. Thus, the younger the infant the more attention should be paid to an adequate supply of water soluble Se, which doesn't require the presence of any carrier protein to protect the spindle cells, in order to create favorable antioxidant retinal environment. According to our results, Se levels in prematures suffering from ROP was particularly low as a result of the diminished maternal supply. The placenta has some barrier function towards Se (3:2) but depending on the maternal level Se does get into the fetal circulation and has positive influence on the fetal antioxidant defence [41].

Several dietary studies have shown that, by world standards, the selenium status of the adult population of Hungary is low [2,11,109]. Close correlation was found between the Se concentration of wheat and human blood [2]. Se concentration of the soil varies widely between geographical areas. The same Hungarian research group reported that the Hungarian rock-soil systems, especially the acid igneous rocks and widely distributed loess and sand formations, where most of the agricultural products are grown, are very low in Se [32]. Our data and results from the relevant literature suggest that Se deficiency as a factor contributing to latent antioxidant deficiency in the Hungarian population plays an important role in the pathogenesis of ROP. Based on this observation, we support the idea that a single dose 'antioxidant cocktail' (Se+vitamin E) given to the high-risk mothers (advanced age, preeclampsia, toxemia, smoking, pregnancy-induced hypertension, etc.) before delivery - as suggested in the literature - might be useful in the prevention of ROP [41]. On the other hand, proper nutrition before and during pregnancy may protect not only the developing fetus from oxidative damage, but also influence the course of pregnancy. In a recent study very low whole blood Se levels and GSH-Px activities were reported in women with miscarriage occurring in the first and second trimesters [126]. In order to improve the Se status of the population Hungarian authors recommend the consumption of bread produced with Se enriched yeast which, according to their results, improve the Se status as soon as within two weeks of supplementation in healthy adults [88]. Another Hungarian group reported the beneficial effect of enteral supplementation of a yeast-selenium on the serum selenium status of premature infants during the first 14 days of life [11].

Apart from the glutathione redox system, free sulfhydryl groups (-SH) in the plasma are significant free radical scavengers. Free –SH groups respond immediately to the oxidative damage, therefore their

concentration reflects the actual antioxidant status of the body. Free -SH groups are mainly provided by the consumption of sulphur-containing aminoacids, such as cystein, methionin, etc. We found that concentrations of free -SH groups were significantly lower in the plasma both of our ROP patients and in their mothers. These findings: –SH depletion and close correlation between the maternal and fetal levels stress again the importance of proper nutrition, especially protein consumption during pregnancy. Based on our data, we suggest, that dietary supplementation with sulphur-containing aminoacids would improve the antioxidant capacity of both the mother and the infant.

Vitamin E is a lipid soluble, chain breaking antioxidant in the plasma. As a scavenger of peroxyl radicals, it is one of the most significant inhibitors of the free radical chain reaction of lipid peroxidation. Low vitamin E status of premature infants was already recognized in 1949 and it was suggested that vitamin E-deficiency might play a role in the etiology of ROP [67]. However, when the role of oxygen was identified in 1953 [14] vitamin E and its possible relation to ROP were forgotten. Support for the vitamin E-deficiency hypothesis came later from ultrastructural investigations [42,51,66]. According to *Hittner*'s observations when retinas of premature infants are exposed to relative hyperoxia, the elevated oxygen tension induces the formation of gap junctions between the adjacent endothelial precursor spindle cells. The appearance of these gap junctions is the first morphologic evidence of oxygen toxicity, resulting in cessation of spindle cell migration and canalisation. The coupling of spindle cells changes these cells from endothelial precursors to sites of synthesis and release of angiogenic factors, which trigger the induction of ROP by abnormal neovascularisation from existing vessels [50]. According to this theory, vitamin E supplementation suppresses gap junction formation by

protecting the spindle cells from the oxidative damage, and thus, reduces the severity of ROP [42].

In our study we evaluated the vitamin E status in our patient and mother groups. The marked decrease of plasma vitamin E concentrations reflected vitamin E deficiency both in our ROP patients and in their mothers. However, plasma concentrations of this antioxidant vitamin were always higher in the mothers. According to the literature vitamin E crosses the placenta only in a 4:1 ratio from maternal to infant blood [42,66]. In our study the ratio of maternal and fetal plasma vitamin E concentration was consistent with this placental barrier function. Other studies indicated that maternal and fetal vitamin E storage occurs only late in gestation, mostly towards the end of the third trimester [16,42-43] thus, preterm babies have a higher incidence of vitamin E deficiency compared to term babies. In our mother groups, plasma vitamin E levels were very low, well under the lowest level of the physiological range, reflecting a general vitamin E deficiency already in the mothers. Although there are natural sources of vitamin E (such as vegetable oils, nuts, grains, green leafy vegetables...etc) we recommend that vitamin E supplementation during pregnancy should be considered in Hungary.

On the other hand, there is still a controversy regarding vitamin E supplementation in preterm infants [17,40,43,46,77-78]. Numerous clinical trials were performed to test the efficacy and safety of vitamin E in suppressing the development of ROP [17,40,43,46,77-78]. This controversy on vitamin E treatment is partly due to the differences in experimental design, the dose of vitamin E and the route of administration applied in the different studies. The initial, rapid intravenous administration resulted in high plasma peak levels (5mg/dl), causing an increased incidence of life threatening side-effects, such as retinal and intraventricular haemorrhages [17,78], sepsis and necrotising enterocolitis. [46,91]. A slower rise in plasma vitamin E concentration

could be achieved by intramuscular administration, but local tissue necrosis and secondary calcification may follow. [93]. According to *Hittner*'s observation -based on animal studies- the oral route of administration, which results in slower rise in plasma concentration, is optimal for retinal vitamin E uptake [40-41]. According to the recommendation of the *American Academy of Pediatrics*, the optimal plasma level of vitamin E in preterm infants is 0.5-2.0mg/dl [3]. Vitamin E supplementation should be initiated in the first hours of life and continued until retinal vascularistion is completed as determined by an indirect ophthalmoscopic examination. Further support for oral vitamin E supplementation comes from a study involving post-mortem measurement of vitamin E levels in human, premature retinas [66]. Baseline vitamin E levels were found to be extremely low. When compared retinal vitamin E levels in vitamin E supplemented and unsupplemented infants, a significant difference in retinal vitamin E levels was found, but only in infants older than 28 weeks gestational age. This finding suggests an insufficient retinal in uptake of vitamin E in the smallest and illest infants as a result of the insufficient amounts of IRBP in the subretinal space [40,42-43]. Thus, vitamin E provides incomplete, age dependent antioxidant protection, which shifted our focus to the already endogenous Se dependent GSH-Px and the beneficial, non-age dependent effect of Se supplementation. There is, however, a synergism between Se and vitamin E: to a considerable extent, the effects of Se deficiency can be overcome by giving excess doses of vitamin E and vice versa [35]. This synergism provides an explanation to our results in the ROP suspect group: both the ophthalmological and Se status improved after per os vitamin E treatment.

Vitamin A, like vitamin E, is an important inhibitor of the free radical chain reaction of lipid peroxidation. *In vitro* studies have shown that various forms of vitamin A can exert antioxidant effects that are more potent than those provided by vitamin E [94]. Adequate vitamin A intake

is essential for successful pregnancy outcome, since normal embryonic development, both cell growth and differentiation depend on the proper maternal supply. In our study, maternal vitamin A levels were in the normal physiological range, reflecting no vitamin A deficiency. There is a controversy in the literature regarding vitamin A supplementation during pregnancy. According to the most recent study on nutrition, one of the four most important forms of malnutrition worldwide is vitamin A deficiency [104]. Subclinical vitamin A deficiency is affects at least 251 million people, including pregnant women. Several clinical trials have shown that supervised vitamin A supplementation in these cases had positive effects on the outcome of pregnancy [4]. Beneficial effects of vitamin A supplementation on pregnancy- induced hypertension have been reported as well [69].

Another research group raised the issue of the effect of maternal smoking on the plasma levels of vitamin A and E: significantly lower levels of vitamin A and E were measured in the placentas of those mothers, who smoked during pregnancy. They suggest supervised antioxidant vitamin supplementation in order to protect newborns from the potentially harmful effect of maternal smoking [52].

On the other hand, excessive dietary intake of vitamin A (e.g. multivitamins) has been associated with teratogenicity in humans, although less than 20 cases were reported over 30 years [4]. There are also several retinoids (vitamin A derivates) that are effective drugs for therapy of skin diseases and some types of cancer, but the application of this class of compounds is forbidden during pregnancy due to their teratogenic activity. Fetal malformations including craniofacial, cardiac, thymic and central nervous system abnormalities were observed as a sequel of isotreninoin treatment (prescribed for severe cystic acne) during pregnancy. [54,119].

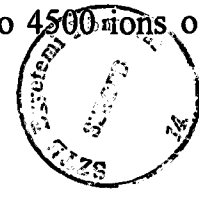
An animal study, which was performed to provide risk assessment to obtain information safe dosage of vitamin A during human pregnancy recommends a dose range of approximately 25000 to 37000 IU/day [36]. Nutritional studies suggest that increasing the amount of Ω3fatty acid in the diet alone, by eating oil-rich fish either fresh or canned as often as twice or three times a week provides optimal protein, vitamin A and D supply during pregnancy [88]. The most recent guideline for vitamin A supplementation during pregnancy suggests that initiation of vitamin A therapy should be carefully examined in each case according to the risk-benefit ratio. The final decision should take into account the estimated vitamin A status of the woman, the availability of vitamin A-rich foods in her diet, and whether her supplementation can be supervised [4].

On the other hand, in spite of the physiological plasma concentrations measured in our mother groups, vitamin A status of our ROP patients was low. Our result was similar to the observation of other authors [21,61,94,118]. They suggest that premature infants have subnormal or low serum retinol concentrations, which have been associated with increased risk of developing chronic lung disease and sepsis. Randomized, controlled trials were undertaken to asses whether vitamin A supplementation beyond that routinely given can reduce the incidence of chronic lung disease [21]. According to their results, supplementing very low birth weight infants with vitamin A is associated with reduction in oxygen requirement among survivors at 36 weeks post-conceptional age. Two studies reported even a reduced incidence of ROP in vitamin A supplemented infants [21]. However, in the most recent systematic review article on vitamin A supplementation *Darlow* suggests that further clinical studies should assess the benefits, safety and acceptability, the optimal dose and route of administration of vitamin A to the high-risk preterm infants [21].

Iron is essential in the human body for the synthesis of a huge range of enzymes and other proteins involved in respiration, oxygen (O_2) transport and several redox reactions. Yet iron is potentially dangerous by having the ability to undergo quick valence changes, which enables this metal to be a powerful catalyst in the production of reactive oxygen species by facilitating the formation of the highly reactive hydroxyl radical (OH^-) from superoxide (O_2^-). [35]. *Sullivan* [106] proposed that iron plays an important role in the pathogenesis of the 'Oxygen Radical Disease of Prematurity'. According to his hypothesis, premature infants are abnormally susceptible to oxygen radical injury because of developmentally low levels of the iron-associated antioxidant proteins (such as the iron-oxidizing ceruloplasmin and iron-binding transferrin) combined with postnatal changes of iron-metabolism.

Birth is a point of transition to the higher oxygen concentration of the extrauterine environment, which is associated with virtual cessation of red blood cell formation. This adjustment has major effect on the neonatal iron metabolism since red blood cell lysis continues or – in cases of hemolysis – increases. Thus, there is an increase in the amount of iron that must be stored, especially in those prematures who require whole blood transfusions or iron supplementation. As a consequence of the developmentally low transferrin concentrations in premature infants, very high transferrin saturations (100%) develop in a very short time. Thus, the plasmas of prematures have no more iron-binding capacity, leading to the appearance of 'free' iron. The presence of this potentially harmful, bleomycin-detectable free iron in the plasma of premature infants has been first reported by *Evans* [25]. Free iron enhances the risk of oxidative injury, especially in premature infants who have greater susceptibility due to their less effective antioxidant systems.

Most intracellular iron is stored in ferritin, which consists of a protein shell and an iron core that can hold up to 4500 ions of iron per



molecule. Iron deposited in ferritin is in an insoluble oxidized from, providing a harmless iron pool for the human body. Plasma ferritin is considered the best measure of total body iron [35]. We therefore measured plasma ferritin concentrations in order to investigate the association between the mother's and preterm infant's iron status. According to our results, marked decrease in plasma ferritin concentration was detectable in all mothers, reflecting iron deficiency in all groups of mothers. The lowest maternal ferritin concentrations, however, were measured in mothers of ROP patients.

In the literature, recent concerns have been voiced about harmful effects of iron supplementation during pregnancy. Questions were raised about the efficacy of iron supplementation, about the side effects of iron treatment, including its potential for oxidation of lipids and DNA [55,92]. It is suggested that the rationale of routine iron supplementation during pregnancy needs to be re-examined [55].

High plasma ferritin concentrations were observed, however, in our ROP patients, reflecting an iron overload in this group. Similar volumes of plasma ferritin levels were observed by *Sullivan*, by detecting ferritin levels in prematures that were more than 10 times the upper limit of normal for adults [107]. In our study, in spite of the mothers' obvious iron deficiency, there was a marked iron overload in the premature infants. In spite of having high plasma ferritin values, these premature infants usually suffer from early anaemia. Thus, they require special therapeutic interventions such as blood transfusions, iron supplementation, etc. The level of free iron immediately before and after blood transfusion in the plasma of preterm infants, suffering from ROP and/or chronic lung disease has been investigated recently [38]. According to the results of this study, plasma levels of free iron were significantly increased after blood transfusion. This finding was specific to preterm infants and was not observed in full term infants after blood transfusion. This finding

suggests that the available iron overloads the premature infant's developmentally limited iron-handling capacity, leading to the oxygen radical disease of prematurity. Several studies were carried out to find a relationship between 'iron-associated risk factors' (e.g. red blood cell transfusion protocol) and the predominant site of the oxygen radical injury (retinal, lung, etc) [24,37,45]. Logistic regression analysis has demonstrated that apart from the gestational age, the frequency of blood transfusions is the only parameter independently associated both with the occurrence of ROP and with its severity [20, 37, 45, 89]. Another prospective, observational study found close correlation between the iron status and the volume of transfused blood and an increased risk for ROP [45].

A recent, retrospective study examined the role of anaemia in the development of ROP. According to their results, infants who remained severely anemic for longer periods of time developed milder ROP, than less anaemic infants who obviously received more transfusions [24]. Thus, iron deficiency may also be protective in certain types of oxygen radical mediated injuries.

In order to prevent iron overload by reducing the need for blood transfusions during the chronic anemia of prematurity, several studies were carried out to assess the efficacy of recombinant human erythropoeitin (EPO) [59,97]. Administration of EPO mobilizes iron from the plasma, decreases iron-saturation of transferrin, increases the number of reticulocytes and increases erythropoiesis. These studies are equivocal in suggesting the administration of recombinant human EPO; at a weekly dose of 600 U/kg because it moderates the course of chronic anemia, appears safe and leads in most cases to the reduction of transfusion requirement in preterm infants. Based on the fact that the frequency of blood transfusions is independently associated both with the occurrence of ROP and with its severity [20, 37, 45, 89], we propose that the

administration of EPO, by reducing the need for transfusions might be useful in the prevention of ROP.

8. CONCLUSION

The aim of our work was to investigate the role of oxidative stress in the pathogenesis of ROP. In order to detect the presence of oxidative stress and find biochemical markers in ROP patients, which explain their special susceptibility to oxidative injury, retrospective and prospective studies were carried out. Our results led us to the following conclusions.

1. The RBC's GSSG/GSH ratio is an adequate parameter to indicate the *in vivo* oxidative stress in the acute phase of ROP. The signs of an acute oxidative stress could only be seen in the 3-months-old or younger patients, supporting Saugstad's hypothesis [90] that ROP is a part (as the retinal manifestation) of the 'Oxygen Radical Disease of Prematurity'.

2. In response an *in vitro* oxidative stress compromised antioxidant defense capacity could be detected in all ROP patients even later in childhood suggesting that ROP probably may develops in those premature infants who have reduced antioxidant defense capacity (either genetic or acquired).

3. Selenium is not only an integral part of GSH-Px enzyme, but also acts as an antioxidant itself by playing a role in the regeneration of vitamin E. The very low selenium levels measured in all our ROP patients both in our retrospective and prospective studies suggest that selenium

depletion in Hungary might play an important role in the pathogenesis of ROP.

4. The fact that during our screening for ROP in our prospective study we could only diagnose 5 cases of mild ROP among 60 high-risk premature infants, leads us to the conclusion that the management of preterm infants at the Perinatal Intensive Care Unit of our University and the secondary treatment at Children's Hospital is very careful.

5. We found that gestational ages and the birth weights of patients diagnosed with ROP were significantly lower, which confirms the importance of immaturity as one of the most significant risk factors for ROP.

6. The close correlation found between the low serum levels of –SH and Se of mothers and babies suggest that dietary supplementation with sulfur containing aminoacids (e.g.methionin, cystein) and Se during pregnancy would improve the antioxidant capacity of premature infants.

7. Considering the fact that marked decrease of plasma vitamin E concentrations were determined both in our ROP patients and in their mothers, we can conclude that an 'antioxidant cocktail' suggested in literature (containing Se and vitamin E) given to the high-risk mothers (advanced age, smoking, toxemia, pregnancy-induced hypertension, etc.) before delivery might be useful in prevention of ROP.

8. According to our results, *per os* vitamin E supplementation seems to have a positive effect on preventing the progression of ROP. Because

of the potential harmful side effects, however, vitamin E administration must be closely monitored by repeated measurements of plasma vitamin E levels.

9. The fact, that in spite of iron deficiency in their mothers, fully saturated iron pools were detectable in the preterm infants, leads us to the conclusion that during the first 6 weeks of life iron overload might be one of the main factors leading to ROP. Therefore we suggest that in the clinical management of premature infants all interventions that may result in excessive iron load (transfusions, iron supplementation, etc.) may contribute to the development of ROP. We propose that the administration of EPO as a treatment option for chronic anemia might be useful in the prevention of ROP by reducing the need for transfusions.

9. MESSAGE

Although the proportion of premature infants who become blind of ROP has decreased, the number of children with visual disability is still high. As an ophthalmologist I have seen retinas getting detached despite the appropriate treatment resulting blindness and phthisis (disfiguring shrinkage) of the affected eye. Even effective and appropriate ophthalmological interventions have side effects: decreased peripheral vision from cryotherapy, transient or permanent cataracts from vitreoretinal surgery, etc. Fortunately, ROP has a tendency for spontaneous regression, but even in these cases infants are predisposed to amblyopia, strabismus and other disorders requiring long-lasting ophthalmological treatment. For this reason, the only solution for retinopathy of prematurity would be prevention. In order to be able to

prevent this disabling neovascular retinopathy we have to be determined in the continuing search for possibly preventable environmental and individual factors leading to ROP. According the *Lancet Editorial*: 'The focus of preventive attention should probably shift away from neonatology towards obstetrics and public health, since it is only by developing effective methods of avoiding premature delivery and extreme low birthweight that we are likely to bring the unexpectedly longrunning saga of ROP to a close '[84].

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