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The hepato-fibrogenic potential of both acute and chronic treatments with paracetamol, ibuprofen, and aspirin in rats

Running title: The hepato-fibrogenic potential of NSAIDs

Nabil Mohie Abdel-Hamid ¹, Mona Mohammed Abdel Hamid ², Ahmed Abdelnaser Mohamed^{3*}

¹Department of Biochemistry, Faculty of Pharmacy, Kafrelsheikh University, Egypt,

²Department of Biochemistry, Faculty of Pharmacy (for Girls), Al-Azhar University, Egypt, Orcid ID: 0000-0002-2810-7016.

³Department of Biochemistry, Faculty of Pharmacy, New Mansoura University, Egypt, Orcid ID: 0000-0003-0666-234X.

***Correspondence:**

Ahmed Abdelnaser Mohamed. Department of Biochemistry, Faculty of Pharmacy, New Mansoura University, Egypt.

Email: Ahmedabdelnaser112@gmail.com. Orchid ID: 0000-0003-0666-234X.

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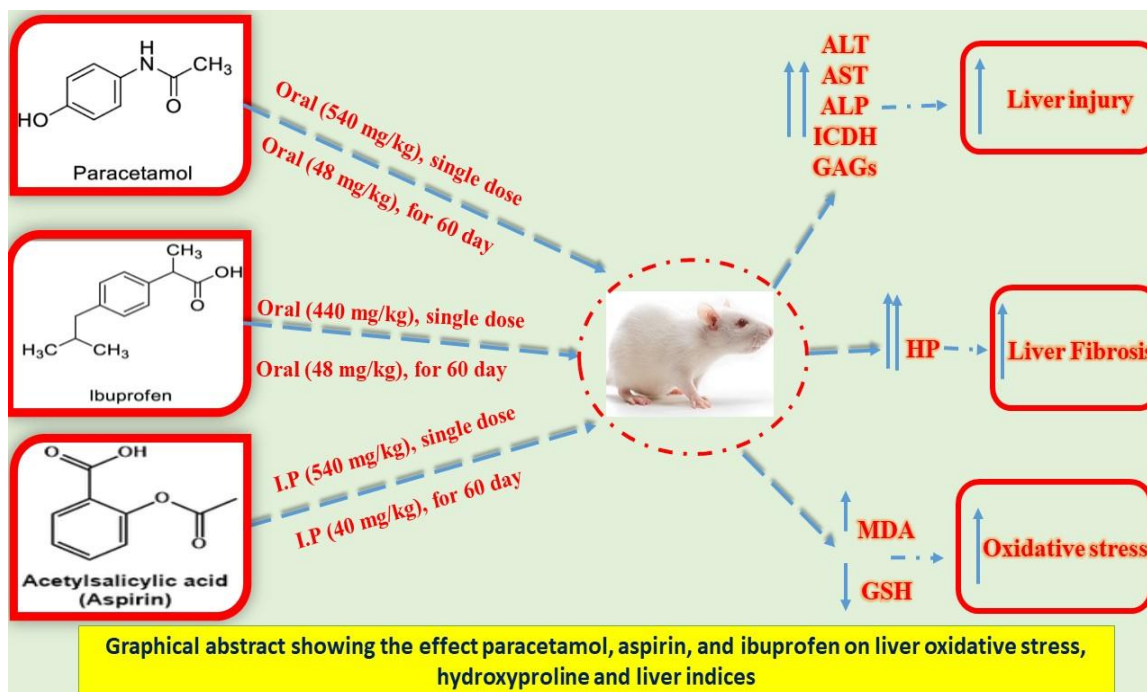
ABSTRACT

Background and purpose: Hepatotoxicity from frequently prescribed drugs has become an evolving health problem. This study was conducted to evaluate the risk of acute and chronic administration of acetaminophen (AAP), ibuprofen (Ibu), and acetylsalicylic acid (ASA). **Methods:** One hundred and twenty male albino rats, were divided into 2 main groups for acute and chronic study. Each group was sub-classified into 5 sub-groups (12 rats for each). Acute study: control (normal saline), AAP (single oral dose, 540 mg/kg, bw), AAP +Zn (APP and Zn, 227 mg/liter drinking water 24 hours before AAP administration), Ibu (single oral dose, 440 mg/kg, bw), and ASA (single intraperitoneal dose, 540 mg/kg, bw). Chronic (period for 60 days): control (normal saline), AAP (single daily doses, 48 mg/kg, bw), AAP +Zn (APP and Zn, 227 mg/liter drinking water for 60 days), Ibu (single daily doses, 48 mg/kg, bw), and ASA (single daily intraperitoneal doses, 40 mg/kg). **Results:** Hepatic aminotransferases, alkaline phosphatase, isocitrate dehydrogenase, serum glycosaminoglycans, tissue hydroxyproline, and malondialdehyde were significantly elevated, but glutathione was significantly decreased, in both acute and chronic treatments in all treated groups. Prior treatment with Zn couldn't change the effects of AAP, except on oxidative stress. Tissue changes after chronic treatment varied from fatty changes to vascular congestions and inflammation. **Conclusion:** We assume that both acute and chronic administration of AAP, Ibu, and ASA have deleterious hepatotoxic and fibrogenic effects on the liver with a non-significant protective role to Zn co-administration with AAP against oxidative stress.

Keywords: Paracetamol; Ibuprofen; Aspirin; Liver; Fibrosis.

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Graphical abstract



Abbreviations: AAP: acetaminophen; ALP: alkaline phosphatase; ALT: alanine aminotransferase; ANOVA: analysis of variance; ASA: acetylsalicylic acid; AST: aspartate aminotransferase; GAGAs: glucose amino glycans; GSH: reduced glutathione; H&E: hematoxylin α eosin; HP: hydroxyproline; Ibu: ibuprofen; ICDH: isocitrate dehydrogenase; MDA: malondialdehyde; NPCs: non-parenchymal cells; NSAIDs: non-steroidal anti-inflammatory; OCT: over the counter; ROS: reactive oxygen species; SEM: standard error of the mean.

Highlights

- Both acute and chronic administration of AAP, Ibu, and ASA have deleterious hepatotoxic and fibrogenic effects on the liver.
- Zn co-administration with AAP showed a non-significant protective role against oxidative stress.
- Dosing and duration of NSAIDs should always be revised for patients with suspected liver diseases.

INTRODUCTION

The liver is the main site for the utmost of the metabolism of drugs that are orally administered due to its histological structure and anatomical proximity to the gastrointestinal tract counting the sinusoidal space and the blood supply from the portal vein, permitting the proper transport of drugs and other xenobiotics (1). It is a complex organ, composed of 60% hepatocytes, parenchymal cells that perform multiple functions, such as metabolism, non-parenchymal cells (NPCs) containing cholangiocytes coating the bile ducts; sinusoidal endothelial cells that form a permeable barrier between both the blood and the space between cells; Kupffer cells, macrophages that reside in the liver; stellate cells, in which fat and vitamin A are synthesized (2). Drug-induced hepatotoxicity is described as hepatic injuries incurred by medication or over-the-counter medications (OTC), herbs, or any other xenobiotic (3-5).

Several clinically relevant drugs have been subjected to prescription restrictions or the incorporation of a black box warning for probable hepatotoxicity. Acetaminophen (AAP) is the most commonly studied hepatotoxic drug. The most commonly hepatotoxicity-associated pharmacological groups of orally administered drugs are nonsteroidal anti-inflammatory (NSAIDs) drugs such as diclofenac and ibuprofen(5).

Although the fact that ibuprofen (Ibu) is one of the most regularly used and safest NSAIDs, there have been cases of Ibu-induced hepatotoxicity. There have been published reports of Ibu hepatotoxicity leading to liver failure, liver transplantation, or death (6). Acetylsalicylic acid (ASA) or aspirin hepatotoxicity is often minor and asymptomatic, while greater dosages can cause encephalopathy with evidence of hepatic malfunction (hyperammonemia and coagulopathy). Reye syndrome is a kind of ASA hepatotoxicity that causes microvesicular fat, lactic acidosis, and hepatic

dysfunction, as well as coma and encephalopathy. Moreover, symptoms of hepatic failure such as elevated serum aminotransferase, hyperammonemia, and encephalopathy, are considerably high, whereas serum bilirubin levels are slightly or only moderately raised (7).

Oxidative stress is caused by the excess production of reactive oxygen and nitrogen species, like free radicals, during redox reactions in the body. There are numerous endogenous and exogenous contributing factors to oxidative stress, which can eventually lead to oxidative damage in various tissues if not appropriately controlled (8). Once NSAIDs are metabolized by tissue peroxidases, they generate pro-oxidant radicals that elevate oxidative stress and thus initiate cytotoxicity (9). Malondialdehyde (MDA) is a thiobarbituric acid reactive substance that is typically produced by human cells as a byproduct of lipid peroxidation. It has been used as a screening tool to evaluate the extent of unsaturated lipid peroxidation associated with free radical-induced oxidative stress (10, 11).

Hydroxyproline (HP) is amongst the most abundant amino acids found in collagen. The amount of HP produced in urine and serum during collagen degradation was associated with fibrosis (12). It has the potential to be employed as a diagnostic marker for fibrotic indices, particularly hepatotoxicity upon NSAIDs' long-run administration and CCl₄ (13).

Isocitrate dehydrogenases (ICDH) are vital enzymes that activate the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), generating NADPH (14). ICDH is predominantly located in the centrilobular region of the liver, so, it is highly elevated in centrilobular injury (15). Total and individual glycosaminoglycans (GAGs), such as sialic acid, and glucosamine (FGA) were estimated in both sera and liver tissues as efficient markers for drug-induced hepatocyte injury (16).

In the current study, we aimed to investigate the possible hepatotoxicity after acute and chronic administration of NSAIDs (AAP, ASA, and Ibu). Zn was tested with the more frequently prescribed NSAIDs; AAP. Hepatotoxicity was examined by monitoring liver enzymes such as ALT, AST, ALP, and ICDH, serum GAGs, hepatic tissue HP, MDA, and GSH along with the histopathological changes after chronic treatment.

MATERIALS AND METHODS

Chemicals

Paracetamol (AAP) was obtained from Glaxo, Egypt, and suspended in water using gum acacia as a suspending agent. Ibuprofen (Ibu) was obtained from XXX and suspended in water using gum acacia as a suspending agent. Aspirin (ASA) was obtained from XXX and dissolved in sterile water (injectable form). Zinc sulfate was obtained from Prolabo and dissolved in drinking water. Other used chemicals were of analytical grade.

Animals

The experiments were carried out on one hundred twenty male albino rats of average weight 190 ± 20 g, supplied from the Animal Farm, Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were kept under constant environmental conditions and observed daily throughout the study period. They were kept in metallic cages with a constant temperature of $25 \pm 2^\circ\text{C}$ and enough ventilation, a 12-hour cycle of light and dark and unrestricted access to drinking and food. Acceptance of the study protocol was taken from the ethical committee of research at the Faculty of Pharmacy, Kafrelsheikh University, Egypt.

Experimental design

After a week of accommodation, animals were divided into 2 main groups for acute and chronic study. Each group was sub-classified into 5 sub-groups (12 rats for each) as follows:

Acute study

- 1. Control group:** rats received only normal saline orally.
- 2. AAP group:** rats received AAP, orally in a single dose (540 mg/kg, body weight).
- 3. AAP +Zn group:** rats received Zinc sulfate (227 mg/liter) in drinking water, 24 hours before AAP administration, using the same dose in group 2.
- 4. Ibu group:** rats received Ibu orally as a single dose of 440 mg/kg, body weight.
- 5. ASA group:** rats received ASA in a single dose of 540 mg/kg, body weight, intraperitoneally (I.P).

Chronic study

- 1. Control group:** rats received only daily normal saline orally.
- 2. AAP group:** rats received a single daily AAP dose (48 mg/kg, body weight, orally) daily for 60 days.
- 3. AAP +Zn group:** rats received Zinc sulfate (227 mg/liter) in drinking water, 24 hours before AAP administration, using the same dosing system as in group 2.
- 4. Ibu group:** rats received a single daily Ibu dose, orally (48 mg/kg, body weight), daily for 60 days.
- 5. ASA group:** rats received single daily I.P doses of ASA (40 mg/kg, body weight) for 60 days.

Collection of the biological samples

Clean capillary tubes were utilized to collect blood samples from retro-orbital puncture of each rat's eyes under light ether anesthesia by the end of the experiment. Upon clotting and centrifugation at 3000 rpm for 15 minutes, sera were instantly frozen at -20°C for biochemical analysis. Afterward, rats were sacrificed via cervical decapitation and their livers were removed, washed with cold saline, blotted by tissue papers, weighed, and split into two parts: the first was immediately directly immersed in 10% buffered formalin for histological examination. The second part was homogenized in buffered saline (10% w/v) and stored at -20°C for biochemical analysis.

Histopathological investigation of liver tissue

Liver specimens were placed in ascending degrees of ethanol for dehydration. They were encased in paraffin wax and sliced into 5 μ m thick. For tissue morphology under a light microscope, sections were stained with Mayer's hematoxylin and Eosin (H&E) stains. Hepatic histological alterations were photographed using a computer system with a digital camera (Nikon digital camera, Japan).

Biochemical analysis

Serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were spectrophotometrically measured based on Reitman and Frankel method (17). Serum alkaline phosphatase (ALP) and isocitrate dehydrogenase (ICDH) activities were measured by kinetic assay according to Jordan (18) and Yamada. et al (19) respectively. Serum total glycosaminoglycans were measured by the colorimetric method according to Schloss (20). Liver homogenates were used for the determination of hydroxyproline (HP) according to the Woessner et al method (21), determination of MDA according to Ohkawa et al method (22), and GSH content was measured according to Beutler et al (23).

Statistical analysis

Data were expressed as mean \pm standard error of the mean (SEM). A one-way ANOVA was used to evaluate the difference between groups, followed by a Tukey's-HSD multiple range post hoc test. The acquired data were statistically evaluated using Graph Pad Prism Software version 6.0, with statistical significance set at $p < 0.05$.

RESULTS

Biochemical analysis

In the acute study, significant increases were observed in all studied serum parameters except levels of both

AST and ALP in the AAP +Zn group showed a significant decrease. Regarding serum total GAGs, a significant increase in both acute and chronic studies was detected in the different studied groups (Table1). In the chronic study, there were significant increases in all serum levels of the studied biomarkers in the different sets, even in the group of AAP +Zn, as well as, ALP activity in the ibuprofen group was non-significantly decreased (Table1).

GSH content in the rat's liver homogenates after 60 days' treatment revealed a significant decrease, while MDA content showed a significant increase in all the studied groups except in AAP +Zn group, no significant differences were observed for GSH as well as MDA contents. Furthermore, HP content was significantly elevated in all the studied groups (Table 2).

1.1. Correlations

A significantly positive correlation was detected between ICDH and GAGs in ASA-treated rats after acute treatment, in AAP + Zn treated rats after chronic treatment, and in Ibu-treated rats after chronic treatment. Similarly, a significantly positive correlation was observed between liver HP and MDA after chronic AAP treatment.

Histopathological examination

Chronic treatment by AAP induced apparent congestion in the central hepatic vein, associated with fatty changes, manifested as signet-ring-shaped cells (Fig. A: 1, 2). Similar changes were also observed after chronic ASA administration (Fig. B: 1). An inflammatory reaction manifested by lymphocytic infiltration was also observed (Fig. B: 2). Chronic Ibu treatment induced less similar effects to AAP with minor fatty change (Fig. B: 1,2).

Table 1: Variations in serum enzyme activities and serum GAGs level after both acute and chronic treatment with AAP, AAP + Zn, ASA, and Ibu (Values are expressed as mean \pm SEM, N= 12):Significant changes from control were considered at $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.001^{***}$.

	Control		AAP		AAP +Zn		ASA		Ibu	
	Acute	Chronic	Acute	Chronic	Acute	Chronic	Acute	Chronic	Acute	Chronic
AST	26 \pm 1	29 \pm 0.3	38 \pm 3 ^{***}	42 \pm 0.9 ^{***}	22 \pm 0.6 ^{**}	70 \pm 2 ^{***}	34 \pm 1 ^{***}	34 \pm 2 [*]	31 \pm 0.4 ^{***}	61 \pm 1 ^{***}
ALT	21 \pm 1	21 \pm 0.3	29 \pm 2 ^{**}	36 \pm 0.6 ^{***}	27 \pm 2 ^{**}	34 \pm 0.8 ^{***}	40 \pm 1	29 \pm .7 ^{***}	31 \pm 1 ^{***}	24 \pm 0.6 ^{***}
ALP	111 \pm 3	111 \pm 2	97 \pm 2 ^{**}	119 \pm 0.8 ^{***}	86 \pm 1 ^{**}	111 \pm 1	183 \pm 1 ^{***}	125 \pm .9 ^{***}	123 \pm 2 ^{**}	102 \pm 0.9
ICDH	8 \pm .1	8 \pm .1	38 \pm 0.3 ^{***}	32 \pm 0.5 ^{***}	10 \pm 0.2 ^{***}	35 \pm 0.8 ^{***}	15.1 \pm 0.2 ^{***}	12 \pm 0.2 ^{***}	30 \pm .5 ^{***}	10 \pm 0.2 ^{***}
Total GAGs	84 \pm 3	84 \pm 3	354 \pm 2 ^{**}	220 \pm 3 ^{**}	238 \pm 2 ^{**}	145 \pm 3 ^{**}	238 \pm 2 ^{**}	454 \pm 3 ^{**}	427 \pm 4 ^{**}	144 \pm 3 ^{**}

Table 2: Variations in hepatic tissue content of GSH, MDA, and HP after chronic treatment with AAP, AAP + Zn, ASA, and Ibu (Values are expressed as mean \pm SEM, N= 12):

Parameters	Control	AAP	AAP +Zn	ASA	Ibu
GSH	4320 \pm 36	3891 \pm 129 ^{**}	3974 \pm 199	3901 \pm 104 ^{**}	3914 \pm 132 ^{**}
MDA	138.6 \pm 4.7	189 \pm 5.7 ^{**}	151 \pm .9	193 \pm 5.2 ^{**}	195 \pm 5.3 ^{**}
HP	8.1 \pm .1	10.4 \pm .1 ^{**}	9 \pm .3 ^{**}	9.1 \pm .15 ^{**}	12 \pm .06 ^{**}

Significant changes from control were considered at $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.001^{***}$.**Table 3:** Correlation between the studied biomarkers in the different studied groups:

Correlated parameters	R	P value
ICDH and GAGs after single dose ASA-treated rats (acute study)	+0.031	< 0.05
ICDH and GAGs after 60 days - AAP + Zn treated rats (chronic study)	+0.048	< 0.05
ICDH and GAGs after 60 days - Ibu treated rats (chronic study)	+0.41	< 0.05
HP and MDA after 60 days AAP treated rats (chronic study)	+0.32	< 0.05

Figure 1: It shows the effect of paracetamol, aspirin, and ibuprofen on liver tissue after 2 months of treatment (H & E, X 300):

The three control sections show homogenous and regular cellular arrangement.

A- The effect of paracetamol on rat liver tissue:

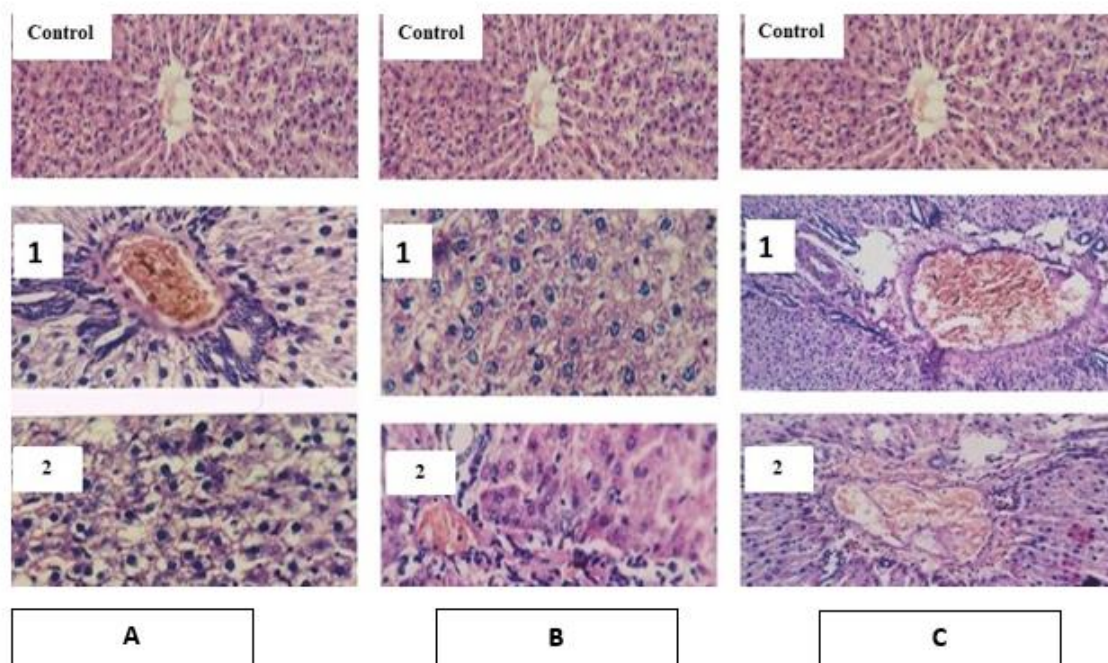
- 1- Congestion of hepatic vein with apparent inflammatory reaction.
- 2- Fatty change.

B- The effect of aspirin on rat liver tissue:

1. Fatty change.
2. Fatty change, congestion of hepatic vein, and inflammatory reaction.

C- The effect of ibuprofen on rat liver tissue:

1. Fatty change.
2. Fatty change, congestion of hepatic vein, and inflammatory reaction.



DISCUSSION

NSAIDs are easily available as over-the-counter (OTC) drugs and are extensively used in enormous amounts every year. Focusing on their adverse effects due to their massive use is an important issue. Hepatotoxicity is considered a common bad impact. Extensive use of NSAIDs was reported to induce liver toxicity (24). AAP is one of the drugs that provoke hepatotoxicity because of the tolerability range of different used doses (25, 26). The overconsumption of NSAIDs has a great

concern regarding liver toxicity since about 10% of drug-induced liver injury cases are associated with NSAIDs (6).

The present study was directed to figure out the hepatotoxic effects of these three NSAIDs at both angles; either through onset overdose (acute study) or continuous daily use (chronic study) as NSAIDs are used all over the world in a wide range of diseases starting from pain, analgesia to cancer. Conventional clinical biomarkers, including ALT, AST, and ALP are

presently helpful to detect drug-induced liver injury, and are fairly precise in predicting liver injury (27). Administration of NSAIDs results in elevated ALT, AST, and ALP activities (28), which correlates with the current study, as the studied groups showed significantly raised AST, ALT, and ALP activities compared to the control except, AST and ALP in AAP +Zn group of acute study and ALP in AAP +Zn and ibuprofen groups of chronic study.

In both acute and chronic groups, our results revealed a significant increase in serum levels of ICDH; which has been stated to be both sensitive and specific to injuries of hepatic parenchymal cells (29).

GAGs are normal constituents existing on the surface of hepatocytes in ordinary conditions (30). The concentration of serum GAGs is a powerful biomarker to measure the level of liver impairment (31). In the present, study total GAGs concentration was significantly elevated for both acute and chronic classifications. In the present work, significantly positive correlations were detected between ICDH and GAGs in Ibu, ASA, and AAP + Zn treated rats after two months of treatment. This indicates that hepatocellular damage is associated with damaged tissues through the destruction of extracellular cementing materials (32).

AAP is converted to the reactive metabolite N-acetyl-p-benzoquinone amine (NAPQI) at normal dosages, which is effectively detoxified via glutathione conjugation. At hazardous levels, higher amounts of NAPQI are generated, resulting in an 80% to 90% depletion of reduced glutathione. Inadequate amounts of reduced glutathione required for NAPQI detoxification result in covalent attachment of the reactive metabolite to cellular proteins and DNA, resulting in elevated cellular damage (26). The generation of drug metabolites by the liver through biotransformation can lead to hepatic damage due to

the formation of noxious or reactive materials such as electrophilic chemicals or free radicals (5, 33).

Chronic NSAIDs administration showed a reduction of GSH and an elevation of MDA contents and tissue contents. These findings follow the reports of Ahmad, et al (34). However, their contents in AAP +Zn group showed non-significant variations. These findings may explain the partial protective antioxidant and anti-inflammatory effect of Zinc as reported by Jarosz, et al (35). Disproportionate oxidative stress is damaging to the host and may result in organ injury. Cytokines such as tumor necrosis factors can be elaborated by oxidative stress, resulting in tissue damage, inflammation, and apoptosis.

In liver stellate cells, oxidative stress-mediated lipid peroxidation can initiate increased collagen production, which is measured chemically by HP tissue content (36). Our results showed a significant elevation of HP content in liver homogenates by the studied NSAIDs, especially after longer periods of treatment. The increase in HP content in liver tissues is directly and significantly correlated with the stage of liver fibrosis (12).

Liver histology is the best tool to state the configuration of hepatic toxicity (37). In the present study, AAP treatment for two months induced marked congestion of the hepatic vein with an inflammatory reaction (Fig. A-1) and a marked fatty change (Fig. A-2) which is manifested as unstained vacuoles with eccentric nuclei. On the other hand, ASA treatment for two months induced marked fatty change and congestion of the hepatic vein, associated with inflammatory reaction and mononuclear infiltration (Fig. B-1). Also, Ibu treatment for two months induced marked hepatic vein congestion (Fig. C -1) and a minor fatty change (Fig. C - 2). The impairment of hepatic fat homeostasis and mitochondrial dysfunction is known to increase the

production of reactive oxygen species that elicit excess cytokine expression (38).

Limitations and forthcoming notices: We will try to apply this design on human samples.

CONCLUSION

Administration of AAP, ASA, and Ibu, either in single large doses (acute use) or therapeutically for a long time showed a deleterious effect on both liver cells (manifested by disturbed liver enzymes), tissue intactness (manifested by depleted GAGs), in addition to fibrogenic potential. Moreover, disturbed tissue antioxidant defense mechanisms may accelerate hepatic tissue damage. The co-administration of Zn to AAP could partially protect hepatic tissue, even though alternate hepatoprotectives may be more helpful than Zn and would be co-administered with all NSAIDs.

Authors Contributions:

Nabil Abdel-Hamid: Conceptualized the work idea. Supervised the practical work. Revised the whole MS and submission. **Mona El-Kady:** Executed part of the experiments and biostatistics. **Ahmed Mohamed:** Executed part of the experiments, wrote the results and drafted the MS. The authors declare that all data were generated in-house and that no paper mill was used

Competing Interests: Authors declare no conflict of interest.

Data Availability Statement: All data are available upon reasonable request.

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Ethical approval: Acceptance of the study protocol was taken from the ethical committee of research at the Faculty of Pharmacy, Kafrelsheikh University, Egypt.

Consent to Participate: Not applicable.

Consent to Publish: All authors are agreeing to publish the accepted manuscript.

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