

## Unusual interleukin-1 and -6 expression in fetal cartilage is associated with placental abnormalities

Franciszek Burdan<sup>1</sup>, Justyna Szumilo<sup>2</sup>, Agnieszka Korobowicz-Markiewicz<sup>3</sup>,  
Katarzyna Dyndor<sup>1</sup>, Michal Szumilo<sup>4</sup>, Robert Klepacz<sup>2</sup>

<sup>1</sup>Experimental Teratology Unit of the Human Anatomy Department; Medical University of Lublin, Lublin, Poland

<sup>2</sup>Clinical Pathomorphology Department; Medical University of Lublin, Lublin, Poland

<sup>3</sup>Pediatric Pulmonology and Rheumatology Department, Medical University of Lublin, Lublin, Poland

<sup>4</sup>Applied Pharmacy Department, Medical University of Lublin, Lublin, Poland

**Abstract:** Unusual expression of interleukin-1alpha, -1beta and -6 was previously found in the epiphyseal cartilage of rat fetuses prenatally exposed to various non-steroidal anti-inflammatory drugs (NSAID, i.e., ibuprofen, piroxicam, tolmetin) and selective cyclooxygenase-2 inhibitor (DFU). The aim of the present study was to evaluate the role of placenta in such phenomenon. Morphology of the organ, thickness of basal and labyrinth layer, immunoeexpression of COX isoenzymes were examined, and confronted with maternal biochemical data and fetal developmental parameters. Higher maternal urea level, as well as lower placental weight and labyrinth thickness were found in the group of fetuses who revealed expression of genes coded the selected interleukins, when compared with the xenobiotic-exposed pups without the selected genes expression and untreated control. A significant correlation between placental weight and maternal total protein or urea level was revealed. Histological changes like inflammatory infiltration and calcification were observed sporadically. Location and intensity of COX-1 staining was similar in all cases. However, more intense COX-2 staining for majority of cells of the basal zone and in dispersed giant cells of the labyrinth was found in inflamed organs. It could be concluded that abnormal expression of the selected interleukins is associated with low placental weight and decrease of its thickness, especially labyrinth zone, as well as with high maternal urea level.

**Key words:** NSAID, cyclooxygenase inhibitor, COX-1, COX-2, placental morphology, pregnancy, developmental toxicity

### Introduction

Anatomical and functional differences between fetal and mature immune system explain their various reactivity [1]. Since the fetal Th<sub>1</sub>/Th<sub>2</sub> cell balance is toward Th<sub>2</sub> cells, the immune system synthesizes mainly Th<sub>2</sub> cytokines, i.e., IL-4, IL-5, IL-9, IL-10, and TGF- $\beta$  [2-4]. Moreover, the production of pro-inflammatory molecules is decreased. Such adaptive mechanisms are responsible for the tolerance between maternal organism and fetoplacental complex, which could be regarded as a semi-allogeneic graft due to high level of paternal antigens [2-5]. In contrast, Th<sub>1</sub>/Th<sub>2</sub> imbalance

could increase Th<sub>1</sub> cytokines synthesis (i.e., IFN- $\gamma$ , TNF, IL-1, IL-2, IL-6) that may convert macrophages and NK cells into lectin-activated killers (LAK). The process is highly accelerated due to catalytic capacity of LAKs. Simultaneously, reduction of orexin-2 (OX-2) and increase of pro-thrombinase (fgl2) synthesis by trophoblastic cells occurs. Fgl2 inhibits OX-2 expression itself and converts pro-thrombin into thrombin that activates IL-8 synthesis by endothelial cells and stimulates neutrophils. Both processes are responsible for a vascular thrombosis and assault of the fetoplacental complex, respectively [3,4]. In such situation, the prolonged placental blood flow termination leads to embryo/fetal malnutrition that could manifest as intrauterine growth retardation (IUGR), developmental abnormalities or even embryo/fetal dead. All those biochemical and immunological anomalies may be influenced by various infectious, physical and psychological agents, as well as toxicants, including drugs [6].

**Correspondence:** F. Burdan, Experimental Teratology Unit of the Human Anatomy Department, Medical University of Lublin, Jaczewskiego 4 Str., PL-20090 Lublin, Poland; tel.: (+48) 603767649, fax.: (+4881) 5328903, e-mail: fb3@wp.pl

An unexpected expression of genes coded IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 was revealed in epiphyseal cartilages in a few rats fetuses in-utero treated with various non-selective (ibuprofen, piroxicam, tolmetin) and selective (DFU\*) cyclooxygenase-2 (COX-2) inhibitors [7]. Interestingly, the expression was found only in drug-treated groups, mostly exposed to low and middle doses, which were well-tolerated by both dams and fetuses (Table 1). It seems that due to immaturity of fetal immune system the only source of those cytokines could be the placenta or maternal circulation, and cytokines could cross the impaired placental barrier [2-4,8]. The present study was undertaken to evaluate the role of placenta in such phenomenon.

## Materials and methods

**Animals and study protocol.** The applied methodology was similar to other developmental toxicological studies and was described in details elsewhere [7,9-12]. Succinctly, 280 (20/group) time-pregnant Wistar CRL:(WI)WUBR rats were intragastrically treated on gestational day (GD) 8-21 with various COX inhibitors. Ibuprofen (8.5, 85.0 and 400.0 mg/kg/dose) and tolmetin (8.5, 85.0 and 850.0 mg/kg/dose) were administered three-times a day, while DFU (0.2, 2.0 and 20.0 mg/kg/dose) and piroxicam (0.3, 3.0 and 30.0 mg/kg/dose) were given once daily. Animals in control groups received the Tween 80 water suspension in volume corresponding to those given (10 ml/kg) in drug-treated groups (Table 1). On GD 21 dams were sacrificed, their blood was collected, and abdominal organs were delivered. The maternal blood was centrifuged and alanine and aspartate aminotransferase activity, as well as urea and total protein level were determined in the sera. Ovarian corpora lutea, resorption sites, dead or live fetuses were counted. Fetuses were removed from uterus, separated from placenta, and routinely teratologically examined [9]. The weight of fetuses and placentas, and the fetal crown-rump length were checked. Skeletal and internal organ morphology, as well as various histological, immunohistochemical [7] and radiological [12] fetal examinations were also performed.

One well-developed, randomly chosen male fetus and its adjusted placenta was taken from 10 randomly selected litters of each experimental group. However, due to maternal mortality, the final number of evaluated litters in groups exposed to the highest doses of tolmetin and ibuprofen was only 4 and 6, respectively.

**Gene expression.** The proximal and distal femoral fetal epiphyses were separated and their RNA was extracted. The expression of genes coded collagen type I and X, COX-1, COX-2, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, osteopontin, osteocalcin, TNF- $\alpha$ , TNF- $\beta$ , L32 and GAPDH was checked using RNase Protection Assay (RPA) with custom multi probe template sets (PharMingen Laboratory; San Diego CA, USA) according to manufacturer directions. The level of mRNA was estimated using computer software TotalLab (BioSystematica, UK), in reference to the mRNA level of the house keeping gene – L32 [7].

**Immunohistochemistry.** All the selected placentas were cut transversally on the level of the umbilical cord adjustment, fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned at 5  $\mu$ m and routinely stained with hematoxylin and eosin (H+E). Immuno-

**Table 1.** Characteristic of litters/fetuses selected to the current study

	n	Dose (mg/kg/day)	Expression of		
			IL1 $\alpha$	IL1 $\beta$	IL6
Control (C) <sup>a</sup>	10	–	–	–	–
Tolmetin	10	25.5	–	–	1
	10	255.0	–	–	–
	4	2550.0	1	1	–
Ibuprofen	10	25.5	2	–	–
	10	255.0	–	–	–
	6	600	–	–	–
Control (Cx) <sup>a</sup>	10	–	–	–	–
Piroxicam	10	0.3	2	1	1
	10	3.0	–	–	–
	10	30.0	–	–	–
DFU	10	0.2	2	2	1
	10	2.0	1	1	1
	10	20.0	–	–	–

<sup>a</sup>C and Cx groups were pulled into one common control group due to lack of statistical differences in both maternal and fetal parameters

histochemical reactions for both COX isoforms were performed on the 4  $\mu$ m slides obtained from the paraffin blocks previously used for histological examination. Monoclonal mouse anti-human antibodies against COX-1 and COX-2 (clones 12E12 and 4H12, respectively; Novocastra; Newcastle, UK) and DakoEnvision<sup>TM</sup>/HRP kit (DakoCytomation, Glostrup, Denmark) were applied. The details of the method were described elsewhere [13]. Slides were evaluated without knowledge of the treatment groups, using light microscope (Olympus BX45; Tokyo, Japan). The thickness of the basal (junctional) zone and the labyrinth of the placenta was measured using the eyepiece micrometer at the objective 10x (Fig. 1). The obtained results were expressed as absolute (in millimeters) and relative values (vs. the whole placental thickness).

**Ethical issues.** All the evaluated samples and data were collected during the previous study that was designed in accordance with international guidelines for teratological studies [9] and fully approved by the Local Bioethical Committee (#372/2002).

**Statistical analysis.** Unlike typical teratological studies, the unit for statistical measurement was the pregnant female, placenta or single, male fetus selected for the genetic and histological evaluation [9]. Homogeneity of distribution was examined using Kolmogorov-Smirnov test. Differences of normal distribution data were analysed by ANOVA and followed by Duncan test. Inhomogeneous distribution data was analysed by Mann-Whitney U test. The nominal scale measures were analyzed by Chi-square test with Yates' correction for independent differences among experimental groups. Spearman rank test was employed to evaluate correlations between various factors. The 0.05 level ( $p < 0.05$ ) of probability was used as the criterion of significance. Data were analyzed using STATISTICA 5.0 on IBM compatible computer.

\* 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone

**Table 2.** Selected maternal, fetal and placental parameters in the control and drug-exposed groups according to ILs expression

	Control	Drug-exposed groups	
		IL absence	IL presence
Number of animals/placentas	20	100	10
Alanine aminotransferase (IU/l)	60.80±8.17	66.00±18.93	59.90±9.79
Aspartate aminotransferase (IU/l)	155.10±38.81	161.64±60.02	139.60±51.24
Total protein (g/l)	61.66±6.35	58.39±5.44	57.63±6.18
Urea (mmol/l)	7.09±0.92	8.27±0.95*	8.01±1.23* <sup>#</sup>
Fetal weight (g)	3.68±0.14	3.60±0.18	3.55±0.11
Fetal length (mm)	37.31±1.34	35.99±5.87	36.46±1.64
Placental weight (g)	0.57±0.62	0.56±0.06	0.47±0.04* <sup>#</sup>
Placental weight/fetal weight (%)	15.54±1.58	15.36±2.19	13.17±1.37* <sup>#</sup>
Absolute thickness (mm)			
placental	2.97±3.16	2.96±0.41	2.62±0.35* <sup>#</sup>
– basal zone	0.72 ±0.87	0.70±0.13	0.71±0.10
– labyrinth	2.25±0.27	2.26±0.34	1.91±0.25* <sup>#</sup>
Relative thickness of labyrinth (%)	75.49±2.37	76.16±3.59	72.98±1.25* <sup>#</sup>

\*p<0.05 vs. control; #p<0.05 vs. IL absence

**Table 3.** The fetal incidence in corresponding to normal<sup>a</sup> (95CI) and abnormal (<95 and >95CI) value of selected maternal and placental parameters in drug-exposed groups according to ILs expression.

		<95CI	95CI	>95CI
Urea (6.66–7.72 mmol/l) <sup>a</sup>	IL absence	4/100	12/100	84/100
	IL presence	2/10	1/10	7/10
Placental weight (0.54–0.60 g) <sup>a</sup>	IL absence	33/100	23/100	44/100
	IL presence	9/10	1/10	0/10
Relative labyrinth thickness (74.39–76.60%) <sup>a</sup>	IL absence	30/100	25/100	45/100
	IL presence	9/10	1/10	0/10

<sup>a</sup>the normal value was calculated as 95CI in the common control group

## Results

For the final statistical analysis data from 130 dams, corresponding fetuses and placentas (including 20 from control groups) was taken.

The urea level was significantly lower in both drug-exposed groups when compare with the control group (Table 2). However, it was higher in the group of dams whose pups exhibited expression of interleukins in the epiphyseal homogenates. Lack of statistical differences was found for other biochemical parameters, as well as both fetal developmental parameters, i.e., the fetal weight and the crown-rump length. Placental weight was significantly lower in the group of fetuses who revealed expression of genes coded selected interleukins in cartilages then in other two groups (Table 2).

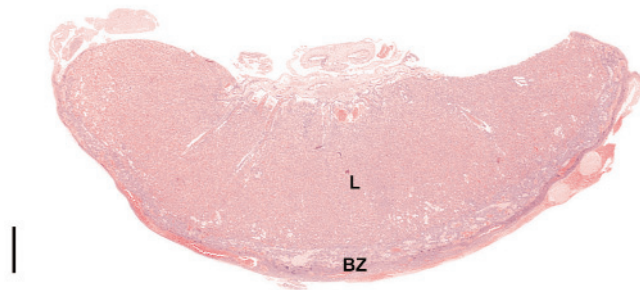
In the group with expression of interleukin genes most of the fetuses were obtained from mothers with higher serum urea level, as well as with low placental

weight and thickness of the labyrinth (Table 3). When maternal and feto-placental parameters were simultaneously evaluated, in the group without interleukin genes expression a statistically significant correlation between placental weight and urea or total protein level was revealed (Fig. 2).

The evaluated placentas showed morphology typical of hemochorial – chorioallantoic ones, with well developed basal zone and labyrinth. The basal zone contained three main types of cells, i.e., undifferentiated, glycogen and giant trophoblasts cells, as well as maternal vascular channels. Whereas labyrinth was composed of dense network of lacunas filled with maternal blood formed by the septa consisted of three layers of trophoblasts and vascular fetal mesenchyma (Fig. 1, 3). In majority of cases mild inflammatory infiltration composed of neutrophils and degenerated/necrotic cells were found in the decidua basalis and nearby basal zone of the placenta. However, in all pla-



centas from group exposed to the highest dose of tolmetin, six from group treated with middle dose of piroxicam, four from control and one from group received middle dose of DFU, the infiltration was severe and observed both on the fetal aspect of the pla-

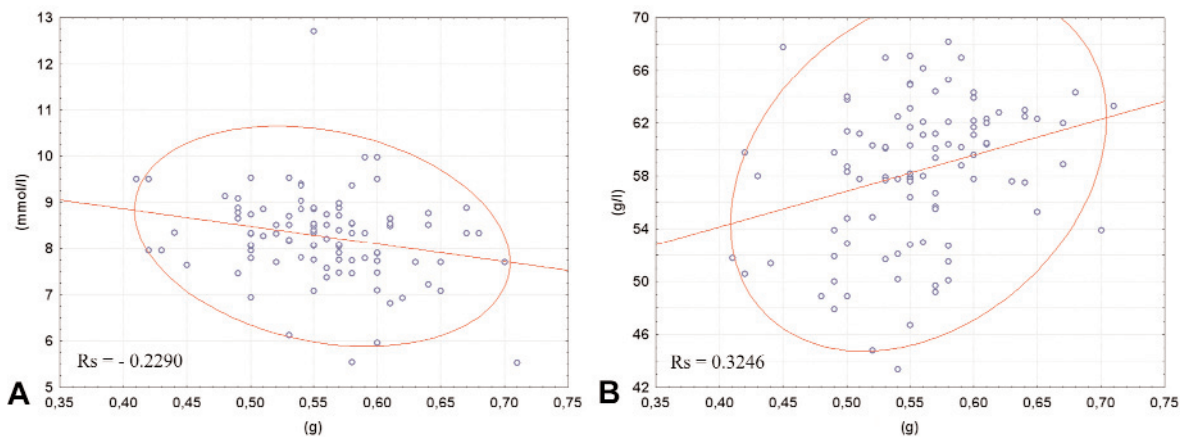


**Fig. 1.** The transversal section of the rat placenta; BZ – basal zone, L – labyrinth (piroxicam 3.0 mg/kg/d H+E; bar – 1 mm).

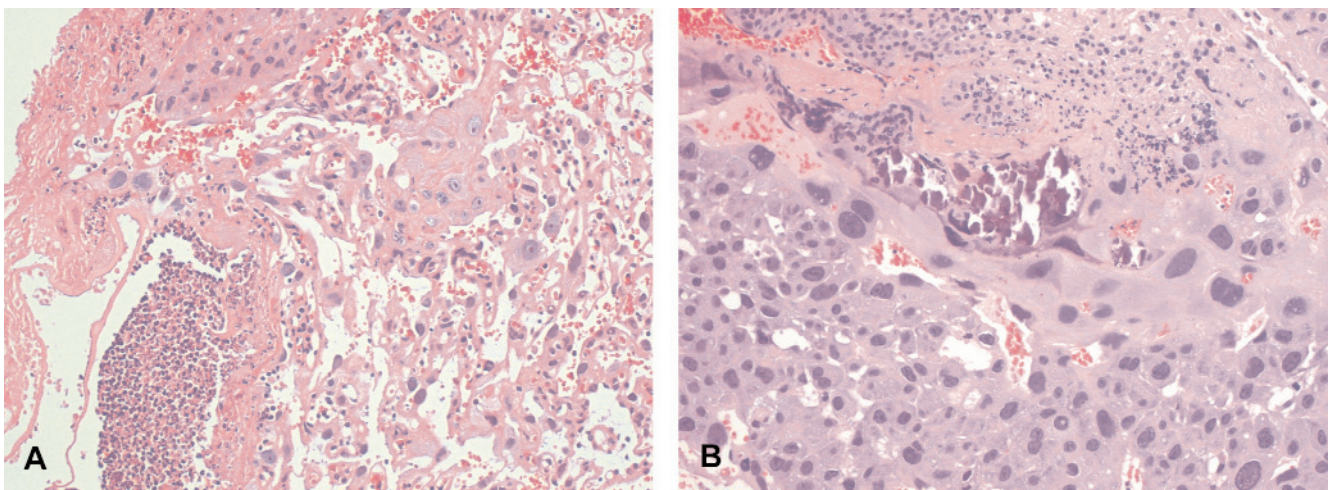
centa and deeply within both zones of the organ (Fig. 3A). Small foci of calcification were noted in the maternal-fetal interface in three pups exposed to middle dose of piroxicam (2) and highest dose of tolmetin (1) (Fig. 3B).

Unlike unaffected thickness of the basal zone, the transversal diameter of the whole placenta and the labyrinth was significantly lower in the group of rats with expression of genes coded selected interleukins (Table 2).

The distribution and intensity of immunostaining for COX-1 in placentas was similar in all groups. Except for erythrocytes, strong cytoplasmic reaction was seen in virtually all cells (Fig. 4A, B). Immunostaining for COX-2 was usually very weak and confined mostly to trophoblasts cells (layer 1) and endothelial cells of the labyrinth (Fig. 4C). However in placentas infiltrated by neutrophils, a strong nuclear staining was also revealed in majority of cells of the basal zone and in dispersed giant cells of the labyrinth (Fig. 4D).

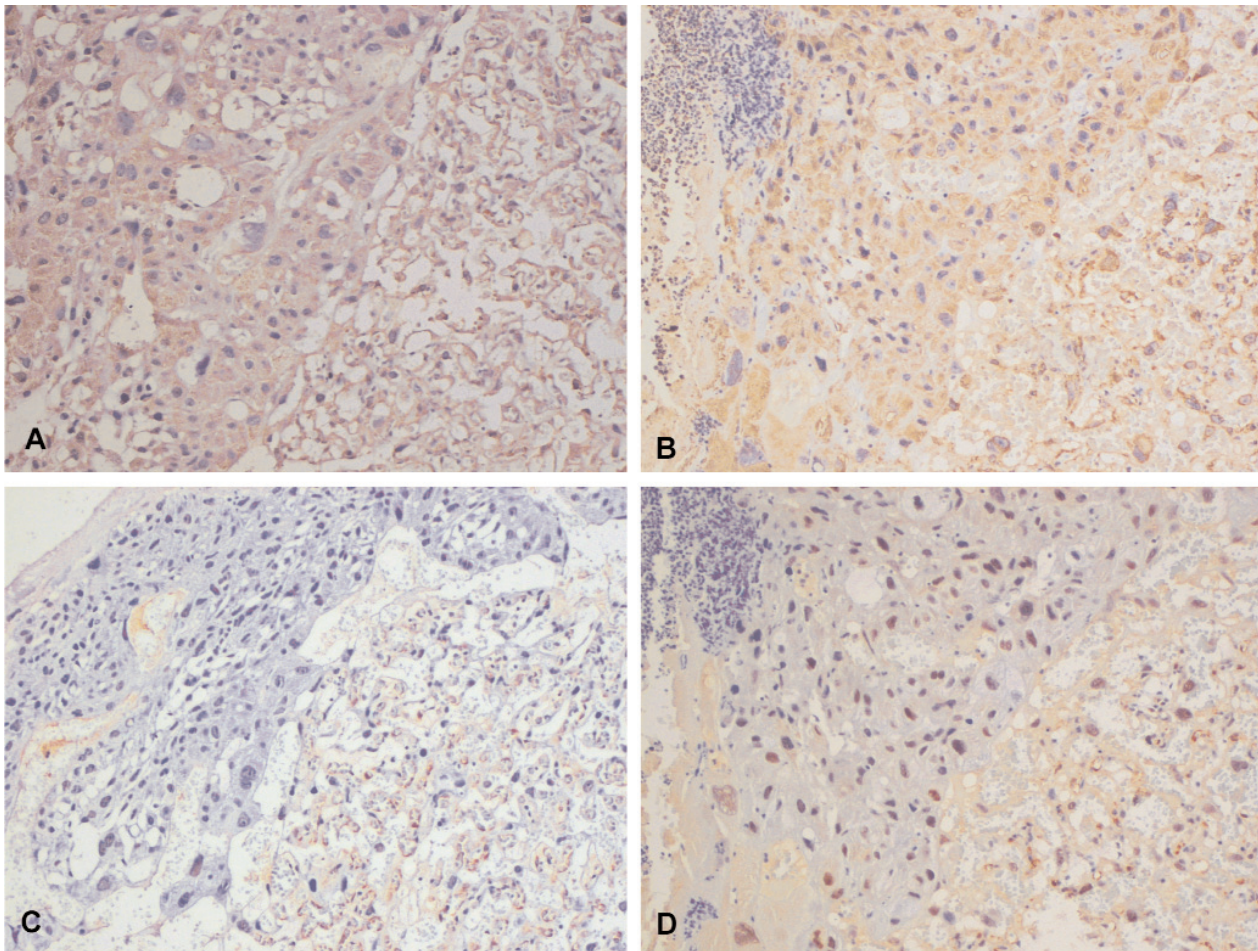


**Fig. 2.** Correlation between placental weight and maternal urea (A) or total protein (B) level in rat fetuses prenatally exposed to cyclooxygenase inhibitors without expression of IL-1 $\alpha$ , IL-1 $\beta$  and IL-6. Rs – Spearman rank R.



**Fig. 3.** Neutrophils on the fetal aspect of the placenta (A) and calcification in the basal zone of the placenta (B) (H+E; A – DFU 2 mg/kg/d, objective 10 $\times$ ; B – tolmetin 2550 mg/kg/d, objective 20 $\times$ ).





**Fig. 4.** Immunohistochemical reaction for COX-1 (A, B) and COX-2 (C, D) in normal placenta (A, C) and infiltrated by neutrophils (B, D) (A, C – ibuprofen 25.5 mg/kg/d; B, D – tolmetin 2550.0 mg/kg/d; DakoEnvision<sup>TM</sup>+/HRP, objective 10x).

## Discussion

The obtained results indicated that unusual expression of *IL-1 $\alpha$* , *IL-1 $\beta$*  and *IL-6* was seen in fetuses delivered by mothers with an abnormal urea level. The group was also characterized by a low placental weight and thinner labyrinth. Histological abnormalities, mostly dense inflammatory infiltration and/or sparse foci of calcifications were revealed occasionally in all three evaluated groups, including common control one. However, in animals exposed to the highest dose of tolmetin, placental changes were found in all the examined litters. On the other hand, placental histological abnormalities were revealed only in four out of 10 organs obtained from fetuses with a positive interleukins expression, as well as in 12/100 organs of pups without genetic abnormalities and exposed to the examined COX inhibitors. A lower frequency (4/20) was found in the common control group. Nevertheless, it has to be stated that mild inflammation and focal necrosis of placenta are physiological findings that could be observed also in healthy organs from GD 17 until partum [14]. Lack of any serious abnormalities typical for well-known placental toxicants,

like methyl parathion was found [15]. The inflammatory process explains the strong immunoexpression of the inducible COX isoform [16,17], while more intensive staining for both, COX-1 and COX-2 of the basal zone in the late gestation is physiological [18].

Interestingly, the previous results suggested that among the whole group of animals exposed in-utero to COX inhibitors, the abnormal urea and/or total protein level was associated with increase incident of postimplantation loss and IUGR, low placental weight, increase of skeletal developmental malformations and variations [19], as well as decrease of vertebrae mineralization [12]. Such data was also partially proved for domestic animals fed with diet rich with degradable proteins that generate a high level of urea in maternal blood [20,21]. However, a maternal toxicity can not be taken into consideration as a leading mechanism that explains interleukin expression, since the phenomenon was observed mostly in fetuses obtained from mothers without gross and histological signs of adverse effects of the examined xenobiotics [10,11].

Although, damage of placental barrier was not proved on histological level, such mechanism is the only one that

could explain positive *IL-1 $\alpha$* , *IL-1 $\beta$*  and *IL-6* expression in fetal cartilages. Since well-vascularised labyrinth was the only affected part of the placenta, the mechanism may be related to homodynamic changes in arteries that supply the organ. Due to high COX activity in endothelial cells and thrombocytes, the examined drugs could affect prostacyclin (PGI) and thromboxan (TXA2) synthesis, respectively. Secondary to PGI/TXA2 modulation, the local haemostatic changes occur, and temporary or permanent vascular occlusion could be generated [16,17,22]. The ischemia-reperfusion (I/R) injury itself is a pivotal mechanism of IUGR and pre-eclampsia [22]. It could also promote pathological changes that generate maternal-fetal blood shunt [6,22]. Yamazaki *et al.* [23] reported that I/R injury increase the placental COX-2 and EP3 receptor expression that may induce IUGR through the prostaglandin E2 (PGE2) stimulation. However, according to the authors the fetal effect was time-dependent since significant changes in fetal weight were revealed not earlier than 5 day after the uterine artery was closed. Furthermore, the thrombo-embolic episodes are well-documented complications of some of the selective COX-2 inhibitors therapy and were the main reason for their withdrawal from the market. A new data suggests that similar adverse effects could be observed in patients taking nonselective compounds, as well [24,25]. It should be also emphasized that COX inhibitors decrease synthesis of PGE2, one of the strongest vasoconstrictor, which stimulates mainly EP3 receptors [16,17]. Because there is a dualistic influence of the examined drugs on haemostase, other studies are needed to find the main mechanism of placental injury. However, without maternal blood and placental interleukin levels, as well as local haemostatic examination such divergences are pure theoretical, since the cytokine source is unknown. An unusual stimulation of the fetal immune system with secondary activation of Th<sub>1</sub> cytokines seems even less likely, especially since histological skeletal changes were observed occasionally in the examined rat population [7]. On the other hand, most of the currently studied fetuses showed a positive expression of TNF [7], that is Th<sub>1</sub> cytokine promoting the immune response to infection [2,26,27]. Nevertheless, since TNF plays a crucial role in various processes such as organogenesis and initiation of partum of the placenta [28], its expression in fetal tissues is physiological through the mid and late pregnancy [27]. Its concentration could be increased by various pathological factors that also stimulate other Th<sub>1</sub> cytokines, like IL-1 and -6, which may damage fetal and mature organs [27,29-31]. Such mechanism was experimentally confirmed by Bell *et al.* [2]. The authors injected lipopolysaccharide into the uterine cervix of pregnant rats and found an increase of TNF concentration in the placenta and the fetal brain, while the level of IL-1 was increased only in the placenta and INF- $\gamma$  exclusively in the brain. Due to the fact that all those factors are respon-

sible for the neuronal degeneration [26,28], it is postulated that Th<sub>1</sub> cytokines may induce congenital abnormalities of the central nervous system. Since TNF and other cytokines do not cross the healthy placenta [32], the infected organ has to be the only source of the Th<sub>1</sub> cytokines, especially TNF and IL-6. The role of intrauterine infection in promotion of the brain congenital abnormalities has been already proved in experimental animal models [33,34], but in none of the study placental morphology was evaluated. However, Silen *et al.* [8] revealed necrosis in both labyrinth and basal zones, as well as in some organs of fetuses delivered by dams interperitoneally injected with native IL-1 $\alpha$  and TNF.

It should be also noted that low placental weight is one of the most commonly affected developmental parameter, observed in various teratological studies [6,9]. Dose-dependent, however insignificant differences were revealed in groups treated with COX inhibitors in the currently examined rat population [10,11] and in some previous studies [6,9,35]. So-called placental toxicity is a major factor explaining developmental complications of arsenic, cadmium, chloroform, enalapril, trypan blue and methyl parathion [6]. All the compounds cause placental necrosis and enzymatic changes that may compromise placental function. Affecting gas exchange, nutrition uptake and abnormal hormonal synthesis were also revealed during exposition to other chemicals, but none of the studies evaluated Th<sub>1</sub> cytokines in the fetal tissues [3,6,9,15].

Lack of the differences in fetal weight and length may be caused by the method used to select the pups for genetic and histological examinations. Since only one well-developed fetus from the litter without any signs of external malformations and IUGR was evaluated, the obtained results could be false negative and other studies have to be taken to completely explain this phenomenon.

On the basis of obtained results, it could be concluded that abnormal expression of selected interleukins is associated with low placental weight, decrease of placental thickness, particularly labyrinth, as well as with high urea level in maternal blood. Other studies, especially multi-generation ones, are required to explain clinical implications of this phenomenon [9,36,37]. An additional evaluation of specific placental hormones and proteins [30,38] will be also desirable for the future human risk assessment [9,30,36,37,39,40]. Histological placental examination seems to be important scientific procedure, especially in cases the organ weight and fetal abnormalities are revealed. Presently, according to international principles for developmental toxicological evaluations it is only an accessory facultative test [9].

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