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Ontogeny of Reactive Nitrogen Species Production by Blood Phagocytes in Pigs

P. ZELNÍČKOVÁ[1,2], M. FALDYNA[1,2], J. ONDRÁČEK[2], H. KOVÁŘŮ[3], F. KOVÁŘŮ[4,5]

[1]Veterinary Research Institute, Department of Immunology, Brno, [2]University of Veterinary and Pharmaceutical Sciences Brno, Institute of Microbiology and Immunology, Brno, [3]First Medical Faculty, Charles University, Prague, [4]University of Veterinary and Pharmaceutical Sciences Brno, Institute of Physiology, Brno, [5]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

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Summary

The aim of this work was to evaluate ontogeny of reactive nitrogen species (RNS) production by peripheral blood phagocytes in pig. Pig fetuses (55 and 92 days of gestation) and postnatal piglets (1, 3, 8, 17, 31 and 41 days after birth) were used. RNS production was measured by fluorescent probes diaminofluorescein-diacetate (DAF-FMDA) and dichloro-fluorescein-diacetate (H2DCFDA). Levels of nitration of cell proteins were established by immunofluorescent detection of nitrotyrosine. Levels of plasma nitrites/nitrates were detected spectrophotometrically by Griess reaction. Nitric oxide production measured by DAF-FMDA in neutrophils decreased during postnatal life. Spontaneous RNS measured by H2DCFDA decreased from 55th day of gestation to the 41st day of life. Phorbol-12-myristate-13-acetate activated production decreased during postnatal life. Production of NO measured by DAF-FMDA in macrophages decreased from the 1st to 41st day after birth. RNS production measured by H2DCFDA in monocytes did not show any significant changes during ontogeny. The level of nitrotyrosine significantly decreased from the 3rd to 17th day. Levels of plasma nitrites/nitrates gradually decreased from the 55th day of gestation to the 41st day after birth. A temporary increase in all parameters occurred after weaning, but without any significance. In conclusion, RNS production has a decreasing trend during ontogeny and is transiently upregulated after weaning.

Key words

Blood phagocytes • Ontogeny • Reactive nitrogen species • Plasma nitrites/nitrates

Corresponding author

Petra Zelnickova, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic, Fax: +420-541-211-229, E-mail: zelnickovap@vfu.cz

Introduction

There is substantial evidence that the porcine immune system develops not only before birth but also during early postnatal life (Zelníčková *et al.* 2006). Development of acquired immunity in pig has recently been a relatively well-explored part of developmental immunology (Trebichavský *et al.* 1996, Kovářů *et al.* 2002). On the contrary, there is very little information about both prenatal and postnatal ontogeny of natural immunity, especially mechanisms of defense against potential infection by phagocytes. The production of reactive oxygen and nitrogen species is one of the most important bactericidal mechanisms of phagocytic cells.

Nitric oxide can be produced by many cell types by action of two types of the enzyme nitric oxide synthase (NOS) (Forstermann *et al.* 1995). Under physiological conditions, only small amounts of NO by the constitutive, calcium-dependent isoform of NOS (cNOS) are produced. NO produced by cNOS plays a role as a regulator of vascular tone. cNOS is expressed mainly by endothelial cells and neurons. On the other hand, inducible NOS (iNOS) expressed by activated phagocytes plays crucial role in production of relatively high amounts of NO, which give rise to other, much more efficient reactive nitrogen species (RNS), e.g. peroxynitrite. RNS ensure the sufficient fight of phagocytes against bacterial pathogens (Fang 1997, Chakravortty and Hensel 2003).

There is very little and controversial information

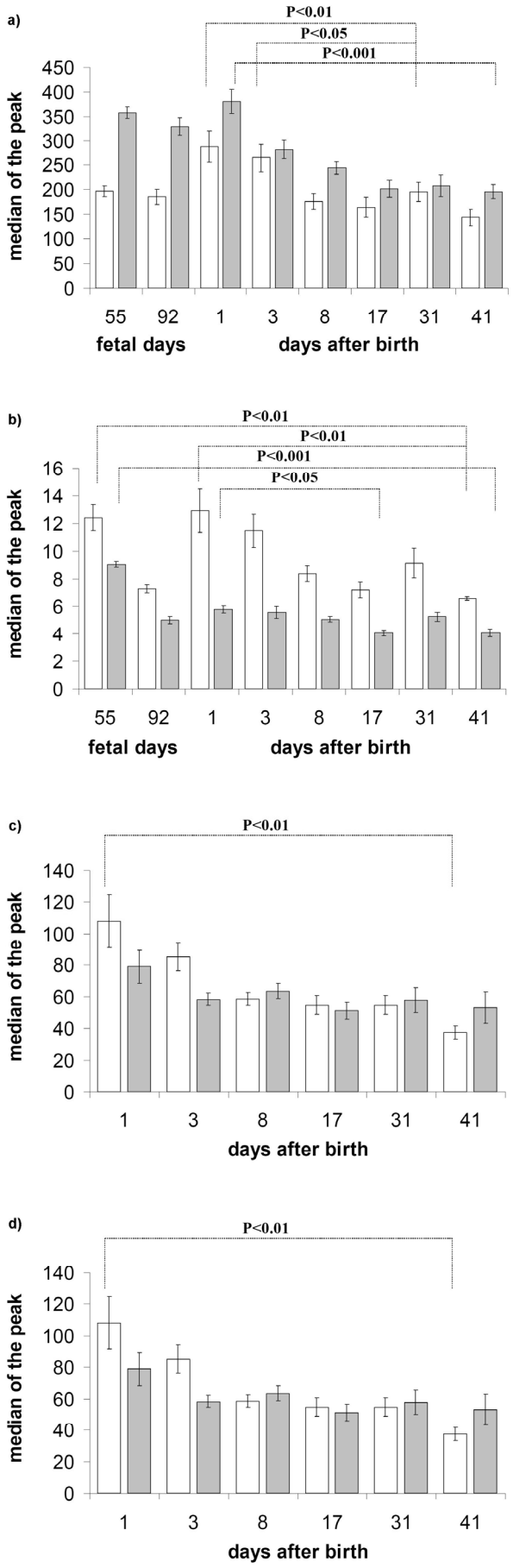

Fig. 1. *In vitro* production of reactive nitrogen species (RNS) by peripheral blood phagocytes during ontogeny in pigs. Nitric oxide detected by diaminofluorescein diacetate (DAF-FM DA, white bars) and other RNS detected by dichlorodihydrofluorescein diacetate (H_2DCF DA, grey bars) were measured in PMA-stimulated neutrophils (a), non-stimulated neutrophils (b), PMA-stimulated monocytes (c) and non-stimulated monocytes (d). RNS production was expressed as the median of the histogram peak in FL1 channel after gating of viable, propidium iodide (FL3 channel) negative and CD14 (FL2 channel) positive cells (final discrimination between monocytes and neutrophils was realized by their light scatter characteristics). Results are shown as mean of medians ± SEM. Significant differences between pairs (Dunn's post-test) are marked in the Figure.

available about ontogeny of RNS production by phagocytes at present. Sherman *et al.* (1996) found that spontaneous, LPS, and IFN-γ stimulated RNS production by rat alveolar macrophages in 3-day-old animals was twice as high as that by older animals (10 days old and adults). On the contrary, Lee *et al.* (2001) found that RNS production by rat alveolar macrophages stimulated in the same way was significantly lower in newborn rats in comparison to adult ones. However, production of RNS by blood phagocytes has not yet been assessed.

More information exists about ontogenesis of plasma nitrite or nitrate production. Plasma nitrite/nitrate may be formed nonenzymatically by ingestion of nitrites with food or may be formed by intestinal flora (Castillo *et al.* 1993). In fasting humans, it arises mostly from endogenously produced nitric oxide (Rhodes *et al.* 1995). Endogenously produced nitric oxide spontaneously changes to nitrite, which converts to nitrate when the reaction is catalyzed by hemoglobin (Ignarro *et al.* 1993). The basal and relatively constant plasma nitrite/nitrate levels are products of cNOS activities (Kleinbongard *et al.* 2003). However these levels can be greatly enhanced during antigenic challenge when iNOS from activated phagocytes become a major source of endogenous nitric oxide (Ergenekon *et al.* 2000).

Ontogeny of plasma nitrite and nitrate levels have been better documented than direct production of RNS by blood phagocytes. However, this is only *via* indirect detection, which may represent production of NO from other sources than blood phagocytes. Unfortunately, the results obtained by different investigators are controversial. While Endo *et al.* (2001) found that levels of plasma nitrites/nitrates increase during the first few days of life in children, Blum *et al.* (2001) found a strong decrease in calves. These data further suggest that there may exist species-specific differences in ontogeny of plasma nitrites and nitrates. As far as we know, there is only one publication referring to increased fetal plasma

nitrate levels in ovine fetuses (Yang *et al.* 1996). Other data related to prenatal ontogeny are limited to a comparison of preterm and term-born children (Honold *et al.* 2000). Moreover, no data about the ontogeny of plasma nitrites/nitrates in pig have been published yet.

Protein tyrosine nitration in neutrophils occurs when RNS, mostly peroxynitrites are produced (Ischiropoulos 1992). Immunohistochemical detection of nitrotyrosine can be used as another method for the evaluation of *in vivo* RNS production.

The aim of the present study was to evaluate production of RNS by peripheral blood phagocytes in pigs during prenatal and postnatal ontogeny.

Methods

Animals and blood sample collection

Piglets on days 55 (7 piglets) and 92 (5 piglets) of gestation, taken from two sows which underwent hysterectomy under halothane inhalation anesthesia, were used in this study. Eight postnatal piglets from two litters were used at the age of 1, 3, 8, 17, 31 and 41 days. The sows were separated from piglets after weaning on day 28 and piglets were then kept together in one pen. The animals were used under the agreement of the Branch Commission for Animal Welfare of the Ministry of Education, Youth and Sports of the Czech Republic.

The blood from fetuses was obtained by puncture of the umbilical cord vein. Peripheral blood from postnatal piglets in the volume of 3-4 ml was collected on heparin (20 IU/ml, Zentiva, Czech Republic) by puncture of the jugular vein.

Assays for determination of nitric oxide production

Flow cytometric measurements

Intracellular fluorescent probes diamino-fluorescein diacetate (DAF-FM DA, Invitrogen, USA) and dichlorodihydrofluorescien diacetate (H_2DCF DA, Sigma-Aldrich, USA) were used for flow cytometric detection of reactive nitrogen species produced by blood phagocytes (neutrophils and monocytes).

The whole blood (60 µl) was mixed with either 15 µl of DAF-FM DA (final concentration 10 µmol/l) or H_2DCF DA (final concentration 5 µmol/l) and 15 ul of phorbol-12 myristate-13 acetate (PMA, Sigma-Aldrich), which was used as stimulator (final concentration 200 nmol/l). All solutions were diluted in HBSS (Sigma Aldrich) in 3 ml FACS tubes and incubated in 37 °C water bath in the dark for 20 min. In blank samples, the fluorescent probes were replaced by HBSS. The reaction was stopped after 20 min and red blood cells were lysed using 3 ml of 5-8 °C cold hemolytic solution (154 mmol/l ammonium chloride, 10 mmol/l potassium bicarbonate) (Sigma-Aldrich). After centrifugation (600 x g, 7 min, 0 °C) and removal of the supernatant, 5 µl of PE-conjugated anti-human CD14 antibody (clone Tük4, Serotec, UK) was added and the solution was incubated in ice-cold water bath in the fridge for 15 min. After the next washing with ice-cold PBS and addition of propidium iodide (Sigma-Aldrich), the samples were immediately measured in flow cytometer (FACS Calibur, Becton Dickinson, USA). Off-line analysis was performed by Summit Software, version 4.0 (Dako Cytomation, Denmark) and WinMDI, version 2.8 (http://facs.scripps.edu/software.html).

For expression and statistical evaluation of results, the median of the positive DAF-FM DA or H_2DCF DA (FL1 channel) peak of either the neutrophil or monocyte subpopulations was evaluated after gating of viable cells (propidium iodide negative cells – FL3 channel). Neutrophils and monocytes were identified according their light scatter characteristics and positivity in FL2 channel (CD14-positive cells).

Spectrophotometrical detection of plasma nitrites and nitrates

Concentrations of plasma nitrites and nitrates produced *in vivo* were detected by the Griess reaction (Sun *et al.* 2003, Trebichavský *et al.* 2001). The blood sample was centrifuged (1000 x g, 10 min, 22 °C) to obtain blood plasma. Then 10 µl of blood plasma diluted 1:5 by deionized water was mixed with 20 µl of 0.31 mol/l PBS (Sigma-Aldrich), 10 µl of 0.86 mmol/l NADHP, tetrasodium salt (Roche, Germany), 10 µl of 0.11 mmol/l flavin adenine dinucleotide, disodium salt (Sigma-Aldrich), and 10 µl of 0.1 IU nitrate reductase from *Aspergillus* sp. (Roche) and incubated in the dark at room temperature for 60 min. Then 60 µl of Griess reagent (1 % sulphanilamide, 5 % H_3PO_4 and 0.1 % 2-(1-naphthylamino ethylamine) (all Sigma-Aldrich) was added to each sample. Samples were measured spectrophotometrically (Lambda 11, Perkin-Elmer, USA) at the wavelength of 540 nm. Concentrations of nitrites/nitrates in each sample (expressed in µmol/l) were read from the calibration curve. The calibration curves were determined using different concentrations of sodium nitrite and sodium nitrate (both Sigma-Aldrich) solutions by the same procedure as blood samples.

Immunofluorescent detection of nitrotyrosine

Nitrotyrosine was detected in neutrophils by indirect immunofluorescent staining (Viera *et al.* 1999). Neutrophils, isolated by discontinuous density gradient Histopaque 1077/Histopaque 1119, were sliced on the cover slips by cytocentrifugation. The cells were fixed in 4 % paraformaldehyde in PBS for 15 min and permeabilized with 0.1 % Triton X-100 for 15 min. Blocking was performed by 10 % donkey serum in PBS for one hour. The samples were then incubated with anti-nitrotyrosine antibodies (Upstate, USA) overnight at room temperature. On the next day, samples were incubated for one hour with FITC conjugated donkey anti-rabbit secondary antibodies. Cell nuclei were stained by propidium iodide. Preparates were mounted with VectaShield H-1000 mounting medium (Vector Laboratories, USA). The intensity of fluorescence for each cell was expressed semiquantitatively as 25 %, 50 %, 75 % and 100 % positivity and then the mean percentage value for all 200 neutrophils was calculated.

Statistical analysis

Age-dependent changes were tested with the non-parametric ANOVA test (Kruskal-Wallis test) and with Dunn's post-test for the comparison of all pairs. All calculations were performed with MS-Excel (Microsoft Corp. Inc.) and Prizma® (Graph Pad Software Inc.) software.

Results

Flow cytometric measurements

Significant changes of RNS production in neutrophils (stimulated as well as nonstimulated) were detected during ontogeny. In monocytes, only stimulated production significantly changed. Some minor changes of RNS production were observed during prenatal ontogeny, but they were non-significant and without any correlation between stimulated and nonstimulated cells, or between DAF-FM DA and H_2DCF DA. Prenatal RNS production was detected in neutrophils only due to a presence of small numbers of white blood cells in blood of pig fetuses. During postnatal period, significant decreases in RNS production by neutrophils (stimulated as well as nonstimulated) and by stimulated monocytes occurred. Moreover, transitional increases in RNS production by neutrophils and by stimulated monocytes occurred after weaning; however, without any significance.

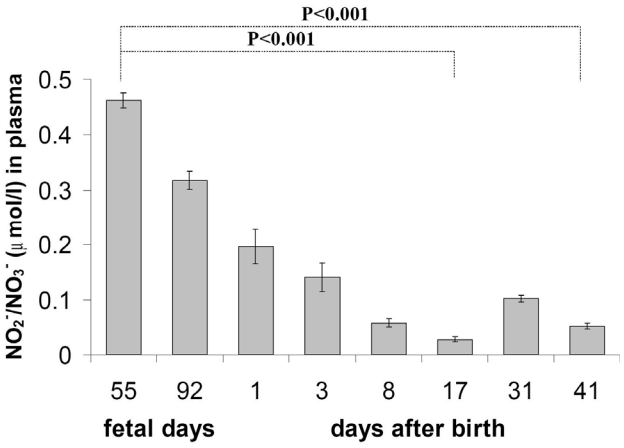

Fig. 2. Level of plasma nitrites/nitrates during ontogeny in pigs. Plasma nitrites/nitrates were measured spectrophotometrically by modified Griess reaction assay at 450 nm. Absorbance was recounted to nitrites/nitrates (expressed in μmol/l) by calibration curve. Results are shown as mean ± SEM. Significant differences between pairs (Dunn's post-test) are marked in the Figure.

Production of RNS by activated neutrophils

Production of RNS significantly changed during ontogeny (Kruskal-Wallis test for DAF-FM DA: $P<0.01$; for H_2DCF DA: $P<0.001$) (Fig. 1a). No significant changes occurred during the prenatal period. Significant decrease was found during postnatal life (Dunn's post-test for DAF-FM DA between days 1 and 31 of age: $P<0.01$; for H_2DCF DA between day 1 and 41 of age: $P<0.001$).

Spontaneous production of RNS by neutrophils

Production of RNS significantly changed during postnatal period (Kruskal-Wallis test for both DAF-FM and H_2DCF DA: $P<0.001$) (Fig. 1b). No significant changes occurred during prenatal period. Significant decrease was found in RNS production during postnatal life (Dunn's post-test for DAF-FM DA between days 1 and 41 of age: $P<0.01$; for H_2DCF DA between days 1 and 17 of age: $P<0.05$).

Stimulated and spontaneous production of RNS by monocytes

Production of RNS (specifically NO measured by DAF-FM DA) significantly decreased during postnatal ontogeny (Kruskal-Wallis test: $P<0.01$, Dunn's post-test between days 1 and 41 of age: $P<0.01$). Postnatal production of RNS measured by H_2DCF DA did not change (Fig. 1c,d).

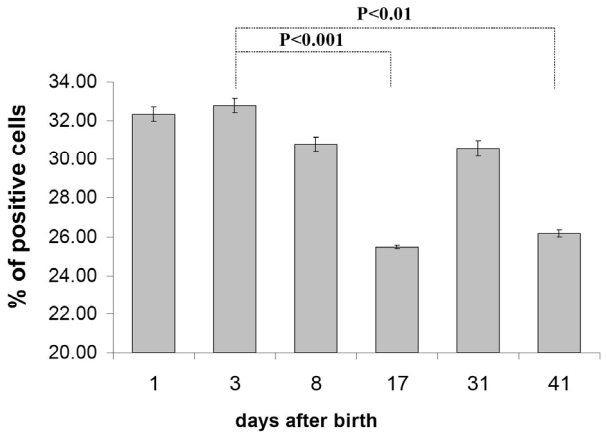

Fig. 3. Levels of nitrotyrosine in neutrophil granulocytes during postnatal ontogeny in pigs. Nitrotyrosine was detected by indirect immunofluorescent staining. Neutrophils were recognized by nucleus morphology after staining with propidium iodide. 200 neutrophils were evaluated. The intensity of fluorescence for each cell was expressed as 25 %, 50 %, 75 % and 100 % intensity of fluorescence and then the mean percentage value for all 200 neutrophils was calculated. Results are shown as a mean of percentage representation ± SEM. Significant differences between pairs (Dunn's post-test) are marked in the Figure.

Spectrophotometrical detection of plasma nitrites and nitrates

Levels of plasma nitrites/nitrates significantly changed during ontogeny (Kruskal-Wallis test: $P<0.001$) (Fig. 2). Gradual decrease occurred from day 55 of gestation to day 41 of postnatal life (Dunn's post-test: $P<0.001$). Plasma nitrites/nitrates levels correlated with NO production by neutrophils during postnatal life including non-significant transient increase after weaning on day 31 of age.

Immunofluorescent detection of nitrotyrosine

The level of nitrotyrosine in neutrophil granulocytes showed significant decrease from day 3 to day 17 ($P<0.01$) and from day 3 to day 41 ($P<0.001$) of age (Fig. 3). Transient increase after weaning, though without significance, correlated with an increase of RNS and ROS production by neutrophils.

Discussion

The aim of the present study was to evaluate production of RNS during prenatal and postnatal ontogeny by peripheral blood phagocytes in pigs. Our previous data (Zelníčková *et al.* 2006) documented that the production of reactive oxygen species by blood phagocytes measured by chemiluminescence also decreased during postnatal ontogeny. In general, it would be more logical to expect that RNS production as a representative bactericidal mechanism in peripheral blood cells will have an increasing trend during ontogeny.

The non-significant increase in RNS production after weaning, which correlated with the increase of plasma nitrites/nitrates levels and content of nitrotyrosine in neutrophils, could be related to the activation of immunity after absence of lactogenic immunity, and consistent with the increase of antigenic pressure.

The production of RNS by neutrophils and monocytes after stimulation correlated with its spontaneous production. The RNS production depends on the amount of iNOS present in RNS-producing cells and on the availability of the substrate (L-arginine). It is likely that the 20 min stimulation with PMA used in the present study is not sufficient for increased iNOS expression, but only for elevation of the RNS production by the increasing substrate availability to present iNOS. It seems to be desirable to detect the content of iNOS in the phagocytosing cells as another parameter, which characterizes the NOS production but there is no commercially available monoclonal antibodies against porcine iNOS.

While there are no data about RNS production by blood phagocytes during ontogeny, more information is available about levels of nitrites/nitrates in blood or urine. However, almost all data arises from human model. The levels of plasma nitrites/nitrates were found to increase from birth to day 5 of life (Endo *et al.* 1996, 2001) with subsequent decrease until day 30 of age (Endo *et al.* 1996). A similar increase of nitrites/nitrates levels in children from days 1 to 4 of age in urine was observed by Tsukahara *et al.* (1997a). Other observations refer to the transient increase of urinary nitrites/nitrates in a later period – an increase from ages 1 week to 1 month with a subsequent decrease to 4 months of age (Tsukahara *et al.* 1997b). The reason for these observations was the developing intestinal flora, which is the source of plasma nitrites/nitrates. Our observations show decreasing plasma nitrites/nitrates during the whole early postnatal period, similar to the data from literature describing plasma levels of nitrites/nitrates in newborn calves (Blum *et al.* 2001).

The principal questions are what is the main source of plasma nitrites/nitrates during ontogeny and why are plasma nitrites/nitrates elevated during the early developmental period?

Decreased levels of plasma nitrites/nitrates

during postnatal ontogeny correlated with decreasing NO production by blood phagocytes. Therefore, the changes in NO production during ontogeny can be caused not only by endothelial NOS (NOS I) (as referred by Tsukahara *et al.* 1997a), but also by inducible NOS II expressed by blood phagocytes.

When experiments concerning ontogeny of RNS and the role of NO production during ontogeny are planned, not only endothelial (NOS I) but also inducible NOS II activity should be included. Constitutive NOS III expressed by neurons should not be omitted either, because changes in cerebral and cerebellar NOS III expression in guinea pigs and rats during ontogeny were found (Lizasoain *et al.* 1996).

Due to the ethical reasons, neither human nor animal studies include nitrites/nitrates status during prenatal period; however, some studies compared preterm and full-term animals, where preterm individuals probably reflect the nitrites/nitrates production before birth. Data from the literature indicate that preterm babies (Tsukahara *et al.* 1997a,b, Honold *et al.* 2000, Dzik *et al.* 2002) and preterm calves (Blum *et al.* 2001) produce higher amounts of nitrites/nitrates than full-term ones. This is in close relation to our findings that pig fetuses in the late phase of gestation (day 92 of gestation) had higher levels of nitrites/nitrates than newborn ones. It should however,be noted, that measurements of nitrites/ nitrates in humans were performed in urine and were recounted to urinary creatinine, whereas measurements in calves were performed directly in the blood plasma. It is questionable whether urinary nitrites/nitrates can fully reflect the levels of plasma nitrites/nitrates because it is known that renal function of neonates is different from that of adults (Hill and Lumbers 1988, Tsukahara *et al.* 1990). One publication, in which nitrites/nitrates were detected only in plasma, disagreed with all of the aforementioned results because no differences between preterm and full-term babies could be found (Biban *et al.* 2001).

No changes between prenatal and newborn production of NO by blood neutrophils were observed in our study, but the levels of nitrites/nitrates decreased during this period. Most plasma nitrites are excreted from the organism through urine. The possibility that the levels of plasma nitrites/nitrates can be increased independently from NO production by restricted removal of nitrites/nitrates from the blood stream should not be omitted.

Some authors have mentioned that increased NO production (expressed as increased level of nitrites/nitrates) is the mechanism of vascular tone regulation (Endo *et al.* 2001) because fetuses have lower blood pressure than postnatal individuals. Why plasma nitrites/nitrates are significantly higher on day 55 of gestation in comparison to day 92 of gestation is not clear, and whether the blood pressure is thus lower on day 55 than on day 92 of gestation remains unknown.

We can conclude that the production of RNS by peripheral blood neutrophils does not change during prenatal development. RNS production by peripheral blood phagocytes has decreasing trend during postnatal ontogeny (except for production of RNS by blood monocytes, which remained unchanged during the postnatal period). There are some similarities in development of RNS production by blood phagocytes and by plasma nitrites/nitrates but we cannot exactly determine if elevated plasma nitrites/nitrates in an earlier period are caused by NO production by peripheral blood phagocytes or if there is some other source of NO (e.g. endothelial cells or neurons). Thus the interpretation of these solitary data for understanding of natural immunity development cannot be exactly established. Further similar studies should be done to obtain more complete view of the RNS production during prenatal and early postnatal period of life.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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