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EXPERIMENTAL STUDY

Hepatocellular and developmental influences of early postnatal indomethacin in mice

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ABSTRACT
OBJECTIVES: The effect of early postnatal indomethacin exposure on hepatocellular and developmental alterations in mice liver was investigated.
METHODS: Pups received IP injections of 0, 25, 50 and 100 mg/kg indomethacin on P0, then killed at P21 and P60.
RESULTS: Indomethacin significantly suppressed body weight at P21, but liver weight significantly decreased only in 25 mg/kg. In contrast, liver weight and liver to body weight ratio significantly increased with increasing dose of indomethacin by P60. The restoration of liver weight was a result of proliferation, as a consequence of a significant increase in the number of uni and bi-nuclear hepatocytes per field in 25 mg/kg at P21 and no evidence of hepatocellular hypertrophy. Indomethacin had a dose-related decrease in number of hepatocytes as the result of hepatocellular hypertrophy confirmed with hepatocytes presenting large cellular and nuclear size in 50 and 100 mg/kg. Moreover, proliferation contributed to the increased liver size, since bi-nuclear hepatocytes and its ratio increased at P21 at first and then decreased by P60 with increasing in dose.
CONCLUSION: Indomethacin has long term effect on liver development in a dose- and time- dependent manner. The hepatocytes during both the liver development and regeneration show significant differences in cell and nuclear number and size *(Tab. 3, Fig. 2, Ref. 48)*. Text in PDF *www.elis.sk.*
Key words: indomethacin, liver, hepatocellular alterations, development alterations, mice.

Abbreviations: P – postnatal day, PG – prostaglandin, PTL – preterm labour, PDA – patent ductus arteriosus, COX – cyclooxygenase, IGF-I – insulin-like growth factor- I, NSAIDs – non-steroidal anti-inflammatory drugs, DILI – drug induced liver injury, HGF – hepatocyte growth factor, PH – partial hepatectomy**.**

Introduction

Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), is commonly used for the treatment of inflammatory diseases such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, tendositis, bursitis, gouty arthritis, inflammatory arthritis, symptomatic patent ductus arteriosus (PDA) and preterm labour (PTL) (1,2,3). The effect of indomethacin is attributed to its ability to non-selectively inhibit cyclooxygenase (COX), the precursor enzyme that synthesize PGs (4, 5). Despite its effectiveness and minimal side maternal effects; it has a potential to cause a number of complications in foetus and neonate (6, 7). The half-life of indomethacin in premature neonates (at least double than in adults) could explain the long-lasting effect of indomethacin after birth (8, 9). Both prenatal and postnatal exposure to indomethacin caused increased neonatal complications including necrotizing enterocolitis (10-12). Also, it has been shown that indomethacin increased oligohydramnios, intraventricular haemorrhage, renal failure (6). In the kidney, early postnatal indomethacin has been reported to cause long term and adverse effects on renal development and function by disturbance in the renal normal biosynthesis of COX and its metabolites (13). Also, it leads to decreasing PGs content, which may play a significant role in alveolar formation and produced abnormality in developing lung structure (14). Therefore, application of NSAIDs such as indomethacin to suppress PG is associated with long-term and adverse effects on postnatal growth and growth factors at the critical time of development. It demonstrated that early postnatal exposure to PG inhibitors such as indomethacin and ibuprofen may result in disturbances in normal maturational events in liver. Since, PGs involved in the regulation of growth and growth factors such as insulin-like growth factors (IGF-1) in liver (15).

It is documented that the absorption, excretion, and metabolism of drugs potentially affect structure and function of liver development (16). Indomethacin, which is metabolized by the liver and undergoes extensive enterohepatic circulation, resulted in a high incidence of gastrointestinal adverse effects (17). Considering that,

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liver is recognized as a major organ for drug metabolism during the foetal period and after birth (18, 19), it might be undergoing suppression of PGs, exhibits disturbance in the growth and developmental processes with an overlap between developmental and pathological processes.

Several analyses of liver development support the view that maturation and development of liver continues into the postnatal period to generate the characteristic tissue architecture of liver (20- 23). Maturation into hepatocytes and bile epithelial cells, hepatocytes proliferation, increase in size of hepatocytes, lobule arrangement and finely increase of liver weight are processes that continue until several weeks after birth (23-27). In addition, polyploidy as a characteristic feature of mammalian liver is associated with late foetal development and postnatal maturation (28, 29). In mice, the architecture of hepatic parenchyma began to resemble that of adult liver after postnatal day 20 (23).

Many hepatic toxic effects of indomethacin were described (17), whereas the effect on liver development has received no attention. Based on the preceding, the present study investigated the hypothesis that early postnatal indomethacin influences liver, while liver development is ongoing. For better understanding of hepatocellular and developmental alterations in mice liver following early postnatal exposure to indomethacin, we studied drug effects on histological and development parameters of liver after postnatal day 20 because the mice liver, is immature, and develops by 60 days postnatal age. We also specifically studied late drug effects at P60 for investigation of remarkable capacity of liver to regenerate after injury.

Materials and methods

Animals

Female and male NMRI mice (25-30 g) were obtained from the Experimental Animal Facility of the Pasteur Institute (Tehran, Iran). All experiments were carried out according to the guidelines of Ethic Committee of Razi University. The animals were maintained in a pathogen-free environment with 12-hour-day/12-hour-night cycle under room temperature and controlled humidity, fed with commercial pellet and water ad libitum. For breeding, in each cage commonly two females and one male were mated for a 5 day periods. The presence of vaginal plug and vaginal smear confirmed pregnancy.

Experimental design

On the day of birth, new-born mice pups were weighed (grams) and randomly assigned to expanded litters of 19 pups/treatment group/ time. Litters were randomly assigned to receive a single intraperitoneal injection of either; 0 (control), 25, 50 and 100 mg/kg indomethacin (Sigma) which were diluted in 0.9 % saline. The animal growth was measured by body and liver weights. At P21 and P60, mice were weighed and following a sacrifice, livers were removed and weighed, immediately.

Liver samples were collected, left and middle lobes were cut and fixed for processing in paraffin blocks. The samples of liver from both lobes were used for histological studies. Serial sections (5 µm) were obtained from blocks placed onto glass slide and then stained with hematoxylin and eosin stain (H&E). The stained sections from lobes were morphologically and morphometrically analysed for assessment of changes in parameters of liver development, including: liver weight, liver/body weight ratio, number of uni- and bi- nuclear hepatocyte, size of hepatocyte cell and nuclear size, formation of lobular pattern and portal triads.

Histological and morphometrical assessments

Formation of normal parenchymal structure with central vein in the centre and the portal triads in the periphery were assessed by H&E -stained histological sections of sample liver (Olympus IX-71, Japan). Morphometric assessments were performed as previously described (30, 31) with minor modifications. Briefly, photomicrographs were captured with 40x objective for analysis of sections. At least one hundred hepatocytes were measured in ten randomly chosen fields/section in five sections in each sample from both medial and left lobes. For each hepatocyte, after taking an image (with a 40x objective), hepatocyte area and diameter, nuclear area and diameter were measured. This allowed us to calculate the cytoplasm area and the nuclear/cytoplasmic ratio from SPSS software.

A standard rectangular grid (270 x 80 μm^2) was placed on the section for the counting of uni- and bi-nuclear hepatocytes (32). Collected five randomly chosen sections of each sample liver from both medial and left lobes and five randomly chosen fields/sections were assessed. The sections of liver were observed under by the light microscope with the 10x objective lens (Olympus IX-71, Japan).

Statistical analyses

The data obtained were analysed by using SPSS (version 20; SPSS Inc.) and expressed as the mean and standard errors of the means (SEM). The morphometric parameters including total body and liver weights, number of cell and hepatocyte cell and nuclear size were computed for each liver. Comparisons of the mean values of the studies indices were done using the One-Way ANOVA and Duncan test. $P < 0.05$ was considered statistically significant.

Results

General features

Examination of animal and organ appearance showed that there were no differences in developmental scheduled times including eye opening, development of fur and ear at P21 and P60 compared to their controls. Observations revealed that organs were in natural positions and the liver was placed in diaphragm region. There was no observable abnormality in the shape of liver and it had four lobes. The liver in mice administered with 50 and 100 mg/kg indomethacin showed a very little discoloration. Only in 100 mg/kg indomethacin at P60, surface of all livers had granular regions with dark colour.

Somatic growth

The results revealed that total body weight significantly decreased with increasing dose of indomethacin at P21 ($p < 0.05$).

Tab. 1. Effect of early postnatal exposure to indomethacin on somatic growth.

Dose* n=19	P21			P60,		
	Body weight (gr)	Liver weight (gr)	Liver/body wt. (ratio)	Body weight (gr)	Liver weight (gr)	Liver/body wt. (ratio)
0	13.25±0.65[a]	0.64±0.04[a]	0.049±0.002[a]	27.25±0.36[a]	1.43±0.02[a]	0.050±0.001[a]
25	7.00±0.37[c]	0.38±0.03[b]	0.055±0.005[a]	26.25±0.45[a]	1.68±0.04[b]	0.062±0.002[b]
50	10.00±0.53[b]	0.52±0.03[a]	0.050±0.003[a]	30.00±0.37[b]	1.80±0.05[c]	0.060±0.002[b]
100	10.25±0.59[b]	0.54±0.02[a]	0.052±0.004[a]	31.00±0.37[b]	1.90±0.02[d]	0.061±0.007[b]

* Dose of indomethacin: 0, 25, 50, and 100 mg/kg, respectively. Results are expressed as the mean ±SEM. [abcd]: Different superscripts indicate significant difference (ANOVA; $p < 0.0.5$).

Despite the similar pattern for the mean liver weight at P21, decrease in liver weight was only significant at 25 mg/kg indomethacin ($p < 0.05$). The changes in liver to body weights ratio were not significant. The total body weight was resolved in 25 mg/kg indomethacin by P60, followed by a significant increase in 50 and 100 mg/kg indomethacin ($p < 0.05$). In contrast to P21, the mean liver weight showed a significant increase with increasing dose of indomethacin at P60 ($p < 0.05$). This was concurrent with liver to body weight ratios (Tab. 1).

Histological and developmental effects

Hematoxylin- eosin (H&E) stained liver sections were used to determine histological changes that occurred in the postnatal mouse liver at P21 and P60. Histological examination of the liver sections in control mice at P21 and P60 exhibited normal parenchymal structure with central vein in the centre and the portal triads in the periphery. Hepatocytes were arranged in plates between the adjacent sinusoids, radiating from the central vein towards the periphery of lobule. Hepatocytes were spherical or polyhedral

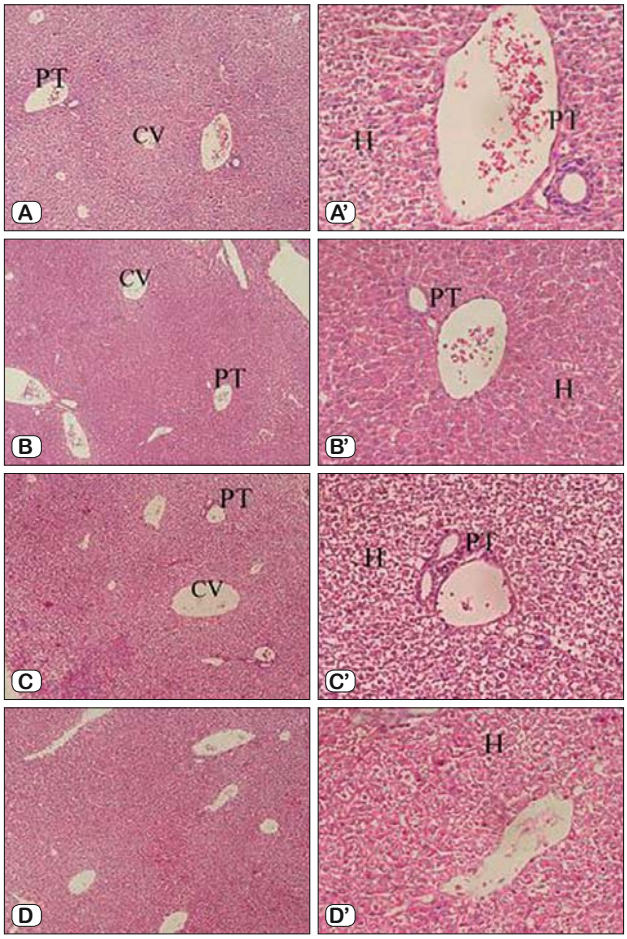

Fig. 1. Representative photomicrographs of liver sections from mice (P21) treated with 0 (A, A′), 25 (B, B′), 50 (C, C′) and 100 mg/kg indomethacin (D, D′). (A-D) low magnification and (A′-D′) high magnification showing: lobular structure consisting of central vein (CV) in the centre, portal triads in the periphery (PT), Hepatocytes (H). (D, D′): showing abnormality in lobular pattern.

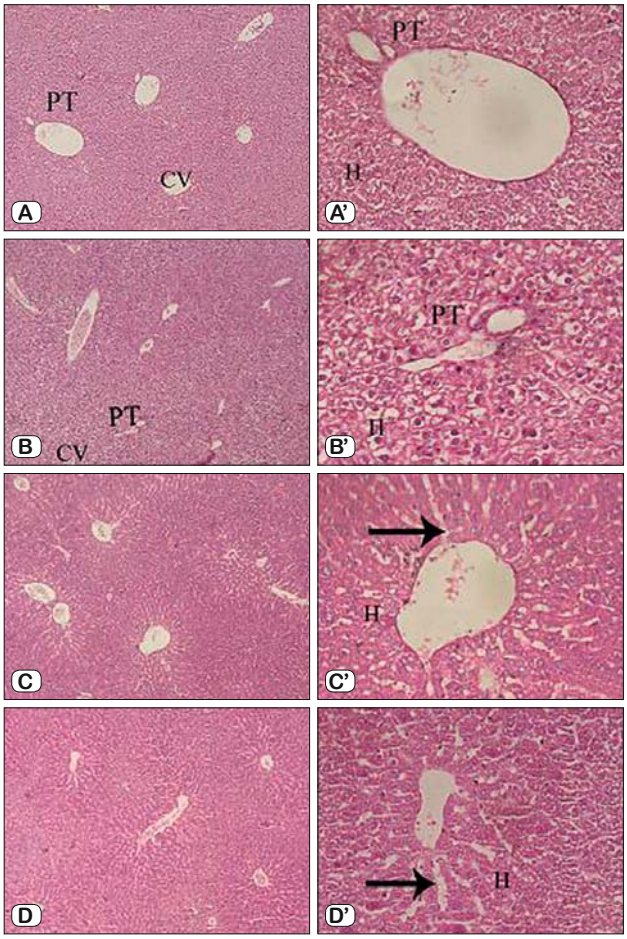

Fig. 2. Representative photomicrographs of liver sections from mice (P60) treated with 0 (A, A′), 25 (B, B′), 50 (C, C′) and 100 mg/kg indomethacin (D, D′). (A-D) low magnification and (A-D) high magnification showing: lobular structure consisting of central vein (CV) in the centre, portal triads in the periphery (PT), Hepatocytes (H), dilated sinusoids (arrow). (C, D, C′, D′): showing abnormality in lobular pattern.

cells. Kupffer cells were observed in the liver sinusoids, these cells and endothelial cells lined the walls of the sinusoids. Treatments of indomethacin at P21 and P60 caused morphological changes including hepatocyte hypertrophy and dilated sinusoids. Also, it showed abnormality in formation of the lobular pattern, especially in portal triads and central vein. The number of portal vein branches increased in abnormal lobular pattern and sometimes the portal triads disappeared. Also, there was an irregularity in sinusoid pattern, hepatocytes cords and central veins. Hepatocellular degeneration such as ballooning of hepatocytes and apoptotic bodies with increasing dose were seen. Taken together, severity of the damage depended in a dose- and time- dependent manner. These changes were more considerable long term at P60 (Figs 1and 2).

Morphometric assessments

The cell number

Morphometric analysis of the liver showed that indomethacin significantly increased the number of hepatocytes in 25 mg/kg compared to the P21 and P60 ($p < 0.05$). However, the number of hepatocytes significantly declined with increasing dose of indomethacin at P21 and P60 ($p < 0.05$). Also, the number of bi-nuclear hepatocytes significantly increased with increasing dose of indomethacin at P21 ($p < 0.05$). This was followed by a significant decrease with increasing dose of indomethacin at P60 ($p < 0.05$). Ratio of the number of bi-nuclear hepatocytes to the total hepatocytes significantly increased in 50 and 100 mg/kg indomethacin with increasing dose of indomethacin at P21 ($p < 0.05$). However, this ratio showed a significant decrease at P60 compared to the control ($p < 0.05$) (Tab. 2).

Hepatocyte cell and nuclear size

Quantitative parameters of hepatocyte area and diameter, nuclear area and diameter, cytoplasmic area, and the nuclear/cytoplasmic ratio in hepatocytes at P21 and P60 are given in the Table 3. Taken together, these results revealed that the mean area and diameter of hepatocytes decreased in 25 mg/kg indomethacin at P21 and P60, but these differences were not significant. In contrast, the mean area and diameter of hepatocytes significantly increased in 50 and 100 mg/kg indomethacin with increasing dose of indomethacin at P21 and P60 ($p < 0.05$). Also, indomethacin resulted in a significant increase in the hepatocytes mean nuclear area and diameter with increasing dose of indomethacin at P21 and P60 ($p < 0.05$). However, there was a downward trend with increasing dose of indomethacin at P21. In addition, indomethacin significantly decreased cytoplasmic area in 25 mg/kg indomethacin at P21 and P60 ($p < 0.05$). In contrast, cytoplasmic area showed a significant increase in 50 and 100 mg/kg indomethacin with increasing dose of indomethacin at P21 and P60 ($p < 0.05$). The nuclear/cytoplasmic ratio significantly increased in 25 and 50 mg/kg indomethacin and significantly decreased in 100 mg/kg indomethacin ($p < 0.05$). These changes were followed by a significant increase at P60 compared to the control ($p < 0.05$).

Discussion

In this report, our findings demonstrated that indomethacin had a profound effect on somatic growth in a dose- and time-dependent manner. Indomethacin suppressed body weight gain at P21, but the effect resolved by P60. However, despite a severe decrease in weight of liver in 25 mg/kg indomethacin, it

Tab. 2. Effect of early postnatal exposure to indomethacin on cell number.

Dose* n=19	P21			P60		
	Hepatocyte cell (n)	Bi- nuclear hepatocyte (n)	Bi-nuclear hepatocytes to hepatocyte cells ratio (%)	Hepatocyte cell (n)	Bi- nuclear hepatocyte (n)	Bi-nuclear hepatocytes to hepatocyte cells ratio (%)
0	139.87±1.98^{a}	5.91±0.24^{a}	4.22±0.003^{a}	67.58±0.97^{a}	12.12±0.36^{a}	18.01±0.006^{a}
25	148.08±1.34^{b}	8.25±0.23^{b}	5.58±0.001^{a}	76.04±0.97^{b}	9.92±0.30^{b}	13.43±0.005^{b}
50	105.91±1.9^{c}	22.29±0.94^{c}	21.03±0.008^{b}	58.08±1.18^{c}	9.00±0.42^{c}	15.64±0.008^{b}
100	88.87±1.20^{d}	33.96±1.01^{d}	38.27±0.011^{c}	46.75±1.73^{d}	7.16±0.18^{d}	15.40±0.003^{b}

* Dose of indomethacin: 0, 25, 50, and 100 mg/kg, respectively. Results are expressed as mean ±SEM.
^{abcd}: Different superscripts indicate significant difference (ANOVA; p< 0.0.5).

Tab. 3. Effect of early postnatal exposure to indomethacin on hepatocyte cell and nuclear size

Dose* n=19	Day	Hepatocyte Area (μm^2)	Nuclear Area (μm^2)	Hepatocyte diameter (μm)	Nuclear diameter (μm)	Cytoplasmic area (μm^2)	Nuclear/ cytoplasmic ratio
0	P21	298.14 ± 2.00^{a}	33.97± 0.30^{a}	19.42 ±0.07^{a}	6.54 ± 0.03^{a}	264.17 ± 1.97^{a}	0.13 ± 0.002^{a}
25		293.87 ± 2.22^{a}	42.36 ± 0.41^{d}	19.26±0.07^{a}	7.29 ± 0.03^{d}	251.50 ± 2. 17^{b}	0.18 ± 0.002^{d}
50		324.36 ± 2.24^{b}	39.43 ± 0.32^{c}	20.25 ±0.07^{b}	7.05 ± 0.03^{c}	284.90 ± 2.20^{c}	0.14 ± 0.001^{c}
100		348.49 ± 2.48^{c}	35.55 ±0.41^{b}	20.99±0.07^{c}	6.66 ± 0.04^{b}	312.95 ± 2.43^{d}	0.12 ± 0.002^{b}
0	P60	452.41± 4.28^{a}	49.56 ± 0.71^{a}	23.89 ± 0.11^{a}	7.86 ± 0.06^{a}	402.85 ± 4.04^{a}	0.12 ± 0.002^{a}
25		443.31 ± 4.44^{a}	55.53 ± 0.83^{b}	23.64 ± 0.11^{a}	8.32 ± 0.06^{b}	387.79 ± 4.33^{b}	0.15 ± 0.003^{b}
50		482.99 ± 3.51^{b}	66.10 ± 0.97^{c}	24.73 ± 0.09^{b}	9.07 ± 0.07^{c}	416.88 ± 3.89^{c}	0.16 ± 0.003^{c}
100		500.75 ± 3.57^{c}	72.28 ± 1.04^{d}	25.19 ± 0.09^{c}	9.49 ± 0.07^{d}	428.47 ± 3.39^{d}	0.17 ± 0.003^{d}

* Dose of indomethacin: 0, 25, 50, and 100 mg/kg, respectively. Results are expressed as mean ±SEM.
^{abcd}: Different superscripts indicate significant difference (ANOVA; p < 0.0.5).

resolved with increasing dose of indomethacin at P21. In contrast, body and the liver weights increased by P60, which was concurrent with the liver to body weight ratios. Similar to this finding, it has been shown that early postnatal indomethacin in rats had latent effect on the somatic growth and finely resulted in a significantly higher body and liver weight. Considering the role of PGs in the regulation of growth as well as the growth hormone and insulin-like growth factors (GH-IGF), higher liver and body weight and the liver to body weight ratios might be due to induced serum and hepatic IGF-I by early postnatal indomethacin, (15). Elevated levels of GH-IGF caused hepatomegaly resulting from hepatocellular hypertrophy and proliferation, with hepatocyte cells presenting large cellular and nuclear size (33). In spite of hepatomegaly in 50 and 100 mg/kg indomethacin at P21, there was a significant decrease in liver weight in 25 mg/kg indomethacin at P21. Previous studies have shown that inhibition of Wnt/β-catenin signalling pathway is a target for NSAIDs as chemo-preventive agents (34). Bernardi et al. reported that indomethacin-loaded lipid-core nanocapsules inhibition of cell growth and proliferation correlated with the inactivation of β-catenin and in glioma cells (35). Previously, it was demonstrated that a high number of proliferating cells was reduced in mice treated with indomethacin after partial hepatectomy (PH) (36). Therefore, it seemed as a serious weight loss in 25 mg/kg at P21 and might due to anti-proliferative effect of drug on immature liver in a period of intense proliferation to reach adult levels, since the activation of β-catenin during postnatal day 0–30 is crucial to stimulate hepatocyte proliferation and to gain a postnatal hepatic size (23). In addition, the activation of B-catenin is crucial for HGF-induced hepatocyte proliferation and biliary differentiation (37, 38). However, the effects were transient and resolved in dose- and time- dependent manner. The restoration of body and liver weights suggest catch-up growth and adopt compensatory evidence. Despite severe decreases in the liver weights in 25 mg/kg indomethacin at P21, there was a significant increase in the nuclear size and the number of uni- and bi-nuclear hepatocytes per field at P21. Meanwhile, there is no evidence of hepatocellular hypertrophy at P21 and P60. This finding further suggests that indomethacin may have triggered a cell proliferation as a regenerative response (17) as confirmed by higher liver and body weight and the liver to body weight ratios in 25 mg/kg indomethacin.

There are several reports that liver restoration and regeneration depends mainly on proliferation (39, 40). Michalopoulos and DeFrances demonstrated that after removal of a portion of the liver by surgery or destroyed by toxic injury, the remnant piece reacted and hepatocytes regenerated liver mass loss via compensatory hyperplasia (41). In addition, hepatocyte proliferation was accompanied by an increase in nuclear size and ploidy of hepatocytes, suggesting that hepatocytes do enter into S phase but some of them fail to enter M phase (27). Therefore, several ploidy classes of tetraploid and octaploid hepatocytes with one or two nuclei emerged as a result of polyploidization in liver (42, 43). Our data showed that despite the increase in ploidy of hepatocytes, a characteristic feature of mammalian liver (28, 29), and increased bi-nuclear hepatocytes and its ratio at first, number of bi-nuclear hepatocytes decreased in the regenerated liver in contrast to developmental events. Since in response to loss of liver mass, bi-nuclear hepatocytes entered into M phase, assembled all condensed chromosome of two nuclei in metaphase and produced two uni-nuclear daughter hepatocytes of higher ploidy. In addition, cell division to generate mononuclear hepatocytes occurs more frequently for binuclear hepatocytes than for mononuclear hepatocytes. Finally, these processes increased both of size and ploidy of hepatocyte (27). Therefore, despite initial anti-proliferative effects of drug on immature liver, hepatocytes entered the cell cycle and underwent reductive divisions to liver regeneration by P60.

In contrast, indomethacin caused hepatomegaly resulted from hepatocellular hypertrophy (44) with hepatocytes presenting large cellular and nuclear size in 50 and 100 mg/kg. It is likely due to induced IGF-1 by early postnatal indomethacin (15, 33). Previous study demonstrated in mice treated with 100, 200, or 400 mg/kg ibuprofen (other NSAIDs) for 2 weeks, liver weights significantly increased and correlated with dose-related increases in hepatocellular proliferation and hepatocellular hypertrophy. However, hepatocellular hypertrophy resulted in a decreased the number of hepatocytes per field (45) due to dose-related increases in size of hepatocytes in 50 and 100 mg/kg indomethacin at P21 and P60.

Present data confirmed that changes in cell number, cell size, nuclear number and nuclear size occurred clearly differed between development and pathological changes (27). The difference between regulation of cell cycle during the development and the regeneration of the liver, the peculiar state of the neonate according to the immaturity at birth and progressive evolution of many metabolic functions, especially hepatic drug metabolism (27, 46) may result in changes in response to indomethacin injury.

Many developmental effects of indomethacin have been described (17). Nagai et al. revealed that administration of very low dose (1.3 mg ip) of indomethacin to male rat pups daily on postnatal days 4-13 caused an abnormal lung structure with diminished alveolar air, increased alveolar duct air, increased mean linear intercept, increased septal wall thickness and diminished the number of alveolar crests (14). In liver, our findings revealed that indomethacin resulted in abnormality in lobular pattern especially in portal triads. Sometimes portal triads are disappearing. Also, it increased the portal vein branches and irregularity in sinusoidal patterns, in dose- and time- dependent manner. Although the causes of the altered lobular structure are not clear, it probably leads to maintain normal protocentral distance (26) and increased the portal vein branches at the lobules may be the result of the adaptive changes of hepatocytes (47). It has been shown that reduced proliferation at the tips of the lobes in mice with genetic disruption of Wilms' tumour suppressor gene caused an abnormal liver lobulation (48). Therefore, abnormality in lobular pattern in response to indomethacin might be due to induced serum and hepatic IGF-I and its ability to inhibit Wnt/β-catenin signalling pathway, since hepatocyte cells during postnatal day 0–30 and biliary cells during P10, 15 and 20 proliferate extensively along with an increase in portal triads (23),

and Wnt/β-catenin signalling pathway plays an essential role in HGF-induced hepatocyte proliferation and biliary differentiation (15, 37, 23, 38, 34). To sum up, early postnatal exposure to indomethacin has long term effect on liver development in a dose- and time- dependent manner.

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