

Evaluation of gene expression level of genes FGF2 and CYP27b1 in male Wister rats treated with indomethacin

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Summary:

The biology department's animal house at Iraq's AL-Qadisiyah University hosted this study. It's part of Iraq's AL-Qadisiyah University, to demonstrate that the Kidney and Liver were subjected to oxidative stress from reactive oxygen species, which led to harmful consequences of the indomethacin medication on molecular level. 40 male Wister rats, aged (2 -3) months, weighing between 200 and 300 grams. Four groups of 10 rats. Control, 5 mg/kg, 7 mg/kg, and 10 mg/kg of indomethacin were given for 21 days to the groups.

After the experiment, the animals were killed by hanging. After twenty-one days ,each of the study groups that received indomethacin therapy saw a statistically significant reduction ($P < 0.05$), which was a decrease in the amount of the cyp27b1 gene, with the level of decrease being largest in the group that received treatment with ten milligrams per kilogram for 21 days. There was no difference ($p < 0.05$) in the expression of the FGF2 gene between individuals in the untreated group and those who were given 5 mg, 7 milligrams, while 10 mg/kg of indomethacin showed significant increase. In conclusion indomethacin cause decrease in the level of cyp27b1 gene, and cause increase expression in FGF2 gene.

Keywords:

Introduction

The nonsteroidal anti-inflammatory medicine known as indomethacin is a dimethyl indole derivative. it is believed that indomethacin's pharmacological impact is mediated by the powerful and nonselective inhibition of the COX enzyme. This is true even if the pharmacological effects of indomethacin are still not fully understood (Katary, 2017).

The kidneys are responsible for the elimination of the vast majority of drugs, as well as the substances that are produced when these medications are metabolized. The capacity of indomethacin to slow down the glomerular filtration rate and increase the amount of urine that is evacuated, may cause a considerable loss in renal function, as reported by de Borst et al. (2012). Ischemia-reperfusion injury (IRI) is a condition that may arise as a consequence of a rapid and transient decline in the amount of blood that flows to a specific organ. This drop can cause a condition known as ischaemia-reperfusion damage (IRI). According to Sharfuddin (2011), IRI is often linked with a powerful inflammatory and oxidative stress response to hypoxia and reperfusion, all of which have an effect on the organ's ability to function normally (Oliver *et al.*, 2002) . It has been shown that renal IRI is a prevalent cause of acute kidney damage and repair, as stated by Zhang et al. (2016). It was initially shown by Villanueva et al. (2008) that FGF2 is expressed early during kidney development, re-expressed in the regeneration phase after I/RI, and that FGF2 helps in the recovery process of I/RI by creating an altered synthesis of morphogens via FGFR2.

On the other hand, The liver is the principal organ that is responsible for the metabolism of several medications. indomethacin down regulate expression of many genes in liver like cyp450 enzyme (LaFramboise *et al.*, 2006). A superfamily of heme-thiolate protein, support the oxidative ,peroxidative and reductive metabolism of such endogenous and xenobiotic substrate (Danielson, 2002).

Material and Method

Animals

Fourty adult male wister rats (*Rattus norvegicus*) average weight were (200-220 gm) and aged (2-3 month), these animals were get from the animal house of the Faculty of science /University of Babel / Iraq. Animals placed in plastic cages with wood chip bedding under controlled and standardized conditions of temperature (22-24 °C) and light/dark (12/12 h) cycles, feed with standard rodent diet and water.

Chemicals

Indomethacin capsule was purchased from pharماسutical(MEDOCHEMIE / CYPRUS) 25mg/kg concentration , phosphate buffer sline (PBS) (Chem cruz / China).

Experimental Design

Animals were conducted in the animal house of the Faculty of the Science/ University of Al- qadisiyah, randomly allocated into Four groups (Each group make of 10 male Wister Rats for 21 days

- Control group gives 3 ml of P.B.S. orally and daily for 21 days.
- Second group gives suspension of (5 mg/kg of indomethacin powder dissolves in P.B.S solution) orally and daily for 21 days
- Third group gives suspension of (7 mg/kg indomethacin powder dissolves in P.B.S solution) orally and daily for 21 days.
- fourth group gives suspension of (10 mg/kg indomethacin powder dissolves in P.B.S solution) orally and daily for 21 days .

Isolation of RNA and Reverse Transcription

Small part from cortex of Kidney and Liver tissue for each sample frozen in liquid nitrogen and kept at -70 °C . mRNA extraction by using extraction kit (ADDBio / Korea) through following the manufacturer's user guide. The concentration and purity of the RNA was measured by using a NanoDrop UV spectrophotometer (Bioneer, Korea). The absorbance was measured at 260 nm for determination of total RNA concentration. Samples with ratio of A260/A280 between 2.0-2.23 were used for reverse transcription. A total of RNA was reversed transcribed to cDNA using the kit from ADDBio (Korea). Reverse transcription to produce cDNA and amplified it using AddScript RT-qPCR Syber master (AddBio, Korea).Reaction mixture 20 µl contain 5 µl of total RNA. The reaction began with 10 °C , then reverse transcriptase 50 °C ,after inactivated it in 80 °C for 5 min.

Quantitative Reverse Transcriptase PCR (RT-qPCR) Preparation

The mRNA levels for FGF2, cyp27b1 and house keeping gene were determined by AddScript RT-qPCR Syber master (AddBio, Korea).

For this purpose, amplifying of the FGF2 and CYP27b1 genes were carried out using the following primers (table 1) these were recruited from (Chena *et al.*,2005 ,Zhu *et al.*,2019).

Table (1) . List of primer sequences for FGF2 and CYP27b1 for real time-qPCR analysis

Primer name	Sequence '5-----3'	Target gene	Reference
FGF2-forward	AGCATCACTTCGCTTCCC GC	FGF2	Zhu et al. (2018)
FGF2-Reverse	GGTTCGCACACACTCCCTTG		
CYP27B1-forward	TTCTCAGACACGATCTATGGCTGT	CYP27B1	Chena et al. (2005).
CYP27B1-Reverse	CTACTGTCTCTGCAGAAAGCGTA		
GAPDH-F	GTGGACCTCATGGCCTACAT	Housekeeping gene	
GAPDH-R	GGATGGAATTGTGAGGGAGA		

QPCR- PCR Master Mix for gene (FGF2, cyp27b1)

The reaction were carried out in 20 μ l of mixture containing 2 μ l cDNA. The thermal cycle condition were 2 min. at 50 $^{\circ}$ C , after, 10 min. at 95 $^{\circ}$ C ,then 15 min. at 95 $^{\circ}$ C with 40 cycles. The point at which the fluorescence intensity exceed stander deviation of baseline fluorescence represented Ct value(measure of amount of specific cDNA ,thus mRNA in the sample. The delta-delta Ct method was used to normalize transcript levels to those of 12S-rRNA mRNA as mentioned by (Schmittgen and Livak, 2008) in which the following formula was employed:

$$2^{-\Delta\Delta CT} = [(CT \text{ gene of interest- } CT \text{ internal control}) \text{ sample A- } (CT \text{ gene of interest- } CT \text{ internal control}) \text{ sample B)].$$

Note: Sample A means one certain group.Sample B means another certain group

Data Analysis

The F-test was used to compare the control group with the treatment group after the results were analyzed using (Mean Standard Error). Using a one-way analysis of variance (ANOVA) and the test (Tukey's multiple comparisons test) at a probability threshold of 0.05. The differences were statistically different from one another. ($p < 0.05$) (Argyrous, 2009).

Result

Molecular analysis

RNA concentration

Figure (1)shows no significant changes ($p < 0.05$) in hepatic RNA concentration between control and treatment groups or between treated groups. Figures (2) demonstrate no significant changes ($p < 0.05$) in kidney RNA concentration between control and treatment groups or between treated groups during 21 days.

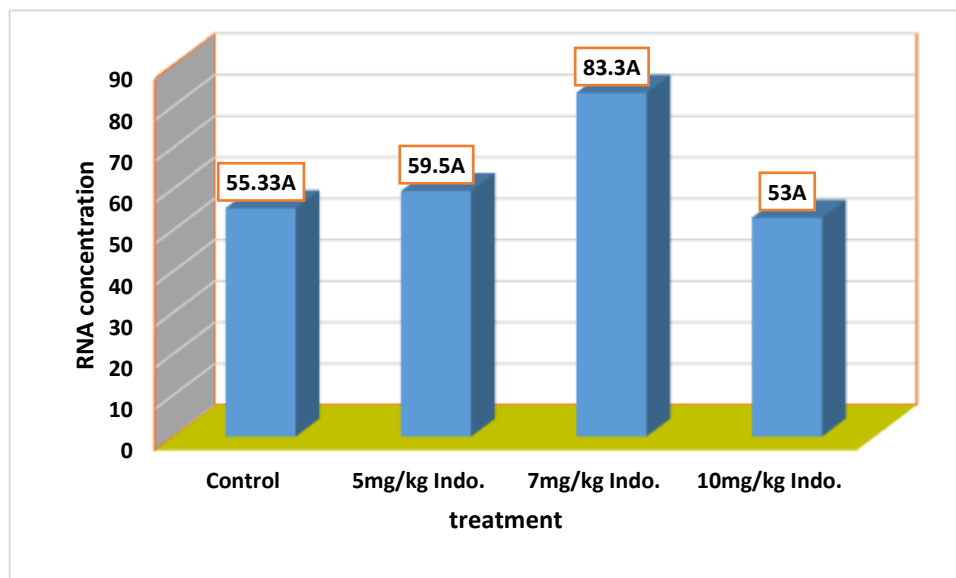


Figure 1: RNA concentration of liver in control and treated groups with dose 5,7,10 mg/ kg indomethacin of male wister rat during 21 days.

(No. of animals=10 for each group, Values are mean \pm SE, Different letters denote to the significant difference ($p < 0.05$)), L.S.D. ($p < 0.05 = 29.11$)

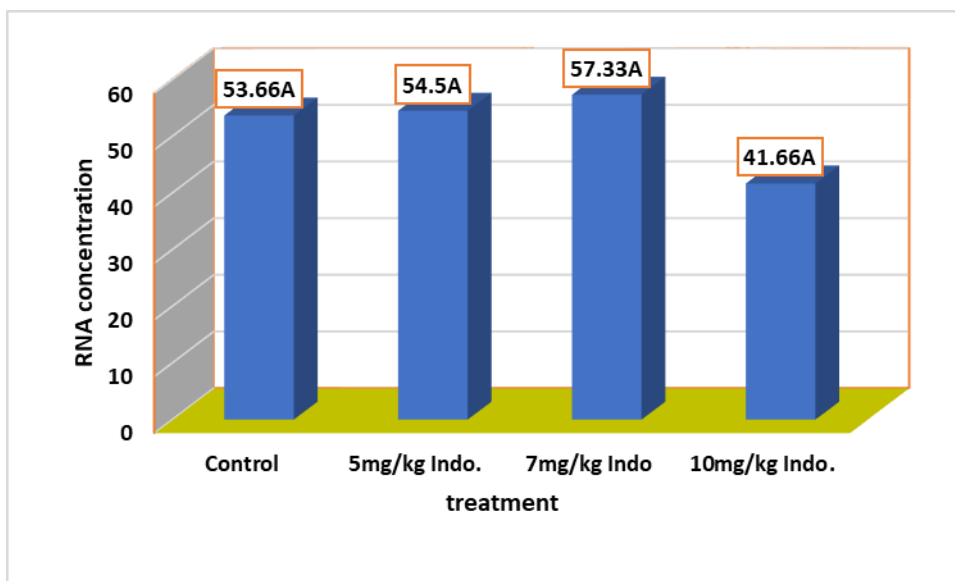


Figure 2: RNA concentration of kidney in control and treated groups with dose 5,7,10 mg/ kg indomethacin of male wister rat during 21 days.

(No. of animals=10 for each group, Values are mean \pm SE, Different letters denote to the significant difference ($p < 0.05$), L.S.D.($p < 0.05$) =34.81)

Analysis of the qRT-PCR gene expression data of FGF2 Gene in Kidney

examined the expression of the FGF2 gene. The results in (Figure 3) revealed marginally or no significant differences ($p < 0.05$) between the control (1 fold) and groups treated with 5 mg (0.5 fold) and 7 mg (0.8 fold) of indomethacin over the course of 21 days, but there was a significant rise ($p < 0.05$) in the regulation of the FGF2 gene in the group treated with 10 mg/kg (3.3 fold) over the course of that same period of time as opposed to the control, 5 mg, and 7 mg/kg groups.

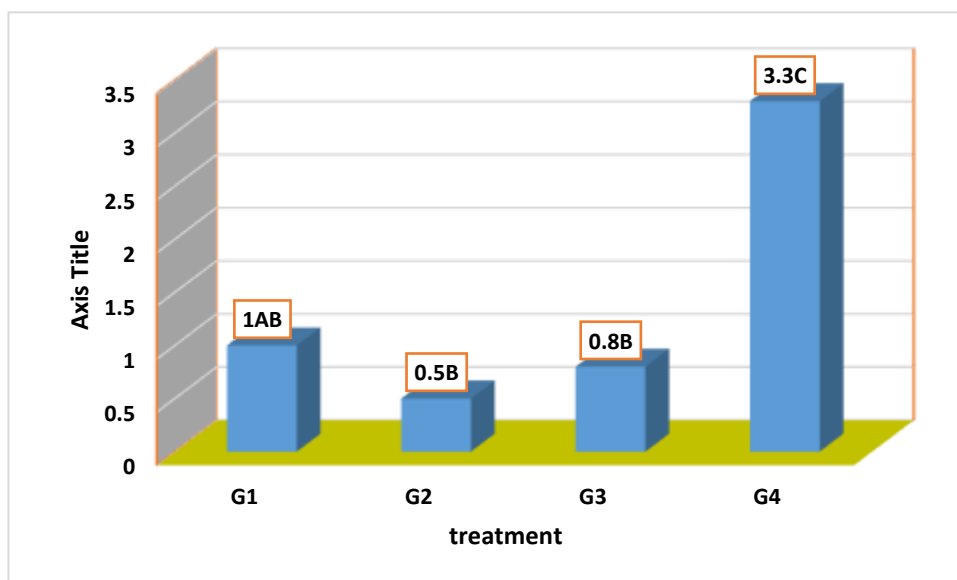


Figure 3: Effect of indomethacin at dose 5,7,10 mg/kg indomethacin on FGF2 gene in Kidney of male wister rat during 21 days.

(No. of animals=10 for each group, Values are mean \pm SE, Different letters denote to the significant difference ($p < 0.05$), LSD ($P < 0.05$)=0.32)

Analysis of the qRT-PCR gene expression data of cyp27b1 in Liver

This study looked at the expression of the cyp27b1 gene. The findings in (Figure 4) showed a substantial down-regulation ($P < 0.05$) of indomethacin treatment groups at 5 mg (0.45 fold), 7 mg (0.45 fold), and 10 mg/kg (0.28 fold) during the course of the trial period of 21 days compared to the control group. Gene expression did not differ significantly ($P < 0.05$) between the groups treated with 5 mg and 7 mg indomethacin

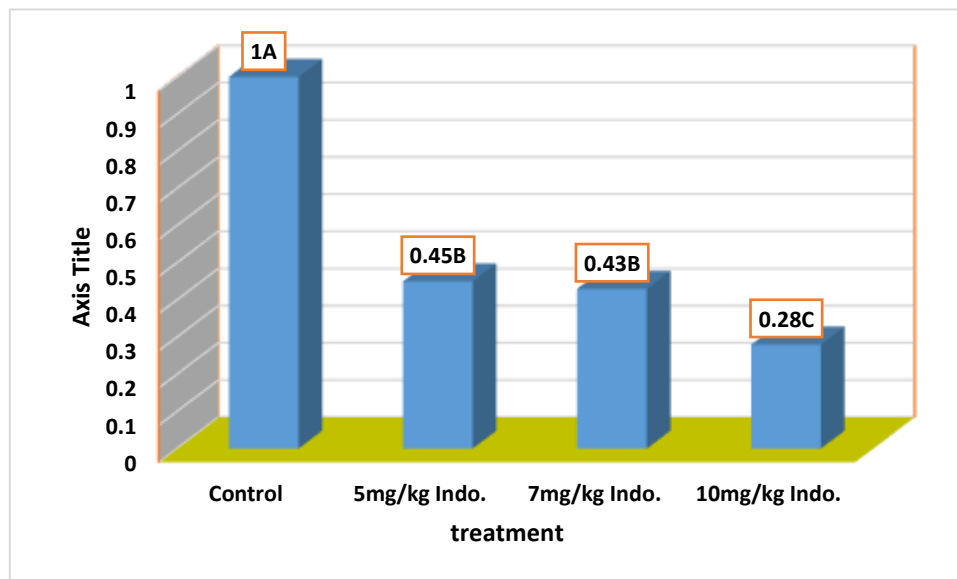


Figure 4: Effect of indomethacin at dose 5,7,10 mg/kg indomethacin on CYP27B1 gene in liver of male wister rat during 21 days.

(No. of animals=10 for each group, Values are mean \pm SE, Different letters denote to the significant difference ($p < 0.05$), LSD ($P < 0.05$), 0.14)

Discussion

Rats given indomethacin exhibited higher renal cortex FGF2 mRNA expression compared to controls (Mucha *et al.*, 2007). Damage from ischemia may result from inhibiting COX-mediated prostaglandin production. According to Lear *et al.* (1990), prostaglandin E2 reduces tubular cell oxygen consumption while widening medullary arteries. Reduced arterial perfusion, vasoconstriction, ischemia, hypoxia, and cortical damage are consequent effects of decreased prostaglandin synthesis (Oliver *et al.*, 2002). According to Rosenberger *et al.* (2003), angiogenesis may be influenced by hypoxia and the various genes that become active in response to low oxygen levels. The elevated levels of VEGF and FGF-2 mRNA might be attributed to indomethacin-induced hypoxia. In kidneys, hearts, and brains, ischemic damage enhances the production of VEGF mRNA and protein (Vannay *et al.*, 2004). Such a protective effect has been shown in a number of individuals with different chronic tubulointerstitial diseases (Choi *et al.*, 2000) and in a rat model of ischemia damage (Matsumoto *et al.*, 2003).

According to Villanueva *et al.* (2006), FGF2 factor may accelerate regeneration and that bFGF can be reexpressed to restore mature kidney function during regeneration, comparable to nephrogenesis during embryonic development.

After a brief period of ischemia, adult kidney cells may once again produce kidney development proteins. One specific morphogen, bFGF, might expedite this process, suggesting that it aids in renal recovery. According to Imgrund *et al.* (1999), bFGF, Ncam, BMP-7, Lim-1, Engrailed, and ZO-1 are required for early metanephric kidney development.

Tie-2 supports vascular integrity, angiogenesis, and nephrogenesis. In contrast to kidneys treated with bFGF, which produced Tie-2 24 hours after I/R, increased its level, and assessed its presence in tubular cells, kidneys injected with saline reexpressed Tie-2 48 hours after I/R. Tie-2 is thought to be important in early mammalian nephrogenesis and vascular development, according to Villanueva (2006). Seven days after I/R, tie-2-positive endothelial progenitor cells were seen (Patschan *et al.*, 2006).

Exogenous FGF2 protects against renal I/RI and dramatically enhances animal lifespan by attenuating numerous I/RI-induced mitochondrial-damaging parameters, according to a research that was published in 2017 by Tan et al., which offered the first experimental confirmation of this phenomena. The expression of Bcl2/Bax has been altered in a pro-apoptotic manner, and caspase-3 is activated. FGF2 is also in charge of maintaining the expression of mitochondrial KATP channels. Therapy with FGF2 reduces the release of HMGB1 that is produced by I/R, as well as signaling mediated by TLRs and the production of inflammatory cytokines, in addition to preserving the renal parenchyma.

On the other hand, Our finding agreed with (Falzon *et al.* , 1985) who mention that the enzymological results indicate that administration of high-doses of indomethacin selectively decreases the hepatic cytochrome P-450 mono-oxygenase system and has a variable effect on several other hepatic enzymes. According to Falzon et al. (1984a), the intestinal ulcers caused by indomethacin may have transferred endotoxins or other substances into the portal vein, which may have contributed significantly to the decline in hepatic cyt. P-450. The direct denaturing action of indomethacin on cyt. P-450 in vitro (although at high concentrations) (Falzon *et al.* 1984b) and the drug's preferred accumulation in the liver in vivo (Rainsford *et al.* 1981) imply it may also have a direct effect on the cytochrome P-450.

Also agree with (Sedrani and EL-Banna, 1987) who said idomethacin alone significantly reduced plasma levels of both 25(OH) D and 1,25(OH)₂D which expressed by cyp27b1 gene. The results of the present investigation clearly indicate that idomethacin, interferes with the normal production of both 25(OH)D and 1,25(OH)₂D in rabbits during early pregnancy, due to the inhibitory effect of Idomethacin on prostaglandin synthesis, since the replacement of a combination of prostaglandins with idomethacin resulted in the restoration of the production of the vitamin D metabolites to their normal levels .PGE₂ and PGF₂α have been reported to stimulate the production of 1,25(OH)₂D in primary chick kidney .cell culture, possibly by a cAMP-mediated mechanism (Trechsel *et al.*, 1980). Study reported that PGE₂ stimulates the production of 1,25(OH)₂D independent of cAMP and PTH in vitamin D-deficient rats (Yamada *et al.*, 1983). Other PG inhibitors have been reported to affect the production of vitamin D metabolites. Aspirin is reported to inhibit the production of 1,25(OH)₂D in vitro due to its inhibitory effect of PG synthesis (Wark *et al.*, 1981).

The fact that the production of progesterone has also been affected by indomethacin treatment, and the fact that progesterone was restored to the control levels by PG treatment, are in support of the earlier reports that PGs may act as mediator in steroidogenesis (Silver and Smith, 1975)

vitamin D deficiency associated with chronic liver disease (Stokes *et al.*, 2013). indomethacin can cause acute hepatitis then developed to chronic auto- immune hepatitis (Abraham *et al.* , 2008).

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