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Precision cut intestinal slices are an appropriate *ex vivo* model to study NSAID-induced intestinal toxicity in rats

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used therapeutic agents, however, they are associated with a high prevalence of intestinal side effects. In this investigation, rat precision cut intestinal slices (PCIS) were evaluated as an *ex vivo* model to study NSAID-induced intestinal toxicity.

Firstly, PCIS were incubated with 0–200 μM diclofenac (DCF), one of the most intensively studied NSAIDs, to investigate whether they could correctly reflect the toxic mechanisms. DCF induced intestinal toxicity in PCIS was shown by morphological damage and ATP depletion. DCF induced endoplasmic-reticulum (ER) stress, mitochondrial injury and oxidative stress were reflected by up-regulated HSP-70 (heat shock protein 70) and BiP (binding immunoglobulin protein) gene expression, caspase 9 activation, GSH (glutathione) depletion and HO-1 (heme oxygenase 1) gene up-regulation respectively. Furthermore, DCF intestinal metabolites, which gave rise to protein adduct but not toxicity, were detected in PCIS.

Secondly, PCIS were incubated with various concentrations of five NSAIDs. Typical NSAID-induced morphological changes were observed in PCIS. The *ex vivo* toxicity ranking (diflunisal > diclofenac = indomethacin > naproxen ≫ aspirin) showed good correlation with published *in vitro* and *in vivo* data, with diflunisal being the only exception.

In conclusion, PCIS correctly reflect the various mechanisms of DCF-induced intestinal toxicity, and can serve as an *ex vivo* model for the prediction of NSAID-induced intestinal toxicity.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), commonly administered for rheumatic and arthritic diseases (Aithal and Day, 2007), are notorious for their high prevalence of side effects in the small intestine including bleeding, ulceration, inflammation or perforation. NSAID-associated enteropathy is a significant clinical issue due to its high morbidity and mortality rates (Davies et al., 2000; Scarpignato and Hunt, 2010). In the present study, rat precision cut intestinal slices (PCIS) were evaluated for their potential to reflect various reported mechanisms of diclofenac-induced intestinal toxicity, and as an *ex vivo* model to predict NSAID-induced toxicity in rat intestine.

Multiple toxic mechanisms have been reported contributing to the NSAID enteropathy, and these mechanisms are shared by most of the NSAIDs. In the present study, one of the most extensively studied NSAID, diclofenac (DCF), was used as a model compound to test the PCIS system. It has been suggested that the topical effects of DCF in the intestine are the result of a multiple hits pathogenesis involving several factors. When the luminal enterocytes are exposed to DCF, electrophile stress (induced by biliary or intestinal metabolites), endoplasmic reticulum (ER) stress, mitochondrial injury, and oxidative stress are induced, which finally lead to cell death (Mahmud et al., 1996; Boelsterli et al., 2013) .

A number of experimental approaches both *in vivo* and *in vitro* have provided evidences for the involvement of multiple mechanisms. *In vivo* studies in rodents indicated that electrophile stress by DCF metabolites formed in the liver and excreted in the bile (Seitz and Boelsterli, 1998; LoGuidice et al., 2012), or intestinal CYP-mediated oxidative metabolism (Zhu and Zhang, 2012), could result in enterocyte protein adduct formation, which were

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; PCIS, precision cut intestinal slices; DCF, diclofenac; ER, endoplasmic reticulum; mPT, permeability transition pore; DMSO, dimethyl sulfoxide; WmE, Williams Medium E; BSA, bovine serum albumin; 4′-OH DCF, 4′-hydroxyl diclofenac; 5-OH DCF, 5-hydroxyl diclofenac; DAG, acyl glucuronide diclofenac; HE, Hematoxylin-Eosin; HSP-70, heat shock protein 70; BiP, binding immunoglobulin protein; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase 1; CYP, cytochrome P450; Chop, CCAAT-enhancer-binding protein homologous protein; HSF1, heat shock transcription factor 1; Bach1, BTB and CNC homologue 1.

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responsible for intestinal ulceration. Other reports suggested that ER stress was involved in NSAID intestinal toxicity, as reduced NSAID enteropathy was found in *Chop* (CCAAT-enhancer-binding protein homologous protein)-knockout and HSF1 (heat shock transcription factor 1)-null mice (LoGuidice et al., 2010; Asano et al., 2009). NSAID induced mitochondrial changes were observed in the rat intestine by electron microscopy (Somasundaram et al., 1997), and DCF-induced enterocyte demise via the mitochondrial permeability transition pore (mPT) opening was found in mice (Ramirez-Alcantara et al., 2009; LoGuidice et al., 2010). Genetic deletion of Bach1 (BTB and CNC homologue 1), a transcriptional repressor of HO-1 (heme oxygenase 1), fully protected mice from an ulcerogenic dose of indomethacin, indicating the involvement of oxidative stress (Harusato et al., 2011).

More insight of the mechanism was gained by *in vitro* approaches using cell lines or isolated mitochondria from several tissues including rat intestinal epithelium, showing NSAID-induced uncoupling of mitochondrial oxidative phosphorylation, the formation of reactive oxygen species, and mPT pore opening-mediated cell death (Lichtenberger et al., 2012; van Leeuwen et al., 2011; Sandoval-Acuna et al., 2012; Nadanaciva et al., 2007; Somasundaram et al., 1997, 2000; Al-Nasser, 2000; LoGuidice et al., 2010). In addition, increased cellular Ca^{2+} concentration, induction of oxidative stress and apoptosis were observed in primary gastric mucosal cells when cultured with NSAIDs (Tanaka et al., 2005; Tsutsumi et al., 2004). Involvement of reactive oxygen species in indomethacin-induced apoptosis was shown in the colon cancer cell line Caco-2 (Omatsu et al., 2009) and in a rat intestinal epithelial cell line (RIE-1) (Omatsu et al., 2010).

Since animal experiments are costly and not in compliance with the 3R (reduction, replacement, refinement) concept, *in vitro* models are needed to study drug-induced enteropathy. The existing *in vitro* cell models are not representative for the intact intestine, each of them only reflects certain isolated aspects of the mechanisms. Therefore, it would be desirable to have a model which can integrate the various aspects of NSAIDs enteropathy (electrophile stress, ER stress, oxidative stress, mitochondrial injury), more closely emulates the intestine, and resembles the *in vivo* situation. Ideally such a model would reflect the multiple toxic mechanisms and be predictive for the potential toxicity of the tested compounds.

PCIS are a multicellular three-dimensional *ex vivo* model. It has been used intensively to study drug metabolism, as well as metabolism inhibition and induction in animals and humans (Martignoni et al., 2006a,b; van de Kerkhof et al., 2005, 2007, 2008; de Kanter et al., 2002, 2004; Khan et al., 2009; Chow et al., 2010). Due to the presence of various cell types in an architectural organization, and the preserved function of metabolic enzymes, transporters, and cofactors, this model closely represents the functional intestine. The PCIS model has therefore the potential to study mechanisms of drug-induced intestinal injury. In addition, by the approach to use both human and animal tissue, it helps gaining a better understanding of species-specific toxicity (de Kanter et al., 2004; Niu et al., 2013). Furthermore, slices can be obtained from duodenum, jejunum, and ileum separately, which make it possible to determine the different sensitivities of these different parts of the intestine.

In the present study, the rat PCIS are evaluated as a model for NSAID-induced intestinal toxicity for the first time. First of all, it was examined whether PCIS could reflect the above-discussed mechanisms underlying NSAIDs enteropathy, using DCF as a model compound. Subsequently, the study was extended to four other NSAIDs (diflunisal, indomethacin, naproxen and aspirin). The toxicity was assessed by determining ATP level and cell death as observed microscopically. *Ex vivo* toxicity ranking of these four compounds and diclofenac was compared with published *in vivo* and *in vitro* data.

2. Materials and methods

2.1. Preparation of rat PCIS

Male Wistar rats, obtained from Charles River (Sulzfeld, Germany), with body weight between 316 g and 422 g, were used in this study. The rats were kept in a temperature and humidity-controlled room with a 12 h light/dark cycle with food and tap water *ad libitum* (Harlan Laboratories B.V., Horst, The Netherlands). The Animal Ethics Committee of the University of Groningen approved the experiments.

PCIS were prepared as described in detail by de Graaf (de Graaf et al., 2010). In brief, rats were sacrificed under anesthesia with isoflurane/O_2. The intestine was preserved in ice-cold oxygenated Krebs–Henseleit buffer (pH 7.4). Jejunum tissue segments were embedded in 3% agarose (Sigma–Aldrich, Steinheim, Germany) in 0.9% NaCl, using a tissue embedding unit. Subsequently, the intestinal segment was sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs–Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCIS (350–450 μm thick and 3–4 mg wet weight) were stored in ice-cold Krebs–Henseleit buffer until incubation.

2.2. Incubation of the PCIS with NSAIDs and metabolism inhibitors

The PCIS were incubated individually in 12 wells plates (Greiner Bio-one GmbH, Frickenhausen, Austria) as described by de Graaf (de Graaf et al., 2010). Each well contained 1.3 ml Williams Medium E (WmE) with Glutamax-I (Gibco, Paisley, UK) supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany) and 50 μg/ml gentamicin (Gibco, Paisley, UK) and 2.5 μg/ml fungizone (amphotericin B) (Gibco, Paisley, UK). The plates were placed in plastic boxes and shaken (90 times per minute) in an incubation cabinet at 37 °C in an atmosphere of 95% O_2 and 5% CO_2.

The stock solutions of diclofenac sodium salt, diflunisal, indomethacin, naproxen and aspirin (Sigma–Aldrich, St. Louis, MO, USA) were prepared in dimethyl sulfoxide (DMSO, VWR, Fontenay-sous-Bois, France) and stored at 4 °C. The DMSO concentration was the same in the treated groups and their corresponding vehicle controls and kept below 0.5%. The applied concentrations of DMSO in this paper did not affect the viability of the PCIS (results not shown). The slices were incubated with diclofenac (50, 100, 200, 500, 1000 μM), diflunisal (50, 100, 500, 1000 μM), indomethacin (50, 100, 500, 1000 μM), naproxen (0.1, 0.5, 2.5, 5 mM) and aspirin (0.1, 0.5, 1, 2, 5, 10 mM).

CYP inhibitors ketoconazole and cimetidine or the UGT inhibitor borneol (Sigma–Aldrich, St. Louis, MO, USA) were used in this study to inhibit the DCF metabolism. PCIS were incubated for 5 h with 200 μM DCF in the presence or absence of ketoconazole (10 μM), cimetidine (5 mM) or borneol (0.5 mM) respectively. The ATP content in the PCIS was measured after the incubation.

2.3. ATP and protein content of slices

The viability of slices was determined by measuring the ATP content after 5 h incubation using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) as described previously (de Graaf et al., 2007). The ATP content was corrected by the amount of protein of each slice. The protein content of the PCIS was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) with bovine serum albumin (BSA, Sigma–Aldrich, Steinheim, Germany) for the calibration curve (de Graaf et al., 2007) .