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

Summary of Ph.D. thesis

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INTRODUCTION

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 (more than 50 recognized risk factors have been reported) 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 production of free radicals overwhelms body's natural antioxidant capacity, resulting in oxidative damage of the susceptible tissues.

AIM OF THE STUDY

On the hypothesis that a special susceptibility of premature infants with ROP does exist (e.g. some kind of trace element or vitamin deficiency causing decreased activity of antioxidant enzymes), retrospective and prospective studies (including maternal biochemical examinations as well) were carried out. The similarity between the erythrocyte (RBC) membrane and the immature retina in their 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 defense mechanisms (glutathione-dependent defense systems, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) etc.) led us to study RBC as indicator of the oxidative status of our 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. Biochemical examinations were carried out on 12 preterm infants at the time of their first ROP screening at the Perinatal Intensive Care Unit, as well as on 14 infants and 24 children suffering from a visual handicap due to preceding ROP at the ophthalmological follow-up clinic.

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. The first ophthalmological examination (ROP screening) was 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. 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.

Maternal examinations

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. The biochemical examination was carried out on 44 mothers at the time of the first ROP-screening.

METHODS

The ratio of oxidized/reduced glutathione (GSSG/GSH) was used as a parameter of *in vivo* oxidative stress. Highly sensitive and specific separate determinations of GSSG and GSH+GSSG concentrations were carried out using the enzymatic recycling method described by *Tietze*. The

degree of GSH stability after an *in vitro* oxidative insult (a method evolved by *Beutler)* and the presence and amount of hemoglobin (Hb) oxidation products (methemoglobin, hemichrome) reflected the antioxidant protective capacity of RBCs. Selenium (Se) concentrations in erythrocytes were determined by the diaminonaphthalene fluorometric method according to *Lalonde*. Plasma concentrations of free sulphydryl groups (-SH) were determined by a spectrophotometric method described by *Koster*. Plasma concentrations of vitamin E (alpha-tocopherol) and vitamin A (retinol) were measured simultaneously by the HPLC method according to *Catignani.* Plasma ferritin concentration, as an indicator of the body's iron pool, was measured by a radioimmunassay method.

RESULTS AND DISCUSSION

Retrospective study

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. 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 (due to oxidative hemolysis) in these prematures with simultaneous active ROP.

In vitro oxidative insult and response: GSH stability test and hemoglobin oxidation

Following an *in vitro* oxidative stress 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. Thus, greater GSH depletion was accompanied by a more extensive oxidation of Hb after a calibrated *in vitro* oxidative stress, reflecting defective GSH recycling not only in active ROP patients but also in infants and children with preceding ROP.

Blood selenium concentration

Very low blood Se levels were measured in all ROP patient groups. Linear relationship has been reported between the RBC's GSH-Px activity and Se concentrations in several studies. Thus, the very low blood Se levels measured by us reflected reduced GSH-Px activity in each age group. Surprisingly, Se status of our healthy controls was low as well in comparison to the Western-European normal Se values, reflecting a general Se deficiency of the Hungarian population.

Prospective study

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, which stresses the importance of immaturity in the pathogenesis of ROP.

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.

Concentrations of free sulfhydryl groups in the prematures and their mothers

Concentration of plasma –SH groups, which is a reliable indicator of the actual antioxidant status of the body, was significantly lower in both of our ROP patients and in their mothers.

Selenium concentrations in the prematures and their mothers

Extremely low RBC Se levels were detected both in the ROP patients and in their mothers. The maternal Se deficiency observed by us 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 28^{th} gestational week, is already detectable in retinas of human embryos weighing more than 160g. For the proper GSH-Px synthesis and enzymatic activity Se is required, which depends on the 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 defense.

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

In the ROP-suspect group of prematures oral vitamin E treatment was started, which 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 biochemical investigation was carried out. Both the concentrations of free sulfhydryl groups and Se were increased after *per os* vitamin E treatment. The well-known synergism between vitamin E and Se provides the explanation to this result.

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. 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. On the other hand, plasma vitamin A concentrations were only lower in those premature infants suffering from ROP.

Plasma ferritin levels

Plasma ferritin concentration, which is an indicator of the body's iron pool, was significantly higher in our ROP patients. Surprisingly, maternal ferritin levels showed the opposite tendency: the lowest plasma ferritin levels were detected in the mothers of the ROP patients, but ferritin levels in the other two mother groups were still lower than that of their babies. Thus, in spite of the mothers' obvious iron deficiency, fully saturated iron pool fully saturated iron pools were detectable in the preterm infants.

CONCLUSIONS

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 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 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 erythropoeitin (EPO) as a treatment option for chronic anemia might be useful in the prevention of ROP by reducing the need for transfusions.

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 a pediatric 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 '.

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