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Original Article

Histopathology and oxidative stress analysis of concomitant misoprostol and celecoxib administration

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Abstract: Nonsteroidal anti-inflammatory drugs (NSAIDs), non-selective or selective inhibitors of cyclooxygenase (COX-1 and -2), reduce pain and inflammation associated with arthritic diseases. Celecoxib, a COX-2-selective inhibitor providing decreased gastric injury relative to non-selective NSAIDs, is commonly prescribed. Misoprostol, a prostaglandin analog, supplements NSAID-inhibited prostaglandin levels. As concomitant celecoxib and misoprostol administration has been shown to intensify renal adverse effects, this article examined the influence of concomitant administration on hepatic histopathology, oxidative stress, and celecoxib concentration. On days 1 and 2, rat groups (n = 6) were gavaged twice daily (two groups with vehicle and two groups with 100 µg/kg misoprostol). From day 3 to day 9, one celecoxib dose (40 mg/kg) replaced a vehicle dose of one group and one group received celecoxib in addition to misoprostol. Livers were harvested on day 10. No hepatic abnormalities were observed denoting a lack of influence by either drug. Also no change in mean biomarker levels was detected. The changes in hepatic celecoxib concentration in the misoprostol-receiving group compared to control were not significant. Thus misoprostol does not influence hepatic celecoxib effects in terms of histopathology, oxidative stress, or celecoxib concentration level at the dosage and duration examined. (DOI: 10.1293/tox.2015-0016; J Toxicol Pathol 2015; 28: 165–170)

Key words: celecoxib, misoprostol, NSAIDs, glutathione, malondialdehyde, histopathology

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed to lessen the pain and inflammation associated with arthritic diseases[1–4]. NSAIDs function through the inhibition of cyclooxygenase (primarily COX-1 and COX-2) reducing the production of prostaglandin (PG), a mediator of both pain and inflammation[3, 5, 6]. COX-1 is known to be primarily involved in homeostatic physiological processes and constitutively produced in numerous tissues; while the COX-2 isoform is inducible and found principally in association with inflammation[7–10]. The majority of NSAIDs inhibit both isoforms of COX and as such are classified as non-selective NSAIDs; however, celecoxib (CEL) is a COX-2-selective inhibitor which provides a more favorable gastric side effect profile compared to traditional non-selective NSAIDS[2, 5, 9–11]. Although achieving a reduction in gastrointestinal injury, CEL administration retains the attribute of nephrotoxicity[11] and presents with some unfavorable cardiovascular effects[12]. The negative renal effects likely stem from the inhibition of COX-2 expressed in the kidneys[13].

Misoprostol (MISO), an analog of PGE_1, has been deployed as a method of alleviating the gastrointestinal side effects associated with non-selective NSAID usage[4, 14–18]. One proposed mechanism of action for this positive effect is that MISO administration replaces the PGE_1 depleted during the course of NSAID usage[15, 19]. As a result of gastric ulcer amelioration, MISO was examined for a similar influence upon the renal side effects of CEL[11]. In that study, we found that the addition of MISO to a CEL regimen exacerbates the renal injury associated with CEL administration.

Free radicals, which are reactive oxygen species created in the process of cellular metabolism, can result in oxidative damage if cellular mechanisms of containment are saturated or depleted[20–23]. Glutathione (GSH) and malondialdehyde (MDA) are compounds which can be used as biomarkers for the detection of oxidative stress[9, 10, 24, 25]. GSH functions as an antioxidant which scavenges free radicals created during metabolism of endogenous or xenobiotic chemicals. Thus observing a decrease in total GSH levels may be an indication of increased oxidative stress. MDA is

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a byproduct of lipid peroxidation by free radicals, thus the observation of increased MDA concentrations within cells points to excessive oxidative stress[1, 23].

The administration of CEL has been shown to have the ability to negatively influence the liver via oxidative stress[9]; however, no study has examined the influence of concomitant MISO administration on the hepatic outcomes of CEL. As concomitant administration of drugs can influence drug tissue accumulation possibly creating toxic conditions, this study examined the hepatic effects of CEL in presence and absence of MISO using livers collected in a previous study[11].

Materials and Methods

Chemicals

Acetonitrile, glacial acetic acid, iso-octane, 2-propanol, sulfuric acid, triethylamine, and water, each being of high performance liquid chromatography (HPLC) quality, were purchased from Fischer Scientific Laboratory (Fair Lawn, NJ, USA). Administered compounds, CEL, MISO, and methyl cellulose, were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada), Cayman Chemical Company (Ann Arbor, MI, USA), and Science Stuff, Inc. (Austin, TX, USA) respectively. The HPLC internal standard (IS), ibuprofen, was procured from Sigma-Aldrich (St. Louis, MO, USA).

Animals and drug administration

All experiments were carried out on male Sprague-Dawley rats (approximately 8 weeks old) in accordance with a protocol approved by the University Committee on Animal Care at East Tennessee State University.

Study design

A detailed description can be found in the previously published article by our group[11]. Briefly, 24 rats were randomized into four groups (n=6). Vehicle (VEH), methyl cellulose suspension, was administered via oral gavage to two groups, VEH+VEH and VEH+CEL (CEL 40 mg/kg), twice daily on days 1 and 2; while the remaining groups, MISO+MISO and MISO+CEL, received two daily doses of MISO (100 µg/kg). Beginning on the third day and continuing to day 9, VEH+VEH received VEH twice daily; VEH+CEL was given a dose of CEL (morning) then VEH (evening); MISO+MISO continued two doses of MISO a day; and MISO+CEL was administered two MISO and one CEL dose each day. Livers were immediately collected and stored in −80 °C following sacrifice on day 10. The CEL dose level was selected based on the ability to significantly reduce electrolyte excretion rates in rats as shown in a previous study[26]; while the MISO dose was taken from a study by Ozer *et al.*[19] in which this dose of MISO was found to provide renal tissue protection against cisplatin-induced injury.

Histopathological evaluation

A portion of each rat liver was collected following partial thaw; immersed in formalin overnight; then embedded in paraffin. Hepatic tissue sections (5 µm) were stained with hematoxylin and eosin (H&E). All sections were examined for portal inflammation, lobular inflammation, hepatocyte injury, and necrosis by a board certified pathologist blinded to the treatment groups.

Oxidative stress assessment

Glutathione measurement: GSH concentrations were quantified using an Arbor Assay GSH Colorimetric Detection Kit Arbor (Ann Arbor, MI). The assay was performed using manufacturer's instructions. Briefly, 10 mg of liver sample was homogenized in 250 µL ice cold 5% 5-sulfosalicyclic acid dehydrate (SSA). Following a 10 minute incubation at 4 °C, samples were centrifuged (14,000 rpm) for 10 minutes at 4 °C. The supernatant was removed and diluted to 1% SSA using manufacturer assay buffer. Samples were then further diluted using manufacturer sample diluent to a sample concentration of 1 µg/µl. The final dilutions were added to a 96 well plate along with the color detection reagent and reaction mix then incubated at room temperature for 20 minutes. The plated samples were read at 405 nm.

Thiobarbituric Acid Reactive Species (TBARS) measurement: Determination of MDA concentration was conducted using a Cayman Chemical Company TBARS Assay Kit (Ann Arbor, MI). According to manufacturer's instruction, the liver sample (25 mg) was homogenized in 250 µL RIPA Buffer with a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO). Following a 10 minute centrifugation (1,600 g) at 4 °C, a 100 µL aliquot of supernatant was added to 100 µL sodium dodecyl sulfate solution and 4 mL color reagent then boiled one hour. The reaction was terminated using a 10 minute incubation on ice then centrifuged (1,600 g) for 10 minutes at 4 °C. The supernatant (150 µL) was then added to the 96 well plate and read at 530 nm.

Chromatographic conditions

Sample preparation: Whole livers were removed from −80 °C, thawed, then a portion of each liver was collected and weighed. Samples were homogenized using a Power-Gen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA) in a 2 mL:1 g water to sample weight ratio.

Analysis equipment and solution preparation: Drug concentration determination was conducted through modification of a method previously reported by Cooper and colleagues[11] using a high performance liquid chromatography (HPLC) system (Shimadzu, Japan) equipped with a LC020AB solvent delivery system, a SIL-20A HT autosampler, a SPD-M20A photodiode detector (254 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). A CentriVap concentrator (Lab Conoco, Kansas City, MO, USA) set at 50 °C was used to evaporate sample organic phase.

A nylon filter (0.5 µm) was used to filter the HPLC mobile phase (MP) for CEL quantification (acetonitrile, water, acetic acid, and triethylamine in a respective ratio of

47:53:0.1:0.03). A CEL standard concentration curve was created using a 100,000 ng/mL stock solution (10 mg CEL dissolved in 100 mL MP). An ibuprofen stock solution of 100,000 ng/mL, also 10 mg in 100 mL MP, was utilized as an IS.

Hepatic celecoxib extraction: Standard curve creation was accomplished using serial dilutions of a standard CEL stock solution ranging from 100,000 ng/mL down to 25 ng/mL. Each concentration (100 µL) was added to a respective blank liver homogenate (100 µL) followed by 100 µL IS. Each sample then received 200 µL 0.6 M sulfuric acid and 5 mL iso-octane propanol (95:5) then vortexed 30 sec. Following a five minute centrifugation (2,500 g), samples were placed in a dry ice/ethanol bath to facilitate organic phase removal to a clean tube. Organic phases were evaporated to dryness then re-suspended in (200 µL) MP. Samples (100 µL) were injected into the HPLC system and drug concentrations were determined using a 15 minute run at a 1 mL/min flow rate. This method produced a lower limit of quantitation of 250 ng/g; a lower limit of detection of 25 ng/g; and a coefficient of variation of 21.5%.

Data treatment and statistical analysis

All values are presented as mean ± standard error of the mean. One way ANOVA with multiple comparisons was used to analyze GSH and MDA concentration values. Hepatic drug concentration values were analyzed using the Student's t-test. Outliers were detected using SPSS software (IBM Corporation, Armonk, NY, USA).

Results

Histopathology

Hepatic histopathological examination revealed that all slides from each treatment group were within normal histological limits with an absence of portal or lobular inflammation, necrosis, or structural changes (Fig. 1).

Oxidative stress

GSH concentration: The detected GSH levels in the VEH+VEH group ranged from 9.66 to 17.97 µmol/g yielding an average concentration of 14.12 ± 1.40 µmol/g. ANOVA revealed no significant difference (p=0.4878), as seen in Fig. 2, among the groups, VEH+VEH, VEH+CEL (12.50 ± 1.41 µmol/g), MISO+MISO (12.68 ± 0.88 µmol/g), and MISO+CEL (11.34 ± 0.90 µmol/g).

MDA concentration: Rats in the VEH+VEH group displayed a range of hepatic MDA concentrations from 3.89 to 9.49 µmol/g. Figure 3 shows that no significant difference (p=0.3589) was detected among the treatment groups, VEH+CEL (9.46 ± 1.65 µmol/g), MISO+MISO (10.53 ± 0.98 µmol/g), MISO+CEL (8.37 ± 0.89 µmol/g), and VEH+VEH (7.53 ± 0.99 µmol/g).

Hepatic CEL concentration

As shown in Table 1, the hepatic concentration of CEL in the absence of MISO was 3.59 ± 2.13 µg/g; while the presence of MISO produced a CEL concentration of 1.02 ± 0.53 µg/g. The change detected in the presence of MISO did not attain significance (p=0.3000).

Table 1. The Hepatic Celecoxib Concentration on Day 10

Group	CEL (µg/g)
VEH+CEL	3.59 ± 2.13
MISO+CEL	1.02 ± 0.53

VEH+CEL: vehicle+celecoxib; MISO+CEL: misoprostol+celecoxib. The values were not significantly different, p>0.05.

Discussion

Previously, we have shown that this dose (40 mg/kg) of CEL produces pathological renal outcomes, in terms of tubular dilatation and necrosis, some of which are amplified in the presence of MISO. Heart tissue was also examined at these doses; however, the tissue was not found to be significantly altered by either drug individually or in combination. In this study, no hepatic histopathological changes were observed; however, Sozer and associates detected portal inflammation and parenchymal necrosis when CEL (5 mg/kg/day) was administered over two weeks[9]. Another study using another dose (5.7 mg/kg/day) given intermittently over 11 days (5 days treatment: 2 days rest: 4 days treatment) saw no change in CEL treated liver sections[27]. These examples may indicate that CEL histopathological changes occur as a result of chronic dosing rather than short term exposure. In regard to the beneficial effects of MISO, a study by Salam and associates found that MISO could lessen the damage caused by carbon tetrachloride which is associated with lipid peroxidation[28]. This information suggests that MISO may have a protective effect; however, as we saw no significant alteration among our groups, we cannot confirm these effects.

Hepatic GSH and MDA were measured to estimate oxidative stress levels. No group in this study presented with a depletion of GSH pointing to equivalent oxidative stress. These results are supported by an *in vitro* experiment using rat liver which showed no significant change in GSH levels upon CEL exposure[8]. While a wide range of MDA concentrations were measured in the control rat livers, the absence of significance difference between the groups suggests no increased lipid peroxidation. In a study conducted using goat liver homogenates, CEL concentrations equivalent to human therapeutic levels showed a significant increase in MDA[1]. Also in a two week twice-a-day (2.5 mg/kg) CEL administration study conducted using young rats, there was an increase in plasma MDA concentration; however, no GSH change in liver was detected [9]. While these results suggest that plasma MDA concentrations may be altered, other studies have shown that CEL administration at therapeutic drug doses does not alter either biomarker in rat livers[8, 27].

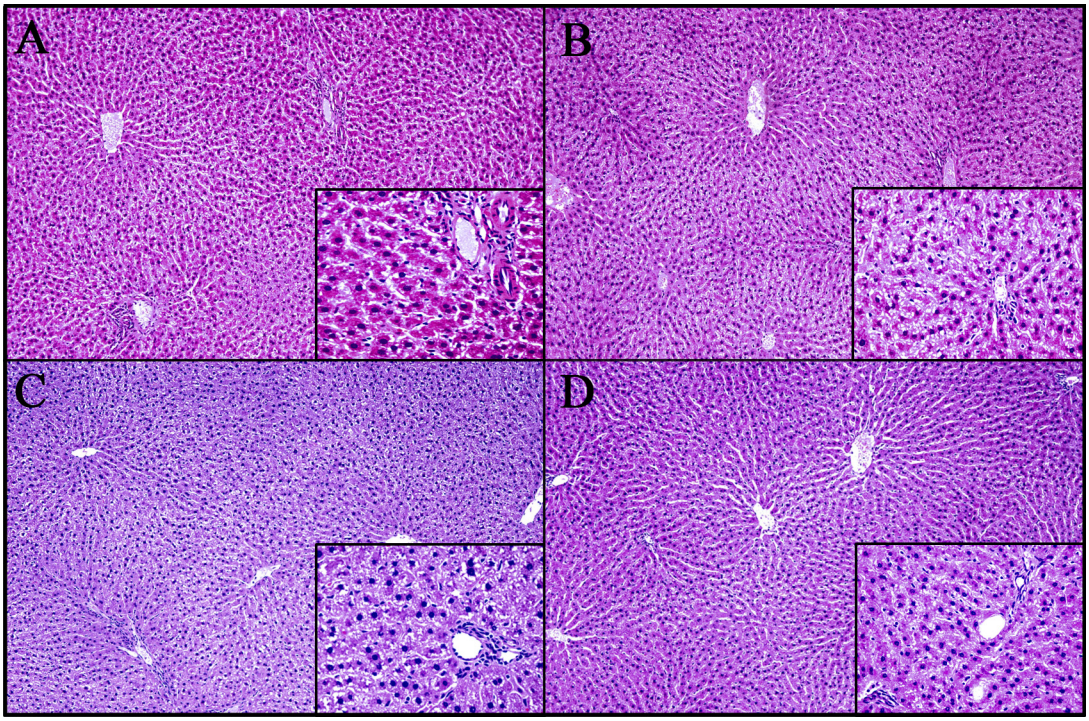

Fig. 1. Liver histopathology. Liver cross sections (H&E stained) from rat groups treated with (A) vehicle+vehicle, (B) vehicle+celecoxib, (C) misoprostol+misoprostol, and (D) misoprostol+celecoxib. 10× magnification with 40× insets

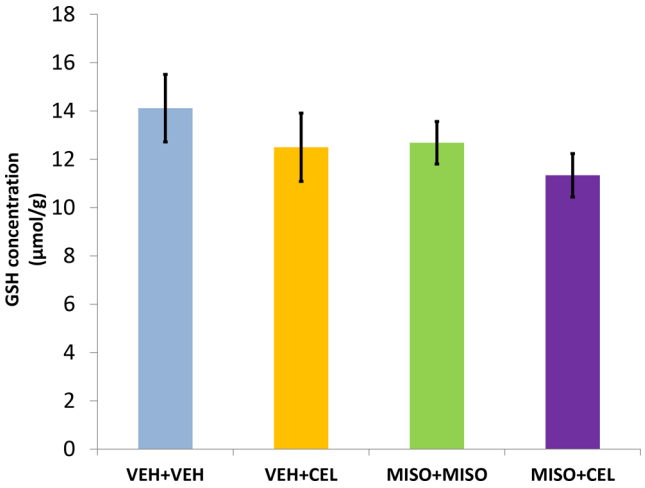

Fig. 2. Hepatic GSH concentration. Effect of control (VEH+VEH), vehicle+celecoxib (VEH+CEL), misoprostol+misoprostol (MISO+MISO), or misoprostol+celecoxib (MISO+CEL) on hepatic GSH concentration. The values were not significantly different ($p>0.05$).

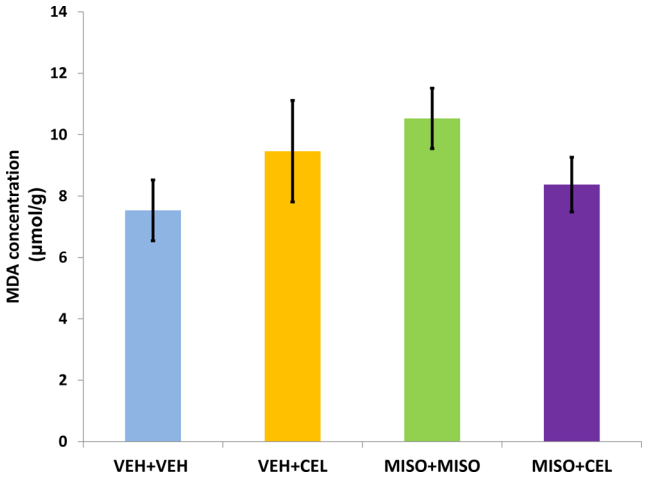

Fig. 3. Hepatic MDA concentration. Effect of control (VEH+VEH), vehicle+celecoxib (VEH+CEL), misoprostol+misoprostol (MISO+MISO), or misoprostol+celecoxib (MISO+CEL) treatment on hepatic MDA concentration. The values were not significantly different ($p>0.05$).

MDA levels in the jejunum were also unchanged upon CEL exposure in a study conducted by Fornai and colleagues[29]. In another study, the addition of MISO prevented an increase in intestinal MDA following ischemia-reperfusion[30]. These protective effects are supportive of the results gathered in this study. There were also no significant changes detected among the groups, which suggests that the drugs do not either individually or in combination elicit more than routine oxidative stress. These results in light of the previous studies suggest that CEL, MISO, or the combination do not alter either MDA or GSH during short term administration.

Although the hepatic CEL concentration was lower in

the MISO+CEL group, no statistically significant difference was found due to high variation within the drug concentrations of the VEH+CEL group. Further studies may be needed to examine the relationship between the two drugs.

Our study had several limitations, one being short treatment duration. As noted earlier some damage was detected following two weeks of dosing[9]. Thus it is possible that some adverse effects are time sensitive appearing only following prolonged exposure possibly after the attainment of steady-state concentrations. Another limitation was the variability of VEH+CEL concentrations. The inclusion of a larger sample size may allow for the detection of a significant change in CEL hepatic disposition.

In conclusion, our results indicate that at the dose and duration examined, neither CEL, MISO, nor their concomitant administration produced hepatic alteration in terms of oxidative stress, hepatic CEL disposition, or hepatic architecture.

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Disclosure of Potential Conflicts of Interest: We have no conflicts of interest.

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