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Association of maternal pancreatic function and foetal growth in rats treated with DFU, a selective cyclooxygenase-2 inhibitor

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Constitutive (COX-1) and inducible (COX-2) cyclooxygenase isoforms have been detected in various mammalian tissues. Their activity is blocked by non-steroidal anti-inflammatory drugs that may induce various side reactions. The aim of the study was to evaluate the effects of DFU, a selective COX-2 inhibitor, on exocrine and endocrine pancreatic function and the immunoexpression of both COX isoforms in maternal and foetal rat pancreases. The compound was administered to pregnant Wistar rats once daily from the 8th to the 21st day of gestation. Glucose level and amylase activity were determined in the maternal sera. Maternal and foetal pancreases were examined histologically. Immunoexpression of COX-1 and COX-2 was also evaluated. Both biochemical parameters, as well as the histological structure of the pancreas were undisturbed in the dams and their foetuses. The maternal glucose level was found to be an important factor for foetal growth. Strong cytoplasmic COX-1 immunostaining was observed in acinar secretory cells, whereas in islets the immune reaction was weak. Endocrine cells also revealed strong cytoplasmic COX-2 staining in the maternal and foetal pancreases. Acinar cells exhibited nuclear reaction, which was strong in the foetal but weak in the maternal pancreases. No differences in COX immunoexpression were found between the DFU-exposed and the control groups in either mothers or foetuses. It should be stressed that DFU administered throughout mid and late pregnancy in rats did not change maternal or foetal pancreatic morphology or immunoexpression of either of the main COX isoforms in the organ.

Key words: coxibs, cyclooxygenase, diabetes, foetus, glucose, NSAID, macrosomia, pregnancy

INTRODUCTION

The two main isoforms of cyclooxygenase (COX), namely constitutive (COX-1) and inducible (COX-2), have been detected in various mammalian tissues and organs. The third one (COX-3), also known as central, has almost exclusively been detected in the central nervous system, in particular structures involved in the general sensory neuronal pathway. The enzyme initiates prostanoid (prostaglandin, prostacyclin and thromboxane) synthesis and its activity is

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inhibited by the specific reversible or irreversible COX inhibitors. The non-selective COX inhibitors (e.g., ibuprofen and naproxen) block both constitutive and inducible isoforms, while the selective ones (e.g., DFU and rofecoxib) inhibit inducible isoform only. However, in higher than therapeutic doses COX-1 inhibition could appear as well [3, 12, 24].

A number of animal and human studies have indicated that a decrease in COX-1 activity induces various side reactions, including gastrointestinal and renal toxicity and others which depend on the blockade of the housekeeping functions of the inducible isoform [3, 12]. However, the constitutive expression of COX-2 has also been found in some organs. It should be noted that high expression of this isoform is typical of foetal organs and decreases rapidly after delivery. In mature tissues the highest expression of COX-2 is characteristic of the macula densa, pancreatic islets and some structures of the central nervous system [12, 28, 31].

In previous studies COX-1 immunoexpression in the pancreas has been limited to acinar secretory cells, while COX-2 was found in secretory cells of the endocrine pancreatic islets and in epithelial cells of the pancreatic ducts and smooth muscle cells of the blood vessels of the pancreatic stroma [6, 21, 31]. On the other hand, Zabel-Langhennig et al. [32] revealed that both COX-1 and COX-2 isoenzymes are synthesised in rat pancreatic acinar cells. The differences are probably secondary to methodology, since the authors examined both isoenzymes on the mRNA level [32]. It was also reported that indomethacin, a non-selective COX-inhibitor, decreased basal, glucose- and glucagon-stimulated acute insulin response [31]. According to Koliopanos et al. [21] pancreatic islets displayed a variable COX-2 staining pattern, which was associated with the distribution of insulin-positive cells and with the clinical diabetes mellitus status. In normal insulin production or latent diabetes COX-2 immunoreactivity was found, whereas in diabetic patients the COX-2 expression was decreased or absent from the pancreatic islets.

The present study was undertaken to evaluate the effects of DFU (i.e., 5,5-dimethyl-3-(fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone), a selective COX-2 inhibitor, on exocrine and endocrine pancreatic functions and immunoexpression of the constitutive and inducible cyclooxygenase isoforms in the rat pancreas during pregnancy. The data were collected during a large scientific project undertaken to evaluate the prenatal toxicity of the examined compound, and the article partially covers foetal findings previously published in detail [8]. Since COX-2 is expressed almost exclusively in islet parenchyma [3, 6, 21, 31], the influence of the maternal glucose level on foetal and placental development was also studied.

MATERIAL AND METHODS

The study was conducted on sexually mature albino rats of the Wistar CRL:(WI)WUBR strain and approved by the Local Bioethical Committee (guideline No.372/2002).

All the animals were obtained from an accredited breeder (Warsaw-Rembertow, Poland), and housed and maintained in an animal care facility. On mating days females (weight 200–250 g) were placed in cages with males (5:2) for approximately 14 hours. The following morning a vaginal smear test was performed to determine if copulation had occurred. The day when sperm was found was designated gestation day 1. Sperm-positive females were randomly selected to the drug-treated and control groups.

DFU (Merck Research Laboratory, a gift from Merck & Co. Inc., Rahway NJ, USA; purity > 99%) was intragastrically administered to pregnant Wistar rats (n = 20/group) once daily from the 8th to the 21st day of gestation in the following doses: 0.2, 2.0 and 20.0 mg/kg/dose. Animals in the control group (n = 20) received the Tween 80 water suspension in a volume that corresponded to the drug-exposed groups (10 ml/kg).

On gestation day 21 the animals were anaesthetised and blood was taken from the beating heart. The dams were sacrificed by decapitation. During the autopsy the internal organs were removed and examined. The foetuses were removed and separated from the placenta. The weights of the foetuses and placentae, as well as the foetal crown-rump lengths, were individually checked. The body mass indices (BMI) were calculated: BMI [kg/m^2] = body weight/(crown-rump length)^2. Comprehensive teratological measurements and observations were performed [8].

The maternal blood was placed in standard plastic test tubes, and centrifuged. The amylase and glucose levels were determined using colorimetric enzymatic methods and commercial laboratory kits by Cormay (Poland).

The maternal and foetal pancreatic samples were fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned at 5 mm and then stained routinely with haematoxylin and eosin.

The immunohistochemical reactions for COX-1 and COX-2 were performed on 4 μm slides obtained

from the paraffin blocks used previously for histological examination. After dewaxing and rehydration the slides were placed for three cycles of heating in a microwave oven (750 W) for 5 min in citrate buffer (0.01 M, pH 6.0) for antigen retrieval. Endogenous peroxidase activity was then blocked with 3% hydrogen peroxide for 5 min and the slides were incubated for 60 min with primary mouse monoclonal anti-human antibodies (Novocastra; Newcastle, UK) against COX-1 (clone 12E12, dilution 1:20) and COX-2 (clone 4H12, dilution 1:200). The next step was incubation with DakoEnvision™+/HRP, Mouse kit (DakoCytomation; Glostrup, Denmark) according to the manufacturer's directions. The specific immune reaction was visualised by 3',3-diaminobenzidine tetrahydrochloride (DAB) (DakoCytomation; Glostrup, Denmark), and finally the sections were counterstained with Mayer's haematoxylin. TBS buffer rinsing was used after each step. The whole procedure was performed at room temperature. In all the cases the appropriate positive and negative controls were performed. Sections treated in the same way but with mouse pre-immune serum without the examined primary antibodies were used as negative controls. For the positive COX-1 and COX-2 controls human colonic mucosa and osteochondroma respectively were applied. Before starting the immunohistochemical study proper, the cross-reactivity with rat tissues was verified. All the slides were evaluated, without knowledge of the group treated, under a light microscope (Olympus BX45; Tokyo, Japan).

Quantitative continuous data were compared between the experimental groups using the Kolmogorov-Smirnov test. Differences in continuous variables were evaluated by the ANOVA, ANOVA Kruskal-Wallis and Student's t tests. If the data were not normally distributed, the Mann-Whitney U test was employed. The nominal scale measures were analysed by the χ^2 test with Yates' correction for independence for differences among treatment groups. On the basis of the normal glucose level, calculated as a 95% coefficient interval (CI) of the glucose level in the control group, the drug-exposed litters were divided into three groups, those below, those over and those at the 95% CI level. Correlations between all the examined parameters were analysed by the Spearman rank R test. A 0.05 level ($p < 0.05$) of probability was used as the criterion of significance.

RESULTS

No maternal mortality was noted in either the drug-treated or the control groups. Maternal body weight gain, as well as food and water intake differed insignificantly from the control group. Except for sporadic enlargement of the intestinal loops and a small amount of peritoneal fluid, no other signs of gastrointestinal toxicity were found during necropsy. However, in a few cases gastric and intestinal injuries of varying severity, including one deeply penetrating ulcer, were revealed microscopically. Mild hepatic and renal abnormalities were also noted during histological examination. A statistically significant decrease in foetal body length observed in the groups exposed to the highest dose of DFU was the only sign of developmental toxicity of the tested substance (Fig. 1). A detailed report on the gastrointestinal, hepatic and renal effects and foetal observations are presented in detail elsewhere [7, 8].

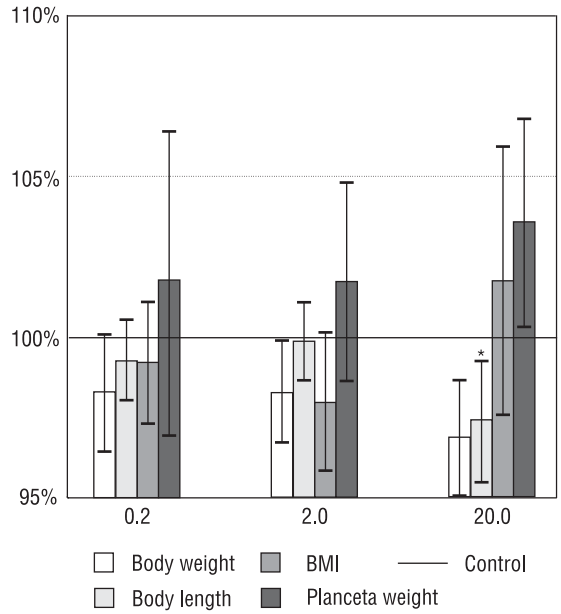

Figure 1. Relative (%; mean ± SD) body weight and length, body mass index (BMI), as well as placental weight in group exposed to DFU [mg/kg] when compared with the corresponding control value (*$p < 0.05$ *vs.* control).

In none of the drug-exposed groups was the glucose level significantly altered when compared with the controls (Table 1). However, a slight increase in glucose level was found in the group treated with the tested compound (Fig. 2). No significant changes in amylase activity were noted.

A statistically significant negative correlation [Spearman rank (Rs) = –0.4581] between maternal glucose level and foetal weight was found in the control group. A positive correlation (Rs = 0.5088) was also revealed between foetal weight and length. A similar but less distinct (Rs = 0.3907)

Table 1. Absolute amylase activity and glucose level in maternal sera in control and DFU-treated groups

	DFU [mg/kg]	Min.	Max.	Mean	SD	Median	p^1	p^2
Amylase [IU/l]	–	3249.00	11714.9	7017.11	2537.47	6948.60	–	–
	0.2	3493.00	11093.4	7256.22	2337.12	7313.90	0.796	0.992
	2.0	3078.90	12697.2	7152.47	2835.52	7390.60	0.870	0.992
	20.0	3441.70	11861.9	7214.35	2676.87	7418.10	0.824	0.992
Glucose [mmol/l]	–	112.00	308.0	217.80	56.33	212.50	–	–
	0.2	165.00	465.0	234.05	78.34	204.50	0.499	0.899
	2.0	143.00	457.0	226.60	71.54	217.00	0.684	0.899
	20.0	145.00	398.0	228.45	63.97	211.00	0.645	0.899

Min. — minimum, Max. — maximum, SD — standard deviation, [1]p value when compared with the control value, [2]p value in the whole group exposed to DFU when compared with the control value

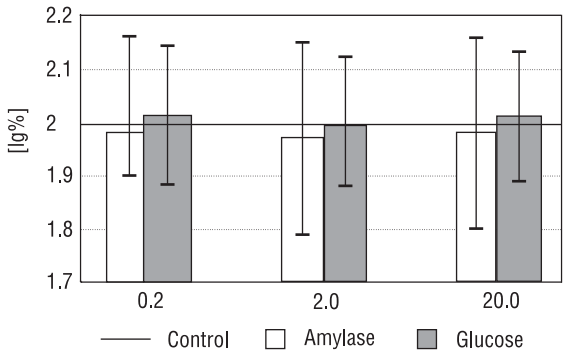

Figure 2. Relative (lg% — logarithm%; Mean ± SD) amylase activity and glucose level in group exposed to DFU [mg/kg] when compared with the corresponding control value.

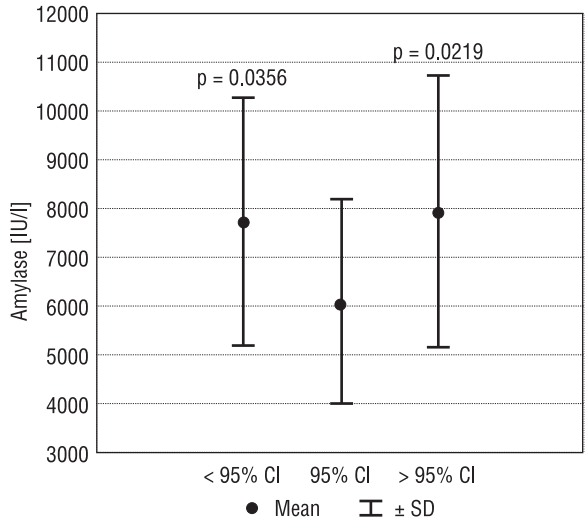

Figure 3. Absolute amylase activity in mothers treated with DFU with low (< 95% CI), normal (95% CI) and higher (> 95% CI) glucose levels (p value *vs*. group with normal glucose level).

correlation between both foetal parameters was observed in the common group that contained all the DFU-treated litters. However, a statistically significant correlation (Rs = 0.2772) was only found between maternal glucose level and foetal/maternal weight ratio. When the whole group was divided into three, corresponding to the normal glucose level (191.44–244.16 mmol/l) calculated as 95% of the coefficient interval of values in the control group, insignificant correlations between maternal and foetal parameters were revealed in litters characterised by glucose levels below or over the control values. The only positive correlation was revealed for foetal length and glucose level (Rs = 0.4932) in litters with the control glucose level. In all the groups examined a significant correlation was seen between BMI and foetal length and/or weight. No significant correlation was consistently noted between placental weight and glucose level and amylase activity. The only statistically significant correlation (Rs = –0.3054) between maternal amylase activity was noted for dam's body weight gain in the common DFU-exposed group. However, it should be stressed that an increase in enzyme activity was found in the drug-treated mothers with lower and higher glucose activity (Fig. 3).

It was also shown that in the control group a greater placental weight was found in litters obtained from mothers with high body weight gain and high glucose levels (Fig. 4A). In the DFU-treated groups a greater placental weight was observed in litters with low glucose levels (Fig. 4B). No similar data were revealed for foetal weight and length in the control groups (Fig. 4C, E). However, a high glucose level and low maternal weight gain predicted high foetal weight, while high foetal length was observed in litters with a high

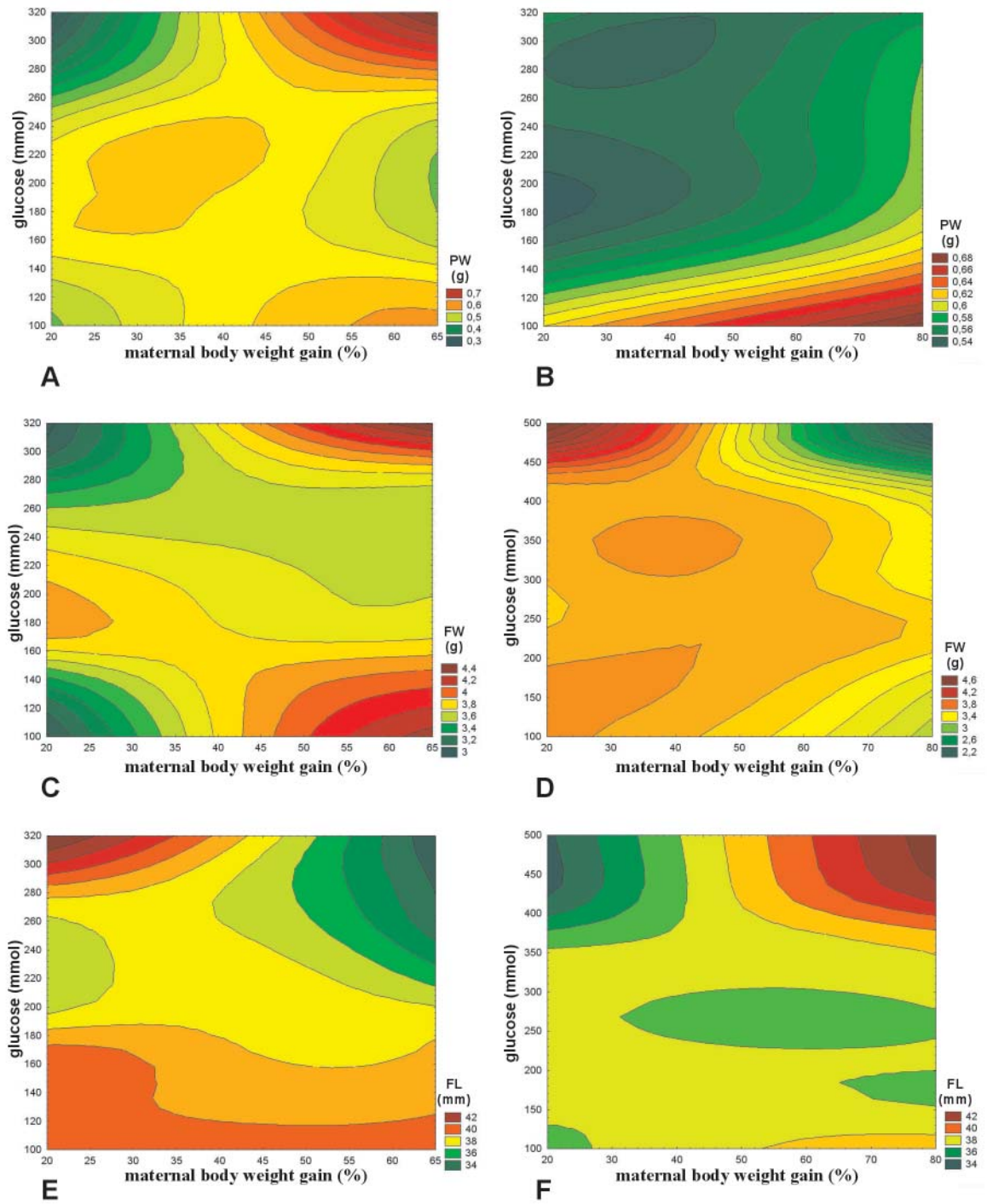

Figure 4. Placental (**A, B**) and foetal (**C, D**) weights [g], as well as foetal length [mm] (**E, F**) in relation to relative maternal body weight gain (%) and absolute glucose level [mmol/l] in control (A, C, E) and DFU-exposed (B, D, F) groups; PW — placental weight; FW — foetal weight; FL — foetal length.

glucose level and a high maternal weight gain (Fig. 4D, F).

The histological structure of the pancreas was found to be undisturbed in all the slides examined from the DFU-exposed and control groups, in both mothers and foetuses (Fig. 5A, B). Strong cytoplasmic COX-1 immunostaining was seen in pancreatic acinar secretory cells, whereas in the endocrine cells of islets the immune reaction was weak (Fig. 5C, D). Endocrine cells were strongly COX-2-positive in the maternal and foetal organs (Fig. 5E, F). In all the cases the staining pattern was cytoplasmic.

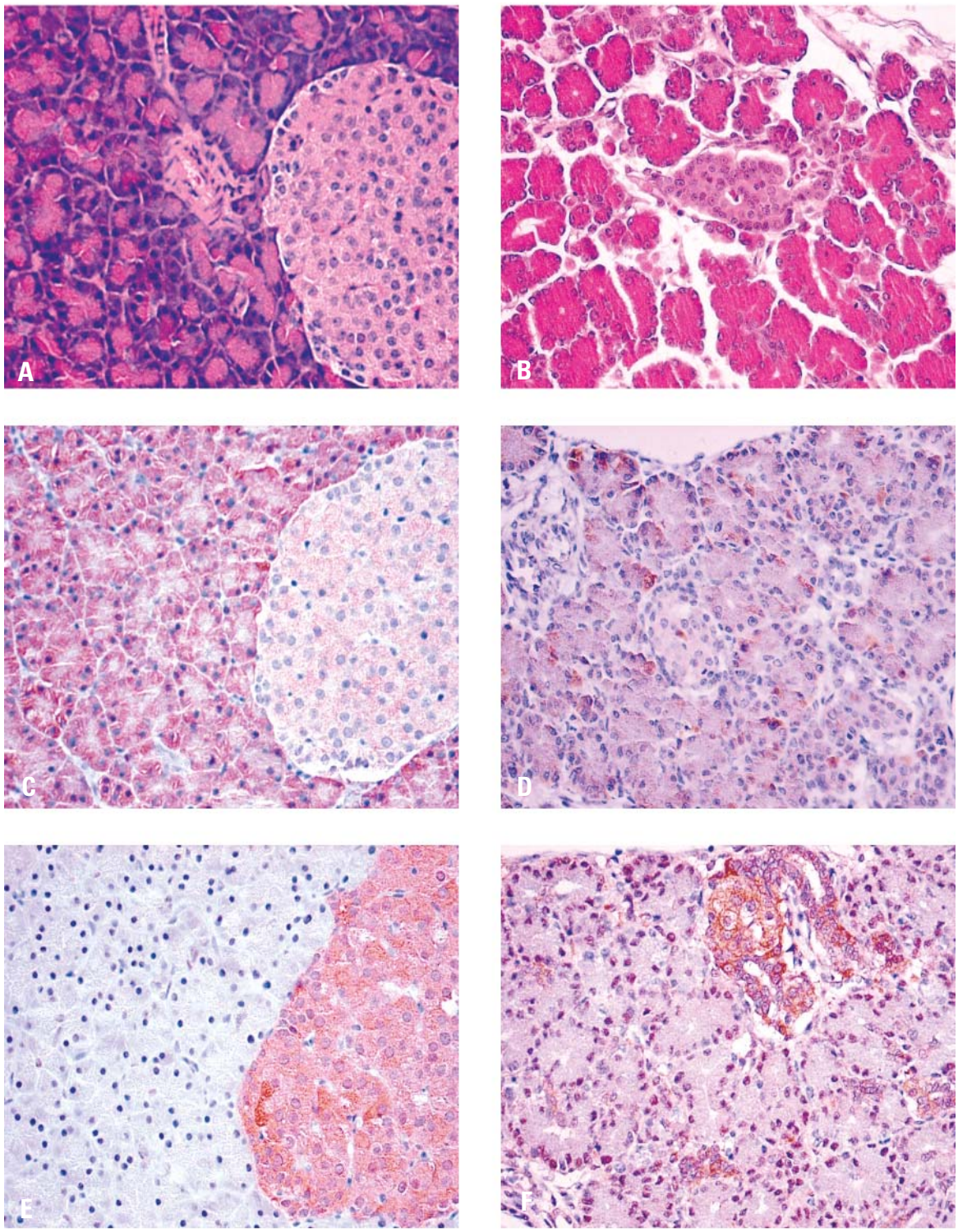

Figure 5. Normal pancreatic morphology in maternal (**A**) and 21-day old foetal (**B**) rat exposed to DFU at a dose of 20.0 mg/kg (haematoxylin and eosin, lens mag. 20×). Immunostaining for COX-1 (**C, D**) and COX-2 (**E, F**) in maternal (C, E) and 21-day old foetal (D, F) pancreas (DakoEnvision™+/HRP; C-F: lens magn. 20×).

Furthermore, acinar cells exhibited a nuclear reaction, which was strong in foetal, while weak and only focal in maternal pancreases. A positive reaction was also observed in the epithelial cells of the pancreatic ducts and the smooth muscle cells of blood vessels. No differences in COX immunoexpression were found between DFU-exposed and control groups in either mothers or foetuses.

DISCUSSION

The study demonstrated that organ morphology and function were undisturbed after administration of a selective COX-2 inhibitor, in spite of the constitutive expression of the inducible isoform in the pancreas. However, since endocrine pancreatic function was monitored only by the glucose level and the morphology of the islets of Langerhans, other more sophisticated methods are desirable to confirm our results. On the other hand, the data obtained verified previous experimental findings on the good tolerability of the tested compound. In the above-cited paper by Burdan et al. [7] a good gastrointestinal, hepatic and renal toxicological profile was demonstrated for both pregnant and non-pregnant rats. Since DFU is still in the pre-clinical study, no similar human data were available in the literature. It should also be noted that, unlike the non-selective COX inhibitor, DFU did not induce severe gastrointestinal changes such as deeply penetrating ulcers, which may result in secondary peritoneal inflammation [7]. Thus the direct effect of local inflammation as a mechanism that may explain an insignificant increase in glucose level cannot be apply [26]. Similar results were also reported in our previous study with another selective COX-2 inhibitor, namely DuP-697, which did not change the morphology and immunoexpression of the constitutive and inducible COX isoforms in the rat pancreas during pregnancy [5]. Biochemical tests were not performed, however. On the other hand, an analgesic and antipyretic mixture containing a non-selective COX inhibitor, prepared with paracetamol, propyphenazone and caffeine in a constant ratio of 5:3:1, increases the amylase activity in non-pregnant female rats exposed to the highest doses of the examined drugs (0.35, 0.21 and 0.07 g/kg respectively) [4]. An insignificant decrease in enzyme activity was noted in other groups of non-pregnant, as well as pregnant animals that were treated with the same or lower doses of the mixture.

As was stressed in the introduction, different pancreatic COX immunoreactivity has already been reported in healthy and diabetic patients, as well as after administration of COX-inhibitors [31]. The roles of prostanoid, especially prostaglandin E_2 (PGE_2), and the COX-related mechanism in the aetiology of diabetes and pancreatitis have already been reported [1, 9, 10, 11, 15, 17, 23]. Histologically, an inflammatory infiltration of the islets of Langerhans is typical of type I diabetes, which is classified as an autoimmune disease [2]. Additionally, inflammatory stimulators such as cytokines, liposaccharide and mitogens increase an otherwise high physiological expression of COX-2 in insulin-secreting cells and secondarily initiate their cytotoxicity [3, 31]. At the same time a low physiological level of COX-1 is not significantly changed [15]. However, chronic hyperglycaemia impairs insulin-secreting cell function and decreases the number and/or size of islets of Langerhans in type II diabetes. This may be the consequence of oversynthesis of interleukin 1 (IL-1) followed by apoptosis of insulin-secreting cells [23]. The pioneer study co-ordinated by Hughes et al. [17] indicated that recombinant human IL-1α was able to induce PGE_2 accumulation in isolated rat islets of Langerhans at concentrations similar to those at which the cytokine inhibits glucose-induced insulin secretion and islet glucose oxidation. Prior studies also showed that a high glucose concentration increased COX-2 expression [26]. The data were partially confirmed in mice treated with NS-398, a selective COX-2 inhibitor, which prevented the onset of diabetes in mice brought on by multiple low doses of streptozotocin [29]. Moreover, COX-2 selective inhibitors such as NS-398 and SC-236 prevent the destruction of insulin-secreting cells initiated by IL-1 [30].

It has also been reported that COX-2 inhibition reduces the severity of pancreatitis and pancreatitis-associated lung injury [1, 10, 11]. With results similar to our data, Alhan et al. [1] demonstrated that celecoxib did not change amylase activity, glucose level or other biochemical parameters and pancreatic morphology in the rat. It was also found that treatment improved lung and renal function, decreased the severity of pancreatic damage and normalised serum levels of IL-6 in cerulean-induced pancreatitis in rats. Similar results were obtained in rats with taurocholate-induced acute pancreatitis treated with parecoxib [10]. However, celecoxib-induced acute pancreatitis and hepatitis have also been found in humans [9].

The present study indicated that prenatal exposure to COX-2 selective inhibitors may affect placental and foetal development. Previously, the dose-dependency had been demonstrated only for foetal length [8]. The results obtained have shown that the best predictive factor for foetal development is not a dose of the compound tested but its effect on pancreatic function, especially its influence on maternal glucose levels, and, though less likely, on amylase activity. In the common DFU-treated group, unlike the control group, a high glucose level increased the incidence of foetal overgrowth (macrosomia).

The data presented above for the influence of the glucose level on foetal and placental development partially confirmed the previous animal and human observations. It was shown that prolonged elevation of the glucose level, a typical sign of diabetes and glucose intolerance, may result in foetal overgrowth, as well as higher perinatal mortality, incidence of respiratory distress and congenital developmental abnormalities such as anencephaly, encephalomeningocoele, caudal regression syndrome, transposition of the great vessels and coarctation of the aorta [16, 20, 22]. During uncomplicated pregnancy the influence of hypoglycaemic factors is much stronger than hyperglycaemic ones. In consequence, higher maternal insulin secretion prevents hyperglycaemia [19]. However, unlike the normal levels of adrenal, noradrenaline, growth hormone and glucagons, which remain similar throughout pregnancy, the higher cortisol level correlates with gestational age. During pregnancy extra placental-relted hyperglycaemic factors, such as lactogen (HPL), prolactin, insulinases (transhydrogenase glutathione insulinase and sulfhydrylprotease, oestrogens and progesterone are also synthesised. HPL and prolactin are characterised by the strongest anti-insulin activity and are blamed for the glucose intolerance [13, 14]. It has also been suggested that during pregnancy the content of insulin receptors decreases, while the insulin-binding protein increases significantly [18, 25]. In spite of the constitutive secretion of foetal insulin at an early developmental stage, the foetus does not regulate hyperglycaemia by increasing insulin secretion owing to the insufficiency of the adenyl cyclase-dependent pathway. Even later, when an extra amount of endogenous insulin is secreted, its total activity cannot normalise the increase in glucose level, since the main role of foetal insulin is to regulate growth [19, 27]. Such activity may explain foetal overgrowth in offspring born to diabetic mothers. Furthermore, both clinical observations in type 1 diabetic pregnancies and preliminary animal experimental studies suggest that even short periods of metabolic perturbation early in pregnancy may affect placental growth and transport functions for the remaining period of pregnancy, thereby contributing to foetal overgrowth. An *in vitro* simultaneous study has demonstrated an up-regulation of placental transport systems for glucose and certain amino acids, as well as placental lipoprotein lipase. In such a situation an up-regulation of inflammatory mediators and *Leptin* genes is also revealed [19]. However, since none of the above-mentioned hormones and enzymes was evaluated in our study, it is not possible to fully explain the phenomena observed.

CONCLUSION

In conclusion, it was found that DFU administered throughout mid and late pregnancy in rats did not change maternal or foetal pancreatic morphology and function, or the immunoexpression of constitutive and inducible cyclooxygenase isoforms in the organ. However, administration of a COX-2 selective inhibitor may affect foetal development in a mechanism that is related to abnormal glucose levels.

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