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Immunoexpression of constitutive and inducible cyclo-oxygenase isoforms in the rat foetal and maternal digestive tract

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Cyclo-oxygenase (COX), which catalyses the conversion of arachidonic acid to prostaglandin endoperoxide and prostanoids, is widely expressed in mammalian organs. The aim of the study was to evaluate the immunoexpression of the constitutive and inducible cyclo-oxygenase isoforms (COX-1 and COX-2 respectively) in the oesophagus, stomach and the small and large bowels of untreated rat dams and foetuses on gestational day 21. The localisation of the COX isoforms was similar in the maternal and foetal organs, although the intensity of the reaction for COX-2 was stronger in the foetuses. Cytoplasmic COX-1 immunostaining was found in myocytes of the muscularis propria, muscularis mucosae and the blood vessels. It was also positive in the endothelial cells, scattered stromal cells of the lamina propria and the ganglion cells of the nerve plexus in the bowels. Apart from the keratinised layer, a strong reaction was revealed in the stratified squamous epithelium of the oesophagus and forestomach. Negative or weakly positive staining was found in the mucus-secreting cells covering the surface, gastric pits and pyloric glands, as well as in the parietal cells and the chief cells. Weakly positive COX-1 immunostaining was observed in epithelial cells of the small intestine crypts, but in some cases enterocytes and goblet cells covering villi were also positive. In the colonic mucosa weak COX-1 staining was typical of the absorptive, and goblet cells. The COX-2 immunostaining was nuclear and/or cytoplasmic. An inconsistent positive reaction was seen in the muscle of the muscularis mucosae, muscularis propria and the blood vessels. Positive staining was also found in scattered stromal cells of the lamina propria and adventitia and the ganglion cells. Weak nuclear staining was found in the stratified squamous epithelium of the oesophagus and forestomach. Unlike the strong foetal reactivity in the epithelial cells of the glandular stomach, a negative or weakly positive reaction was seen in the maternal parietal and/or mucous-secreting surface stomach cells. Some epithelial cells of the crypts both in the small and large bowel were also COX-2 positive. In conclusion, constitutive and inducible COX isoforms were

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detected in the digestive tract of pregnant female and in foetuses. COX-1 was the predominant isoform in both the adult and foetal organs. (Folia Morphol 2008; 67: 24–31).

Key words: cyclo-oxygenase, COX-inhibitor, lactation, NSAID, pregnancy, prostaglandin-endoperoxide synthase, oesophagus, stomach, small bowel, large bowel

INTRODUCTION

Numerous experimental and clinical studies have shown that cyclo-oxygenase inhibitors, also known as non-steroidal anti-inflammatory drugs, may damage the gastrointestinal mucosa. The harmful effect is mediated by various mechanisms including topical injury, increased expression of intercellular adhesion molecules, thromboxane depletion, and probably the most important, inhibition of prostaglandin synthesis [12, 22]. 6-keto-prostaglandins increase epithelial cell proliferation, stimulates mucin, bicarbonate and phospholipid secretion, which protects the gastroduodenal mucosa against hydrochloric acid, pepsin and bile salts. Unlike that of gastroduodenal injury, the pathogenesis of oesophageal, jejunal and ileal mucosa injury is still unclear. It is suggested that the drugs, in spite of prostaglandin synthesis blockade, directly uncouple oxidative metabolism in the mitochondria of the enterocytes. This leads to cell damage and an increase in intestinal mucosal permeability, thus permits bacteria and bile from the lumen to penetrate the intestinal wall and to cause deep damage [19, 22].

Synthesis of prostaglandins, as well as of other prostanoids depends initially on cyclo-oxygenase (COX) activity, also known as prostaglandin-endoperoxide synthase (EC 1.14.99.1). Two main isoforms of the enzyme have been detected so far. COX-1 is constitutively expressed in most of the tissues and catalyses prostanoid synthesis, which is believed to support the physiological functions of the organs. COX-2, in contrast, is induced by various pathological factors, including stress, proinflammatory cytokines, growth factors and endotoxins [22]. However, it has also been detected physiologically, for instance in the brain, kidney, lung, male and female reproductive organs, placenta and some foetal tissues [4, 6, 9, 22, 24, 25]. Recently, a new isoform, COX-3, has also been revealed in the central nervous system. This is regarded as a post-translational modification of COX-1 [3], but there are also data linking the isoform to COX-2 [28].

Although there have been a number of studies showing COX expression in the gastric mucosa, enzyme localisation has not been studied extensively in the oesophagus or the small and large intestines, especially in foetuses. The aim of this study was to evaluate the immunoexpression of the constitutive (COX-1) and inducible (COX-2) isoforms in selected organs of the digestive tract at the end of pregnancy in rat dams and offspring.

MATERIAL AND METHODS

Sexually mature albino rats of the Wistar CRL:(WI)WUBR strain obtained from a commercial breeder (Warsaw-Rembertów, Poland) were used. The rats were acclimated for at least two weeks, housed and maintained in an animal care facility. On mating days, females weighing 200–250 g were placed in cages with males (5:2) for approximately 14 hours. The following morning a vaginal smear was performed to determine if copulation had occurred. The day when sperm was found was designated as gestation day 1 (GD1). Sperm-positive females were randomly taken for the examined group (n = 8). No xenobiotics were administered during the study.

On gestation day 21 the females were sacrificed. The foetuses were delivered by caesarean section as standard for teratological investigation [5]. All the foetuses were sexed and a single non-malformed male and female were randomly taken from each litter. Samples of the maternal and foetal oesophagus, forestomach and glandular stomach, the proximal part of the jejunum, the distal part of the ileum, and the transversal colon were taken during autopsy. The organs from the adult animals were opened longitudinally, placed on a cork board, and fixed in 10% buffered formalin. The foetal organs, except for the stomach, were fixed without previous opening. The appropriate samples were then embedded in paraffin blocks, sectioned at 5 μm and stained routinely with haematoxylin and eosin (H & E) and alcian blue/periodic acid Schiff (AB/PAS). In cases of any significant pathological change samples were withdrawn from the study.

The immunohistochemical reaction for COX-1 and COX-2 was performed on 4 μm slides obtained from the paraffin blocks used previously for histological examination. After being dewaxing and rehydrating 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 blocked with 3% hydrogen peroxide for 5 min and the slides were incubated for 60 min with the primary monoclonal mouse anti-human antibodies (Novocastra; Newcastle, UK) against COX-1 (clone 12E12, dilution 1:20) and COX-2 (clone 4H12, dilution 1:200). As the next step incubation with DakoEnvision^{+TM}/HRP, Mouse kit (DakoCytomation, Glostrup, Denmark) was applied according to the manufacturer's directions. The specific immune reaction was visualised using 3',3-diaminobenzidine tetrahydrochloride (DakoCytomation) 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. The appropriate positive and negative controls were prepared. Sections treated in the same way but with mouse pre-immune serum instead of the primary antibodies examined were used as negative controls. For the positive COX-1 and COX-2 controls, human skin and colonic mucosa were applied, respectively. Before the proper immunohistochemical study was begun, cross-reactivity with rat tissues was verified [4, 6, 7]. All the slides were evaluated using a light microscope (Olympus BX45, Tokyo, Japan).

RESULTS

In all cases COX-1 immunostaining was exclusively cytoplasmic. In the maternal samples a strong positive reaction was usually found in the striated or smooth muscle of the muscularis propria as well as in the myocytes of the muscularis mucosae and the blood vessels (Fig. 1A, 2A, 3A, 4A). It was also positive in the endothelium, ganglion cells of the nerve plexus, and in the germinal centres of the lymphoid follicles seen in the bowel mucosa. Scattered stromal cells with COX-1 reactivity were found in the lamina propria and the adventitia (subserosa). Apart from the keratinised layer, a strong reaction was revealed in the stratified squamous epithelium of the oesophagus and the forestomach (Fig. 1A). However, staining intensity in the epithelial cells of the glandular stomach varied greatly from case to case. They varied from completely negative to weakly positive in the mucus-secreting cells covering the surface, the gastric pits and the pyloric glands, as well as in the parietal and chief cells of the fundic glands (Fig. 2A). Weakly positive COX-1 immunostaining was also observed in the epithelial cells of the small intestine crypts. However in some cases, especially in the samples taken from the jejunum, enterocytes and goblet cells lining the intestinal villi were positive as well (Fig. 3A). In the colonic mucosa weak COX-1 staining was typical of absorptive and goblet cells both in crypts and on the surface (Fig. 4A).

The staining pattern of the non-epithelial cells in the foetal samples was similar to that observed in the adult animals. However, in the epithelial cells the COX-1 reaction was much stronger than in the maternal samples of the corresponding organs and comprised a majority of cells (Fig. 1B, 2B, 3B, 4B).

COX-2 immunostaining with applied monoclonal antibody was nuclear and/or cytoplasmic. The former was noted mainly in the epithelial cells. In the maternal samples an inconsistent positive reaction was seen in the striated or smooth muscle of the muscularis mucosae, muscularis propria and blood vessels (Fig. 1C, 2C, 3C, 4C). Positive staining was typical of the scattered stromal cells of the lamina propria and adventitia of the oesophagus and bowels, as well as of the ganglion cells. Weak nuclear staining was found in the stratified squamous epithelium of the oesophagus and forestomach (Fig. 1C). In the maternal glandular stomach the reaction in the epithelial cells was negative or, in some cases, single weakly COX--2-positive parietal and/or mucous-secreting surface cells were revealed (Fig. 2C). Weak COX-2 immunoexpression was also observed in some epithelial cells, mostly at the base of the crypts of the small bowel or, rarely, in epithelial cells covering the villi (Fig. 3C). A similar weak reaction was noted in some epithelial cells lining the crypts and colonic surface (Fig. 4C).

In the foetal samples of all the examined organs the COX-2 immunostaining pattern was usually similar to that of the adults, but in the glandular stomach and colon the intensity of the staining, especially in the epithelium, was stronger and involved a majority of epithelial cells (Fig. 1D, 2D, 3D, 4D).

There were no differences between male and female foetuses in the immunoreactivity of either isoform.

DISCUSSION

The study presents the immunoreactivity of both COX isoforms in the oesophagus, stomach, small intestine and colon in pregnant female rats and their 21-day old foetuses. The results are similar to previous observations concerning COX-1, while the COX-2

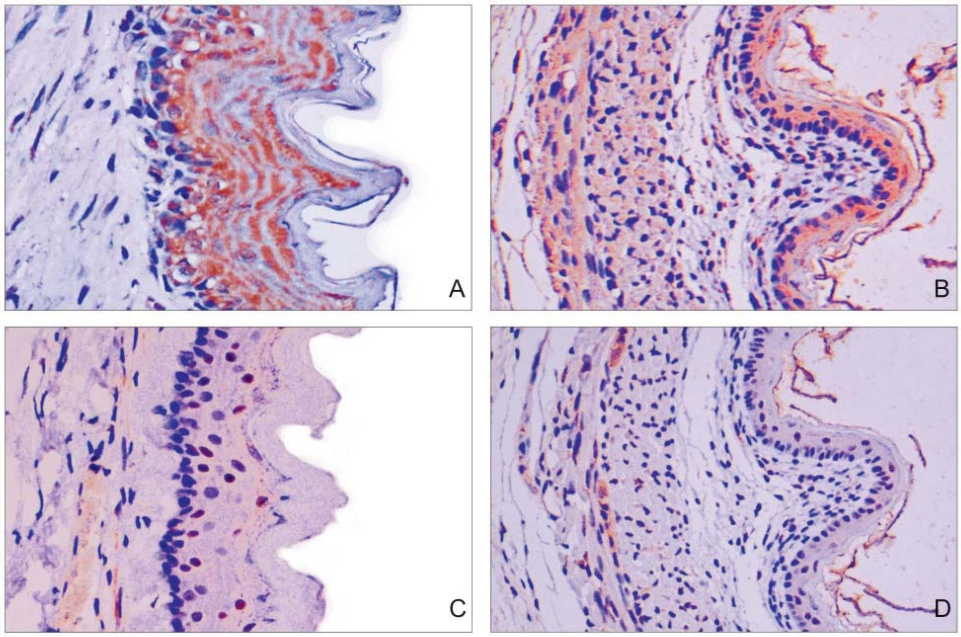

Figure 1. Oesophageal immunoexpression of COX-1 (**A, B**) and COX-2 (**C, D**) in maternal (**A, C**) and female foetal (**B, D**) samples (DakoEnvision^{+TM}/HRP; objective magnification A–D: 20×).

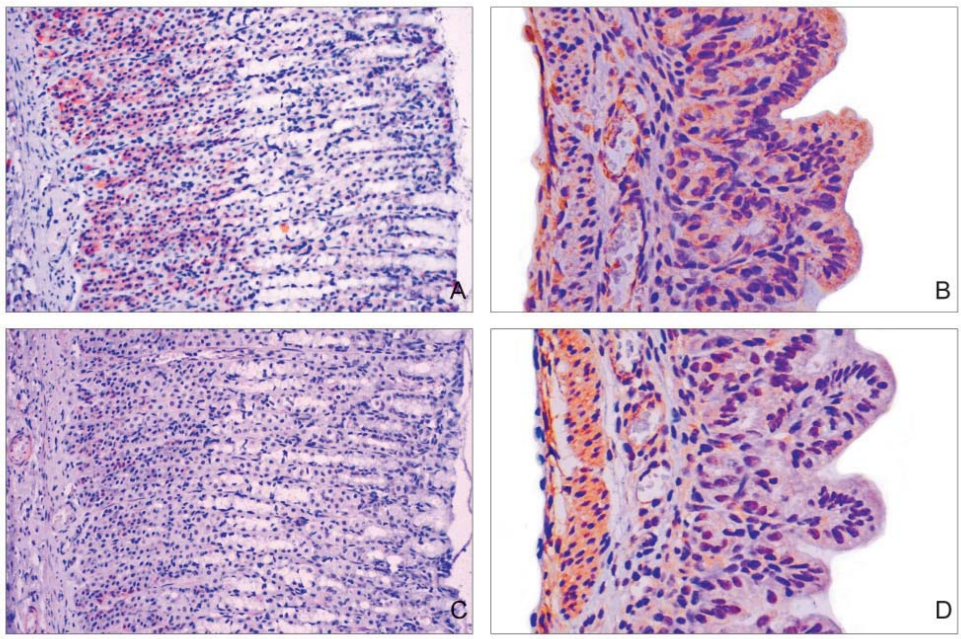

Figure 2. Gastric immunoexpression of COX-1 (**A, B**) and COX-2 (**C, D**) in maternal (**A, C**) and female foetal (**B, D**) samples (DakoEnvision^{+TM}/HRP; objective magnification A, C: 10×; B, D: 20×).

immunoreaction was much stronger and/or observed in organs and cells that were COX-2 negative in previous studies (Table 1).

In 1991 Mikkelsen et al. [18] localised cyclo-oxygenase in all parts of the intestine using polyclonal COX antibody. Their results were similar to ours; immunostaining was observed in most smooth muscle cells but the reaction was much stronger in the muscularis mucosae than in the longitudinal and circular muscle layers. Comparable expression was

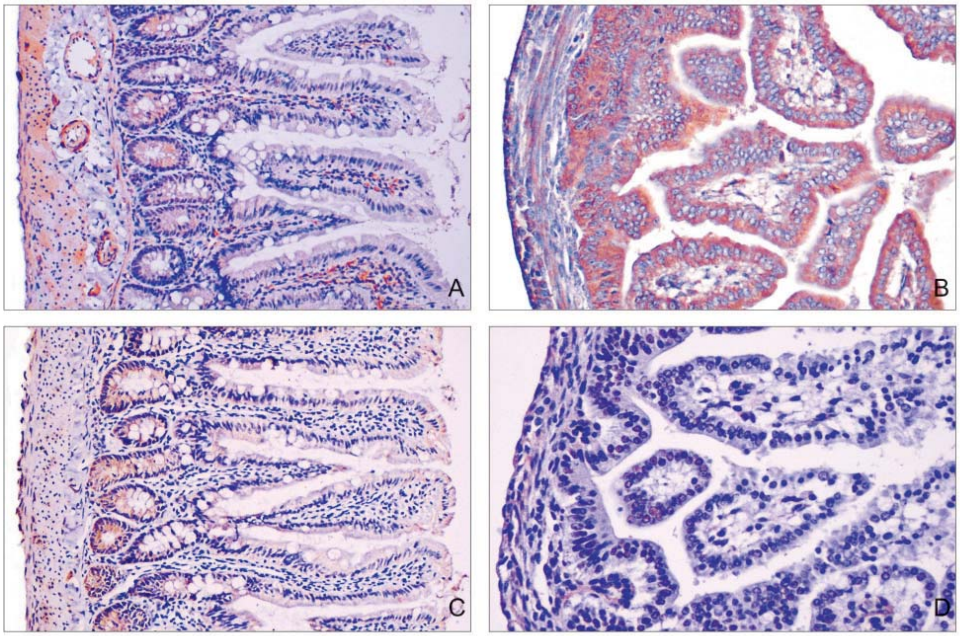

Figure 3. Enteric immunoexpression of COX-1 (**A, B**) and COX-2 (**C, D**) in maternal (**A, C**) and female foetal (**B, D**) samples (DakoEnvision^{+TM}/HRP; objective magnification A, C: 10×; B, D: 20×).

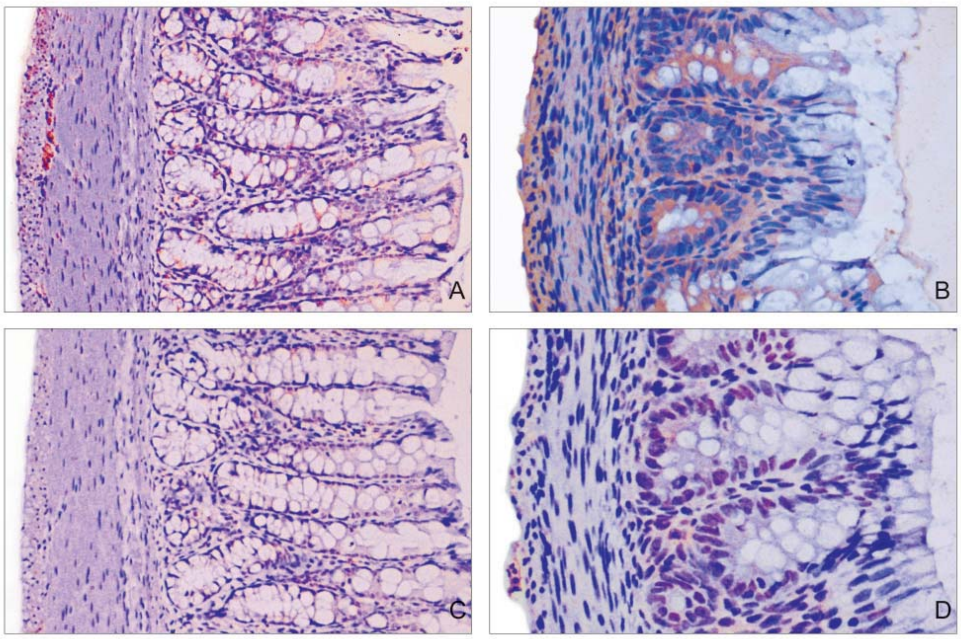

Figure 4. Colonic immunoexpression of COX-1 (**A, B**) and COX-2 (**C, D**) in maternal (**A, C**) and female foetal (**B, D**) samples (DakoEnvision^{+TM}/HRP; objective magnification A, C: 10×; B, D: 20×).

observed in endothelial cells and in unidentified cells in the subserosa. These observations were partially confirmed later by immunohistochemistry with monoclonal antibodies, as well as a variety of molecular techniques. On the basis of northern and western blot analyses [29], co-expression of COX-1 and COX-2 mRNA was detected in the canine jejunum. Similar data were obtained for horse [20, 27] and rat [1, 2, 16] samples. Tomlinson et al. [27] reported up-regulation of both COX isoforms where there was intestinal

Table 1. Intra-anatomical and inter-species physiological localisation of constitutive (COX-1) and inducible (COX-2) cyclo-oxygenase isoforms in selected organs of the digestive tract*

	COX-1							COX-2					
	Dog	Horse	Human	Monkey	Rat			Dog	Horse	Human	Monkey	Rat	
					A-NP	A-P**	F**					A-NP	A-P**	F**
Oesophagus	NE	NE	NE	NE	NE	+***	+***	NE	NE	NE	NE	NE	+***	+***
Stomach	+		+	+	+	+	+	+[29]		+[26]		+[15]	+	+
Duodenum	+		+	+	+	NE	NE						NE	NE
Jejunum	+	+	+	+	+	+	+	+[29]	+			+[16]	+	+
Ileum	+		+	+	+	+	+						+	+
Caecum	+		+	+	+	NE	NE					+	NE	NE
Colon	+		+	+	+	+	+	+		+	+		+	+

*Modification from Radi and Khan [20], a new data was added and adequate references were cited in square brackets; **own results presented in this paper; ***both oesophagus and forestomach; A-NP — adult non-pregnant, AP — adult pregnant, F — foetus, NE — not evaluated, (+) — positive expression and/or immunoexpression of corresponding gene or protein, respectively

ischaemic injury in horses. On the other hand, ventromedial hypothalamus-lesioned rats revealed an increase in COX-1 mRNA and a decrease in COX-2 mRNA levels in the jejunal mucosa 6 and 12 hours after the operation [16]. However, the levels of both isoforms increased after 24 hours. This phenomenon was accompanied by increased proliferation of the epithelial cells of the small intestine, probably as a consequence of excessive prostaglandin E2 synthesis. Such results were also confirmed by an experiment with lipopolysaccharide administration [1]. In contrast, Beubler et al. [2] reported that cholera toxin did not exert any effect on the constitutive expression of COX-1 and COX-2 mRNA in the rat jejunum. However, later studies showed that the toxin enhanced the COX-2 level, which remained unaffected by dexamethasone [1]. A low physiological level of COX-2 was previously found in the region of gut-associated lymphoid tissue in the rat caecum [17].

Data regarding the immunolocalisation of the constitutive and inducible COX isoforms in the oesophagus and small and large bowel are sparse. However, there are a number of papers describing COX-1 and COX-2 localisation in the gastric and duodenal mucosa. Generally COX-1 immunoexpression in various part of the digestive tract is limited to the mucosal epithelium, vascular endothelium and smooth muscle cells of the tunica muscularis. Iseki [15] observed a strong immunoreactivity for COX-1 in the rat mucous neck cells of the gastric gland. A weak reactivity of COX-1 was also found in the mucous cells of the cardiac and pyloric glands of the stomach, as well as of Brunner's glands of the duodenum. The immunoreactivity of COX-2 was revealed in the surface mucous cells in both the fundic and pyloric gastric regions. Such observations were also confirmed in canines [29].

In normal human gastric mucosa COX-2 mRNA expression was absent. However, strong expression was observed in *Helicobacter pylori*-positive gastritis, moderate in inflamed gastric mucosa, but with no signs of bacterial infection [13]. The opposite results were presented by To et al. [26]. In the gastric mucosa of healthy humans COX-1 was detected in stromal cells in the lamina propria, while focal and weak immunostaining for COX-2 was seen only in the foveolar epithelium. In the case of ulceration the immunoreactivity of COX-1 was significantly increased in the cells of the lamina propria in the ulcer edges, but COX-2 was strongly expressed in the hyperplastic foveolar epithelium. At the ulcer base there was a strong expression of COX-1 and COX-2 in the

macrophages, myofibroblasts and the endothelial cells of the granulation tissue. Similarly, Chan et al. [11] reported localisation of COX-1 in the mononuclear inflammatory, endothelial and smooth muscle cells of the lamina propria, whereas COX-2 was reported in the foveolar and glandular epithelium in gastritis associated with *Helicobacter pylori*. In contrast to the previously cited article [26] COX-2 but not COX-1 was elevated in the infected mucosa. Recent studies conducted by Gudis et al. [14] using a western blot analysis showed COX-1 protein expression only in superficial cells of an acute ulcer, while COX-2 was shown in both an acute and a healed ulcer. Furthermore, strong COX-1 and COX-2 immunoexpression was found in fibroblasts and macrophages of the ulcer base. COX-2 expression was seen in both the fibroblasts of an ulcer and in epithelial cells and also in the scattered mast cell-like cells and neuroendocrine-like cells.

It is worth mentioning that there are large variations between different organs and species in the levels of both COX isoforms. Seibert et al. [21], who evaluated the level of COX-1 protein in various parts of the gastrointestinal tract of dogs, found that the level in the gastric mucosa was 10-times higher than that in the jejunum and ileum. Inter-species comparison showed that the highest expression of COX-1 in the small intestine was observed in monkeys, humans, rodents and dogs, respectively [17]. Such differences may explain the oversensitivity of rats, mice and dogs to relatively low doses of non-selective COX inhibitors.

In a recently published summary [20] COX-1 expression in adult non-pregnant rats was observed in the stomach, duodenum, jejunum, ileum, caecum and colon, while COX-2 reactivity was detected physiologically only in the caecum (Table 1). Gene expression and/or protein immunoexpression were also demonstrated in the same organs in untreated adult canines, primates and humans. Contrary to our observations and some of the papers cited above [15, 16, 26, 29], the authors concluded that the COX-2 isoform is almost absent in these species except for low levels in the caecum of rats and in the colon of dogs, monkeys and humans.

There are no available data on expression of the COX isoforms in the foetal gastrointestinal system as there are for other foetal organs such as the brain [22], cartilage [9, 24], heart [25], kidney [6, 24], lungs [4], pancreas [7] and skin [24, 25]. On the other hand, enzyme expression in the maternal tissues does not fully cover the non-pregnant state. Since pregnancy and early lactation alter the hormonal status [23], it strongly influences the translation and transcription of genes coded in both constitutive and inducible cyclo-oxygenase isoforms [10]. Secondary to all these changes, the morphology and physiology of the maternal digestive tract can be modified during both pregnancy and lactation [23]. The high physiological expression of both constitutive and inducible COX isoforms explains the higher level of maternal toxicity, mostly gastrointestinal complications, previously reported with non-selective COX inhibitors in pregnant rats [8, 20].

CONCLUSION

In conclusion, it should be stressed that the constitutive and inducible isoforms of cyclo-oxygenase were detected immunohistochemically in the oesophagus, stomach, ileum, jejunum and colon of pregnant female rats and their 21-day old foetuses. Additionally, COX-1 was the predominant isoform in the organs of both dams and offspring.

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