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Celecoxib or diclofenac hepatic status in the presence or absence of rebamipide

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Abstract. – **OBJECTIVE:** Utilization of nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, can produce gastrointestinal ulceration. Thus, cyclooxygenase-2-selective inhibitors, such as celecoxib, and protective agents (e.g. rebamipide) have been employed to alleviate harmful NSAID effects. This study sought to explore the influence of rebamipide on the hepatic outcomes following administration of two commonly prescribed NSAIDs.

MATERIALS AND METHODS: Rats were given either vehicle or rebamipide (30 mg/kg) orally twice daily for two days, then on the third day respective groups were dosed with either vehicle, celecoxib (40 mg/kg), or diclofenac (10 mg/kg) in addition to a respective dose of vehicle or rebamipide. Livers were collected on day 4 following euthanasia. Hepatic tissue was examined via histopathology and assayed for oxidative stress and specific NSAID concentration.

RESULTS: The liver sections were found to be free from structural changes. Oxidative stress biomarkers, reduced glutathione and malondialdehyde, were discovered to be unaltered among the groups tested. The hepatic NSAID concentrations were not significantly affected by the presence of rebamipide.

CONCLUSIONS: The concomitant administration of rebamipide does not influence the hepatic condition of rats administered either celecoxib or diclofenac at the dosages and over the time course examined.

Key Words:

Celecoxib, Cyclooxygenase, Diclofenac, Liver, NSAIDs, Rebamipide.

genase (COX) which exists in two primary isoforms, COX-1 (a constitutively produced enzyme) and COX-2 (an enzyme upregulated during the inflammatory response), is the therapeutic drug target inhibited by NSAID administration. As COX is inhibited, the anti-inflammatory and analgesic effects of NSAIDs are produced through a reduction in prostaglandin³⁻⁶.

NSAIDs are classified based on COX isoform selectivity. Most NSAIDs fall into the category of non-selective inhibitors (e.g. diclofenac), which show no preferential inhibition of isoforms; while those NSAIDs which inhibit COX-2 with greater specificity are labeled COX-2-selective inhibitors, such as celecoxib^{1,6-8}. NSAIDs are known to produce gastrointestinal adverse effects; thus, various methods of side effect alleviation have been devised from the use of co-administered drugs, misoprostol, histamine H₂-receptor antagonists, and proton pump inhibitors, to the use of COX-2-selective inhibitors which have a lesser gastric adverse effect profile compared to non-selective inhibitors^{1,2,9}.

Although not approved by the Food and Drug Administration for usage in the United States, rebamipide is a gastroprotective agent used in Japan for the prophylaxis of NSAID-induced gastritis by stimulating prostaglandin synthesis and thereby protecting the gastric mucosa against damage¹⁰⁻¹³. The use of rebamipide along side NSAID administration has been shown to prevent gastric damage; furthermore, it has been discovered that rebamipide suppresses celecoxib-induced apoptosis, which may explain its protective effects against ulcers, along with an effectiveness against Behcet's syndrome^{11,14}. Although used as a gastroprotectant, rebamipide administration has been associated with gastrointestinal side effects, such as diarrhea or nausea^{12,14}.

Introduction

A wide range of conditions from arthritis to muscular strains are treated using nonsteroidal anti-inflammatory drugs (NSAIDs)^{1,2}. Cyclooxy-

As both NSAIDs have shown the ability to elicit oxidative stress¹⁵⁻¹⁷, thus these parameters will be examined. Increased oxidative stress is a detrimental condition which can lead to cellular or tissue dysfunction. Produced during routine cellular metabolism and handling of xenobiotics, free radicals are various reactive molecules which can damage cellular structure^{18,19}. Reduced glutathione (GSH) present within cells typically acts as an antioxidant which sequesters reactive species; while malondialdehyde is formed as a result of peroxidation of lipids by free radicals^{20,21}. Thus, both GSH and malondialdehyde can be used as biomarkers for the detection of changes in oxidative stress levels^{15,22,23}.

Celecoxib and diclofenac are metabolized in the liver primarily by cytochrome P-450 (CYP) 3A4 and CYP2C9^{7,24}; while rebamipide undergoes hydroxylation by hepatic CYP3A4 in humans^{13,25}. Although both celecoxib and diclofenac have shown the capability of causing changes in the liver^{15,26}, no study has been conducted to investigate the effects of rebamipide on the hepatic outcomes of either NSAID. The influence of concomitant rebamipide on the liver during celecoxib or diclofenac administration was examined using liver samples collected in the course of a previous study conducted by our laboratory⁴.

Materials and Methods

Chemicals

All HPLC grade chemicals (acetonitrile, glacial acetic acid, iso-octane, 2-propanol, sulfuric acid, triethylamine, and water) were obtained from Fischer Scientific Laboratory in Fair Lawn, NJ. Drug compounds, celecoxib, diclofenac, rebamipide, and methyl cellulose, were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada), MP Biomedical (Solon, OH, USA), Tokyo Chemical Industry, Co., Ltd (Tokyo, Japan), and Science Stuff, Inc. (Austin, TX, USA), respectively. The HPLC internal standards (IS), ibuprofen and flufenamic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and Drug Administration

Experiments were conducted using male Sprague-Dawley rats according to a protocol approved by the University Committee on Animal Care at East Tennessee State University, TN, USA.

Study Design

A complete description of the study design is presented in a prior study completed in our lab⁴. Briefly, rats were randomized into six groups. On the first and second days, 3 groups received oral doses of vehicle and 3 groups received rebamipide (30 mg/kg), twice daily. On the third day, the vehicle groups received another dose of vehicle then after 10 minutes a single dose of 40 mg/kg celecoxib (vehicle+celecoxib), 10 mg/kg diclofenac (vehicle+diclofenac), or vehicle (vehicle+vehicle). Also on day 3, the rebamipide groups received another dose of rebamipide then after 10 minutes a dose of celecoxib (rebamipide+celecoxib), diclofenac (rebamipide+diclofenac), or vehicle (rebamipide+vehicle). Rats were euthanized on day four and harvested livers were kept at -80 °C until analysis.

The celecoxib dosage was selected based upon the highest anti-inflammatory effect seen in a previous study²⁷. This dosage has also been shown to significantly alter urinary electrolyte levels²⁸. The 10 mg/kg per day dose of diclofenac was chosen as a therapeutic equivalent to the 40 mg/kg celecoxib dose. A twice-a-day 30 mg/kg rebamipide dose has been reported as a prophylactic of gastric lesions during aspirin administrations²⁹.

Histopathological Examination

In preparation for examination of histopathology, liver sections (5 µm) were embedded in paraffin and stained with hematoxylin and eosin. A certified pathologist blinded to the treatment groups evaluated the liver sections for tissue necrosis and inflammation.

Oxidative Stress Assessment

Reduced-Glutathione Measurement

An Arbor Assay GSH Colorimetric Detection Kit (Ann Arbor, MI, USA) was utilized to determine the hepatic levels of GSH. Experiments were carried out in accordance with manufacturer's instructions.

Thiobarbituric Acid Reactive Species Measurement

The concentration of malondialdehyde present in each rat liver was investigated using a Cayman Chemical Company Thiobarbituric Acid Reactive Species Assay Kit (Ann Arbor, MI, USA). The assay was conducted according to the manufacturer's instructions.

Chromatographic Conditions

Sample Preparation

In order to assay the livers for drug concentration, a sample was collected from each liver and weighed. A 2:1 ratio of water in microliters to liver sample weight in milligrams was homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA).

Analysis Equipment and Solution Preparation

Hepatic NSAID concentrations were assayed using a HPLC system (Shimadzu, Tokyo, Japan) equipped with a LC020AB solvent delivery system, a SIL-20A HT auto-sampler, a SPD-M20A photodiode detector (celecoxib; 254 nm and diclofenac; 280 nm), a CBM-20A communication bus, a DGU-20A3 vacuum degasser, and a CTO-20A column oven (C18 analytical column, 100 × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA for celecoxib and C18 analytical column, 50 × 4.6 mm, 2.6 μm; Shimadzu, Tokyo, Japan for diclofenac). The sample organic phases were evaporated to dryness using a CentriVap concentrator (Lab Conoco, Kansas City, MO, USA) set at 50 °C.

The mobile phase for celecoxib was composed of acetonitrile, water, acetic acid, and triethylamine in a respective ratio of 47:53:0.1:0.03. The mobile phase was filtered using 0.5 μm nylon filters. The celecoxib standard curve was created using a stock solution consisting of 10 mg of celecoxib in 100 mL mobile phase giving a 100,000 ng/mL concentration. The IS (internal Standard) for celecoxib was ibuprofen dissolved in mobile phase (10 mg in 100 mL mobile phase) yielding a 100,000 ng/mL concentration.

The diclofenac mobile solution consisted of 0.5 μm nylon filtered acetonitrile, water, and acetic acid in a 50:50:0.25 ratio. The diclofenac stock solution was 50,000 ng/mL diclofenac dissolved in methanol (10 mg in 200 mL methanol). An IS stock solution was composed of flufenamic acid (10 mg in 10 mL acetonitrile then further diluted 100 fold) in a concentration of 10,000 ng/mL.

Hepatic Celecoxib Extraction

Hepatic celecoxib levels were determined using a previously reported method modified for liver³⁰. The celecoxib stock solution was serially diluted in mobile phase (100,000, 25,000, 5,000, 2,500, 1,000, 500, 250, 100, 50, and 25 ng/mL) to create a standard calibration curve. One hun-

dred microliters of the respective concentrations and 100 μL IS were added to 100 μL of blank liver homogenate. Samples were extracted using 0.6 M sulfuric acid (200 μL) and 5 mL iso-octane propanol (95:5) and vortexed for 30 seconds. Samples were centrifuged for five minutes at (2,500 g) then placed in a dry ice/ethanol bath to freeze the aqueous phases. Organic phases were removed to a clean tube then evaporated to dryness. Following reconstitution in 200 μL mobile phase, 100 μL were injected into the HPLC system with 15-minute runs with a 1 mL/min flow rate. This method yielded a 25 ng/g lower limit of detection (LLD) and a lower limit of quantitation (LLQ) of 250 ng/g with coefficient of variation (CV) of 21.5%.

Hepatic Diclofenac Extraction

HPLC detection of diclofenac was conducted using a liver-specific modification of a method described by el-Sayed et al³¹. Diclofenac stock solution was diluted with methanol to create the standard curve (50,000, 25,000, 10,000, 5,000, 2,500, 1000, 500, 250, 100, 50 ng/mL). Briefly, 100 μL of blank liver homogenate were spiked with a corresponding 100 μL standard concentration. Fifty microliters of IS was then added to all followed by 2 mL acetonitrile. Following a one minute vortex, samples were centrifuged at 2,500 g for 15 minutes. Organic phases were removed to a clean tube then evaporated. Samples were reconstituted with 200 μL mobile phase then 100 μL was injected into the HPLC system with a flow rate of 0.75 mL/min over 15 minutes. The diclofenac assay provided a LLD of 50 ng/g and a LLQ of 100 ng/g with a CV of 3.49%.

Statistical Analysis

Presented as mean ± standard error of the mean, biomarker data was analyzed with one-way ANOVA and hepatic drug concentrations, celecoxib and diclofenac, were examined for significance using the Student's *t*-test. The drug concentration data was also tested for outliers using SPSS software (IBM Corporation, Armonk, NY, USA). *p* < 0.05 was considered statistically significant.

Results

Histopathology

Upon examination for histopathology, the liver tissue sections from all experimental and control

groups were found to present without portal or lobular inflammation, alteration in structure, or necrosis (Figure 1). Sections were declared to be within normal histological limits.

Oxidative Stress

GSH Concentration

Hepatic GSH levels detected in the vehicle+vehicle group ranged from 8.30 to 10.38 $\mu\text{mol/g}$ and presented with an average concentration of $9.81 \pm 0.39 \mu\text{mol/g}$. The mean GSH concentration values for each group are shown in Figure 2. The ANOVA did not detect any significance amongst the experiment groups ($p = 0.5779$).

Malondialdehyde Concentration

Quantification of malondialdehyde in the vehicle+vehicle group showed an average of $8.85 \pm 0.46 \mu\text{mol/g}$ and a range of 7.65 to 10.33 $\mu\text{mol/g}$. Figure 3 presents the mean values of malondialdehyde found within the groups. ANOVA re-

vealed no significant difference among the groups ($p = 0.0579$).

Hepatic Celecoxib Concentration

Table I shows the mean celecoxib concentration in the liver for the vehicle+celecoxib and rebamipide+celecoxib groups. No significance was detected in the difference between group concentrations ($p = 0.8367$).

Hepatic Diclofenac Concentration

The mean diclofenac concentrations quantified in liver samples from both the vehicle+diclofenac and rebamipide+diclofenac groups are displayed in Table II. The presence of rebamipide did not produce a significant difference ($p = 0.4010$) in hepatic diclofenac concentration.

Discussion

In our study, the histopathological examination did not reveal any structural changes in the rat livers from any examined group. Similar celecox-

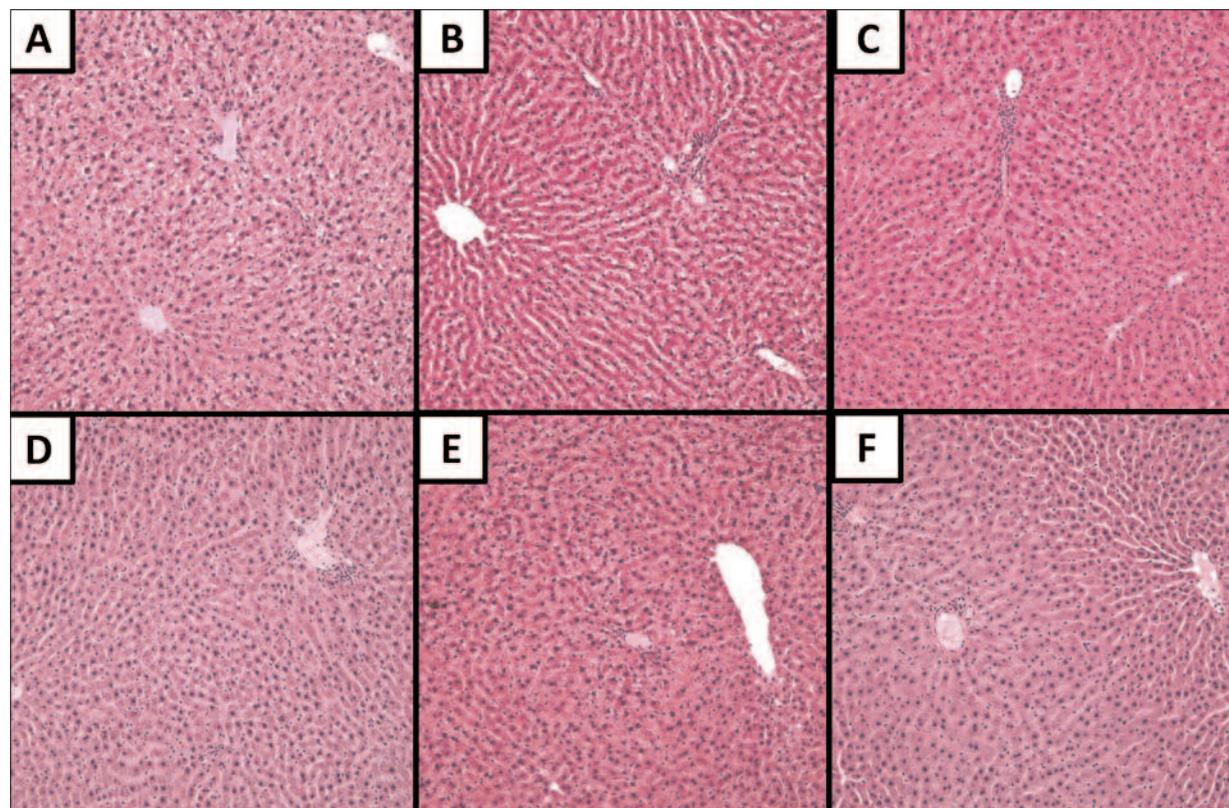


Figure 1. Rat hepatic cross sections (H&E stained) from rat groups treated with **(A)** vehicle+vehicle (n = 5), **(B)** vehicle+celecoxib (n = 6), **(C)** vehicle+diclofenac (n = 9), **(D)** rebamipide+vehicle (n = 7), **(E)** rebamipide+celecoxib (n = 7), and **(F)** rebamipide+diclofenac (n = 5). 10 \times magnification.

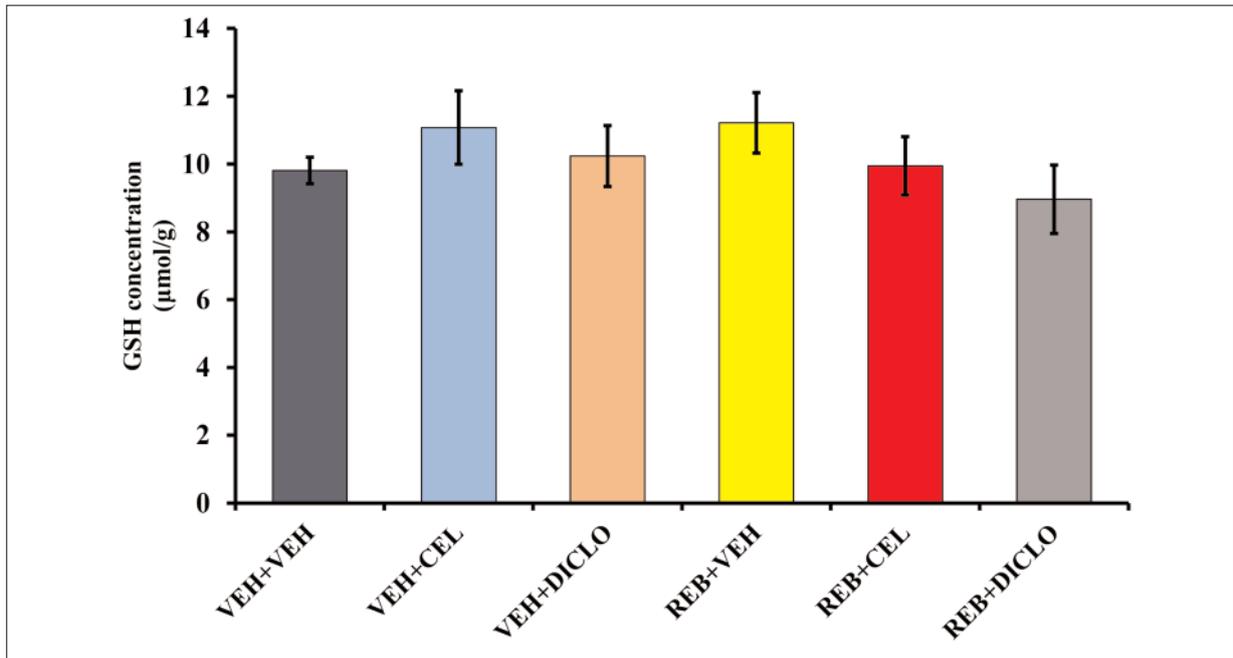


Figure 2. Effect of vehicle+vehicle (VEH+VEH, n = 5), vehicle+celecoxib (VEH+CEL, n = 5), vehicle+diclofenac (VEH+DICLO, n = 9), rebamipide+vehicle (REB+VEH, n = 6), rebamipide+celecoxib (REB+CEL, n = 7), and rebamipide+diclofenac (REB+DICLO, n = 6) on hepatic GSH concentration. Values not significantly different ($p > 0.05$).

ib results were observed by Ekor et al³² when dosing rats with 5.7 mg/kg celecoxib over an 11 day period in a design of 5 days dosing: 2 days rest: 4 days dosing. However, in a two week study using 2.5 mg/kg celecoxib twice-a-day, conducted by Sozer's group, inflammation and

necrosis were both observed in the rat livers¹⁵. Thus, exposure time may have an influence on adverse event occurrence.

While we saw no liver changes in the diclofenac dosed rats, a two-day study consisting of a daily dose of 50 mg/kg intraperitoneal diclofenac pro-

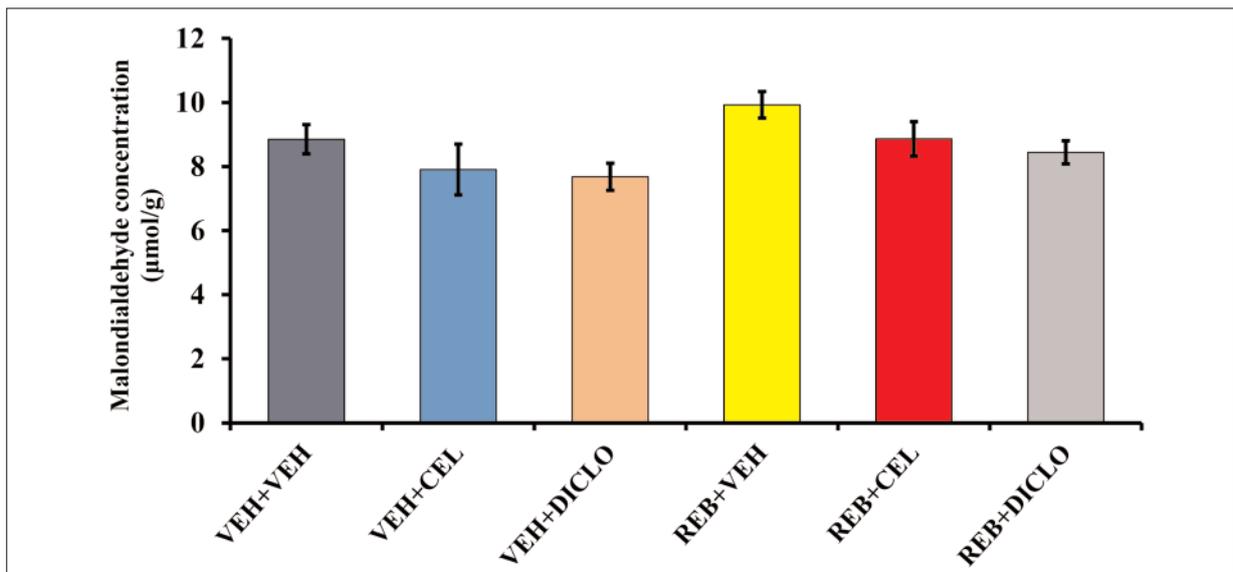


Figure 3. Effect of vehicle+vehicle (VEH+VEH, n = 5), vehicle+celecoxib (VEH+CEL, n = 6), vehicle+diclofenac (VEH+DICLO, n = 9), rebamipide+vehicle (REB+VEH, n = 6), rebamipide+celecoxib (REB+CEL, n = 8), and rebamipide+diclofenac (REB+DICLO, n = 5) on hepatic malondialdehyde levels. Values not significantly different ($p > 0.05$).

Table I. The hepatic celecoxib concentration at 24-hours post-dose.

Group	Celecoxib (ng/g)
Vehicle+celecoxib (n = 6)	3,280.05 ± 585.80
Rebamipide+celecoxib (n = 6)	3,577.81 ± 1,263.05

Values not significantly different, $p > 0.05$.

duced severe histopathological injury¹⁶. Diclofenac also showed significant degeneration of hepatocytes, cytoplasmic eosinophilia, and sinusoidal dilatation compared to control groups in a 1 mg/kg a day study following 10 days of treatment¹⁷. Our results suggest that a single dose of either celecoxib or diclofenac may not produce pathological alterations within a 24-hour period.

There was no observation of increased oxidative stress as observed through depleted GSH. The GSH results concerning celecoxib administration are supported by a study performed by Kirkova et al³³ which saw no significant GSH change in rat liver *in vitro* following incubation with 1 mM celecoxib and the study by Ekor et al³² in which no hepatic GSH level change was detected. Administration of 150 mg/kg diclofenac did not alter GSH levels in mouse liver at 3 or 17 hours post-dose²⁶, which supports the absence of short-term damage even at a high dose. However, in another study¹⁶, Wistar rats were administered 50 mg/kg intraperitoneal diclofenac for two days and the liver GSH levels in the diclofenac group were significantly reduced compared to control.

Although the absence of an elevated malondialdehyde in our study level indicated an absence of intensified oxidative stress, an increase in plasma malondialdehyde was detected following celecoxib dosing in the Sozer et al study¹⁵. An oral dose of 100 mg/kg diclofenac elevated plasma malondialdehyde levels almost fourfold³⁴ and erythrocyte levels of malondialdehyde were in-

Table II. The hepatic diclofenac concentration at 24-hours post-dose.

Group	Diclofenac (ng/g)
Vehicle+diclofenac (n = 4)	2,246.20 ± 641.26
Rebamipide+ diclofenac (n = 6)	3,259.51 ± 944.46

Values not significantly different, $p > 0.05$.

creased 4 days following 10 days of diclofenac dosing¹⁷. While both compounds have the ability to alter the oxidative stress profile of plasma, our results indicate that neither celecoxib nor diclofenac alter the oxidative stress levels in rat livers following a single dose.

As the hepatic concentration of each NSAID was not significantly altered, rebamipide did not seem to influence the liver distribution of either drug. This outcome was expected for celecoxib as no pharmacokinetic interaction in plasma was noted in an earlier pharmacokinetic interaction study using intravenous dosing of celecoxib, diclofenac, and rebamipide. However, that study showed that diclofenac plasma concentrations were increased when rebamipide was concomitantly administered³. Although increased hepatic diclofenac concentrations were observed in our study, the difference did not achieve statistical significance.

There were some limitations in this study. The first being the brevity of drug dosing which may have limited the observation of adverse effects. Treatment duration could be a factor in the absence of negative effects observed elsewhere. Second, high variation within the rebamipide plus diclofenac group concerning hepatic diclofenac concentration may have masked a pharmacokinetic interaction. A larger study population may have shown a significant change in hepatic concentration. A third limitation is a higher CV value for celecoxib due to interfering peaks at low concentrations.

Conclusions

The administration of celecoxib, diclofenac, rebamipide, or the drugs in combination, over the duration and at the dosages tested, do not produce hepatotoxic outcomes such as histopathological changes or increased oxidative stress. In addition, rebamipide does not alter the hepatic concentration of either NSAID. These outcomes may be important to the understanding of utilizing these NSAIDs as short-term therapies for pain and inflammation.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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