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ERYTHROCYTE DAMAGE IN NEWBORN BABIES CAUSED BY HYPERBILIRUBINAEMIA AND HYPOXIA

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Erythrocyte damage of newborn babies suffering from hyperbilirubinaemia and hypoxia was compared with a control group. In the cases of serum bilirubin level higher than physiological icterus lipid peroxidation of erythrocytes decreased probably due to the antioxidant effect of bilirubin. Moreover, an increase in potassium and protein outflow from patients' red blood cells was observed indicating a membrane damage both in hyperbilirubinaemic and hypoxic groups. Superoxide dismutase activity of serum and erythrocytes did not show significant difference in patients compared with healthy newborns. However, the low serum coeruloplasmin level in the hypoxic group and the low serum transferrin level of babies both with hypoxia and hyperbilirubinaemia suggest an insufficient antioxidant defence against free radicals.

INTRODUCTION

The role of hyperbilirubinaemia is a very thoroughly studied area of neonatology. However, we could find only a few data about the effect of bilirubin on erythrocytes /1,3,10/. During our previous study on the antioxidant system of premature babies /6/ arose the question: what is the influence of hyperbilirubinaemia and hypoxia on erythrocytes. In order to answer this question we measured lipid peroxidation (LPO), K^+ outflow as well as protein release in red blood cell suspension of healthy, hyperbilirubinaemic and hypoxic newborns. In addition, the level of three components of serum antioxidant system - superoxide dismutase, coeruloplasmin and transferrin - was investigated in all the three groups.

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MATERIALS AND METHODS

Patients

Twenty-five healthy newborns with birth weight 3378 ± 507 g (SD) and gest. age 38.8 ± 1.8 weeks (SD) were enrolled into the control group (I.). Birth weight of 30 hyperbilirubinaemic newborns (II.) was 1805 ± 655 g (SD), their gestational age was 31.8 ± 3.4 weeks (SD), and their serum bilirubin level (145-320 μm/l) was higher than that of physiological icterus.

The birth weight of 10 newborns (III.) - suffering from hypoxia - was 2010 ± 1244 g (SD), their gestational age was 33.2 ± 4.0 weeks. Their blood gas parameters during the last 24 hours before drawing blood were the following: oxygen saturation was below 85 %, pCO_2 was higher than 40 Hgmm (AVL 995 Automatic Blood Gas Analyser). All the patients received usual perinatal medical treatment in our perinatal intensive care unit.

Lipid peroxidation (LPO) in erythrocytes

Freshly drawn heparinised blood was washed three times with 10 volume of 0.15 M NaCl solution centrifuged at $0^{o}C$ for 10 min, with 2500 r.p.m. The parameters a.-c. (Table I) are referred to the hematocrit of this 3-times washed erythrocyte suspension. One ml from 10 % hemolysate of this suspension was left to stand for 2 hours at $0^{o}C$. After adding 1 ml of 15 % trichloroacetic acid (TCA) to haemolysate malon-dialdehyde - an end-product of lipid peroxidation - was measured /4,15/ with 0.5 ml 1 % thiobarbituric acid (in 0.05 M NaOH) at $100^{o}C$. After having determined the differences in absorbance between 532 and 600 nm, the MDA concentration was calculated using the molar extinction coefficient given by Sinnhuber and Yu /12/ as 1.56×10^{5}.

In vitro inhibition of lipid peroxidation (LPO) in erythrocytes

To the three times washed healthy erythrocyte suspension 25-250 μm/l bilirubin (Reanal) was added in 0.15 M NaCl solution (containing 20 % ethanol to gain better solubility). The mixture was left to stand for 1 hour at $0^{o}C$ and erythrocytes were centrifuged at $0^{o}C$ (10 min, 2500 r.p.m.). (The treatment of erythrocytes with 0.15 M NaCl - containing 20 % ethanol - did not cause any considerable inhibition effect in LPO). Measurement of LPO in these erythrocytes was carried out as described above.

Protein and K^{+}- outflow from erythrocytes

0.2 ml 0.15 M NaCl solution was added to 0.2 ml of three times washed erythrocyte suspension. The protein content of the supernatant was estimated at t=0 time and after 2 hours incubation at $0^{o}C$ according to Lowry's method /7,9/. Concentration of K^{+} in the same supernatant samples was determined with a Radelkis OP-266/1 type biological Alkali Microanalyser. Superoxide dismutase activity in serum and erythrocytes was determined with method described by Misra and Fridovich /8/.

Serum transferrin and coeruloplasmin level was measured with a Beckman II-type Immunochemistry Analyser.

TABLE I

Newborn babies	I. Healthy (25)	II. Hyperbilirubinaemic (30)	III. Hypoxic (10)
Birth weight (g)	3378 ± 507 (SD)	1805 ± 655 (SD)	2010 ± 1244 (SD)
gest. age (week)	38.8 ± 1.8 (SD)	31.8 ± 3.4 (SD)	33.2 ± 4.0 (SD)
a. LPO in erythrocytes (μM/l)	1.99 ± 0.26	0.91 ± 0.33	1.61 ± 0.31 (n.s.)
b. Protein outflow from erythrocytes (mg/ml)	0.06 ± 0.02	0.132 ± 0.022	0.138 ± 0.04
c. K^+ outflow from erythrocytes (mM/l)	0.81 ± 0.12	1.13 ± 0.17	2.7 ± 0.70
Serum antioxidants:			
coeruloplasmin in se. (mg/l)	180 - 450	273 ± 125 (n.s.)	133.8 ± 67.3
transferrin in se. (mg/l)	2040 - 3600	1996 ± 655	2114 ± 574

n.s. no significant difference
LPO lipid peroxidation

RESULTS

To determine the effect of hyperbilirubinaemia and hypoxia on erythrocytes of newborns we investigated the following parameters in the case of 25 healthy (group I), 30 hyperbilirubinaemic (group II) and 10 hypoxic newborn babies (group III) (Table I).

a.) The amount of thiobarbituric acid reactive substances, which formed from washed and incubated erythrocytes during lipid peroxidation, was significantly lower in hyperbilirubinaemic newborns (0.91 $\pm$ 0.33 μM/l) than in the healthy group (1.99 $\pm$ 0.26 μM/l) ($p < 0.001$ with unpaired t-test). In the case of hypoxic newborns this value (1.61 $\pm$ 0.31 uM/l) did not show a significant difference in comparison with the healthy group (Table I).

In vitro incubation of normal erythrocytes with different amount of exogenous bilirubin resulted in dose dependent decreasing in lipid peroxidation (LPO). Thus, a 250 μM/l of exogenous bilirubin solution was able to reduce LPO with 30 % (Fig.1).

b.) Protein outflow from the washed erythrocyte in vitro proved to be significantly higher with 0.132 $\pm$ 0.022 mg/ml in hyperbilirubinaemic group (II) than in the healthy group (I) with 0.06 $\pm$ 0.02 mg/ml ($p < 0.05$ with unpaired t-test, and $p < 0.01$ with Mann-Whitney's test). This parameter was significantly higher in the case of hypoxic newborns (III). with 0.138 $\pm$ 0.04 mg/ml.

c.) Potassium ion outflow changed similarly to protein outflow. K^+ outflow was higher (1.13 $\pm$ 0.17 mM/l) in the case of hyperbilirubinaemia (II) than in the healthy group (I) with 0.81 $\pm$ 0.12 mM/l ($p < 0.01$ with Mann-Whitney's test, and $p < 0.01$ with Student's t-test referring to log of the mean values of K^+ outflow). In the hypoxic group (III.) this value was considerably higher (2.7 $\pm$ 0.70 mM/l) comparing with the healthy group (I).

d.) Superoxide dismutase activity in the serum and erythrocytes of newborn babies did not show significant difference in the I. II. and III. groups.

e.) Serum coeruloplasmin level in the hyperbilirubinaemic group (273 ± 125 mg/l) did not show significant difference related to normal range (180-450 mg/l). Serum coeruloplasmin level was significantly lower (133.8 ± 67.3 mg/l) in the case of hypoxic babies (III.) on the 4th day of life ($p < 0.001$). A wide range of coeruloplasmin reference values can be found in the literature /2,11/.

f.) Serum transferrin level was significantly lower in the hyperbilirubinaemic group(1996 ± 655 mg/l) as related to the normal range: 2040-3600 mg/l ($p < 0.01$). Similarly, in the hypoxic group (III.) this value was significantly low, 2114 ± 574 mg/l on the 4th day of life ($p < 0.01$ calculated with one sample t-test).

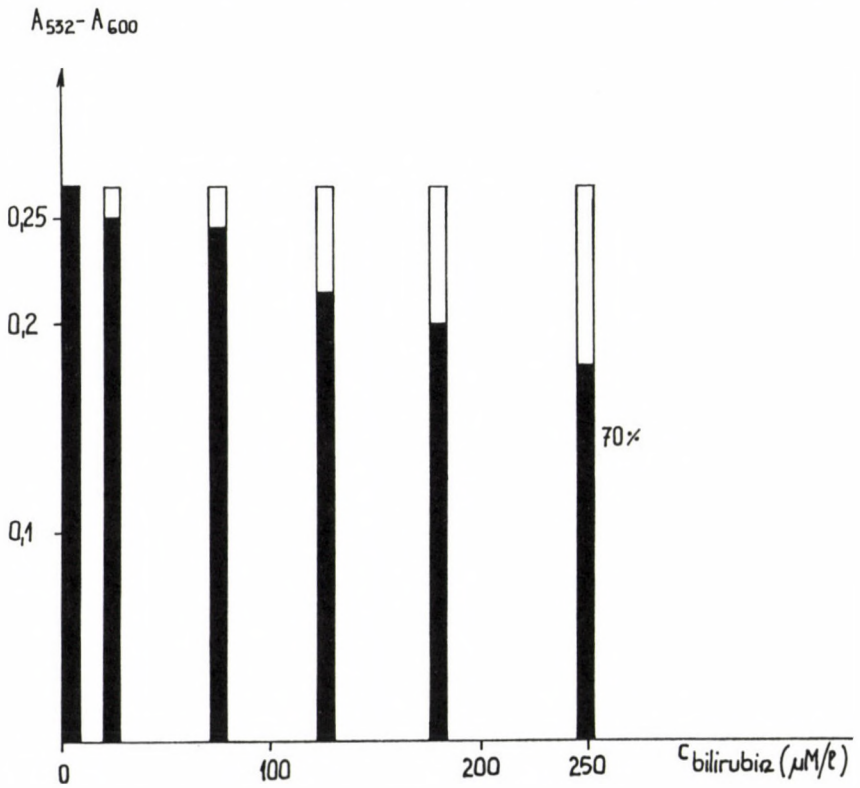

Fig. 1. Dose-dependent lipid peroxidation in erythrocytes of healthy newborn babies following 1 h in vitro incubation with exogenous bilirubin at 0°C. (Mean of three determinations)
$A_{532} - A_{600}$: absorbance of thiobarbituric acid reactive substances

DISCUSSION

In vivo and *in vitro* decrease in erythrocyte lipid peroxidation of newborn seems to support the theory - well-known in literature /14/ - that bilirubin possesses a remarkable antioxidant property beside its cytotoxic effect. The base of antioxidant effect is that bilirubin can donate its H atom attached to the C-10 of the tetrapyrrole ring, forming the resonance stable bilirubin radical. This radical is able to react with oxygen as well as alkyl-peroxyl radicals resulting in nonradical products. In this way bilirubin, as an effective free radical scavenger reduces the chain-breaking of unsaturated lipids in the membrane. This can be the reason for decreasing erythrocytes membrane lipid peroxidation in the hyperbilirubinaemic newborns as well as *in vitro* test.

Probably other erythrocyte surface membrane compounds (e.g. proteins, glycoproteins) are more sensitive to free radicals than lipids. We suppose that membrane damage initiated by free radicals and some interaction between bilirubin and erythrocytes' cell surface ingredients resulted in an increased degree of protein and K^+ outflow from erythrocytes. On the basis of our investigation (using the method of E.J. van Kampen /5/) a considerable portion of the released proteins proved to be haemoglobin fragments. This protein and K^+ release might follow an erythrocyte membrane damage in hyperbilirubinaemic and in hypoxic group, too.

Our present and previous study being in accordance with the results of Sullivan /13/ showed that in case of newborn babies, particularly in prematures the low level of serum coeruloplasmin and transferrin cannot protect erythrocytes effectively against free radical attack.

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