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Urinary excretion rates of 8-oxoGua and 8-oxodG and antioxidant vitamins level as a measure of oxidative status in healthy, full-term newborns

TOMASZ DZIAMAN[1], DANIEL GACKOWSKI[1], RAFAL ROZALSKI[1], AGNIESZKA SIOMEK[1], JAROSLAW SZULCZYNSKI[2], ROMUALD ZABIELSKI[3], & RYSZARD OLINSKI[1]

[1]*Department of Clinical Biochemistry, Collegium Medicum, Nicolaus Copernicus University, Karlowicza 24, PO-85-092 Bydgoszcz, Poland,* [2]*Department of Neonatology, City Hospital in Bydgoszcz, Szpitalna 19, PO-85-168 Bydgoszcz, Poland, and* [3]*Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Nowoursynowska 159, PO-02-766 Warsaw, Poland*

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Abstract

The aim of the present study was to evaluate the oxidative status in healthy full-term children and piglets. Urinary excretion of 8-oxoGua (8-oxoguanine) and 8-oxodG (8-oxo-2′-deoxyguanosine) were determined using HPLC/GS/MS methodology and concentrations of vitamins A, C and E with HPLC technique. The levels of 8-oxoGua in urine samples were about 7–8 times higher in newborn children and piglets when compared with the level of adult subjects, while in the case of 8-oxodG the difference was about 2.5 times. The levels of vitamin C and E in umbilical cord blood of newborn children significantly depend on the concentration of these compounds in their mother's blood. However, the values of vitamin C in human's cord blood were about 2-times higher than in respective mother blood, while the level of vitamin E showed an opposite trend. The results suggest that: (i) healthy, full-term newborns are under potential oxidative stress; (ii) urinary excretion of 8-oxoGua and 8-oxodG may be a good marker of oxidative stress in newborns; and (iii) antioxidant vitamins, especially vitamin C, play an important role in protecting newborns against oxidative stress.

Keywords: *Newborns, oxidative stress, free radicals, 8-oxoguanine, 8-oxo-2′-deoxyguanosine, antioxidant vitamins*

Introduction

Reactive oxygen species (ROS) can damage different kinds of biomolecules including proteins, lipids and DNA. Therefore, series of primary antioxidant defenses have evolved that protect organisms against these lesions. However, in humans as well as in other mammals during early life the antioxidant systems are poorly developed [1,2] Moreover, partial pressure of oxygen in the extrauterine environment is much higher than in the womb [3] and a sudden increase in oxygenation after birth may expose the neonate to oxidative stress [4]. Thus, inadequate antioxidant capacity and increased formation of ROS after birth may be responsible for damage of cellular components in newborns. Therefore, we need a better understanding of oxidative processes in growing infants to help us better design interventions with antioxidant therapy.

Although some studies suggest that increased oxidative stress in newborns may be responsible for

Correspondence: Ryszard Olinski, Department of Clinical Biochemistry, Collegium Medicum, Nicolaus Copernicus University, Karlowicza 24, PO-85-092 Bydgoszcz, Poland. Fax: +48 52 341-5933. E-mail: ryszardo@cm.umk.pl

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many neonatal diseases [5], the specific biochemical markers which can predict the onset of ROS-induced damage are largely undefined. Determination of urinary excretion rates of oxidatively modified nucleosides/bases can be used to assess oxidative DNA damage and as a general index of oxidative stress on the level of the whole organism. One of the most reliable assays for this determination is the methodology which involves an HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection (HPLC/GC/MS) [6]. In addition to unequivocal identification of the analysed compounds and high sensitivity, the isotopically labelled internal standards used in this approach allow compensation for eventual losses of the analysed products. It is also worth mentioning that, in addition to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) detection, which is the only analysed product in the case of such commonly used techniques as ELISA (Enzyme-Linked ImmunoSorbent Assay) and HPLC/EC methods, MS-based methodologies also enable measurement of the modified base—8-oxo-7,8-dihydroguanine (8-oxoGua) [6,7]. Using HPLC/GC/MS methodology we have found that urinary excretion of 8-oxoGua and 8-oxodG does not depend on diet in the case of humans and mice and reflects oxidative DNA damage [7,8]

It is generally accepted that 8-oxoGua excised from DNA by cellular repair is excreted into the urine without further metabolism [9]. It has been reported that the presence of the modified nucleoside 8-oxodG in urine represents the primary repair product of the oxidative DNA damage *in vivo*, presumably by the nucleotide excision repair (NER) [10,11]. Alternatively, 8-oxodG in urine could derive from sanitation of the cellular nucleotide pool by MTH1 (MutT Homologue) directed pathway [12]. However, it should be remembered that the products of NER and MTH1 both require further processing to result in 8-oxodG. Moreover, there is little evidence that 8-oxodG is a product of DNA repair itself [13]. Oxidatively damaged DNA bases are mostly repaired by the base excision repair pathway (BER), although some components of the nucleotide excision repair pathway may also play a role in the repair of some oxidized bases in DNA [14,15].

It is also not clear how the newborns can cope with oxidative stress. Since antioxidant vitamins may play a major role in neutralizing ROS we decided to check the possible changes in vitamins A (retinol), C (ascorbic acid) and E (α-tocopherol) concentration in blood plasma of the newborns. However, for humanitarian reasons, it is not possible to take blood frequently from a healthy infant or newborn child. Therefore, we have chosen an animal, a piglet, as a model for this kind of study. Of the higher animals, the pig is considered as a suitable model because of the similarity in size and function of pig organs to human organs [16,17]. There are also similarities between pigs and humans concerning oxidative DNA damage (reflected in similar levels of urinary 8-oxoGua and 8-oxodG) and metabolic rates [16,17].

The aim of the present study was to evaluate the oxidative status in healthy full-term newborn children and piglets. Urinary excretion of 8-oxoGua and 8-oxodG were determined using HPLC/GS/MS methodology. In addition, concentrations of vitamins A, C and E with HPLC technique were analysed in umbilical cord blood in newborn children and their mothers as well as in the blood of newborn piglets.

Materials and methods

Human studies

A group of 20 newborn, healthy full-termed babies, after vaginal delivery, were studied. Among them there were 10 female and 10 male babies. Mean birth weight was 3.407 kg $\pm$ 0.408 SD.

A group of 20 mothers was also studied. Blood samples were obtained from newborns, from the umbilical vein immediately after cord clamping and by venipuncture from the mothers group.

In the case of urine analyses, 45 healthy adult subjects (mean age of 30 years) were also included. The study was approved by the medical ethics committee of the Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland (in accordance with Good Clinical Practice, Warsaw 1998) and all the patients (or their mothers) gave informed consent.

Animal studies

The experiments and treatments were conducted in compliance with the European Union regulations concerning the protection of experimental animals. The Local Ethics Committee approved the study protocol. A total of 72 male and female neonatal piglets (Polish Landrace $\times$ Pietrain) from 12 different litters were used. The piglets were delivered in term, healthy and without complications and were kept with their sows until sacrificed. The piglets were fed by their sows and had free access to infrared lamps. Six piglets from each litter were randomly allocated into 6 age groups and killed on day 1 (non-suckling neonates), 2, 4, 7, 14 or 28 of their life by suffocation in CO_2. After midline incision urine was harvested by a bladder needle punction.

Plasma preparation for vitamin analyses

Blood samples were collected in heparinized Vacuette® tubes and centrifuged for 10 min, at 1800 $\times$ g, at 4°C to obtain plasma. The heparin-plasma samples were stored at -85°C for a maximum period of 3 months.

Urine analysis

A spontaneously voided urine samples were collected. Standard, sterile, adhesive plastic bags were used for urine collection. Urine samples were frozen at $-85^{\circ}C$ and stored for no more than 1 month.

Exactly 0.5 nmol of ($^{15}N_3$, ^{13}C)-8-oxoGua, 0.05 nmol of ($^{15}N_5$) 8-oxodG and 10 µl of acetic acid (Sigma, HPLC grade, concentration 99%) were added to 2 ml of human or pig urine. Isotopic purity of the applied standards was 97.65% and 99%, respectively. After centrifugation (2000 × g, 10 min), supernatant was filtered through a Millipore GV13 0.22 µm syringe filter and 500 µl of this solution was injected onto HPLC system. In our previous study [7] isotopically labelled internal standards of unmodified compounds (1 nmol of ($^{13}C_3$) Gua and 1 nmol of ($^{15}N_5$) dG) were added to the urine samples to monitor fractions containing both these compounds and to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside. Isotopic purity of the applied standards was 96.4% and 98.0%, respectively.

Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to the method described by Gackowski et al. [7].

GC/MS analysis was performed according to the method described by Dizdaroglu [18], adapted for additional ($^{15}N_5$) 8-oxoGua analyses (m/z 445 and 460 ions were monitored).

Plasma analysis and sample preparation

To precipitate proteins, aliquots (200 µl) of freshly prepared or freshly thawed plasma samples were mixed with 200 µl of HPLC-grade water and 400 µl of ethanol. For the vitamins extraction, 800 µl of hexane were added and mixed for 30 min. Then, 600 µl of upper layers (hexane) were collected, dried in Speed-Vac system and dissolved in 150 µl of mobile phase with 0.5% (m/v) BHT for the vitamins stabilization. An aliquot of 20 µl of this solution was injected into the HPLC system.

Standard and control serum samples, with known vitamin E (α-tocopherol), vitamin A (retinol) concentrations, were purchased from Chromsystems and prepared as plasma samples.

Chromatography

The HPLC system consisted of a GP 40 gradient pump, GINA 50 autosampler (both from Dionex) and Jasco FP-920 fluorimetric detector was used for vitamin E (α-tocopherol) and vitamin A (retinol) quantification. Samples were separated in an isocratic system on column Atlantis DC (5 µm, 150 mm × 4.6 mm) with guard column, both from Waters. The mobile phase, containing 85% (v/v) of acetonitrile and 15% (v/v) of methanol, at a flow rate of 1.5 ml/min, was used. The effluent was monitored with fluorimetric detection (ex. = 340 nm, em. = 472 nm for retinol and ex. = 290 nm, em. = 330 nm for α-tocopherol) and analysed by Chromeleon 6.6 software.

Determination of plasma vitamin C (ascorbic acid), uric acid by HPLC technique was described previously [19].

Statistical analysis

All results are expressed as means ± SD. The STATISTICA (version 6.0) computer software (StatSoft, Inc, Tulsa, USA) was used for the statistical analysis. For normal distribution, variables were analysed by 'Kolmogorov-Smirnow' test with Lillefors correction. Student's *t*-test for dependent samples was carried out to compare the variables between groups. Correlation coefficients were determined by the Pearson's test. Statistical significance was considered at $p < 0.05$.

Results

Human studies

The mean endogenous levels of vitamin C in the plasma of the newborns' group and their mothers' reached the mean values of 98.6 ± 11 µM and 44.6 ± 9.2 µM, respectively (Figure 1A). The difference was statistically significant. As has been shown in Figure 1B, vitamin E level was significantly reduced in the plasma of neonates when compared with their mothers' groups. The mean values were 8.2 ± 4.9 µM and 50.1 ± 19.8 µM, respectively (Figure 1B). No statistical differences in vitamin A concentration were found between the studied groups (Figure 1C). The mean values of vitamin A concentration were 0.6 ± 0.2 µm (babies) and 0.9 ± 0.4 µm (mothers).

The mean value of uric acid in the plasma of the newborns' group was 309.9 ± 75.0 µM, while in their mothers the level reached a concentration of 299.1 ± 75.7 µM.

Statistically significant correlations were found between babies and their respective mothers concerning the plasma antioxidant vitamins (with the exception of vitamin A) and uric acid concentrations (Figure 1).

The levels of 8-oxoGua in urine from human newborn samples were significantly elevated from day 4 to day 61, while in the case of 8-oxodG from day 1 to day 92, compared with levels measured in normal adults (6.82 ± 3.32 nmol/mmol creatinine and 1.29 ± 0.51 nmol/mmol creatinine, respectively, for 8-oxoGua and 8-oxodG) (Figure 2). For 8-oxoGua the level declined significantly at day 92. However, at days 1 and 2, creatinine concentrations and the levels of 8-oxoGua and 8-oxodG were very similar to concentrations characteristic for adults.

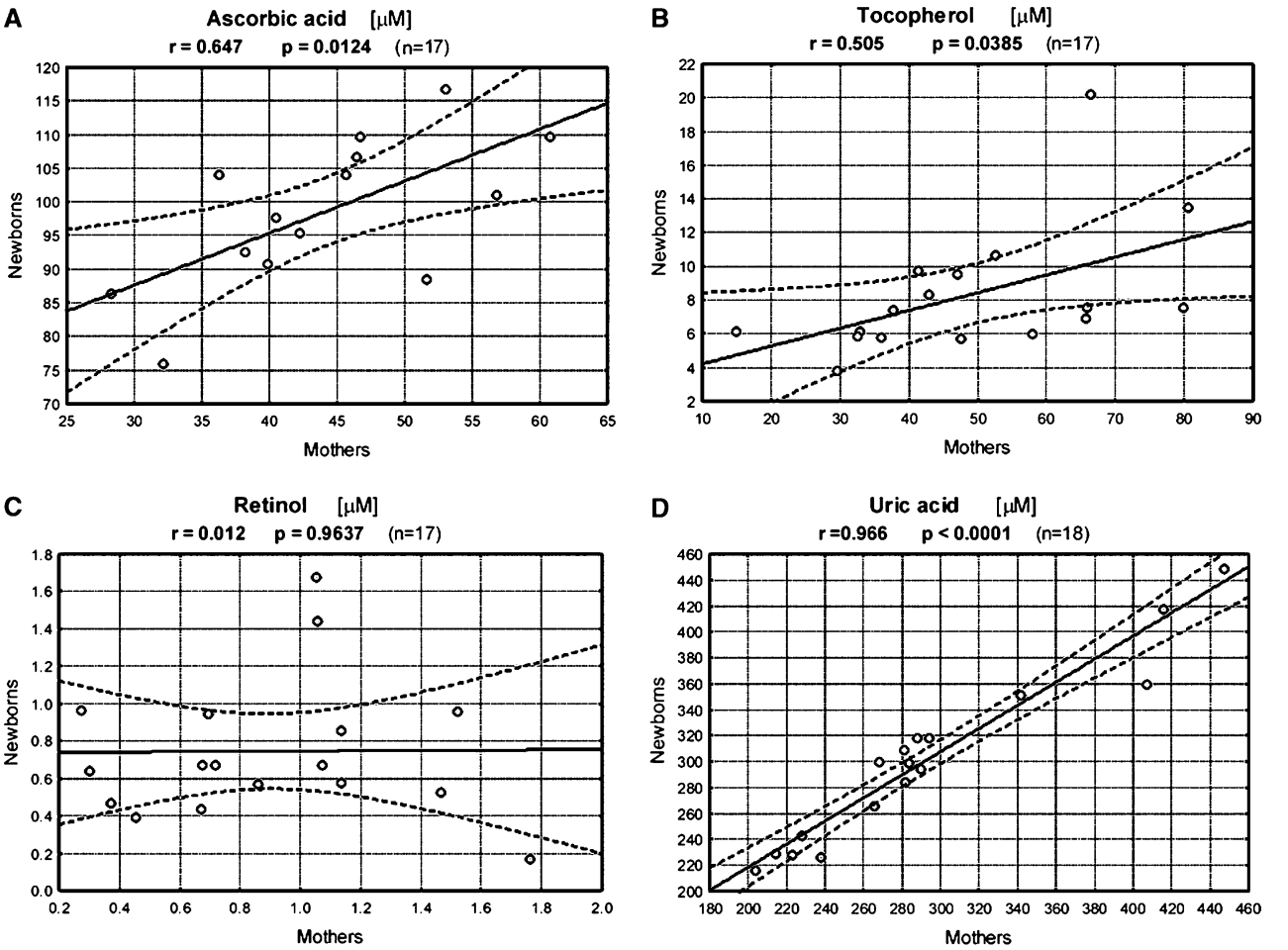

Figure 1. Plasma antioxidant values in mothers and their respective newborn children. Significant correlation between mother and newborn was seen for vitamin C ($r = 0.647$; $p = 0.012$), vitamin E ($r = 0.505$; $p = 0.039$) and uric acid ($r = 0.966$; $p < 0.001$).

Therefore, at these days the values possibly represent those of the mother (for a specific discussion of this issue see Matos et al. [20]). The values of creatinine concentration were: for adults 9.83 ± 3.71 and for newborns at 1st and 2nd day of their life 5.96 ± 5.43 and 10.02 ± 4.58 μM, respectively.

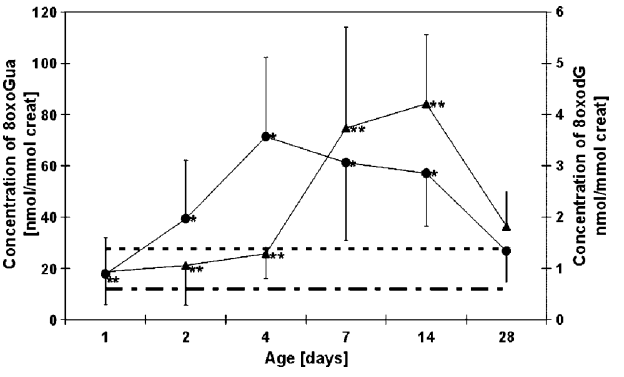

Figure 2. Urinary excretion rates of 8-oxoGua and 8-oxodG in full-term healthy newborn children. * changes of 8-oxoGua significantly different from the normal adults' values; ** changes of 8-oxodG significantly different from the normal adults' values; error bars indicate SDs. ● children values of 8-oxoGua; –- normal adult values of 8-oxoGua; ▲ children values of 8-oxodG; --- normal adult values of 8-oxodG.

Later on, starting from day 4, creatinine concentrations were stable and characteristic for infants. Respective values at days 4, 7, 21, 62 and 92 were 1.16 ± 0.38, 1.2 ± 0.71, 1.04 ± 0.71, 0.75 ± 0.6 and 1.11 ± 0.48 μM.

Animal studies

Concentration of vitamin C and E in plasma showed a significant rise over the initial value (day 1) between the 2nd and 4th day of age and a subsequent decline later on while vitamin A peaked at the 14th day reaching a plateau thereafter (Figure 3).

The levels of 8-oxoGua in urine samples showed significant increase from the 2nd day to the 14th over the value characteristic for adult animals, while in the case of 8-oxodG the increase was observed from 7–14 days, declining significantly at day 28 (Figure 4). There were no significant differences in creatinine values among newborns concerning their age. Similarly to the human study, creatinine concentration at the 1st day mirrored that characteristic for adults (respective values were 11.13 ± 6.41 and 13.01 ± 8.89 μM), while later on the creatinine concentrations were characteristic for newborns and their

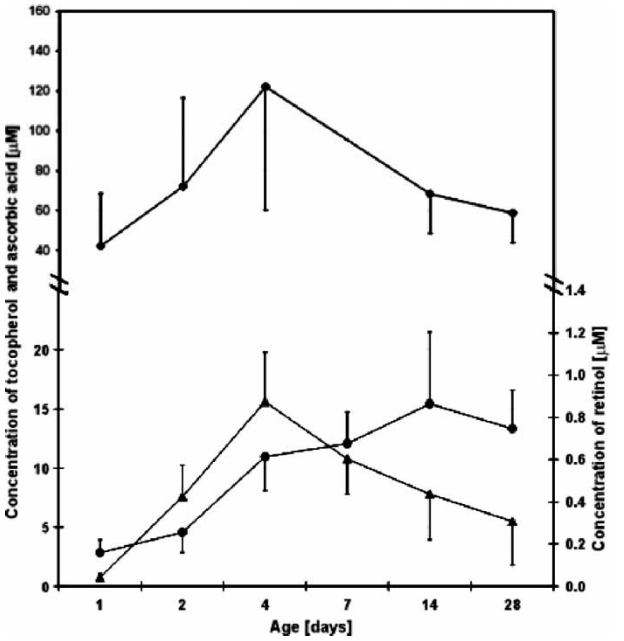

Figure 3. Plasma antioxidant vitamins in newborn pigs. ● retinol; ▲ tocopherol; ◆ ascorbic acid.

respective values at days 2, 4, 7, 14 and 28 were: 3.93 ± 3.18, 2.45 ± 1.68, 2.19 ± 1.06, 3.84 ± 1.96 and 4.37 ± 2.04 μM.

Discussion

Our experiments demonstrated that levels of 8-oxoGua and 8-oxodG excreted with urine were significantly higher in healthy full-term children soon after birth than in normal adults. Similarly, in the case of piglets value of urinary modifications in newborns was significantly elevated when compared with adult pigs. However, in the case of pigs the maximum values of 8oxoGua were reached earlier, i.e. 4 days after birth, decreasing gradually later on (Figure 4). It is likely that

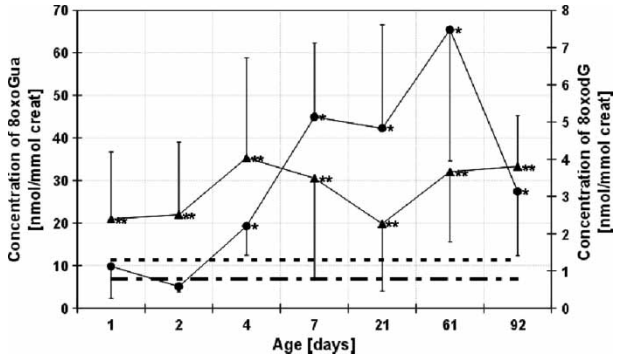

Figure 4. Urinary excretion rates of 8-oxoGua and 8-oxodG in newborn pigs. * changes of 8-oxoGua significantly different from the normal adults' values; ** changes of 8-oxodG significantly different from the normal adults' values; error bars indicate SDs. ● piglet values of 8-oxoGua; –- normal adult pig values of 8-oxoGua; ▲ piglet values of 8-oxodG; --- normal adult pig values of 8-oxodG.

this difference between humans and pigs mirrors the growth velocity difference between the species, since the pig is a rapidly growing animal during the initial post-natal days, doubling its weight each week. These increases do not reflect changes in creatinine concentration since this parameter was relatively stable during the observed time range in both species.

In previous works urinary excretion rates of 8-oxodG were measured in human neonates with median gestation of 28 weeks and with very low birth weight [21]. The reported values were about 10-times higher than those found in our study. One reason for these differences may be that in previous studies pre-term children were included in the study group. More likely the inconsistency between our results and the aforementioned work may be explained by the different methodological approach, for example in the Matsubasa et al. [21] study 8-oxodG level was determined with ELISA. However, it has been shown that ELISA estimates were higher than HPLC or HPLC/GC/MS estimates. This in turn suggests that the monoclonal antibody used in the ELISA kit is not sufficiently specific for the detection of 8-oxodG [22]. It is possible that the antibody used in the ELISA kit may be cross-reactive towards 8-oxoGua. Therefore, the changes detected in the aforementioned work may describe 8-oxodG plus 8-oxoGua excretion. Our results, which are based on our methodology that discriminate between 8-oxoGua and 8-oxodG, demonstrated more distinct changes in urinary 8-oxoGua level than that of 8-oxodG, suggesting that the modified base is a better marker of oxidative stress. A similar phenomenon was described by us for cancer patients [23].

The observed shape of the curve of the excretion rates of both modifications (Figure 2) may reflect the growth velocity of human neonates which are higher in the first weeks of age than at any time thereafter [24]. Neonates who are growing rapidly have a higher metabolic rate than adults. High metabolic rate, in turn, requires a high level of mitochondria respiration and subsequent higher production of ROS, which are responsible for the formation of modifications analysed in our work. Indeed in our previous study highly significant, positive correlations between specific metabolic rates and excretion rates for all the analysed modifications were found [16].

Since the urinary excretion rate of oxidative DNA damage is a gross measurement of the whole-body level of oxidative stress, our findings clearly indicate that newborns are coping with excess production of ROS. Results of numerous studies concerning humans as well as other mammals suggests that newborns are at higher risk for ROS induced damage [25–30]. However, most of the results were indirect comparisons of antioxidant capacity and/or plasma malondialdehyde measurements between newborns and adults. Also the majority of works were devoted to the study of oxidative stress in pre-term neonates.

Our findings indicate that oxidative stress is characteristic for full-term, healthy newborns and the most dangerous period is the first 2 months of life. In this context it is worth mentioning that the majority of infant deaths observed during the first year occurred in the first month of life [31]. Oxidative stress was postulated to be implicated in several newborn pathological conditions with the common term 'oxygen radical disease of neonatology' [32]. There may be several causes of elevated oxidative status characteristic for newborns: (i) difficulties in adapting to ambient oxygen since there is a 5-times difference between oxygen pressure concerning intra- and extra-uterine environment [33]; (ii) antioxidant mechanisms are impaired in newborns in comparison with adults [1,2]; (iii) level of free iron pool, which catalyse Fenton reaction, may be increased [30]; and (iv) as was mentioned above, the high metabolic rate characteristic for newborns requires a high level of mitochondria respiration, which in turn is responsible for subsequent higher ROS production.

Antioxidant vitamins can scavenge ROS and counteract oxidative stress. Therefore, concentrations of vitamins A, C and E were determined at different time points in blood plasma of newborn piglets. Concentration of vitamin C in plasma rose sharply and peaked 4 days after birth, decreasing thereafter, reaching a plateau characteristic for an adult organism. Also changes in vitamin E concentrations followed a similar pattern with observed milder slope of decrease, while vitamin A level remained stable after reaching a maximum 14 days after the birth. This stability of vitamin A may be linked to the paramount importance of this compound for lung development, because the surfactant proteins are selectively regulated by retinoic acid [34]. It is likely that the observed rapid increase in antioxidant vitamin concentration is a response to excess production of ROS, which is associated with a sudden increase of oxygen pressure soon after birth. It is also likely that vitamin C plays the major role in this phenomenon since its concentration increased in newborn piglets from 42 to an unprecedented concentration of 122 μM. It should also be remembered that vitamin C acts in synergy with tocopherols (vitamin E) by regenerating tocopheroxyl radical to tocopherol [35,36]. Vitamin C (ascorbic acid) is a major aqueous-phase antioxidant. Pigs as well as other species can synthesize ascorbic acid due to activity of the enzyme L-gulono-gamma-lactone oxidase (EC 1.1.3.8). This enzyme is missing in primates and humans. The neonatal pig is born with a moderate body reserve and blood concentration of vitamin C [37] and its synthesis begins thereafter [38,39]. Therefore, newborn pigs can respond to stress conditions with increasing synthesis of this antioxidant vitamin. Since these antioxidant vitamins are effective free radical scavengers the observed decrease in their concentration (Figure 3) suggests that they are partially used up as the first line of defence against oxygen radicals produced soon after the birth. We have also measured concentrations of antioxidant vitamins and uric acid in the cord blood of neonates and compared these values with the respective levels of their mothers. The levels of vitamin C in umbilical cord blood significantly depend on the concentration of this compound in their mother blood (correlation coefficient = 0.647). However, the values in cord blood were about 2-times higher than in the respective mother blood, most likely due to active transport across placenta [32]. This finding once again underlines the importance of vitamin C as the major defense line against oxidative stress in newborns. Lipid soluble vitamins such as vitamin A and E cross the placenta to a lesser extent [32] and the concentration of α-tocopherol in umbilical cord blood is significantly lower than in their mothers (Figure 1). Nevertheless, the level of vitamin E in the blood of newborns significantly correlates with their respective mothers' concentrations. This finding suggests that the transport of vitamin E across the placenta is limited to certain low concentration values. However, apparently this low concentration of vitamin E is sufficient for a normal embryonic/foetal development. Our data are in agreement with recent findings which demonstrated, using a mouse model, that vitamin E is indispensable for the placentation but not necessary for the embryonic development itself [40].

Uric acid in physiological concentration is regarded as a main antioxidant and not only does it efficiently scavenge free radicals but it has also been shown to stabilize vitamin C in human serum and reduce consumption of vitamin E and β-carotene [41]. The presented results show that concentrations of uric acid in cord blood are almost exactly the same as the corresponding values of their mothers. This, in turn, suggests passive transport of this compound across the placenta.

Collectively our data suggest that healthy newborns are experiencing elevated oxidative status, mostly during the first weeks of their life. Full-term breast-fed infants could cope with this oxidative stress due to the consumption of great amounts of antioxidants present in mother's milk [42,43]. However, in certain cases the increased oxidative stress in newborns may be a factor responsible for many neonatal diseases [5]. Our study demonstrated an importance of antioxidant vitamins, especially vitamin C, as a defense line against oxidative stress in newborns. Therefore, in the case of a selected group of infants, when oxidative stress is expected to be higher (preterm, low weight) it would be reasonable to consider antioxidant supplementation with additional nutrients rich in antioxidant vitamin C and E. The presented results also suggest that measurement of

urinary 8-oxoGua and 8-oxodG may be a useful non-invasive marker which can predict oxidative stress in newborns/infants.

Differences between the patterns of changes between antioxidant vitamins and oxidative DNA damage modifications in piglets (Figures 3 and 4) suggest that the vitamins only partially take part in the protection of DNA against ROS-induced damage. Similarly, supplementation data demonstrated the lack of clear protection effect of antioxidant vitamins on oxidative DNA damage [44]. Apparently, other components of antioxidant defense may be involved in DNA protection. Therefore, it is possible that the decline in urinary excretion of 8-oxoGua in children, at day 92 (Figure 2), may depend on increased activity of antioxidant enzymes (e.g. reported increase activity of catalase (CAT) and superoxide dismutase (SOD), which peaked around the 3rd month of life [33].

Summing up, the presented results demonstrated that: (i) healthy, full-term newborns are under potential oxidative stress which is reflected in an elevated level of urinary excretion of 8-oxoGua and 8-oxodG when compared to the values characteristic for adult organisms, (ii) it is possible that antioxidant vitamins, especially vitamin C, play an important role in protecting newborns against stress, (iii) pigs can serve as good models to study oxidative stress in newborn children.

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