

Effect of Adrenomedullin and Omega-3 Polyunsaturated Fatty Acids on Celecoxib - Induced Acute Hepatic Injury in Experimental Rats

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Abstract

Celecoxib as a nonsteroidal anti-inflammatory agent has hepatotoxicity, increase both oxidative stress and proinflammatory cytokines. The present study investigated the effect of adrenomedullin and omega-3 polyunsaturated fatty acids in celecoxib - induced acute hepatic injury in experimental rats. Fifty Sprague-Dawley rats were divided into five groups; control, celecoxib treated group, celecoxib and adrenomedullin treated group, celecoxib and omega 3 treated group, celecoxib, adrenomedullin and omega 3 treated group. Liver functions tests, hepatic oxidants and antioxidants parameters, plasma nitric oxide levels, serum proinflammatory cytokines, plasma PGE2 and adrenomedullin were measured and also histopathological examination was done. celecoxib significantly impaired liver functions, increase hepatic oxidants, decrease hepatic antioxidants, increase proinflammatory cytokines, decrease PGE2 and increase adrenomedullin ,moreover showing necrosis in histopathology. Adrenomedullin and omega-3 PUFAs improved liver functions, decrease oxidative stress, decrease cytokines and PGE2 but nitric oxide level was significantly increased by adrenomedullin whereas decreased by omega-3 PUFAs. There was insignificant change in serum albumin in all groups. Histopathological examination revealed that most of the hepatocytes appeared with normal colored eosinophilic cytoplasm and vesicular basophilic nuclei. It could be concluded that celecoxib had an oxidant stress effect in addition to increase proinflammatory cytokines and consequent acute hepatic insult. The development of hepatic injury was celecoxib dose dependent. Adrenomedullin and omega-3 polyunsaturated fatty acids could alleviate the acute hepatic injury, oxidative stress, decrease proinflammatory cytokines and by turn recovery of hepatic morphology and functions.

Introduction

Excessive dose exposure to various pharmacological or chemical substances may induce acute liver injury either by direct damage or immune-mediated which characterized by oxidative stress, inflammation and derangement of hepatic function [1]. The inflammatory mediators may include reactive oxygen species (e.g., superoxide & nitric oxide), lipid mediators (e.g., prostaglandins (PGs) and peptide mediators (e.g., cytokines) [2].

The cyclooxygenases are required for the formation of prostaglandins, which act like hormones in controlling many of the functions of the body. Celecoxib (celebrex) is one of the cyclooxygenases 2 (Cox-2) inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs) which selectively inhibit Cox-2. Hepatic cyclooxygenase-2 expression increases in toxins-induced liver diseases. Celecoxib is effective in pain relieving when used at therapeutic doses [3]. An overdose of celecoxib has been associated with liver injury in humans and experimental animals and has worsened the liver damage [4].

A potent vasodilating peptide, adrenomedullin (ADM), a 52 amino acid peptide, has been isolated from human pheochromocytoma [5], also it has been found to be expressed in various organs, including the liver [6]. It can act as both a hormone and a cytokine (often termed a "hormokine" [7]. Adrenomedullin is upregulated in liver diseases to counteract the disease process with its physiological actions including anti-oxidative effects [8]. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) induce the production and secretion of ADM but the effect of ADM on cytokines remains unclear. Conversely,

Pintér et al., postulated that ADM induces the downregulation of inflammatory cytokines in cultured cells [9]. Furthermore, Shinya et al., suggested that ADM down regulates inflammatory processes in a variety of different colitis models [10]. In addition, oxidative stress can be triggered by various inducers, including over dose of NSAIDs which can induce acute inflammation associated liver tissue damage [11]. Furthermore, it has been suggested that adrenomedullin could also exert a protective action to the liver, through its vasodilator, apoptosis suppression and anti-oxidative actions. [12]. In current study, there is an attempt to clarify the effect of ADM on the production of inflammatory mediators, including cytokines and oxidant substances.

Omega-3 polyunsaturated fatty acids (PUFAs) comprise a family of unsaturated fatty acids that consist of α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) which are abundantly present in fish oil and has anti-inflammatory and antioxidant properties protecting oxidative stress-induced toxic injuries [13]. Moreover, Omega-3 has many other effects, such as reducing hepatic injury; i.e. the protective effect of omega-3 has been reported to inhibit the oxidative stress in hepatic ischemia-reperfusion injury in rat [14]. They were also reported to have efficacy in ameliorating various toxin-induced hepatocellular damage [15]. The omega-3 fatty acids are generally safe and well tolerated and have not been implicated in causing serum enzyme elevations or clinically apparent liver injury. An inflammation-dampening effect has been observed with increased omega-3 fatty acid supplementation in several inflammatory diseases.

The aim of this study is to investigate the effect of ADM and omega 3 PUFAs on celecoxib –induced acute hepatic injury in experimental rats.

Materials & Methods

Experimental Animals

Fifty Sprague-Dawley male rats (weighing 320 ± 30 gm) were used in the experiments. Animals were housed [Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University ,Egypt] in separate cages at 25±2°C °C with relative humidity of 50 ± 10 % under 12 hour's light and dark cycle. Rats were maintained on a standard diet and water was freely available for 2 weeks acclimatization period before the test. All experimental animals were handled in accordance with the standard guide of laboratory animal care of the United States National Institutes of Health (NIH, 1978). All animal procedures were performed in accordance with protocols approved by the Institutional Review Board (IRB) of Mansoura faculty of Medicine, Mansoura University, Egypt.

Chemicals

Adrenomedullin (ADM) is a synthetic peptide, produced by SIGMA CHEMICAL CO . St.Louis , USA. It is dissolved in distilled water. Celecoxib (celebrex) is produced by Pfizer (New York, NY, USA) . Omega-3 poly unsaturated fatty Acids (omega-3 PUFAs) in fish oil . The fatty acid composition of the Marincap capsule [Marin-cap 500 mg, Kocak Pharma, Turkey] was EPA 18 % and DHA 12 %

Experimental design

Experimental rats were divided into 5 groups, consist of 10 rats in each group as follow;

1. **Group I** (control group), rats were injected sodium citrate buffer (pH 4.5) as a single intraperitoneal injection.
2. **Group II** (celecoxib group), rats received a single dose (1 g/kg B.W. ip) dissolved in sodium citrate buffer(pH 4.5) [16]
3. **Group III** (celecoxib + ADM): as group II but rats received s.c injection of ADM at a dose of 100 ng/kg/day for 14 days (17).
4. **Group IV** (celecoxib + omega 3 PUFAs): as group II but rats received omega 3 PUFA at a dose 0.3 g/kg/day by gavage for for 14 days [18, 19].
5. **Group V** (celecoxib + ADM+ omega 3 PUFAs): as group III but rats received omega 3 PUFA at a dose 0.3 g/kg/day by gavage for for 14 days

Blood samples collection and liver harvesting

By the end of experiment, rats were anaesthetized using pentobarbital anesthesia (60 mg/kg, ip), blood samples were collected from rats via cardiac puncture, and an anticoagulant (EDTA) was added to tube containing 5 ml blood which centrifuged at 2,000 g for 1 min at 4°C to separate plasma from erythrocytes. Plasma was immediately frozen and stored at -80 °C until assayed. Also , the serum was obtained by a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1,000 x g. Assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Histopathological examination of the liver tissues

Also, the abdomen of the rat was opened via midline laparotomy and liver samples were harvested from all animals, and they were divided into 2 sets; one set is prefixed in 10% neutral buffered formalin for 24 hours and prepared by the usual tissue handling for paraffin blocks preparation. Sections of 3-4 μm thickness were stained with Hematoxylin and Eosin (H&E), examined and photographed. The other set was used to prepare a liver homogenate by taking parts of the livers obtained from the rats of all groups, freshly washed and homogenized with an ultrasonic homogenizer in cold phosphate buffer, pH 7.4, containing ethylene-diaminetetra- acetic acid (EDTA). The supernatant obtained from each sample was used for SOD ,CAT , GSH-Px and MDA analysis [20].

Assay of liver functions parameters

Serum bilirubin is measured in serum according to Mallory H.T. and Evelynk.A., [21] .Prothrombin time (PT) was measured using calcium rabbit brain thromboplastin technique (Diagen Diagnostic Reagent Ltd, Oxon, UK). [22]. Serum aspartate aminotransferase (AST) , alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using a commercial kit purchased from BioTron Diagnostics (Hemet, CA) as specified by the manufacturer [23]. This ELISA kit is a sandwich enzyme immunoassay for the in vitro quantitative measurement [24].Hepatic lactic dehydrogenase (LDH) was measured by harvesting the cells and centrifuged at $900\times g$ for 5 min. The activity of LDH was determined spectrophotometrically from the changes in absorbance at 560 nm, using 0.18 mM NADH and

0.72 mM pyruvate as substrates in 50 mM phosphate buffer [25].

Assay of markers of oxidative stress in liver

Hepatic malondialdehyde MDA , super oxide dismutase SOD levels were measured in liver homogenates using superoxide dismutase assay kit (SOD) (Thiobarbituric Acid Reactive Substances, TBARS Assay kit, Cayman Chemical Item Number 10009055 as recommended by the manufacturer) [26]. Hepatic catalase activity (CAT) was measured by Beutler's method (Beutler E, ed.(1973): Red cell metabolism, a manual of biochemical methods. New York:Grune & Stratton, inc;76-85).Hepatic glutathione peroxidase activity (GSH-Px) was measured by glutathione colorimetric assay kit [27]. Hepatic reduced glutathione (GSH) in cell lysates was determined according to Chen et al. [28]. Fluorescence was measured in a Wallac 1420 microplate reader (Perkin Elmer, Waltham, MA, USA) where the excitation and emission wavelengths were 350 and 420 nm, respectively.

Plasma nitric oxide (NOx) measurement

Plasma concentration of NOx was studied by chemoluminescence method using commercially available colorimetric assay (Roche, Cat No 1746081)[29].

Assay of serum pro- inflammatory cytokines (IL-1, IL-6 and TNF-alpha)

Serum samples from rats and control at 14 day after surgery were analyzed for IL-1, IL-6 and TNF- α .These cytokines were measured by ELISA kits (The Biosource International Cytoscreen™, Camarillo, CA, USA) according to instructions in the package insert.

Assay of plasma prostaglandins E2 (PGE2)

Plasma prostaglandin E2 (PGE2) was estimated by using titerzyme PGE2 enzyme immunoassay kit, manufactured by Perspective Biosystems.

Assay of plasma adrenomedullin (ADM)

Plasma ADM concentration was measured by enzyme immunoassay (Phoenix Pharmaceuticals Inc. Harbor Boulevard, Belmont, California 94002) after extraction through the Sep-pak C-18 column supplied by the manufacturer [30].

Statistical analysis.

The obtained data were represented as Mean \pm SD. Comparison for parametric data was done by analysis of variance (ANOVA) followed by turkey's post hoc analysis. $P < 0.05$ was considered significant.

Results***Animal survival***

By the end of experiment, four rats died during the experiment. The final numbers were, group I=10, group II=8, group III=9, group IV=9 and group V=10.

The effect of adrenomedullin and omega 3 polyunsaturated fatty acids on liver functions tests in celecoxib – induced acute hepatic injury in experimental rats.

There were significant ($P < 0.001$) increase in total serum bilirubin (mg/dl), prothrombin time (sec.), serum AST (IU/L), serum ALT (IU/L), ALP (IU/L) and hepatic LDH (IU/L) in group II in comparison to group I. Meanwhile, there were significant ($P < 0.01$) decrease in these parameters in group III in relation to group II as a result of

adrenomedullin administration. Furthermore, there was evident and significant ($P < 0.001$) improvement (decrease) in the same parameters in group IV in comparison with groups II and III as a result of administration of omega 3 PUFAs. In addition, there was a significant ($P < 0.001$) decrease in same parameters in group V in comparison to groups II and III as a result of effect of both adrenomedullin and omega 3 PUFA in celecoxib – induced acute hepatic injury. There was insignificant change in serum albumin (g/dL) in all groups (II, III, IV and V) in comparison to group I (table 1).

The effect of adrenomedullin and omega 3 polyunsaturated fatty acids on hepatic oxidants, antioxidants parameters and plasma nitric oxide level in celecoxib – induced acute hepatic injury in experimental rats.

There was a significant ($P < 0.001$) increase in oxidant parameters (hepatic MDA (nmol/gm tissue) and plasma NOx ($\mu\text{mol/L}$)) while significant ($P < 0.001$) decrease in hepatic antioxidant system (SOD (U/mL), CAT (mmol/mL), GSH-Px (mol/mL) and GSH ($\mu\text{g/mg}$ protein)) in group II as compared with group I. On the other hand, there was a significant change in these parameters (decrease hepatic MDA and plasma NOx and increase SOD, CAT, GSH-Px and GSH) in groups III, IV and V in comparison to group II (table 2).

Effect of adrenomedullin and omega 3 polyunsaturated fatty acids on serum proinflammatory cytokines in celecoxib – induced acute hepatic injury in experimental rats.

There was a significant ($P < 0.001$) increase in serum IL-1 ($\text{pg}\cdot\text{mL}^{-1}$), IL-6($\text{pg}\cdot\text{mL}^{-1}$) and TNF- α ($\text{pg}\cdot\text{mL}^{-1}$) in groups II as compared with group I. While there was significant decrease in group III

as compared with group II. Wherever, there was a significant ($P < 0.001$) decrease in groups IV and V in comparison to group II (figure 1).

Table 1 Liver functions tests in studied groups

Variables	Group I(n=10)	Group II(n=8)	Group III(n=9)	Group IV(n=9)	Group V(n=10)
Total serum bilirubin (mg/dl)	0.18 \pm 0.05	4.71 \pm 0.52 ^a	2.51 \pm 0.42 ^{ab}	0.84 \pm 0.57 ^{bc}	0.58 \pm 0.43 ^{bc}
Serum albumin(g/dL)	3.82 \pm 0.12	3.57 \pm 0.64	3.45 \pm 0.42	3.71 \pm 0.24	3.77 \pm 0.46
Prothrombin time (sec.)	15.53 \pm 0.32	21.01 \pm 0.11 ^a	20.82 \pm 0.20 ^a	15.52 \pm 0.21	15.41 \pm 0.21
Serum AST (IU/L)	123.85 \pm 5.17	171.95 \pm 15.41 ^a	152.73 \pm 12.45 ^{ab}	123.55 \pm 5.14 ^{abc}	101.94 \pm 11.46 ^{abcd}
Serum ALT (IU/L)	30.65 \pm 1.92	144.55 \pm 4.75 ^a	65.61 \pm 1.84 ^{ab}	37.64 \pm 4.72 ^{bc}	32.63 \pm 4.52 ^{bc}
Serum ALP (IU/L)	90.49 \pm 4.75	136.49 \pm 9.14 ^a	110.64 \pm 9.71 ^{ab}	91.64 \pm 9.64 ^b	79.39 \pm 4.58 ^{bc}
Hepatic LDH (IU/L)	892.57 \pm 141.17	1468.14 \pm 152.26 ^a	1298.71 \pm 167.15 ^{ab}	719.43 \pm 119.02 ^{abc}	608.43 \pm 129.05 ^{abcd}

The values were expressed as mean \pm SD. Results were considered significant if $p < 0.05$.^a Significant compared with control group I, ^b Significant compared with group II, ^c Significant compared with Group III and ^d Significant compared with Group IV

Table 2 Hepatic oxidants , antioxidants parameters and plasma nitric oxide level in studied groups

Variables	Group I(n=10)	Group II(n=8)	Group III(n=9)	Group IV(n=9)	Group V(n=10)
Hepatic MDA (nmol/gm tissue)	3.55 \pm 0.32	8.94 \pm 0.31 ^a	4.11 \pm 0.35 ^b	3.90 \pm 0.35 ^b	3.47 \pm 0.22 ^b
Plasma NOx ($\mu\text{mol/L}$)	71.14 \pm 33.41	130.36 \pm 24.27 ^a	150.13 \pm 20.82 ^{ab}	81.71 \pm 5.42 ^b	90.65 \pm 6.39 ^b
Hepatic SOD (U/mL)	22.50 \pm 1.52	10.54 \pm 1.23 ^a	19.27 \pm 1.73 ^b	27.28 \pm 1.62 ^{bc}	28.47 \pm 1.52 ^{bc}
Hepatic CAT (mmol/mL)	7.27 \pm 0.24	2.83 \pm 0.15 ^a	5.90 \pm 0.35 ^{ab}	6.93 \pm 0.24 ^{bc}	7.84 \pm 0.31 ^{bcd}
Hepatic GSH-Px (mol/mL)	0.42 \pm 0.05	0.21 \pm 0.07 ^a	0.40 \pm 0.03 ^b	0.51 \pm 0.03 ^{bc}	0.47 \pm 0.05 ^b
Hepatic GSH ($\mu\text{g/mg}$ protein)	92.31 \pm 4.62	30.73 \pm 5.43 ^a	87.32 \pm 6.12 ^b	100.3 \pm 5.14 ^{bc}	102.35 \pm 4.13 ^{abc}

The values were expressed as mean \pm SD. Results were considered significant if $p < 0.05$.^a Significant compared with control group I, ^b Significant compared with group II, ^c Significant compared with Group III and ^d Significant compared with Group IV

The effect of adrenomedullin and omega 3 polyunsaturated fatty acids on plasma prostaglandins E2 and adrenomedullin in celecoxib – induced acute hepatic injury in experimental rats.

There was a significant ($P < 0.001$) decrease in plasma PGE2 (pg/ml) in group II in relation to control group while significant increase in groups III, IV and V in comparison to group II. On the other hand , there was a significant($P < 0.001$) increase in plasma adrenomedullin (ng/ml) level in groups II, III,IV and V in comparison with control group while a significant increase of adrenomedullin in group III as compared with group II. In addition, there was a significant

increase in group V as compared to group IV (figure 2).

The effect of adrenomedullin and omega 3 polyunsaturated fatty acids on histopathological specimens of the liver tissue in celecoxib – induced acute hepatic injury in experimental rats

The figure 3 .I (control group) showed the normal liver architecture with normal central vein and blood sinusoids. Figure 3.II (celecoxib treated group) showed extensive interstitial hemorrhage, areas of cloudy swelling and necrosis. The central veins appeared congested and most of the hepatocytes showed signs of degeneration with vacuolated cytoplasm and pyknotic nuclei. Inflammatory cell

infiltration can be seen intervening between the hepatocytes. Figure 3.III (celecoxib -ADM) showed less marked hepatocytes degenerative signs, congestion of the central veins and inflammatory cell infiltration. Figure 3.IV (celecoxib –omega 3 PUFAs) showed a milder degree of liver tissue affection, central veins are still congested and the hepatocytes

showed less degenerative changes. Figure 3.V(celecoxib- ADM-omega 3 PUFAs) showed clear central veins and scarce inflammatory cell infiltration in addition, most of the hepatocytes appeared with normal colored eosinophilic cytoplasm and vesicular basophilic nuclei (figure 3, H&E, x400).

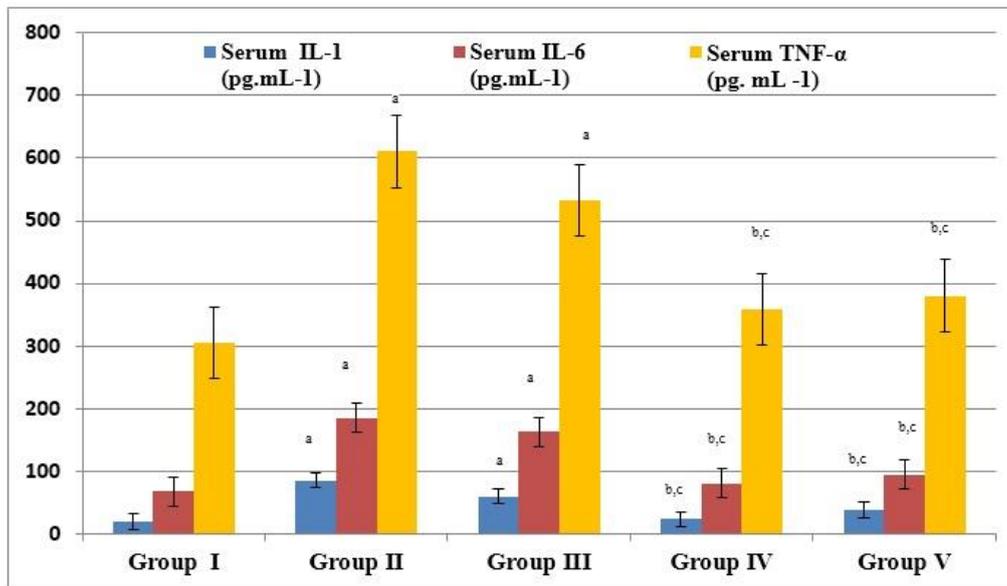


Figure 1 Serum proinflammatory cytokines in studied groups. ^a Significant compared with control group I, ^b Significant compared with group II, ^c Significant compared with Group III and ^d Significant compared with Group IV

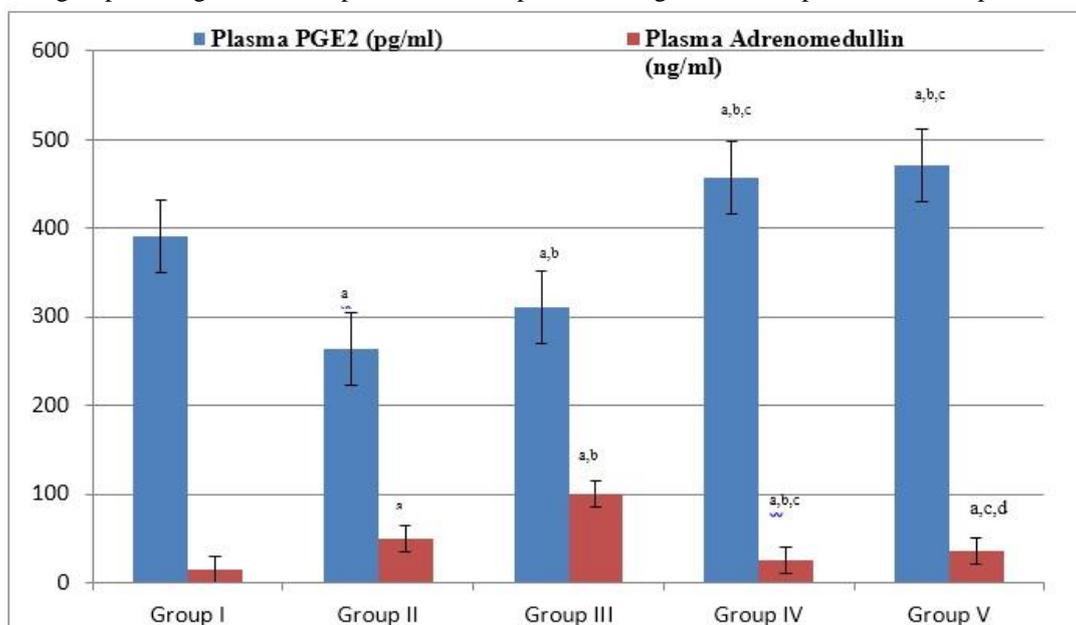


Figure 2 Plasma prostaglandins E2 and adrenomedullin levels in studied groups. ^a Significant compared with control group I, ^b Significant compared with group II, ^c Significant compared with Group III and ^d Significant compared with Group IV

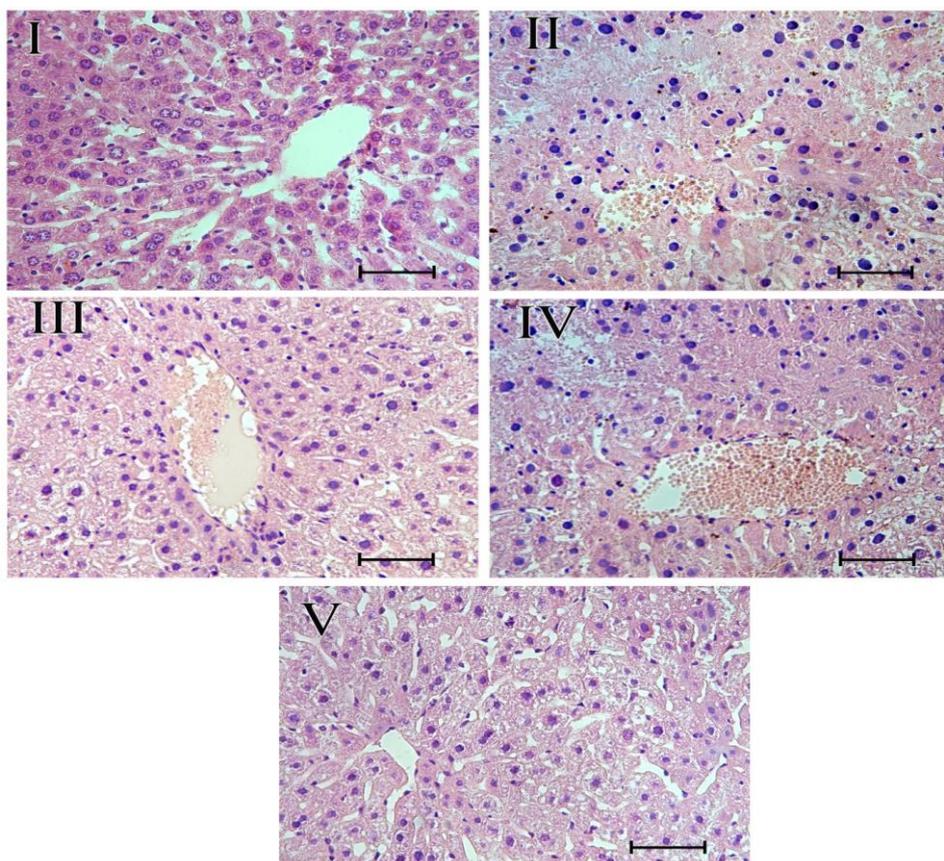


Figure 3: Histopathological results in different studied groups. *Figure 3.I* (control group) showed the normal liver architecture with normal central vein and blood sinusoids. *Figure 3.II* (celebrex treated group) showed extensive interstitial hemorrhage, areas of cloudy swelling and necrosis. The central veins appeared congested and most of the hepatocytes showed signs of degeneration with vacuolated cytoplasm and pyknotic nuclei. Inflammatory cell infiltration can be seen intervening between the hepatocytes. *Figure 3.III* (celebrex -ADM) showed less marked hepatocytes degenerative signs, congestion of the central veins and inflammatory cell infiltration. *Figure 3.IV* (celebrex -omega 3 PUFAs) showed a milder degree of liver tissue affection, central veins are still congested and the hepatocytes showed less degenerative changes. *Figure 3.V* (celebrex - ADM -omega 3 PUFAs) showed clear central veins and scarce inflammatory cell infiltration in addition, most of the hepatocytes appeared with normal colored eosinophilic cytoplasm and vesicular basophilic nuclei. **Hx&E(x400).**

Discussion

Liver injury was induced in rats using high dose of celecoxib. The main pathogenic mechanisms responsible for hepatic injury caused by overdose of celecoxib are oxidative stress and more expression of inflammatory cytokines [31]. So intervention of these pathways could protect against acute liver injury

Regarding the postulated mechanisms involved in celecoxib induced acute hepatic injury; *First*, there are a number of enzymatic and non-enzymatic sources of ROS in the liver injury, including

xanthine oxidase activity, lipid peroxidation, peroxidase, nitric oxide synthase (NOS), NADPH oxidase [32] and accelerated generation of hydroxyl radicals ($\bullet\text{OH}$) in liver of rats [33]. Furthermore, the oxidation of sulfhydryl groups of cytochrome and depletion of reduced glutathione (GSH) occur causing inadequate detoxification mechanism and accumulation of malondialdehyde (MDA) within hepatocytes [34] causing increased permeability of mitochondrial membrane and cell death. [35]. The involvement of oxidative stress in liver injury was demonstrated in this study by a

significant increased MDA in liver tissues and plasma NOx. Moreover, there were deficient in hepatic antioxidant enzymes ;superoxide dismutase (SOD) , catalase (CAT) , glutathione peroxidase (GSH-Px) and associated decrease in reduced glutathione (GSH) resulting in increased production of O₂• and H₂O₂ [36]. Moreover, Hui et al. [37] denoted that celebrex accelerates hepatic necrosis in models that involve severe oxidative stress which was associated with significant derangement of the liver functions tests in our study .it was apparent that total serum bilirubin , prothrombin time, serum AST, ALT, ALP and hepatic LDH were significantly increased (P <0.001) in group-II while no significant difference of serum albumin was found between the other four groups compared with the control group.

In addition, as a participation of immune system, hepatic infiltration by neutrophils , as shown in our histopathological findings, is an early response to tissue injury, cellular stress or systemic inflammation. This accumulated neutrophils in liver damage sense chemotactic signals from the hepatic parenchyma, which leads to neutrophil extravasation and contact with hepatocytes. This triggers complete neutrophil activation with prolonged adherence-dependent oxidative stress and degranulation. The oxidants diffuse into hepatocytes and initiate intracellular oxidative stress, mitochondrial dysfunction and, finally, cause oncotic cell death [38]. This cell death was demonstrated in our histopathological results, correlates with the oxidative stress [38].

One of the other important free radicals involving in the pathogenesis of celecoxib -induced liver injury are reactive nitrogen species produced during oxidative stress, which include the radicals

nitric oxide (NOx⁻) and nitrogen dioxide (NO₂⁻), as well as the non radical peroxynitrite (ONOO⁻), nitrous oxide (HNO₂), and alkyl peroxynitrates (RONOO). Of these NOx⁻ have been the most widely investigated in the liver injury [39]. Increased NOx and associated increased production of cytokines raise the susceptibility to hepatotoxicity induced by celecoxib and was correlated with the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) in hepatic tissue.

Second, celecoxib -induced hepatotoxicity stimulates the release of proinflammatory cytokines, such as IL-1, IL-6 and TNF-α [40] which correlates with the results of current study assuming the incrimination of immune function in rats with acute liver injury. TNF- α is significantly elevated in sera of acute hepatic injury rats [40] as demonstrated in our work.

Third, in drug-induced liver injury, the drug or its metabolite binds to cellular proteins to form hapten-carrier conjugates forming drug- protein adducts. Hapten-like autoimmunity may involve the phagocytosis, processing and presentation of altered proteins by antigen presenting cells, activation of T-helper cells, induction of hapten-specific cytotoxic T cells and the production of autoantibodies by B cells against target antigens in the liver, including the drug (hapten) [41]. Also, the binding of a celecoxib to the P-450 enzyme acts as an immunogen, activating T cells and cytokines production [42] and activation of the apoptotic pathways by the TNF-α receptor may trigger the cascade of intercellular caspases, which results in programmed cell death as manifested in our study by increase in circulating markers of

liver damage and extensive interstitial hemorrhage, areas of cloudy swelling and necrosis [42].

Celecoxib inhibits cyclooxygenase 2 (COX-2), an enzyme that metabolizes arachidonic acid to a class of oxidized fatty acids called prostaglandins [43] especially PGE2 which can directly or indirectly act as anti-inflammation by suppressing NF κ B activation [43]. Furthermore, COX-2-dependent electrophile oxo-derivative molecules have been shown to modulate the anti-inflammatory action via activation of nuclear factor erythroid-2-related factor-2 (Nrf2) - dependent antioxidant response element (ARE) [44].

Interestingly, in this study we found that markers of liver injury ; hepatic enzymes as AST, ALT , ALP ,LDH , MDA and cytokines were elevated in the rats that received toxic dose of celecoxib [45].

The leakage of intracellular enzymes (LDH) into the plasma is an obvious sign of hepatic injury, being an index for hepatic necrosis which was assured by our histopathological results, due to the disturbance caused in the transport functions of hepatocytes [45]. These results supported the concept that COX2 and PGE2 are anti-inflammatory; the effect was blocked by celecoxib. So the rationale of this present research aimed to study the effect of adrenomedullin and omega 3 PUFA in celecoxib – induced liver injury in experimental rats.

Furthermore, cytokines such as tumor necrosis factor alpha (TNF- α) has been linked to hepatotoxicity and the inflammatory response as mentioned before and are key factors in liver inflammation and increased serum ALT levels specifically via a NF κ B pathway [46].

Adrenomedullin (ADM) is widely known as a potent vasodilator and increased during hepatic injury to counteract the effect of oxidative stress and cytokines. This concept was driven us to study the effect of exogenous ADM in celecoxib - induced hepatic injury. The endogenous antioxidant potential of ADM improved liver and the mitochondrial dysfunction by attenuating the celecoxib -induced excessive oxidative stress and prevention of GSH depletion [47]. Current results in groups III and V showed the attenuation of oxidative stress (decreased MDA and NO x) by ADM .This support the degradation of free radicals by activation of scavenger systems including non-enzymatic antioxidants (e.g., GSH) as well as enzymatic activities (e.g., SOD, CAT and GSH-Px) [47]. Also, the ADM showed significant decrease in AST and LDH in comparison to group II. A greater increase in the cytokines was observed in the group II than in groups treated with ADM. Although the precise mechanisms by which ADM regulates redox systems have not yet been clarified, Kim et al., have shown that ADM elevated cellular GSH levels via an up-regulation of its rate-limiting synthetic enzyme, γ -glutamate-cysteine ligase (γ -GCL) [48]. However, ADM suppresses ROS production through different signaling pathways, such as the activation of the cAMP-protein kinase A pathway in mesangial cells and the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase via the nitric oxide-cGMP signalling pathway [49].

ADM maintained GSH level in celecoxib – induced hepatic injury almost to the levels of normal as demonstrated in our study. GSH plays an important role in preserving membrane integrity

and in assuring the reduced state of the protein; therefore, deletion of GSH provokes cellular damage [50]. Under physiological conditions the reduced form of GSH is 10- to 100-fold more common than the oxidized form, but the ratio is decreased by oxidative stress. The redox status of GSH is controlled by GSH-Px which catalytically converts GSH from the reduced to the oxidized form or vice versa, respectively [50]. The current findings in our work clearly showed that ADM protected against acute hepatic injury, suppressed increase of MDA by modulation of GSH.

An additional mechanism for ADM rather than its antioxidant effect is the down regulation of inflammatory cytokines and alleviation of inflammatory processes in [51]. ADM can attenuate the increase in IL-1, IL-6 and TNF- α improving disturbed liver functions induced by celecoxib and in other part, ADM significantly reduces inflammatory mediators [51].

Inflammatory agents such as NOx may be involved in some part of the regulation of liver cell dysfunction [52]. Increased NOx enhances ADM to initiate cytokine production. Also cytokines stimulate ADM gene expression which was attenuated by Nomega-nitro-L-arginine methyl ester (L-NAME). In current study, there was a significant increase of ADM associated with NOx. These findings suggest that NOx is a direct activator of ADM gene expression in a variety of cell types and that inflammatory cytokines stimulate ADM expression via both NOx - dependent and -independent mechanisms. The stimulatory effect of NOx appears to not be related to the classic guanylate cyclase-cGMP pathway. These results indicate that adrenomedullin augments NOx synthesis in these cells under

cytokine-stimulated conditions, at least partially through a cAMP-dependent pathway [53]. In the present study ADM levels correlated with NOx and cytokines levels. It was probably due to the fact that elevated NOx levels in cases of acute liver injury might further stimulate a vicious cycle [52]. As blood flow is increased, the inflammatory cascade potentiates excessive cytokine production and MDA formation, with varying degrees of tissue damage. Cellular signaling by ADM is mediated via cyclic AMP/protein kinase A, nitric oxide/cyclic GMP, phosphoinositide 3-kinase/Akt, and/or the extracellular-signaling-regulated kinase pathway. It was suggested that increased cytokines such as TNF- α and IL6 in hepatic injury is the stimulus for the production of ADM by vascular smooth muscle, endothelial cells and human hepatic stellate cells (HSC), a perisinusoidal pericytes. Also HSCs have functional receptors for ADM, the stimulation of which increase NOx [54]. The current work showed a significant decrease in MDA in rats administered ω -3 PUFAs. In addition, combination therapy of both ADM and ω -3 PUFAs significantly decreased MDA in rats given ADM or ω -3 PUFAs alone. Meanwhile, ω -3 PUFAs are metabolized in the liver by beta-oxidation and broken down locally, usually into short chain fatty acids. They have little effect on hepatic cytochrome P450 or drug transporter activity[55].

Enzymatic lipid mediators of ω -3 PUFAs retard oxidative stress and inhibit inflammation. ROS are constantly produced as by-products of normal cellular metabolism or from exposure to stimuli, such as chemicals reacting with DNA, ROS can cause various DNA lesions including oxidized bases, single-strand breaks and double strand

breaks. Cells produce multiple ROS scavengers and ω -3 PUFAs increased mRNA levels of antioxidant molecules to defend themselves against such oxidative threats Otterbein et al. [56]. Moreover, ω -3 PUFAs regenerate thioredoxin, a potent anti-oxidant molecule, using NADPH [57]. These data indicate that ω -3 PUFAs induce the expression of these antioxidants, reduce ROS, and as a result, diminish DNA damage of the cell.

Further findings in the present study were; omega 3 PUFAs treatment for 14 days significantly reduced serum AST, ALT, ALP and LDH levels in celecoxib -treated rats with specific preference for ALT [58]. This is an indication of the stabilization of plasma membrane, as well as repair of hepatic tissue damage induced by celecoxib toxicity. Also, the ω -3 PUFAs have been shown to reduce hepatic inflammation as shown in current study which revealed that there was a significant decrease in serum IL-1, IL-6 and TNF- α .

The relative contributions of eicosapentaenoic (EPA) and docosahexaenoic (DHA) might also be important in determining the effect of fish oil [59]. In another study by González et al. , DHA supplementation led to increased formation of DHA-derived lipid mediators such as 17-HDHA and protectin D1, which were able to protect the liver from drug-induced inflammatory damage and can suppress TNF- α secretion from cultured murine macrophages [60].

The ameliorating effect of ω -3 PUFAs on biochemical tests (total serum bilirubin, AST, ALT, ALP ,hepatic LDH) in the model of celecoxib -induced hepatic injury may be attributable to inflammatory cytokine down-regulation via ω -3 PUFAs content. Omega-3 PUFAs can be oxidized and the oxidized

derivatives can act directly on inflammatory cells via surface or intracellular receptors which include transcription factors like peroxisome proliferator activated receptors , oxidation can occur to the non-esterified form of the PUFA or to PUFAs esterified into more complex lipids including circulating or cell membrane phospholipids and intact lipoproteins such as low density lipoprotein (LDL) assuring the suitable environment for membrane protein function. In addition, membrane phospholipids are substrates for the release of non-esterified PUFAs intracellularly , the released PUFAs can act as signaling molecules, ligands (or precursors of ligands) for transcription factors, or precursors for biosynthesis of lipid mediators which are involved in regulation of many cell and tissue responses, including aspects of inflammation and immunity[61]. Thus, changes in membrane phospholipid fatty acid composition can affect the function of cells involved in inflammation by; change in the physical properties of the membrane; effects on cell signaling pathways, either through modifying the expression, activity of membrane receptors or modifying intracellular signal transduction mechanisms that lead to altered transcription factor activity and changes in gene expression; alterations in the pattern of lipid mediators produced, with the different mediators having different biological activities and potencies. These fatty acids are carried in the bloodstream at much higher concentrations in more complex lipids (triglycerides, phospholipids, cholesteryl esters) within lipoproteins. Many of the cell types involved in inflammatory responses express lipoprotein receptors (e.g., LDL receptor, very low density lipoprotein receptor, scavenger receptors)

and so are able to take up intact lipoproteins, subsequently utilizing the fatty acid components. Thus, lipoproteins may affect inflammatory cell function, perhaps due to their component fatty acids [62].

The decreased inflammatory response by ω -3 PUFAs in our work was associated with decreased plasma IL-1, IL-6 & TNF- α levels. EPA inhibited the specifically celecoxib -induced TNF- α production by cultured monocytes [63].

In general arachidonic acid-derived eicosanoids act in a pro-inflammatory way, although this is an over-simplification since it is now recognised that PGE₂, for example, has both pro- and anti-inflammatory effects, and that another eicosanoid derived from arachidonic acid, lipoxin A₄, is anti-inflammatory. Animal studies have shown a direct relationship between arachidonic acid content of inflammatory cell phospholipids and ability of those cells to produce PGE₂ [64].

Also mitogen-activated protein kinases (AMPK) regulates phosphorylation in omega 3-mediated protection against celecoxib, which is consistent with previous finding by Lin that ω -3 PUFAs protects hepatocytes from inflammation through the AMPK -mediated inductions of COX-2 [65]. Also, ω -3 PUFAs and their specific lipid mediators can reduce the activity of inflammatory processes. EPA or fish oil decreased celecoxib -induced activation of nuclear factor kappaB (NF- κ B) in monocytes, which leads to a decreased production of TNF- α perhaps due to decreased activation of MAPK [65]. Omega-3 PUFAs could also dampen the inflammatory response in liver tissue, probably by regulating Kupffer cell activation and suppressing cytokine production. Celecoxib binds to Kupffer cells and activates

them, resulting in a liberation of large amounts of cytokines, such as IL-1, IL-6 and TNF- α . TNF- α is a critical mediator of liver damage in this model as evidenced by decreased production of pro-inflammatory cytokines and significantly lower serum ALT [66]. Moreover, the formation and the molecular effect of ω -3 PUFAs has identified a G protein-coupled receptor-specific pathway involved in NF- κ B down-regulation by the ω -3 PUFAs -derived resolvin E1, which in turn could also down-regulate NF- κ B-induced TNF- α formation. Moreover, ω -3 PUFAs can give rise to lipid mediators (e.g. resolvins and protectins) which are anti-inflammatory or might exert an anti-inflammatory effect via competitive inhibition of the omega-6 PUFA-derived pro-inflammatory eicosanoids [67]. In addition, there was an observed associated decrease in LDH, a known inflammatory marker with ω -3 PUFAs administration [68].

Interestingly, ADM and omega 3 inhibited the c-Jun N-terminal kinase (JNK), both pharmacologically and genetically, results in improved survival, lower TNF- α receptors, and cell death. Theoretically, inhibition of the innate immune response, JNK-induced apoptosis may be of benefit in decreasing massive hepatocyte apoptosis (69).

Finally, the principal findings in our study were that celecoxib induced acute hepatic injury and the increased of biomarkers of hepatic injury, oxidative stress indicators and cytokines formation. On the other hand, ADM and omega-3 PUFAs protected liver against injury caused by celecoxib; decreased levels of biomarkers of liver injury, inhibited biomarkers of oxidative stress and augmented the antioxidant system. Importantly,

there was high variation in effects of ADM and Omega 3 on levels of cytokines ; Omega- 3 PUFAs specifically decreased their levels significantly more than ADM. Also, the increased level of NOx associated with ADM was observed. Furthermore, Omega 3 PUFAs significantly increased PGE2 as compared with ADM. Regarding plasma adrenomedullin concentrations have been reported to be high in all celecoxib – induced hepatic injury groups in comparison to control group.

Conclusion

It can be concluded that our study provided additional evidence that rats with high dosage of celecoxib leads to acute hepatic injury. On the light of this study, it was clear that adrenomedullin and omega -3 PUFAs treatment and their accompanying their antioxidant and anti-inflammatory effects have a beneficial impact in the reversal of liver functions. The mechanisms outlined in this paper and oxidative stress and cytokines cycle may furnish the basis for therapeutic approaches to ameliorating the hepatic injury caused by drug toxicity. The relation between adrenomedullin & omega -3 PUFAs in one side and COX2 & PGE2 in another side needs more investigation and is still to be elucidated. Also immunohistochemistry will be needed to confirm the histopathological results. Finally, the combined therapy with antioxidants and anti-inflammatory agents may be leads to satisfactory results in drug toxicity.

Conflicts of interest

No conflicts of interest.

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