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Diclofenac

Pharmacology, Uses and Adverse Effects

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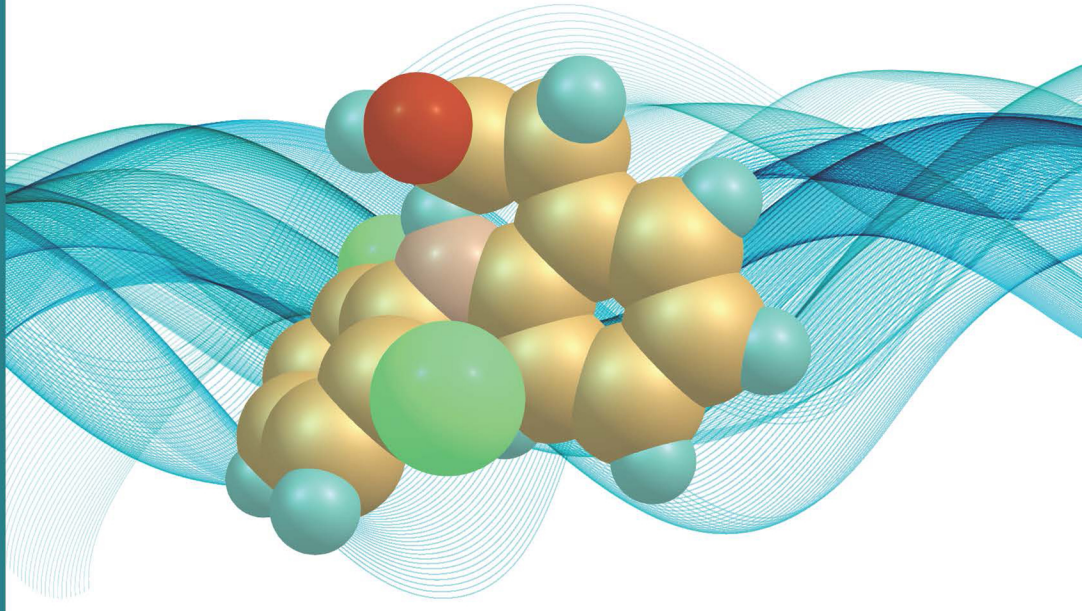

Eugenia Yiannakopoulou
Editor

NOVA

DICLOFENAC

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EUGENIA YIANNAKOPOULOU
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NOTICE TO THE READER

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PREFACE

Although the non steroidal anti-inflammatory agent diclofenac is an old drug, there is ongoing research interest on potential novel indications of the drug, on adverse drug reactions of diclofenac and on the management of these adverse drug reactions. This book aims to contribute knowledge on novel aspects of the pharmacology of diclofenac. Thus, the book provides evidence on three fields: (i) potential novel indications of diclofenac, especially in oncology, (ii) adverse events of diclofenac and (iii) modulation of gastrointestinal and cardiovascular toxicity of diclofenac.

The book titled 'Diclofenac: Pharmacology, Uses and Adverse Events' is focused on the above areas, contributing evidence on:

- the anticancer effects of diclofenac,
- the modulation of stress response by diclofenac,
- rare severe adverse reactions of diclofenac,
- thyroid effects of diclofenac,
- maternal and fetal adverse reactions of diclofenac and
- the modulation of gastrointestinal
- the modulation of cardiovascular toxicity of diclofenac.

All the chapters are well-written and structured and appropriately referenced. The authors present their own research work and at the same time they provide a well-informed literature review.

The book will be interesting for researchers with interest in diclofenac, academic teachers, medical doctors, pharmacologists, pharmacists, and medical students.

Chapter 1 - Non steroid anti-inflammatory agents (NSAIDs) represent the most commonly used medication worldwide. Currently, NSAIDs are the most promising agents in development for cancer chemoprevention. In addition there is evidence for their chemotherapeutic potential. NSAIDs, including diclofenac apart from inhibiting cyclo-oxygenase enzymes are known to target multiple pathways that are implicated in carcinogenesis. Thus these agents could play a role in the chemoprevention or adjuvant therapy of malignant tumors. Although, in general, NSAIDs have common mechanisms of action, the anticancer effects are not the same for all the chemical entities. In addition, the mechanisms of anticancer activity are not common for all the NSAIDs. Diclofenac is an old drug and there is growing interest on its potential role in the field of oncology. Diclofenac exerts its action by inhibiting prostaglandin synthesis via inhibition of cyclo-oxygenase I and cyclo-oxygenase-II. Furthermore, novel cyclo-oxygenase independent mechanisms of actions have been suggested for diclofenac including the inhibition of substrate P, inhibition of peroxisome proliferator activated receptor gamma (PPARgamma), blockage of acid-sensing ion channels, alteration of interleukin-6 production, and inhibition of N-methyl-D-aspartate (NMDA) receptor hyperalgesia. This chapter reviews the anticancer effects of diclofenac. The chapter is structured in four parts. In the first part, the pharmacology of diclofenac is presented. The second part is focused on human data that support the possible role of diclofenac as a chemo-preventive and chemotherapeutic agent. In the third part experimental *in vitro* and *in vivo* evidence on the anticancer effects of diclofenac is presented. The fourth part is devoted to the mechanisms of anticancer effect of diclofenac. Antitumor effect of diclofenac has been mainly attributed to the cyclo-oxygenase I and II inhibition. However, it has also been well evidenced that diclofenac inhibits tumour cell growth and

proliferation by cyclo-oxygenase independent mechanisms. The molecular pathways that can be targeted by diclofenac have not been fully elucidated. However, it is evident that diclofenac is a multi-target agent with anticancer action, that is expressed via multiple mechanisms. This multitarget approach renders diclofenac a promising agent, that should be further investigated for the chemoprevention or adjuvant treatment in the field of oncology. In fact, there are experimental data from different cancer cell types, that support the notion of diclofenac repurposing in oncology. In conclusion, the anticancer effect of diclofenac can be interpreted via multiple mechanisms. This multitarget approach could render diclofenac a putative agent in oncology for chemoprevention as well as for adjuvant treatment. Importantly the anticancer action of diclofenac has been evidenced in aggressive tumours including glioblastoma and pancreatic adenocarcinoma. In the context of drug repurposing, future research efforts are needed for the development of diclofenac as an anticancer agent possibly in synergy with well established treatment modalities.

Chapter 2 - Every condition that threatens the state of homeostasis is considered a stress, and causes leading to it are termed stress factors. Stress response is the term used for the reactions that follow the recognition of the stress factor aiming to protect the organism from the stress factor and avoid danger. Stress signal detection leads to activation of signal pathways, with subsequent induction of gene transcription, protein translation and alteration of cell energy state. Thus, mild stress response protects the organism from stressors in the environment. However, if stress response is exaggerated, it results to occurrence of disease. Stress response is implicated in the pathophysiology of a number of diseases, including atherosclerosis, cardiovascular disease, heart failure, carcinogenesis. Thus, there is growing research interest in the design of drugs that target stress response. In addition, there are old drugs that are currently investigated for their possible effect on stress response. Non steroidal anti-inflammatory agents comprise a chemically heterogenous group of medications including aspirin and salicylates as well as selective and non selective inhibitors of cyclo-oxygenase. The main actions of these agents include anti-inflammatory, analgesic and antipyretic actions, while aspirin and salicylates are also

known for their antiplatelet action. Aspirin and salicylates are known to modulate stress response in pro-caryotic organisms as well as in eukaryotic cells. Diclofenac is a well known non steroidal anti-inflammatory agent with analgesic and anti-inflammatory properties, that are due to the inhibition of the action of cyclo-oxygenase. Recently, there is evidence supporting that cyclo-oxygenase independent mechanisms are implicated in the pharmacology of diclofenac, including the inhibition of the glycolysis of tumor cells, the modulation of apoptosis, the modulation of stress repsonse. In that aspect, there is experimental evidence indicating that diclofenac is able to modulate stress response, including heat shock, oxidative stress response. Diclofenac has been evidenced to modulate heat shock response in the eukaryotic organism S.cerevisiae. In addition low concentrations of diclofenac act as a mild oxidative stressor inducing mild oxidative stress response that promotes cellular protection from a subsequent lethal stressor. This chapter presents experimental evidence suggesting modulation of stress response by diclofenac. The mechanisms underlying the effect of diclofenac on stress response are also presented. Clinical implications of targeting stress response by diclofenac including cancer chemoprevention are also discussed. Although the adverse effects of diclofenac could limit its long term use as a chemo=preventive agent, the combination of diclofenac with other chemo=preventive agents could provide synergistic favourable effects with lower frequency of adverse events.

Chapter 3 - Diclofenac is a non steroidal anti-inflammatory agent of the phenylacetic class. Diclofenac is implicated in a number of adverse events affecting different systems. Although the gastrointestinal and cardiovascular adverse events associated with diclofenac are well documented, diclofenac is also associated with serious adverse events, including severe allergic reactions, eosinophilic pneumonia, aseptic meningitis, immune hemolytic anaemia, immune thrombocytopenia, immune neutropenia, hepatic injury, renal injury. Severe allergic reactions to diclofenac include anaphylactic reaction, anaphylactic shock, Kounis syndrome. Even fatal allergic reactions to diclofenac have been reported. Although severe allergic reactions are more common after intramuscular and intravenous administration, there are also reports of severe allergic reactions after oral, subcutaneous and rectal

administration. Although serious adverse events of diclofenac are rare, diclofenac is one of the most commonly used non steroidal anti-inflammatory agents worldwide. Importantly, diclofenac is included in the over-the counter medicines in most countries. Furthermore, diclofenac is often used for the treatment of postoperative pain in an opioid sparing approach. Diagnosis of severe allergic reactions as well as other rare reactions is quite difficult in the postoperative patient. Thus, clinicians should be aware of the serious adverse events associated with the use of diclofenac in order to be able to recognize them promptly and treat them properly. Health organizations should reconsider the policy of the over the counter use of the non steroidal anti-inflammatory agents. Patients should be well informed about the severe adverse reactions associated with the use of diclofenac.

Chapter 4 - Diclofenac, a commonly prescribed non steroidal anti-inflammatory agent has well known adverse events including hepatological, gastrointestinal, renal and cardiovascular adverse events. Besides them, diclofenac affects thyroid function tests. Importantly, recently published research suggests that diclofenac is a thyroid receptor antagonist. This chapter will be structured in three sections. The first section will focus on the mechanisms of action of drugs on thyroid function tests. The second section will review evidence on the effect of diclofenac on thyroid function tests. The third session will focus on the clinical implications of the thyroid effects of diclofenac especially for patients treated with thyroid replacement therapy. Non steroidal anti-inflammatory agents are known to affect the results of the thyroid function tests, by binding to thyroid hormone binding proteins and releasing free T3 and free T4. The transient elevation of free hormones leads to a sequence of alterations in the thyroid function. It is not really known if the effect of NSAIDs on thyroid function is a class effect or if the effect differs among different drugs. Given that NSAIDs encompass a broad class of medications with different chemical structures, it is straightforward that the effect of NSAIDs on thyroid function would be different for the different drugs. A number of studies demonstrate that diclofenac affects thyroid hormone measurements. However, the majority of the data are derived from short term administration of diclofenac in healthy

volunteers. There is paucity of data on the effect of diclofenac on thyroid hormone measurements in the case of patients treated for hypothyroidism or in the case of patients submitted to thyroidectomy and treated with thyroxin replacement therapy. Knowledge of the thyroid effects of diclofenac is necessary for endocrinologists, endocrine surgeons and rheumatologists, because it will affect therapeutic decisions. Failure to recognize thyroid effects of diclofenac in clinical practice may lead to unnecessary diagnostic tests as well as to incorrect treatment of euthyroid patients. Clinicians should always correlate the blood measurements with the clinical picture of the patient. A detailed history of short and long term drug use should always be included in the evaluation of thyroid hormone measurements. Special attention is needed in the case of patients submitted to thyroidectomy, as diclofenac use may affect thyroid replacement therapy.

Chapter 5 - The use of non-steroidal anti-inflammatory drugs (NSAIDs) like diclofenac is common among the general population and also among pregnant women. Although highly effective, its administration during pregnancy is limited due to its association with multiple side effects. These are mainly due to its mechanism of action. The resulting prostaglandin inhibition has several effects on the fetus, depending on the type of agent, the dose and duration of therapy, the period of gestation, and the time elapsed between maternal NSAID administration and delivery. Fetal complications include alteration of renal perfusion with the consequent oligohydramnios and premature closure of the ductus arteriosus that may lead to the production of persistent pulmonary hypertension and tricuspid regurgitation. For all these reasons, the Food and Drug Administration (FDA) has advocated cautious use of this drug during pregnancy. NSAIDs should be given in pregnancy only if the maternal benefits outweigh the potential fetal risks, at the lowest effective dose and for the shortest duration possible. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac during pregnancy, the possible adverse effects and safety.

Chapter 6 - Nowadays non-steroidal anti-inflammatory drugs (NSAIDs) represent the most widely used drug in medical practice in the treatment of a wide variety of inflammatory and painful processes. Indeed, some of these

drugs can be bought without prescription. Their use during pregnancy has also increased, in spite of the recent findings. Indications of use of these agents are gynecological disorders like ectopic pregnancy, acute pelvic inflammatory disease, complications of ovarian cysts, endometriosis, dysmenorrhea and fibroids. Abdominal pain during pregnancy is also a relatively common symptom and may require treatment with analgesia. It may reflect anatomical and physiological changes of the pregnant state. Various obstetric conditions such as placental abruption, clinical chorioamnionitis, threatened preterm labour and uterine rupture present with acute abdominal pain. Pregnancy may also increase predisposition to certain clinical conditions like urinary tract infection, which in turn may present with abdominal pain. In conclusion, diclofenac is used in the treatment of a wide variety of gynecological and obstetric conditions. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac, possible adverse effects and its safety. For this purpose, the authors have conducted a literature research of clinical trials and other recent prospective studies on diclofenac in different areas.

Chapter 7 - The cyclooxygenase (COX) inhibitor diclofenac (DCF) is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) for managing pain and inflammation. The COX enzymes are key enzymes responsible for the conversion of arachidonic acid to prostaglandin E_2 (PGE_2) and other chemical mediators which enhance pain and inflammation but generally protect gastrointestinal (GI) tract. Reduction in the level of PGE_2 by DCF makes the GI tract vulnerable to ulceration. Interestingly, cytochrome P450 (P450) enzymes generate analgesic and anti-inflammatory epoxyeicosatrienoic acids (EETs) from cell membrane-derived arachidonic acids, ω-6 fatty acids. Similarly, P450s also generate anti-inflammatory epoxydocosapentaenoic acid (EDPs) from docosahexaenoic acid (DHA) and other anti-inflammatory epoxy fatty acids from related ω-3 lipids. Inducers of P450s including anti-ulcer medicine omeprazole increase the level of epoxy fatty acids and can reduce inflammation. The epoxy fatty acids are labile to metabolism by soluble epoxide hydrolase (sEH). Inhibition of the sEH alleviates pain and inflammation associated with GI ulcers. The authors have focused their

discussion on anti-ulcer effects of a soluble epoxide hydrolase (sEH) inhibitor (sEHI) N-[1-(1-oxopropyl)-4-piperidinyl]-N′-[4-(trifluoro-methoxy) phenyl]-urea (TPPU) in DCF-induced gastric and intestinal ulcers and on the anti-ulcer effects of the proton pump inhibitor omeprazole (OME). The sEHIs can also decrease pain, potentiate anti-inflammatory effects of NSAIDs and decrease the ulcerative potential of NSAIDs. Co-formulation of TPPU with low dose DCF can reduce the GI side effects. These side effects of NSAIDs act to counter the very benefits offered by these drugs. Possibly, by blocking these side effects of NSAIDs, the authors can make NSAIDs even more effective and be able to use them at lower doses. The authors also discussed about anti-ulcer effects of many other pharmacological agents. The Δ^9-tetrahydrocannabinol (THC), steroid (dexamethasone), and inhibitors of fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), JNK, β-glucuronidase, and cyclophilin D exhibit anti-ulcer effect against NSAIDs including DCF.

Chapter 8 - Diclofenac, like many other non-steroidal anti-inflammatory drugs (NSAIDs), is associated with a cardiotoxic effect. Although the reduction in the level of thromboxane/prostaglandin A2 has been considered as a risk factor, the participation of other factors is involved. Recent studies suggest that chronic treatment of diclofenac to mice at a dose of 10 mg/kg for 28 days induced proteasomal dysfunction with a parallel increase in the level of polyubiquitinated protein and oxidized proteins in the heart. Similarly, acute dosing of diclofenac to mice at the dose of 100 mg/kg for 18 h also caused proteasomal dysfunction in the heart. Incubating cardiac (H9c2) cells with diclofenac (5 μM) also resulted in proteasomal dysfunction. Diclofenac dose-dependently increased the generation of reactive oxygen species (ROS) and induced cell death in cardiac cells. Diclofenac also decreased mitochondrial complex III activity and mitochondrial membrane potential. Overall, current experimental data suggest that diclofenac causes significant increases in ROS as well as mitochondria and proteasome dysfunction, which are likely to be associated with the cardiotoxic effect of diclofenac.

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Chapter 1

ANTICANCER EFFECTS OF DICLOFENAC: A MULTITARGET APPROACH

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ABSTRACT

Non steroid anti-inflammatory agents (NSAIDs) represent the most commonly used medication worldwide. Currently, NSAIDs are the most promising agents in development for cancer chemoprevention. In addition there is evidence for their chemotherapeutic potential. NSAIDs, including diclofenac apart from inhibiting cyclo-oxygenase enzymes are known to target multiple pathways that are implicated in carcinogenesis. Thus these agents could play a role in the chemoprevention or adjuvant therapy of malignant tumors. Although, in general, NSAIDs have common mechanisms of action, the anticancer effects are not the same for all the chemical entities. In addition, the mechanisms of anticancer activity are not common for all the NSAIDs. Diclofenac is an old drug and there is

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growing interest on its potential role in the field of oncology. Diclofenac exerts its action by inhibiting prostaglandin synthesis via inhibition of cyclo-oxygenase I and cyclo-oxygenase-II. Furthermore, novel cyclo-oxygenase independent mechanisms of actions have been suggested for diclofenac including the inhibition of substrate P, inhibition of peroxisome proliferator activated receptor gamma (PPARgamma), blockage of acid-sensing ion channels, alteration of interleukin-6 production, and inhibition of N-methyl-D-aspartate (NMDA) receptor hyperalgesia. This chapter reviews the anticancer effects of diclofenac. The chapter is structured in four parts. In the first part, the pharmacology of diclofenac is presented. The second part is focused on human data that support the possible role of diclofenac as a chemo-preventive and chemotherapeutic agent. In the third part experimental *in vitro* and *in vivo* evidence on the anticancer effects of diclofenac is presented. The fourth part is devoted to the mechanisms of anticancer effect of diclofenac. Antitumor effect of diclofenac has been mainly attributed to the cyclo-oxygenase I and II inhibition. However, it has also been well evidenced that diclofenac inhibits tumour cell growth and proliferation by cyclo-oxygenase independent mechanisms. The molecular pathways that can be targeted by diclofenac have not been fully elucidated. However, it is evident that diclofenac is a multi-target agent with anticancer action, that is expressed via multiple mechanisms. This multitarget approach renders diclofenac a promising agent, that should be further investigated for the chemoprevention or adjuvant treatment in the field of oncology. In fact, there are experimental data from different cancer cell types, that support the notion of diclofenac repurposing in oncology. In conclusion, the anticancer effect of diclofenac can be interpreted via multiple mechanisms. This multitarget approach could render diclofenac a putative agent in oncology for chemoprevention as well as for adjuvant treatment. Importantly the anticancer action of diclofenac has been evidenced in aggressive tumours including glioblastoma and pancreatic adenocarcinoma. In the context of drug repurposing, future research efforts are needed for the development of diclofenac as an anticancer agent possibly in synergy with well established treatment modalities.

Keywords: diclofenac, chemoprevention, anticancer effect, adjuvant therapy, modulation of oxidative stress, multi-target approach

1. INTRODUCTION

Non steroid anti-inflammatory agents (NSAIDs) represent the most commonly used medication worldwide. Currently, NSAIDs are the most

promising agents in development for cancer chemoprevention. In addition there is evidence for their chemotherapeutic potential. NSAIDs, including diclofenac apart from inhibiting cyclo-oxygenase enzymes are known to target multiple pathways that are implicated in carcinogenesis (Richter et al. 2001). Thus these agents could play a role in the chemoprevention or adjuvant therapy of malignant tumors. Although, in general, NSAIDs have common mechanisms of action, the anticancer effects are not the same for all the chemical entities. In addition, the mechanisms of anticancer activity are not common for all the NSAIDs. Diclofenac is an old drug of the phenylacetic acid class, one of the oldest NSAIDs, being in use since 1976 (Davies and Anderson 1997). Diclofenac is indicated for the treatment of fever pain in rheumatoid arthritis and musculoskeletal conditions as well as for the treatment of postoperative pain. In addition, diclofenac is available as a gel formulation for the treatment of actinic keratosis, that is a precancerous lesion.

Although cancer treatment has been substantially improved in the last years, there is still need for novel treatments. As the development of novel drugs is a really demanding process that takes a long of time, recently there is interest in repurposing in oncology old drugs. There are a number of advantages in this approach including the known pharmacodynamics, pharmacokinetics and safety issues of old drugs. There is growing interest on the potential role of NSAIDs including diclofenac in the field of oncology (Chou et al. 2004, Grösch et al. 2004, Harris et al. 2005, Harris et al. 2006, Harris et al. 2007, Harris 2009, Forget et al. 2014, Forget et al. 2013, Yiannakopoulou 2015, Pandey et al. 2019).

2. PHARMACOLOGY OF DICLOFENAC

2.1. Mechanism of Action

Diclofenac exerts its action by inhibiting prostaglandin synthesis via inhibition of cyclo-oxygeanase I and cyclo-oxygenase-II. Studies in humans, have shown that diclofenac results in 49% reduction of the activity of cyclo-

oxygenase I and in 98% reduction of the activity of cyclo-oxygenase-II (van Hecken et al. 2000). In addition, diclofenac inhibits phospholipase A2 both *in vitro* and *in vivo* (Mäkelä et al. 1997). Phospholipase A2 generates arachidonic acid and lysophospholipids that generate pro-inflammatory eicosanoids and platelet activating factor. Furthermore novel cyclo-oxygenase independent mechanisms of actions have been suggested for diclofenac including the inhibition of substance P release, modulation of peroxisome proliferator activated receptor gamma (PPARgamma) activity, blockage of acid-sensing ion channels, alteration of interleukin-6 production, and inhibition of N-methyl-D-aspartate (NMDA) receptor hyperalgesia (Papworth et al. 1997, Adamson et al. 2002, Bishop-Bailey and Wray 2003, Nixon et al. 2003, Lee et al. 2004, Ayoub SS et al. 2009, Gan 2010, Vellani et al. 2013).

2.2. Pharmacokinetics

Diclofenac, when given orally is rapidly absorbed and almost completely distributed to plasma and tissues. Drug accumulation after repeated dosing within the normal therapeutic range is not common. Diclofenac binds extensively to plasma albumin. The area under the plasma concentration-time curve of diclofenac is proportional to the dose for oral doses between 25 to 150 mg (Davies and Anderson 1997). Peak plasma concentration following a single 50 mg enteric coated diclofenac sodium tablet is 5.0 μM, attained in around 2 hours. The potassium salt of diclofenac is absorbed more rapidly, and a 50 mg tablet reaches a peak plasma concentration of 3.8 μM in 20–60 minutes. Terminal half-life is 1.8 hours after oral dosing. Diclofenac is eliminated following biotransformation to glucoroconjugated and sulphate metabolites which are excreted in urine. The major primary metabolite of diclofenac is 4′-hydroxydiclofenac (4′-OH diclofenac), with 3′-OH- and 5′-OH-diclofenac being minor metabolites (Stierlin and Faigle 1979, Stierlin et al. 1979). Both diclofenac and its hydroxylated metabolites undergo glucuronidation and sulphation. A small amount of the drug is eliminated unchanged. The excretion of conjugates

may be related to renal function. Conjugate accumulation occurs in end-stage renal disease; however, no accumulation is apparent upon comparison of young and elderly individuals. Dosage adjustments for the elderly, children or for patients with various disease states (such as hepatic disease or rheumatoid arthritis) may not be required. Clinically important drug interactions have been demonstrated for a number of drugs including aspirin (acetylsalicylic acid), lithium, digoxin, methotrexate, cyclosporin, cholestyramine and colestipol.

2.3. Dosage

Diclofenac is the most frequently used NSAID in low, middle, and high income countries, and it is available over the counter in most countries. Diclofenac, is available as a sodium or potassium salt. Diclofenac can be used in tablet, gel/emulsion, injection and suppository forms. The dosing regimen of diclofenac varies by format and indication. Typical doses for rheumatic disease and musculoskeletal disorders vary between 75 and 150 mg, divided to two or three doses, orally or rectally. Diclofenac is also indicated for the treatment of post-operative pain administered with diclofenac injections, either intramuscularly or intravenously, at a dose of 75–150 mg, with a maximum of 150 mg in 24 hours. Diclofenac is also licensed in gel formulation for the treatment of actinic keratosis. The gel formulation contains diclofenac sodium 3% in a sodium hyaluronate base and is applied twice daily for 60–90 days in patients presenting with actinic keratosis.

2.4. Toxicity

Most of the adverse events of diclofenac are attributed to the inhibition of both isoforms of cyclooxygenase I and II. Diclofenac is a non-selective inhibitor of both isoforms of the cyclooxygenase enzyme (COX-1 and COX-2), However, diclofenac reduces cyclo-oxygenase II activity by 98%. The

gastrointestinal toxicity of diclofenac is well established. Diclofenac has also been reported to be associated with non gastro-intestinal haemorrhage (Salemis 2009, Michalevicz and Seligsohn 1982, Yiannakopoulou 2011).

Common side effects include abdominal pain, constipation, diarrhoea, dyspepsia, flatulence, heartburn, nausea and heatdache. Less common side effects include rash, itching, bloating, GI ulceration, oedema and dizziness. Rare but serious adverse events include GI bleeding, anaemia, liver failure, pancreatitis and pneumonia. As with all non steroidal anti-inflammatory agents, long-term use of diclofenac has been associated with an increase in the risk of cardiovascular events, particularly myocardial infarction and stroke. Diclofenac is contra-indicated in patients with previous history of hypersensitivity to aspirin or any other NSAID, suffering from congestive heart failure, ulcerative colitis or other inflammatory bowel condition, active GI ulcer or bleeding. It is also recommended that diclofenac should be avoided in the final trimester of pregnancy and caution be exercised during lactation.

3. Anticancer Effect of Diclofenac

3.1. Epidemiological Studies

A number of epidemiological studies have shown protective role of aspirin and the other NSIADs against breast cancer (Kwan et al. 2007, Harris et al. 2003).

A number of epidemiological studies have shown protective role of diclofenac against breast cancer. In a relevant study, Hung et al. investigated the effect of NSAIDs including diclofenac in breast cancer risk. Women suffering from autoimmune disease and having been treated with NSAIDs including diclofenac for at least 3 months were followed from the first day of diagnosis of autoimmune disease until 2013. A total of 12 331 NSAID users and 12 331 non-NSAID users were included in this study after 1:1 individual matching. The authors concluded that the incidence of new-onset breast cancer in NSAID users was significantly decreased in users taking

selective cyclooxygenase 2 inhibitors including diclofenac, ibuprofen and piroxicam (Hung et al. 2018). However, there are also negative epidemiological studies in breast cancer patients. Thus, a prospective cohort study concluded that postdiagnostic prescriptions for aspirin or other NSAIDs including diclofenac had little or no association with breast cancer recurrence. However, prediagnostic use of the drugs could reduce the incidence of breast cancer recurrence (Cronin Fenton et al. 2016)

In addition, epidemiologic data have shown that chronic intake of traditional nonsteroidal anti-inflammatory drugs could reduce the incidence of colorectal cancer.

The protective effect of diclofenac has also been investigated in breast carcinoma. In a retrospective analysis of a single centre cohort including 720 breast cancer patients has concluded that intraoperative use of diclofenac has been associated with improved disease free survival and overall survival in conservative breast cancer surgery (Forget et al. 2014).

4. EXPERIMENTAL DATA

The anticancer effect of diclofenac has been investigated in a number of tumors including colorectal carcinoma, esophageal cancer, pancreatic adenocarcinoma, prostatic adenocarcinoma, glioblastoma. Diclofenac has been shown to have chemopreventive effect in experimental colon cancer in rats (Ghanghas et al. 2016). In addition, diclofenac has been shown to inhibit the growth of murin C-26 colon carcinoma cells (Fallkowski et al. 2003). Furthermore, diclofenac has been shown to have antiproliferative effect against human colon cancer cells (Hixson et al. 1994). Furthermore, diclofenac has been shown to attenuate Wnt/ β catienin signalling pathway in colon cancer cells by activation of NF-kappaB (Cho et al. 2005).

In addition, topical application of diclofenac in 2.5% hyaluronan has been shown to inhibit basal cell carcinoma, actinic keratosis, and murine colon-26 adenocarcinoma cell growth *in vivo* (Harper 1994, Rivers et al. 1995, Seed et al. 1997, Seed et al. 1997). Diclofenac has been reported to decrease mRNA-VEGF levels via COX inhibition in cultured esophageal

cancer cell lines (von Rahden et al. 2005). In addition diclofenac has been shown to inhibit he growth of HeLa cell, mammary cell carcinoma, rhabdomyosarcoma and fibroblast cell lines in the culture media (Al Nimer et al. 2015).

Glioblastoma is a particularly aggressive tumor, characterized by highly infiltrative cells, high mitotic activity and angiogenesis. Current treatments are practically ineffective. Although combined treatment approaches are applied, the median survival time following diagnosis is limited to 14.6 months with standard therapy. Thus, novel treatment modalities are urgently needed. Diclofenac has been reported to reduce proliferation, colony formation and migration of human glioblastoma cells by downregulating Wnt β-catenin/Tcf signalling pathway (Sareddy et al. 2013). In addition, diclofenac has been reported to inhibit lactate formation and to counteract local immune response in murine glioma cells (Chirasani et al. 2013). Combined treatment with metformin and diclofenac has been reported to have synergistic anti proliferative and anti-migratory effect in glioblastoma cancer cell lines (Gerthofer et al. 2018). Furthermore, diclofenac has been reported to inhibit migration and proliferation of human glioma cells by inhibition of STAT-3 signaling and downstream modulation of glycolysis (Leidgens et al. 2015).

Pancreatic adenocarcinoma is the more lethal common cancer usually diagnosed at an advanced stage with locally advanced unresectable disease or metastasis at the time of diagnosis. It is the fourth most common cause of cancer related death in Europe and United States. The pancreatic adenocarcinoma typically has a poor prognosis with a 5-year survival rate quite low of about 1–4%. In fact, the incidence of pancreatic ductal adenocarcinoma equals with mortality. Current therapies are largely ineffective and are associated with high levels of toxicity. Undoubtedly there is need for new therapies for the treatment of pancreatic adenocarcinoma. Repurposing aspirin or diclofenac for chemoprevention and/or adjuvant treatment of pancreatic adenocarcinoma would be really interesting. Diclofenac has been reported to reduce proliferation of pancreatic acinal cells upon inflammatory injury and mitogenic stimulation (Bombardo et al. 2018). Mayorek et al. have reported that diclofenac inhibited tumor growth

in a murine model of pancreatic cancer by modulation of the levels of VEGF (Mayorek et al. 2010).

The anticancer effect of diclofenac has also been evidenced in neuroblastoma. Indeed, diclofenac inhibited neuroblastoma growth *in vivo* in established neuroblastoma xenografts in nude rats. *In vitro*, diclofenac presents synergistic action with arachidonic acid in the induction of neuroblastoma cell death (Johnsen et al. 2004, Johnsen et al. 2005).

Diclofenac has also been reported to act synergistically with other treatment modalities. Thus, it has been reported that topical diclofenac enhanced radiation sensitivity via enhancement of TRAIL in human prostate adenocarcinoma xenograft model (Inoue et al. 2013). In addition, combination chemoprevention with diclofenac, calcipotriol and difluoromethylornithine has been reported against non melanoma skin cancer in mice (Pommegaard et al. 2013). Synergistic interaction of metformin, diflunisal and diclofenac has been reported in the inhibition of proliferation and the induction of apoptosis in acute myeloid leucemia cell lines (Renner et al. 2018).

5. Mechanisms of Anticancer Action of Diclofenac

Antitumor effect of diclofenac has been mainly attributed to the cyclo-oxygenase I and II inhibition. However, it has also been well evidenced that diclofenac inhibits tumor cell growth and proliferation by cyclo-oxygenase independent mechanisms. The molecular pathways that can be targeted by diclofenac have not been fully elucidated. However, it is evident that diclofenac is a multi-target agent with anticancer action, that is expressed via multiple mechanisms. This multitarget approach renders diclofenac a promising agent, that should be further investigated for the chemoprevention or adjuvant treatment in the field of oncology. In fact, there are experimental data from different cancer cell types, that support the notion of diclofenac repurposing in oncology.

Diclofenac can inhibit glycolysis of tumor cells (Gerthofer et al. 2018, Chirasani et al. 2013). Relevant research data suggest that diclofenac is a possible inhibitor of the outward export of lactate (Gottfried et al. 2013). Diclofenac is assumed to reduce extracellular lactate levels not only by the direct inhibition of outward transport, but also by influencing gene expression. In addition diclofenac induces apoptosis by modulating both intrinsic and extrinsic pathways (Fecker et al. 2010). Activation of mitochondrial apoptosis pathways in squamous cell carcinoma cells by diclofenac has been related to upregulation of Bad as well as downregulation of Mcl-1 and Bcl-w (Robust et al. 2012). Diclofenac has been demonstrated to activate caspase cascade in squamous cell carcinoma cell lines (Fecker et al. 2007). Furthermore, diclofenac has been reported to modulate transcription factors in tumor cells including MYC, a transcription factor that plays a key role in the regulation of cell growth, proliferation and apoptosis (Gottfried et al. 2013). Modulation of oxidative stress response by diclofenac could be another putative mechanism of its antitumor action (Yiannakopoulou 2005, Yiannakopoulou et al. 2005, Zoubair et al. 2016). Diclofenac, as an acetic acid derivative leads to the generation of free oxygen radicals (Galati et al. 2002, Osičková et al. 2014). Low levels of free oxygen radicals modulate apoptosis. Thus, diclofenac could enhance apoptosis through the modulation of oxidative stress. Indeed, in a relevant paper, Cecere et al. have reported that diclofenac induced apoptosis in the neuroblastoma cell line SH-SY5Y through the reduction of protein and enzymatic activity of superoxide dismutase (Cecere et al. 2010).

In conclusion, the anticancer effect of diclofenac can be interpreted via multiple mechanisms. This multitarget approach could render diclofenac a putative agent in oncology for chemoprevention as well as for adjuvant treatment. Importantly the anticancer action of diclofenac has been evidenced in aggressive tumours including glioblastoma and pancreatic adenocarcinoma. In the context of drug repurposing, future research efforts are needed for the development of diclofenac as an anticancer agent possibly in synergy with well established treatment modalities.

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Chapter 2

TARGETING STRESS RESPONSE WITH DICLOFENAC

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ABSTRACT

Every condition that threatens the state of homeostasis is considered a stress, and causes leading to it are termed stress factors. Stress response is the term used for the reactions that follow the recognition of the stress factor aiming to protect the organism from the stress factor and avoid danger. Stress signal detection leads to activation of signal pathways, with subsequent induction of gene transcription, protein translation and alteration of cell energy state. Thus, mild stress response protects the organism from stressors in the environment. However, if stress response is exaggerated, it results to occurrence of disease. Stress response is implicated in the pathophysiology of a number of diseases, including atherosclerosis, cardiovascular disease, heart failure, carcinogenesis. Thus, there is growing research interest in the design of drugs that target stress response. In addition, there are old drugs that are currently investigated for

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their possible effect on stress response. Non steroidal anti-inflammatory agents comprise a chemically heterogenous group of medications including aspirin and salicylates as well as selective and non selective inhibitors of cyclo-oxygenase. The main actions of these agents include anti-inflammatory, analgesic and antipyretic actions, while aspirin and salicylates are also known for their antiplatelet action. Aspirin and salicylates are known to modulate stress response in pro-caryotic organisms as well as in eukaryotic cells. Diclofenac is a well known non steroidal anti-inflammatory agent with analgesic and anti-inflammatory properties, that are due to the inhibition of the action of cyclo-oxygenase. Recently, there is evidence supporting that cyclo-oxygenase independent mechanisms are implicated in the pharmacology of diclofenac, including the inhibition of the glycolysis of tumor cells, the modulation of apoptosis, the modulation of stress repsonse. In that aspect, there is experimental evidence indicating that diclofenac is able to modulate stress response, including heat shock, oxidative stress response. Diclofenac has been evidenced to modulate heat shock response in the eukaryotic organism S.cerevisiae. In addition low concentrations of diclofenac act as a mild oxidative stressor inducing mild oxidative stress response that promotes cellular protection from a subsequent lethal stressor. This chapter presents experimental evidence suggesting modulation of stress response by diclofenac. The mechanisms underlying the effect of diclofenac on stress response are also presented. Clinical implications of targeting stress response by diclofenac including cancer chemoprevention are also discussed. Although the adverse effects of diclofenac could limit its long term use as a chemo=preventive agent, the combination of diclofenac with other chemo=preventive agents could provide synergistic favourable effects with lower frequency of adverse events.

Keywords: stress response, aspirin, salicylates, diclofenac, heat shock, oxidative stress response, pharmacological modulation

1. INTRODUCTION

Every condition that threatens the state of homeostasis is considered a stress, and causes leading to it are termed stress factors. Stress response is the term used for the reactions that follow stress and aim to protect the organism and avoid danger. Stress signal detection leads to activation of signal pathways, with subsequent induction of gene transcription, protein translation and alteration of cell energy state (Toone and Jones 1988,

Moskiwa et al. 1999, Kultz et al. 2003, Estruch 2000, Ruis and Schuller 1995). Stressors such as heat, ischemia and radiation can induce different cellular responses according to their intensity and duration (Jaattela 1999). Stress response may be mild or severe. Mild stress response is defined as the response of the organism to stressors that are not lethal (Verbeke et al. 2002, Verbeke et al. 2001, Verbeke et al. 2000). Mild stress response is beneficial for the organism, favouring accommodation to severe stress response. In case of enhanced intensity or duration of the stressor, cellular apoptosis is induced and if the stressor is more potent, it leads to cellular necrosis (Jaattela 1999). Several stress responses have been well characterized including heat shock response, oxidative stress response, endoplasmin reticulum stress response, surgical stress response.

Thus, mild stress response protects the organism from stressors in the environment. However, if stress response is exaggerated, it results to occurrence of disease. Stress response has been implicated in the pathophysiology of number of diseases, including atherosclerosis, arterial hypertension, heart failure, diabetes, carcinogenesis, multiple sclerosis, Alzheimer disease, Parkinson disease, pancreatitis, ischemia reperfusion injury, non alcoholic fatty liver disease, pre-eclampsia (Arrigo 1999, Kojoda and Harrison 1999, Touyz 2005, Reiter et al. 2004, Butterfield and Boyd-Kimball 2005, Gawrieh et al. 2005). Modulation of stress response could be a therapeutic approach for a number of diseases. Thus, there is growing research interest in the design of novel drugs or evaluation of old drugs that target stress response.

2. NON STEROIDAL ANTI-INFLAMMATORY AGENTS

Non steroidal anti-inflammatory agents comprise a chemically heterogenous group of medications including aspirin and salicylates as well as selective and non selective inhibitors of cyclo-oxygenase. The main actions of these agents are anti-inflammatory, analgesis and antipyretic while aspirin and salicylates are also known for their antiplatelet action (Tegeder et al. 2001, Verbung et al. 2001, Amann and Peskar 2002, Galati

et al. 2002). Non steroidal anti-inflammatory agents are among the most commonly prescribed medications worldwide (Brooks and Day 1991, Szewczyk and Woijtezak 2002). NSAIDs are quite effective for the relief of inflammation and pain due to arthritis (Steimeier 2000). In addition, NSAIDs are indicated for the treatment of dysmenorrhoea, malignant pain, neoplasmatic fever, hemicrania (Camu et al. 2002, Reynolds et al. 2002). Nowadays, it has been recognized that aspirin, salicylates and other non steroidal inflammatory agents could act as chemopreventive agents against colorectal carcinoma (Gungar et al. 2018, Ghangas et al. 2016, Ghangas et al. 2016, Pantziarka et al. 2016, Pommergaard et al. 2013, Lebman et al. 2013, Kaur et al. 2011, Kaur et al. 2010, Vaish et al. 2010). Celecoxib, a COX-II inhibitor has been approved by FDA for chemoprevention of colorectal carcinoma in patients appearing with familial polyposis, a genetic syndrome due to a mutation of the onco- suppressor gene APC (adenomatous polyposis coli). Diclofenac has also been evidenced to have chemopreventive action against colorectal cancer (Kaur et al. 2011, Kaur et al. 2010, Kaur et al. 2010, Sanyal and Kaur 2010, Kaur and Sanyal 2010).

Although the inhibitory effect of aspirin and of the other non steroidal anti-inflammatory agents on cyclo-oxygenase has been well recognized, there are a number of cyclo-oxygenase independent mechanisms of action, that could be implicated in the chemopreventive and anticancer properties of aspirin and NSAIDs. These mechanisms include the inhibition of transcription factors, the regulation of epigenetic targets, the modulation of lymphangiogenesis, as well as the modulation of stress response. (Yiannakopoulou E 2005, Yiannakopoulou E 2007, Yiannakopoulou et al. 2005, Yiannakopoulou 2014, Yiannakopoulou and Tyligada 2009, Yiannakopoulou 2012). Aspirin and the other salicylates, especially in high doses are well recognized inhibitors of I(kappa)B kinase-beta, an enzyme complex, that is involved in the propagation of the cellular response to inflammation and also part of the upstream NF-κB signal transduction cascade (Yin et al. 1999). Cyclo-oxygenase independent mechanisms of action have also been reported for diclofenac (Yiannakopoulou E 2011, Brueggemann et al. 2009, Adamson et al. 2002).

3. Aspirin and Salicylates and Modulation of Stress Response

Aspirin and salicylates are known to modulate stress response in procaryotic organisms as well as in eukaryotic cells (Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2009). Experimental studies suggest that salicylates, aspirin and the other non steroidal anti-inflammatory agents have both pro-oxidant and anti-oxidant actions. The antioxidant actions of salicylates have been linked with the beneficial effects of these agents, while, their pro-oxidant actions have been associated with the adverse reactions of these drugs. However, this approach is superficial and does not take into account the beneficial effect of low level pro-oxidant action. Low levels of pro-oxidants modulate cell signalling pathways. Salicylates are known to modulate cell signalling pathways, possibly through their pro-oxidant action.

The mechanisms of antioxidant action of salicylates are not well delineated. Sodium salicylate acts as a chemical trap against hydrogen peroxide radicals, the most detrimental reactive oxygen species, thus reducing ischemia/reperfusion injury (28, 29). In addition, non steroidal anti-inflammatory agents including indomethacin and sulindac have been reported to scavenge free oxygen radicals exerting thus a protective effect against cellular oxidative stress. Although salicylates trap free oxygen radicals, this action is weak and does not seem to interpret the anti-oxidant action of salicylates. Evidence suggests that aspirin and salicylates enhance the activity of cellular protective antioxidant mechanisms. In particular, aspirin elicits nitric oxide release by a direct activation of the endothelial NO synthase. In addition, aspirin and salicylates downregulate superoxide production and enhance GSH dependent antioxidant mechanisms. Furthermore, salicylic acid functions as a signalling molecule in plants involved in the expression of a number of genes. Other non steroidal inflammatory agents including diclofenac have been evidenced to modulate stress response. Sulindac has been shown to protect against oxidative stress by initiating a preconditioning response. Since modulation of stress response

by aspirin and the other NSAIDs are regulated by cyclo-oxygenase independent mechanisms, it is not straightforward that aspirin and the different NSAIDs modulate stress response through the same mechanisms.

4. DICLOFENAC AND MODULATION OF STRESS RESPONSE

Non steroidal anti-inflammatory agents including diclofenac have also been reported to modulate stress response (Yiannakopoulou 2005, Yiannakopoulou et al. 2005).

4.1. Diclofenac and Heat Shock Response

All the organisms have an optimal temperature for growth. High environmental temperature is considered as a physical stressor by all the organisms. When environmental temperature rises above the highest normal level for each organism, cellular proliferation ceases and a number of toxic effects appear. Heat shock response has been extensively studied in the unicellular eukaryotic organism S.cerevisiae. S. cerevisiae is an ideal experimental model for the investigation of the modulation of stress response by medications (Hoon et al. 2008, Yasokawa and Iwahashi 2010). The advantages of yeast over mammalian cellular systems are its straightforward genetic accessibility, cost-effectiveness, and rapid growth. In addition many of the mechanisms underlying toxicity and resistance to chemicals and other environmental stresses are conserved in S.cerevisiae (Mager and Winderickx 2005).

In the unicellular eukaryotic organism S.cerevisiae normal temperature ranges between 23°C and 35°C (Davidson and Schiestl 2001). Both mild thermal stress and heat shock can be studied in S.cerevisiae. When temperature rises to 37°C-43°C for 30 min-2h, mild heat stress ensues (Costa et al. 1993, Boy-Marcotte et al. 1999, Tyligada et al. 1999, Sugiyama et al. 2000, Domitrovic et al. 2003). Conditions of heat shock (severe thermal stress) are created when temperature rises to 48°C-54°C for 5min to half an

hour (Tyligada et al. 1999, Sugiyama et al. 2000, Davidson and Schiestl 2001, Domitrovic et al. 2003). Under experimental conditions of mild heat stress, the cells of S.cerevisiae continue to grow and obtain resistance to subsequent lethal heat stress.

Sodium diclofenac has been evidenced to modulate heat shock response in S.cereviesiae. under the following experimental conditions.: mild thermal stress was induced by exposure of S. cerevisiae to 37°C for 2 hours (Tyligada et al. 1999); acute heat shock was induced by exposure of S.cerevisiae to 53°C for 30 min. Control cell cultures were exposed to 27°C for 22 hours. Cell cultures were submitted to heat shock either without preconditioning or after prior preconditioning to mild heats stress for 2 hours. Sodium diclofenac was dissolved in water and was administered in concentrations 0.0005-0.3 mM. The minimum inhibitory concentration for diclofenac was 0.3 mM and the minimum fungicidal concentration was 1,2 mM (Yiannakopoulou 2005). Treatment with diclofenac was performed either during mild thermal stress or during heat shock. Diclofenac administered in concentrations up to 0.02 mM during heat shock did not affect cell viability in comparison with control cells that had not been treated with drug. On the contrary the treatment of control cell cultures with 0.04 mM diclofenac during heat shock, resulted in statistically significant increase of cell viability in comparison with the viability of control cells during heat shock. Treatment with 0.15 and 0.3 mM diclofenac resulted in statistically significant reduction of viability of cell cultures submitted to heat shock. When cell cultures submitted to preconditioning to mild thermal stress were treated with 0.005- 0.02 mM diclofenac during heat shock, no statistically significant effect on cell viability was observed, while treatment of the above cell cultures with 0.04 – 1.5 mM diclofenac during heat shock resulted in statistically significant increase of cell viability. High dose of diclofenac i.e., 0.3 mM diclofenac reversed the phenomenon of acquired thermo-resistance resulting in reduced cell viability.

Treatment of control cell cultures with 0.005-0.2 mM diclofenac during mild thermal stress had no effect on cell viability, while treatment of cell cultures with 0.04 mM diclofenac under the same experimental conditions resulted in statistically significant increase of cell viability in comparison

with the viability of cell cultures submitted to mild heat shock without treatment with any agent. Higher concentrations of diclofenac 0.008-0.3 mM reversed thermo-resistance acquired by mild heat stress resulting in statistically significant reduction of cell viability in comparison with the viability of the cells submitted to mild thermal stress without treatment with any medicine. The above experimental data led to the conclusion that the effect of diclofenac when administered in concentrations lower than the minimum inhibitory concentration and lower than the minimum fungicidal concentration either 2h before or during heat shock was dependent on the concentration of diclofenac and on the exposure of cell cultures to mild thermal stress. When administered during mild thermal stress, diclofenac reversed thermo-resistance in lower concentrations than the concentrations that reversed thermo-resistance of the cells that were preconditioned to mild thermal stress suggesting effect of diclofenac on the phenomenon of acquired thermo-resistance. On the contrary, when administered to cells submitted to heat shock without prior preconditioning to mild thermal stress, diclofenac induced thermoresistance only in the concentration of 0.04 mM either when administered 2h prior to heat shock or during heat shock suggesting protective action of diclofenac against heat shock without preconditiong effect of diclofenac against heat shock.

In the literature there are limited data on the effect of diclofenac on heat shock. However, diclofenac is a derivative a acetic acid and there is evidence suggesting that acetic acid acts as a stress factor for S.cerevisiae (Piper et al. 2001, Samanfar et al. 2017). It is well known that incubation of cell cultures of S.cerevisiae under low concentrations of weak organic acid induces stress response characterized by the induction of two membrane proteins Hsp30 and Pdr 12 (Piper et al. 1998). The effect of diclofenac on heat shock modulation could also be interpreted by the effect of diclofenac on cellular redox homeostasis.

4.2. Diclofenac and Oxidative Stress Response

Cellular oxidative stress, results from an imbalance between pro-oxidant and anti-oxidant mechanisms due to either increased production of free oxygen radicals and/ or deficiency of antioxidant mechanisms. Under physiological conditions, in a normal cell, there is continuous production of free oxygen radicals. It is well established that free radicals are products of normal cellular metabolism and have a dual role that could be either beneficial or harmful for the cell (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2007). Low levels of free oxygen radicals modulate cell signalling, initiate pathways of cellular stress response, mediate cellular differentiation, gene transcription, cellular proliferation and apoptosis. On the other hand, high levels of free oxygen radicals are toxic for the cells due to oxidative damage to cellular constituents as DNA, lipids, proteins, sugars. The cells have developed antioxidant mechanisms that ensure the redox homeostasis, the balance between pro-oxidant and antioxidant substances. The antioxidant mechanisms include the antioxidant enzymes and the non enzymatic antioxidants (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2007). Oxidative stress in implicated in the pathophysiology of a number of diseases. Thus, there are ongoing research efforts on the modulation of oxidative stress response (Liu et al. 2018, Yiannakopoulou et al. 2012, Yiannakopoulou and Tyligada 2009, Yiannakopoulou and Tyligada 2006). Pharmacological modulation of oxidative stress response is quite crucial (Yiannakopoulou 2012, Yiannakopoulou and Tiligada 2006).

Aspirin, salicylates have been evidenced to modulate oxidative stress response (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2009, Yian et al. 2016, Gondor et al. 2016, Enayat and Banerjee 2009).

The most direct evidence on the protective effect of salicylates against oxidative stress is based on experimental data from S.cerevisiae (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2009). The experimental conditions were the following: Post

logarithmic cell cultures of Scerevisiae were exposed to hydrogen peroxide for 1 h. The authors investigated the effect of long term pre-treatment, the effect of short term pre-treatment with salicylates as well as the effect of exposure to salicylates or diclofenac during the oxidative stress. For chronic pre-treatment, cells were exposed to salicylates for 22 hours prior to oxidative stress. For the short term treatment cells were exposed to salicylates one hour prior to the oxidative stress. Experiments under the above experimental condition have shown that treatment with low dose aspirin confers long term resistance against hydrogen peroxide induced oxidative stress in yeast (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2009). Modulation of stress response by salicylates is due to the effect of salicylates on cell signalling pathways as well as to the pro-oxidant- and anti-oxidant effects of salicylates (Yiannakopoulou 2005, Yiannakopoulou 2007, Yiannakopoulou and Tyligada 2009, Oliveira et al. 2018, Wróbel et al. 2017, Rivas-Estilla et al. 2012).

The evidence on the protective effect of diclofenac against oxidative stress is limited. However, it is well confirmed that high concentrations of diclofenac act as a stress factor and induce cellular stress response. The toxicity of diclofenac is largely attributed to the generation of free radicals by diclofenac (Osičková et al. 2014, Galati et al. 2002). Thus, it could be expected that exposure to low concentrations of diclofenac could confer protection against lethal oxidative stress through the induction of antioxidant mechanisms. Indeed, in a relevant study, Zoubair et al. reported that diclofenac protected mice from hydroxide peroxide induced oxidative stress through the induction of antioxidant mechanisms. Oxidative stress was induced through the intraperitoneal injection of hydrogen peroxide. Diclofenac treatment increased the levels of antioxidant enzyme and restored redox homeostasis in mice (Zoubair et al. 2016).

CONCLUSION

Experimental evidence suggests that pharmacological modulation of oxidative stress response is feasible and might alter the natural history of relevant pathological states The non steroidal anti-inflammatory agent diclofenac has been evidenced to modulate stress response. Although relevant data are derived from experimental studies, modulation of stress response by diclofenac is also possible in humans, taken into account the redox properties of diclofenac. Diclofenac leads to the generation of free radicals. Low concentrations of free radicals behave as a mild stressor that enables preconditioning to a subsequent lethal stressor. Stress response is implicated in a number of diseases included carcinogenesis. Modulation of stress response by diclofenac could have clinical implications in chemoprevention. Although the adverse effects of diclofenac could limit its long term use as a chemo-preventive agent, the combination of diclofenac with other chemo=preventive agents could provide synergistic favourable effects with lower frequency of adverse events.

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Chapter 3

DICLOFENAC AND SEVERE ADVERSE REACTIONS

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ABSTRACT

Diclofenac is a non steroidal anti-inflammatory agent of the phenylacetic class. Diclofenac is implicated in a number of adverse events affecting different systems. Although the gastrointestinal and cardiovascular adverse events associated with diclofenac are well documented, diclofenac is also associated with serious adverse events, including severe allergic reactions, eosinophilic pneumonia, aseptic meningitis, immune hemolytic anaemia, immune thrombocytopenia, immune neutropenia, hepatic injury, renal injury. Severe allergic reactions to diclofenac include anaphylactic reaction, anaphylactic shock, Kounis syndrome. Even fatal allergic reactions to diclofenac have been reported. Although severe allergic reactions are more common after intramuscular and intravenous administration, there are also reports of severe allergic reactions after oral, subcutaneous and rectal administration. Although

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serious adverse events of diclofenac are rare, diclofenac is one of the most commonly used non steroidal anti-inflammatory agents worldwide. Importantly, diclofenac is included in the over-the counter medicines in most countries. Furthermore, diclofenac is often used for the treatment of postoperative pain in an opioid sparing approach. Diagnosis of severe allergic reactions as well as other rare reactions is quite difficult in the postoperative patient. Thus, clinicians should be aware of the serious adverse events associated with the use of diclofenac in order to be able to recognize them promptly and treat them properly. Health organizations should reconsider the policy of the over the counter use of the non steroidal anti-inflammatory agents. Patients should be well informed about the severe adverse reactions associated with the use of diclofenac.

Keywords: diclofenac, severe allergic reactions, anaphylaxis, Kounis syndrome, anaphylactic shock, immune hemolytic anaemia, immune thrombocytopenia, immune neutropenia, hepatic injury

1. INTRODUCTION

Diclofenac is a non steroidal anti-inflammatory agent of the phenylacetic class. Diclofenac is implicated in a number of adverse events affecting different systems. Although the gastrointestinal and cardiovascular adverse events associated with diclofenac are well documented, diclofenac is also associated with serious adverse events, including severe allergic reactions, eosinophilic pneumonia, aseptic meningitis, immune hemolytic anaemia, immune thrombocytopenia, immune neutropenia, hepatic injury, renal injury. As diclofenac is one of the most commonly used non steroidal anti-inflammatory agents worldwide, clinicians should be aware of these adverse events in order to be able to recognize them promptly and treat them properly.

2. SEVERE ALLERGIC REACTIONS

Non steroidal anti-inflammatory agents are the second most commonly implicated medications causing anaphylaxis. This could be partially

attributed to the mechanism of action of NSAIDs. NSAIDs modulate arachidonic acid metabolism. Arachidonic acid metabolism takes place via the cyclo-oxygenase and lipo-oxygenase pathway. Both pathways result in the production of mediators of immunological and inflammatory reactions. Any blockade of cyclo-oxygenase pathway, shunts the metabolism to the direction of lipo-oxygenase pathway resulting in increased production of leucotrienes (Robinson et al. 1986). Diclofenac, a non steroidal anti-inflammatory agent has been implicated in the occurrence of severe allergic reactions including anaphylaxis, anaphylactic shock, Kounis syndrome. Dicolofenac sodium is widely used as opioid sparing agent for the treatment of post-operative analgesia. Roots of diclofenac administration are oral, intramuscular, intravenous, subcutaneous, rectal. Anaphylaxis has been reported after diclofenac administration by all these routes (Sen et al. 2001). Although severe allergic reactions are rare, diclofenac is the most often used non aspirin non steroidal aniti-inflammatory agent worldwide. Importantly, diclofenac is included in the over-the counter medicines in most countries. Therefore, clinicians should be aware of the severe allergic reactions associated with the use of diclofenac and patients should be informed accordingly.

2.1. Anaphylaxis

Anaphylaxis is a serious and occasionally fatal adverse event. The American College of Allergy, Asthma and Immunology describes anaphylaxis as an acute systemic reaction caused by immunoglobulin E (IgE)-mediated immunological release of mediators from mast cells and basophils to allergenic triggers, such as food, insect venoms, latex, and medication (Lieberman et al. 2005). Anaphylaxis includes anaphylaxis and anaphylactoid reaction. The difference between the two entities lies in the aetiology. Anaplylaxis is an IgE mediated reaction while anaphylactoid reaction is not associated with immunoglobulin E. However, the onset, clinical picture and the severity of both entities is similar. Clinical features of anaphylaxis include increased vascular permeability, vasodilation,

hypotension, tachycardia, bronchospasm, interstitial pneumonitis, abdominal pain, urticaria, angioedema, and even shock (Foucher et al. 1997). Anaphylactic reaction to diclofenac has been well described.(Schäbitz et al. 2001, Grass et al. 2004, Singh et al. 2011, Jha et al. 2015). It should be emphasized that anaphylactic reaction to diclofenac can appear in patients who have never been exposed to diclofenac before.

Anaphylactic reaction to intravenous diclofenac mimicking pulmonary embolism has been described (Singh et al. 2001). In a relevant case report, a 25 year old primigravida female reported tightness in the chest, palpitations and shortness of breath 20-25 min after the starting the infusion of intravenous diclofenac. The differential diagnosis included pulmonary embolism or anaphylactic reaction to intravenous diclofenac. Even fatal anaphylactic reaction to diclofenac has been reported after intramuscular injection of diclofenac in a 45 year old man for the treatment of low back pain. The man collapsed 15 min after injection and he went into coma twenty minutes after successful resuscitation. He never recovered from coma (Alkhawajah et al. 1993).

Diclofenac is contraindicated in patients with known hypersensitivity to diclofenac. Diclofenac should not be given to patients, who have experienced asthma, urticaria, or other allergic-type reactions after taking aspirin or other NSAIDs. Furthermore diclofenac induced asthma has also been reported (Sharir 1997, Gupta et al. 2000).

Anaphylactic shock by diclofenac has been well described (Hadar et al. 2000, Colak et al. 2014, Colak et al. 2015). Anaphylactic shock secondary to injection of diclofenac sodium can be treated successfully with intramuscular injection of adrenaline (Colak et al. 2014).

2.2. Kounis Syndrome

The concurrence of chest pain and allergic reactions accompanied by clinical and laboratory findings of classic angina pectoris caused by mast cell activation induced by allergic and anaphylactic reactions is sometimes referred to as Kounis syndrome (Kounis and Zavras 1991, Kounis and

Zavras 1996, Kounis 2016). The pathophysiology of Kounis syndrome is based in the coronary artery vasospasm due to o release of vasoactive mediators secondary to mast cell degranulation. ST-segment elevation myocardial infarction is a rare complication of anaphylactic reactions, but can occur even in patients with angiographically normal coronary arteries due to contraction of the coronary arteries. In addition, Kounis syndrome can result from the destabilization of atherosclerotic plaque. Medications including non steroidal anti-inflammatory drugs, antibiotics, antineoplastic agents, radiological contrast agents, bee stings, shellfish and coronary stents have been implicated in Kounis syndrome (Ilhan et al. 2009, AV-Tejedor et al. 2011, Tummala Gunes et al. 2017). Kounis syndrome can be also triggered by diclofenac (Tiwari et al. 2013. Tummala et al. 2013). The diagnosis of Kounis syndrome is facilitated by the clinical signs and symptoms of the anaphylactic reaction. Although rarely, Kounis syndrome can also be presented as asystole and or cardiogenic shock. However, it should be emphasized that Kounis syndrome has been reported without any dermatological signs suggesting allergic reaction. Thus, a high index of suspicion is needed. The treatment of Kounis syndrome is not standardized. The recommended treatment includes therapeutic strategies that target both the acute coronary syndrome and anaphylaxis. Physicians should be aware of this complication so that they recognize it early and treat it properly. The temporal relationship of the acute coronary syndrome with diclofenac digestion should always be taken into account. ECG examination is recommended to be done in all patients with anaphylactic reactions for the diagnosis of an occasional acute coronary syndrome.

3. Other Severe Adverse Reactions

3.1. Eosinophilic Pneumonia

Eosinophilic pneumonia is a syndrome, first described in 1989, characterized by pulmonary infiltrates with eosinophilia. Eosinophilic pneumonia may be primary (idiopathic) or secondary. Causes of secondary

eosinophilic pneumonia include drugs and toxins (Bartal et al. 2018).. Eosinophilic pneumonia is a rare and serious adverse drug reaction induced by non steroidal anti-inflammatory agents, including diclofenac (Goodwin and Glenny 1992). There are two described case reports of eosinophilic pneumonia induced by oral diclofenac (Khalil et al. 1993, Krabansky et al. 2018). In the first case, a 64 year old woman presented with weakness, dyspnoea and fever three days after ingestion of diclofenac for treatment of pain (Krabansky et al. 2018). Diagnostic work up included computed tomography scan that revealed bilateral interstitial infiltration and broncho-alveolar lavage that showed an elevated level of eosinophils. Drug induced eosinophilic pneumonia was diagnosed after excluding other possible diagnoses. In the second case report, a 67 year old man taking diclofenac sodium presented with dyspnea, cough, erythema multiforme, and a diffuse pulmonary interstitial infiltrate associated with eosinophilic alveolitis (Khalil et al. 1997).

3.2. Aseptic Meningitis

Drug induced aseptic meningitis is an uncommon adverse drug reaction induced by a number of medications including nonsteroidal anti-inflammatory drugs, antimicrobials, intravenous immunoglobulin, intrathecal agents, vaccines (Jolles et al. 2000, Hopkins and Jolles 2005). The most plausible pathogenetic mechanism involves immunological hypersensitivity to the drug, most likely type III and type IV hypersensitivity. Diclofenac has been implicated in drug induced aseptic meningitis (Codding et al. 1991, Seaton and France 1999, Chasan et al. 2003).

3.3. Immune Hemolytic Anemia, Immune Thrombocytopenia, Immune Neutropenia

Diclofenac has frequently been implicated as the cause of immune hemolytic anemias and less frequently of immune thrombocytopenia

(Salama et al. 1996, Meyer et al. 2003, Ahrens et al. 2006, Mayer et al. 2015). Drug induced immune hemolytic anemia is rare with an estimated incidence of 1-4 cases per million individuals per year. However, the true incidence might be higher as this adverse event may be misdiagnosed as idiopathic autoimmune hemolytic anemia (Salama and Mayer 2014). Drug induced hemolytic anemia is due to the formation of drug dependent antibodies and /or of autoantibodies. The autoantibodies bind to the red blood cells either in the presence or in the absence of the sensitizing drug and cause Fc mediated extravascular hemolysis. The drug dependent antibodies bind to red blood cells only in the presence of the drug causing complement mediated intravascular hemolysis. Drug-dependent antibodies are investigated by testing drug-treated red blood cells (RBCs) or by testing RBCs in the presence of a solution of drug. Drug-independent antibodies are serologically indistinct from idiopathic warm autoantibodies and cannot be defined or excluded by serologic testing (Leger et al. 2014).

Drugs most often implicated in drug induced hemolytic anemia include second and third generation cephalosporins, methyldopa, rifampicin, piperacillin, diclofenac. A number of case reports of diclofenac induced immune hemolytic anemias have been published (Kramer et al. 1986, Salama et al. 1991, Bougie et al. 1997, Laidlaw et al. 1997, de Quirós et al. 1997, Jürgensen et al. 2001, Meyer et al. 2003, Ahrens et al. 2004). In most cases, patients have recovered after several weeks of appropriate treatment and diclofenac discontinuation. However, there are also fatal cases of diclofenac induced immune hemolytic anemia. (Heuft et al. 1990). In a case control study, that investigated the possibility of drug induced hemolytic anemia among cases of new onset immune hemolytic anemia, diclofenac was found to be associated with an odds ratio of 3·1 (CI 1·3-7·0) with the occurrence of immune hemolytic anemia (Garbe et al. 2014). Diclofenac induced immune hemolytic anemia is usually acute and is commonly associated with renal failure (Ahrens et al. 2006). Importantly, diclofenac induced immune hemolytic anemia can be easily misdiagnosed, as diclofenac dependent antibodies react with red blood cells only in the presence of metabolites of diclofenac (Johnson et al. 1993, Sachs et al. 2004).

Diclofenac induced severe thrombocytopenia has also been reported (Kramer et al. 1986, Cledes et al. 1988, Epstein et al. 1990, George and Rahi 1995, Kim and Covacs 1997, Abraham 2011) Diclofenac induced severe neutropenia has been reported as another potential serious adverse effect of the use of diclofenac. Although the mechanisms are not fully elucidated, drug-induced immune neutropenia occurs when drug-dependent antibodies form against neutrophil membrane glycoproteins and cause neutrophil destruction. Patients may be asymptomatic. Clinical manifestations of drug induced immune neutropenia include fever, chills, and infections resulting in death if left untreated. Drugs most often associated with neutropenia include dipyrone, diclofenac, ticlopidine, calcium dobesilate, spironolactone, antithyroid drugs including propylthiouracil, carbamazepine, sulfamethoxazole- trimethoprim, lactam antibiotics, clozapine, levamisole, and vancomycin. Neutrophil drug-dependent antibodies can be detected through specific examinations including flow cytometry, monoclonal antibody immobilization of granulocyte antigens, enzyme-linked immunosorbent assay, immunoblotting, granulocyte agglutination, and granulocytotoxicity.

Patients who are receiving diclofenac and develop symptoms of either thrombocytopenia or neutropenia should have a complete blood count, and if this diagnosis is confirmed, the drug therapy should be stopped (Kim and Kovacs 1995).

3.4. Diclofenac Induced Hepatotoxicity

Diclofenac causes liver injury among chronic drug users (Schapira et al. 1986, Helfgott et al. 1990, Vilà Santasuana et al. 1997, Bogić et al. 1997, Hackstein et al. 1998, Bhogaraju et al. 1999, Dierkes-Globisch et al. 2000, Sallie et al. 2001, Nezic et al. 2012). Serum liver blood tests may be elevated in up to 15% of patients, but are greater than 3 times the upper limit of normal in only 2% to 4% of patients. In a prospective clinical trial involving 17,289 patients, diclofenac was shown to be often associated with aminotransferase elevations. (Laine et al. 2009). A relevant study

investigating the incidence and presentation of drug induced liver injury in the general population of Iceland, reported that diclofenac was the second more common agent causing drug induced liver injury (Bjornsson et al. 2013). Mechanisms of diclofenac induced hepatotoxicity include mitochondrial injury, oxidative stress, formation of protein covalent adducts of reactive metabolites of diclofenac and immune mediated mechanisms (Gill et al. 1995, Masubuchi et al. 2002, Galati et al. 2002, Lim et al. 2006). Furthermore, diclofenac metabolites have been reported to induce apoptosis in hepatocytes by alteration of mitochondrial function and generation of reactive oxygen species (Gómez-Lechón et al. 2003).

Diclofenac induced hepatotoxicity ranges from asymptomatic elevation of transaminase activity to significant liver disease. Clinical manifestations include jaundice preceded by abdominal pain, anorexia, nausea, vomiting and malaise. Acute liver failure can also result from diclofenac use. Even fatal cases of diclofenac induced hepatitis have been reported in the literature (Breen et al. 1986). The time to onset of liver injury has been reported to range between one week and a year after beginning diclofenac treatment. Complete recovery with normalization of transaminase levels has been reported one to four months after discontinuation of diclofenac. Topical forms of diclofenac have been associated with a low rate of serum enzyme elevations (Daniels et al. 2019). In a recent publication, the authors reported the case of a 79 year old woman who presented with elevated liver transaminases. Normalization of serum enzymes was noticed after discontinuation of diclofenac gel 1% (Daniels et al. 2019). However, it should be emphasized that sever reversible hepatitis has been reported even after percutaneous administration of diclofenac. Thus, clinical judgement should guide the prescription of topical forms of diclofenac.

3.5. Diclofenac Induced Kidney Injury

Furthermore, kidney injury can be caused among chronic drug users. The incidence of diclofenac induced nephrotoxicity is estimated at about 3%. Diclofenac alters renal function due to the inhibition of prostaglandin

synthesis resulting to reversible ischemia. Oxidative stress has also been implicated in diclofenac induced nephrotoxicity due to the production of free oxygen radicals and subsequent apoptotic death of renal cells. Diclofenac induced nephrotoxicity is more common among patients with co-morbidities, old patients and dehydrated patients. Co-treatment with diuretics, ACE inhibitors, aminoglycosides increases the risk of diclofenac induced nephrotoxicity.

Diclofenac induced kidney injury includes acute kidney injury/tubulonecrosis, interstitial nephritis, membranous nephropathy, hyponatremia, hypokalemia, hypertension, papillary necrosis and analgesic nephropathy (Wolters et al. 1985, Campistol et al. 1989, Cicuttini et al. 1989, Garrouste et al. 1990, Tattersall et al. 1992, Rubbio Garcia et al. 1992, Cucurull et al. 1994, Révai and Harmos 1999, Poux et al. 2000, Mohammed and Stevens 2000, Galesic et al. 2008, Inoue et al. 2008, Dhanvijay et al. 2013). Interstitial nephritis has been commonly attributed to a delayed hypersensitivity response to diclofenac, and nephrotic syndrome results from changes in glomerular permeability mediated by prostaglandins and other hormones. In the case of nephrotic syndrome, the glomerular lesion is commonly the 'minimal change' lesion and usually is associated with acute interstitial nephritis. Nephrotic syndrome frequently causes venous thromboembolic complications. Arterial thrombosis has also been reported in a case of diclofenac induced nephrotic syndrome (Huang et al. 2004).

4. Clinical Implications

Diclofenac, a commonly used non steroidal anti-inflammatory agent is implicated in adverse drug reactions from different systems. Although the gastrointestinal and cardiovascular adverse events of diclofenac are well documented, diclofenac has also been implicated in severe allergic reactions including anaphylactic reaction, anaphylactic shock, Kounis syndrome, autoimmune hepatitis, kidney injury. Even fatal reactions to diclofenac have been reported. Taking into account, that diclofenac is administered for the treatment of post-operative pain, diagnosis of the above adverse events in the surgical patient is quite challenging. Clinicians should be aware of the

serious adverse events associated with the use of diclofenac in order to be able to recognize them promptly and treat them properly. Health organizations should reconsider the policy of the over the counter use of the non steroidal anti-inflammatory agents. Patients should be well informed about the severe adverse reactions associated with the use of diclofenac. Allergic patients should be recognized and diclofenac use should be avoided especially in the outpatient setting. Severe adverse events to diclofenac should be systematically reported so that awareness is augmented and treatment standardized.

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Chapter 4

THYROID EFFECTS OF DICLOFENAC

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ABSTRACT

Diclofenac, a commonly prescribed non steroidal anti-inflammatory agent has well known adverse events including hepatological, gastrointestinal, renal and cardiovascular adverse events. Besides them, diclofenac affects thyroid function tests. Importantly, recently published research suggests that diclofenac is a thyroid receptor antagonist. This chapter will be structured in three sections. The first section will focus on the mechanisms of action of drugs on thyroid function tests. The second section will review evidence on the effect of diclofenac on thyroid function tests. The third session will focus on the clinical implications of the thyroid effects of diclofenac especially for patients treated with thyroid replacement therapy.

Non steroidal anti-inflammatory agents are known to affect the results of the thyroid function tests, by binding to thyroid hormone binding proteins and releasing free T3 and free T4. The transient elevation of free hormones leads to a sequence of alterations in the thyroid function. It is not

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really known if the effect of NSAIDs on thyroid function is a class effect or if the effect differs among different drugs. Given that NSAIDs encompass a broad class of medications with different chemical structures, it is straightforward that the effect of NSAIDs on thyroid function would be different for the different drugs.

A number of studies demonstrate that diclofenac affects thyroid hormone measurements. However, the majority of the data are derived from short term administration of diclofenac in healthy volunteers. There is paucity of data on the effect of diclofenac on thyroid hormone measurements in the case of patients treated for hypothyroidism or in the case of patients submitted to thyroidectomy and treated with thyroxin replacement therapy. Knowledge of the thyroid effects of diclofenac is necessary for endocrinologists, endocrine surgeons and rheumatologists, because it will affect therapeutic decisions. Failure to recognize thyroid effects of diclofenac in clinical practice may lead to unnecessary diagnostic tests as well as to incorrect treatment of euthyroid patients. Clinicians should always correlate the blood measurements with the clinical picture of the patient. A detailed history of short and long term drug use should always be included in the evaluation of thyroid hormone measurements. Special attention is needed in the case of patients submitted to thyroidectomy, as diclofenac use may affect thyroid replacement therapy.

Keywords: diclofenac, thyroid, thyroid function tests, thyroid replacement therapy

1. INTRODUCTION

Thyroid hormones, triiodothyronine (T3) and tetraiodothyronine (T4) circulate in blood bound to proteins and only a small amount of them is free and carries out the biological functions. Only 0.02% of T4 and 0.3% of T3 circulate in the blood in the free form. Tests used to measure circulating thyroid hormone concentrations include total thyroxine (T4), total triiodothyronine (T3), free thyroxine index, free T4 (FT4), and free T3 (FT3). Total T4 and total T3 measurements are less accurate because several medications can interfere with the results. These medications include estrogen and estrogen-containing birth control pills, tamoxifen citrate, fluouracil, mitotane, androgens, anabolic steroids, nicotinic acid, and glucocorticoids. The integrity of the pituitary negative feedback system is

evaluated by measuring the levels of thyroid stimulating hormone (TSH). The measurement of the total or of free T3 is useful for the diagnosis of hyperthyroidism especially if FT4 levels are normal and TSH suppressed.

2. DRUGS AND THYROID FUNCTION

Dopamine agonists, levodopa, bromocriptine, glucocorticoids (>0.5 mg/day dexamethasone, 100 mg/day hydrocortisone) can acutely suppress TSH to lower than normal but still detectable levels, while in overt hyperthyroidism TSH levels are commonly undetectable. Dopamine antagonists such as metoclopramide hydrochloride can increase TSH but usually at levels lower than 10 U/L. In addition amiodarone and iodinated contrast media increase TSH but still at levels lower than 10 U/L. Amiodarone is a potent class III antiarrhythmic and also possesses beta adrenoreceptor blocking properties. Amiodarone is very rich in iodine, with a 100-mg tablet containing an amount of iodine that is 250 times the recommended daily iodine requirement. Amiodarone-induced thyroid dysfunction occurs because of both its iodine content and the direct toxic effects of the compound on thyroid parenchyma. Patients treated with amiodarone can present with altered thyroid hormone profile without thyroid dysfunction (Loh 2000). In addition, amiodarone can cause either hyperthyroidism or hypothyroidism (Harjai and Licata 1997). Amiodarone-induced hyperthyroidism is occurs more commonly in iodine-deficient regions of the world. On the other hand, amiodarone-induced hypothyroidism is more common in iodine-sufficient areas (Basaria and Cooper 2005).

Tamoxifen has been evidenced to affect thyroid hormone measurements in postmenopausal breast cancer patients (Zidan and Rubenstein 1999, Kostoglou-Athanassiou et al. 1998, Anker et al. 1998, Mamby et al. 1995).

The interference of medications with thyroid hormone measurement is based on the following mechanisms (Davies and Franklyn 1991):

- Displacement of thyroid hormone from thyroid binding globulin
- Inhibition of conversion of T4 to T3
- Drug induced thyroid disease
- Interference with thyroid hormone metabolism
- Interference with thyroid hormone absorption
- Interference with thyroid hormone transport.
- Interference with the close inverse-feedback between circulating thyroid hormones and TSH

It is well known that a number of medications displace thyroid hormones from their protein binding sites inducing thus transient increase in free thyroid hormone concentrations and suppression of TSH levels (Haugen 2009). This is the main mechanism of drug intereference with thyroid hormone measurements. However, this is not the sole mechanism. Medications may sometimes interfere with the intestinal absorption of levothyroxine, primarily by forming an insoluble complex with the thyroid hormone in the intestinal lumen (Ananthakrishnan et al. 2008, Csako et al. 2001, John-Kalarickal et al. 2007, Singh et al. 2000, Singh et al. 2001). A number of commonly used drugs, such as bile acid sequestrants, ferrous sulphate, sucralfate, calcium carbonate, aluminium-containing antacids, phosphate binders, raloxifene and proton-pump inhibitors, have been reported to affect the absorption of levothyroxine (Benvenga et al. 1987, Liwanpo and Hershman 2009, Skelin et al. 2017). On the other hand, numerous drugs such as glucocorticoids, dopamine, fenclofenac, furosemide and diphenylhydantoin may modify the close inverse-feedback relationship between circulating thyroid hormones and TSH (Stockigt et al. 1985). Such effects could involve altered hypothalamic TRH secretion, a direct effect on TSH production by the thyrotroph, alterations in circulating free thyroid hormone concentrations, or changes in thyroid hormone uptake by the thyrotroph (Lim et al. 1996).

3. Non Steroidal Anti-Inflammatory Agents and Thyroid Function

3.1. Mechanisms of Interference of NSAIDs with Thyroid Function

Non steroidal anti-inflammatory drugs (NSAIDs) commonly prescribed for their analgesic effects in patients suffering from osteoarthritis and rheumatoid arthritis have well known adverse effects including gastrointestinal, renal, cardiovascular and hepatological effects (Scanzello et al. 2008). In addition, NSAIDs have endocrinological adverse events. Thus, NSAIDs are known to affect the results of the thyroid function tests, by binding to thyroid hormone binding proteins and releasing free T3 and free T4 (Isaacs and Monk . 1980, Bartha 1971, Carlson et al. 1999, Allen and Taylor 1980, Cremoncini et al. 1984). The transient elevation of free hormones leads to a sequence of alterations in the thyroid function. Evidence suggests that the transient increase of free T3 and free T4 lead to the transfer of T4 and T3 into intracellular sites thus resulting in temporary TSH suppression. This transient TSH suppression transiently reduces thyroid hormone release. The time sequence of these events is apparently drug and dose dependent, i.e., it has been shown that a very large single dose of salsalate can affect thyroid hormone measurements for at least several days. Awareness of these effects is quite crucial for the avoidance of wrong therapeutic decisions especially in patients submitted to thyroidectomy. However, data on the effect of NSAIDs on thyroid hormone measurements are rather limited and cannot easily be generalized in large populations. Relevant studies are quite heterogenous in terms of study design, type of investigated NSAID agent, dose and duration of treatment as well as in terms of the investigated population. In the majority of the studies, the investigators have studied the effect of salicylic acid or mefenamic acid derivatives on thyroid hormone measurements (Christensen 1959, Wolff et al. 1961, Ingbar 1963). For example, it has been reported that administration

of a single dose of aspirin acutely increases free thyroid hormone concentrations by 2- to 3-fold (Larsen 1972).

Salicylates alter thyroid function primarily by competition with thyroid hormones for low affinity binding sites on serum thyroid binding proteins (Larsen 1972, Lim et al. 1988, Munro et al. 1989, Wang et al. 1999, Goussis and Theodoropoulos 1999). Thus, T4 binding to hormones is inhibited and free thyroid hormone concentrations are increased. However, other mechanisms have also been reported i.e., inhibition of hepatic 5í-monodeiodination, competition with plasma membrane, cytosolic or nuclear membrane binding, decreased uptake of iodine by the thyroid gland (Dussault et al. 1976, Chalmers et al. 1993, Lim et al. 1996, McConnell 1999, Koiqumi et al. 1984, Liwanpo and Hershman 2009). The clinical relevance of these mechanisms has not been delineated. Especially it has not been clarified if the above mentioned mechanisms affect the interference of salicylates with thyroid function tests.

3.2. Animal Data

Although it is clearly known that animal data cannot be extrapolated to humans, there are experimental data that confirm interference of NSAIDs with thyroid hormone measurements in dogs and horses (Daminet et al, 2003, Daminet and Ferguson 2003, Sauve et al. 2003, Panciera et al. 2006). Sojka et al. have investigated the effect of phenylbutazone in blood concentrations of triiodothyronine, total thyroxine and free thyroxine in horses. Phenylbutazone administered at a dosage of 4.4 mg/kg every 24 hours, for 7 days significantly decreased T4 and fT4 concentrations, but did not significantly affect T3 concentrations (Sojka et al. 1993).. Daminet et al. have reported that acetylsalicylic acid and ketoprofen affect thyroid hormone measurements in dogs (Daminet et al. 2003). However, Panciera and Johnston have reported no effect of etodolac on thyroid function tests in dogs. (Panciera and Johnston 2002).

3.3. Human Data

Koizumi et al. investigated the effect of mefenamic acid on thyroid hormone measurements. The authors reported that a single oral dose of mefenamic acid significantly depressed plasma thyroxine (T4) within 3 h in man, while. Plasma free fractions of T4 and tri-iodothyronine (T3) significantly increased. In addition, mefenamic acid depressed plasma T4 within 3 h in thyroidectomized, T4-maintained rats (Koizumi et al. 1984). In another study it was reported that mefenamic acid decreased total T4 by 20% and TSH by 45% at 24 h of administration of the drug.

In another study, Samuels et al. investigated the effect of NSAIDs on thyroid hormone measurements in 25 healthy volunteers. In order to elucidate the effect of treatment duration, the investigators designed a single dose study and a 1week study. In the single dose study, the participants received a single dose of salicylic acid, salsalate, meclofenamate, ibuprofen, naproxen or indomethacin at 8.00 h. Blood measurements were performed for TSH, total and free T4, total and free T3, at different time points including 0, 1, 2, 3, 4h and 8h after the drug administration. In the one week study, the subjects were exposed to one of the six drugs for one week. Blood measurements for thyroid hormones and TSH were performed daily at 8h Thyroid hormone measurements were not affected by ibuprofen, naproxen or indomethacin when administered either as a single dose or for one week. Single dose aspirin or salsalate decreased total and free thyroid hormone measurements (Samuels et al. 2003).

In another study, the authors measured thyroid hormones at baseline and after 24 hours and 72 hours in n eight subjects taking a therapeutic dose of salsalate 1,500 mg twice daily. The therapeutic doses of salsalate significantly decreased serum concentrations of total T4 and total T3 to about 75% of baseline levels after 3 days of salsalate use. The levels of TSH were also suppressed (McConnell 1999). In a cohort of 14 euthyroid patients taking salsalate for various rheumatic diseases for a mean period of 44 weeks, blood levels of thyroid hormones were measured. Abnormalities were observed in routine thyroid function tests similar to those observed in central hypothyroidism. Decreases of total T4 concentrations of about 20 to

40% were observed. However, all subjects remained clinically euthyroid (McConnell 1992). Similar decreased of total T4 concentrations after salicylate treatment have been reported by other authors ((Surks & Stievert, 1995). The effect of fenclofenac on thyroid function has been investigated in a number of studies, that provide evidence on the modulation of thyroid function by fenclofenac due to competitive inhibition by fenclofenac of binding of thyroid hormones to thyroid binding proteins (Capper et al. 1981, Humphrey et al. 1980, Issaks and Monk 1980, John et al. 1983, Kurtz et al. 1981, Pearson et al. 1982, Taylor et al. 1983, Ratcliffe et al. 1980).

Apart from the above cited articles that provide evidence on the modulating effect of NSAIDs on thyroid function, there are also studies, that suggest no effect of these medications on thyroid function. In a relevant study, Croxson et al. reported that after administration of indomethacin 200 mg daily for 2 days to four euthyroid volunteers no significant effect on serum triiodothyronine or thyroxine was observed and no consistent alteration of serum TSH was caused (Croxson et al. 1977). In addition, Bishnoi et al. have reported that serum T4 and T3 measurements were not affected in patients treated with diflunisal, ibuprofen, indomethacin, piroxicam, or sulindac (Bishnoi et al. 1994).

However, the effect of a number of NSAIDs has not been broadly investigated. Furthermore, there is limited evidence on the effect of newer NSAIDs i.e coxibs on thyroid hormone measurements. In addition, most studies investigating the effect of NSAIDs have been performed before the development of current assays for the measurement of TSH, free T4 and free T3.

4. DICLOFENAC AND THYROID FUNCTION

It is not really known if the effect of NSAIDs on thyroid function is a class effect or if the effect differs among different drugs. Given that NSAIDs encompass a broad class of medications with different chemical structures, it is straightforward that the effect of NSAIDs on thyroid function would be different for the different drugs. The effect of diclofenac on thyroid function

has been investigated in a number of studies (Bishnoi et al. 1994, Aoyama et al. 1990, Topliss et al. 1988, Fowler et al. 1982, Barlow et al. 1993, Barlow et al. 1996, Sternad et al. 1993, Kasono et al. 2001, Kinouchi et al. 2016, Klopčič et al. 2018). All these studies demonstrate that diclofenac affects thyroid hormone measurements. However, the majority of the data are derived from short term administration of diclofenac in healthy volunteers. There is paucity of data on the effect of diclofenac on thyroid hormone measurements in the case of patients treated for hypothyroidism or in the case of patients submitted to thyroidectomy and treated with thyroxin replacement therapy.

In a relevant study, Bishnoi et al. performed a cross-sectional survey at Veterans Affairs and University hospitals including eighty nine patients taking NSAIDs and 22 control subjects not taking NSAIDs, in order to investigate the effect of commonly prescribed non steroidal anti-inflammatory drugs on thyroid hormone measurements. Blood measurements included total T4, free T4, total T3 and TSH. Blood levels of TSH were normal in all subjects. Patients treated with salsalate had lower T4 measurements, while T3 measurements were depressed in patients treated with salsalate, diclofenac sodium, and naproxen (Bishnoi et al. 1994).

Nadler et al. have investigated the effects of a single dose of aceclofenac on thyroid function and thyroid hormone binding in 18 healthy volunteers. TSH and free thyroid hormone measurements were performed prior to and 2 hours after single dose aceclofenac treatment. Based on the measurements T3 protein binding was significantly affected, and particularly, there was significant decrease in T3 binding on thyroid binding globulin and a significant increase of albumin-bound T3. No effect was observed in all the other thyroid hormone measurements (Nadler et al. 2000).

Thus, in a relevant study, Aoyama et al, 8 male and 2 female volunteers were given a single dose of 50 mg diclofenac sodium. The concentrations of total T4, total T3 and free T4 were measured ninety minutes after the administration of the drug, Based on the data, the concentrations of total T 3, total T 4 and free T 4 were significantly decreased, but the concentrations of free T 3 and the levels of T 3 uptake, % free T 3 and % free T 4 were

increased. In a following step of the study, diclofenac sodium was added to serum in vitro, resulting in an increase of the levels of free T 3, free T 4 as well as of T 3 uptake, suggesting that diclofenac sodium inhibited the binding of T 3 and T 4 competitively to the binding protein with subsequent increase of the levels of free T3 and free t4 (Aoyama et al. 1990).

Apart from human data, there are also environmental data from aquatic organisms that evidence effect of diclofenac on thyroid function (Saravanan et al. 2014).

The mechanism underlying the interference of diclofenac with thyroid function tests is not clearly delineated. As all the other NSAIDs, diclofenac displaces thyroid hormones from their protein binding sites inducing thus transient increase in free thyroid hormone concentrations and suppression of TSH levels. However, novel research data suggest additional possible mechanisms that could explain the interference of diclofenac with thyroid function tests. In that aspect, diclofenac has been reported to be thyroid receptor beta antagonist (Zloh et al. 2016). Thyroid hormone receptors are nuclear receptors that control transcription and thus have various effects in the cells of the human body. Thyroid hormone receptors regulate physiological processes including development, growth, metabolism, cardiac function. Furthermore, diclofenac has been reported to inhibit thyroid hormone transporters, including OATP1C1 (Westholm et al. 2009). Recently, several transporters capable of thyroid hormone transport have been identified (Hagenbuch 2007). It is known that conversion of thyroid hormone by the intracellular deiodinases requires transport of thyroid hormones within the cell. Cellular entry is also required for binding of thyroid hormones to the nuclear receptors. Thus, apart from displacement of diclofenac from thyroid binding proteins, other mechanisms might account for the modulatory effect of diclofenac on thyroid function tests.

5. Clinical Implications

Diclofenac may cause serum changes in the blood concentrations of thyroid hormones. If these alterations are not recognized, clinicians may be

directed to incorrect therapeutic decisions. For example, in patients presenting with moderate degrees of hypo- and hyperthyroidism thyroid hormone levels may be spuriously normal. On the other hand, diclofenac use may wrongly lead to false diagnosis of thyroid disease in euthyroid patients (Wenzel 1981; Wenzel 1996).

Thus, clinicians should always correlate the blood measurements with the clinical picture of the patient. A detailed history of short and long term drug use should be always be included in the evaluation of thyroid hormone measurements. Special attention is needed in the case of patients submitted to thyroidectomy, as diclofenac use may affect thyroid replacement therapy (Kargin et al. 2014). Patients submitted to thyroidectomy should be educated of on the possible interference of drugs with thyroid hormone replacement therapy. They should be instructed to avoid over the counter drug use. In case that thyroid hormone measurements do not correspond to the clinical picture, diclofenac use should be discontinued and thyroid hormone measurements should be repeated.

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Chapter 5

THE USE OF DICLOFENAC IN PREGNANCY: FETAL AND NEONATAL EFFECTS

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ABSTRACT

The use of non-steroidal anti-inflammatory drugs (NSAIDs) like diclofenac is common among the general population and also among pregnant women. Although highly effective, its administration during

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pregnancy is limited due to its association with multiple side effects. These are mainly due to its mechanism of action. The resulting prostaglandin inhibition has several effects on the fetus, depending on the type of agent, the dose and duration of therapy, the period of gestation, and the time elapsed between maternal NSAID administration and delivery. Fetal complications include alteration of renal perfusion with the consequent oligohydramnios and premature closure of the ductus arteriosus that may lead to the production of persistent pulmonary hypertension and tricuspid regurgitation. For all these reasons, the Food and Drug Administration (FDA) has advocated cautious use of this drug during pregnancy. NSAIDs should be given in pregnancy only if the maternal benefits outweigh the potential fetal risks, at the lowest effective dose and for the shortest duration possible. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac during pregnancy, the possible adverse effects and safety.

Keywords: diclofenac, ductus arteriosus, non-steroidal anti-inflammatory drugs (NSAIDs), oligohydramnios, pregnancy

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used among the general population in the treatment of a wide variety of inflammatory, painful, febrile and rheumatic processes.

Nowadays NSAIDs represent the most widely used drug in medical practice. Indeed, some of these drugs can be bought without prescription (Aker 2015) and their use has dramatically increased in recent years among patients of all ages, ranging from the newborn to the adults (Musu 2011). Their use during pregnancy has also increased, in spite of the emerging findings; even short-term administration of NSAIDs during the late pregnancy period is correlated with a significant increase of adverse effects (Musu 2011).

Diclofenac is an antiphlogistic drug, derived from non-steroidal phenylacetic acid, closely related to indomethacin. After oral administration, diclofenac undergoes first-pass metabolism which decreases its systemic bioavailability to 50% and its elimination half-life is about 2 hours (Cassina

2010). Until now, there has been little evidence concerning the side effects of diclofenac ingestion during pregnancy (Auer 2004).

Although highly effective, its administration during pregnancy is limited due to its association with multiple side effects. These are mainly due to its mechanism of action. NSAIDs block the synthesis of prostaglandins through inhibition of different cyclooxygenase (COX) isoenzymes. These enzymes are responsible for the metabolism of arachidonic acid (Auer 2004). The principal mode of action of diclofenac is its non-selective inhibition of COX-1 and COX-2. In addition to the desired effect of reducing inflammation, non-selective COX inhibitors also inhibit gastric, platelet, and renal production of prostaglandin (Ostensen 1996).

Prostaglandins are vital to fetal development, including nephrogenesis, as demonstrated by the presence of COX isoforms in the fetal kidney. COX-1 is present in the glomerulus, in collecting duct cells, interstitial cells, endothelial cells, and the smooth muscle cells of pre- and postglomerular vessels, while COX-2 is present in endothelial and smooth muscle cells of the arteries and veins and intraglomerularly in podocytes (Kömhoff 1997). The effect and side effects of NSAIDs depend on the isoforms of cyclooxygenases that they preferentially or selectively inhibit. The use of selective COX-2 inhibitors has been shown to result in a significant dose-dependent increase in renal vascular resistance and a decrease in renal blood flow (Prévot 2004). These adverse effects are more marked during the third trimester due to an increase in the expression of COX enzymes, particularly COX-2 (Ostensen 2004).

The main source of information on the safety of NSAIDs in pregnancy comes from its indication in the treatment of polyhydramnios and to prevent preterm labour, because of its tocolytic properties (Karadeniz 2013, Ostensen 1996). NSAIDs have previously been widely used in these pregnancy-related conditions. Since the seventies, NSAIDs have been used as effective tocolytic agents. Indomethacin has been the reference drug, delaying delivery for at least 48 hours and up to 7-10 days. However, their administration in pregnancy may cause adverse embryo-fetal and neonatal effects as they cross the placenta (Siu 2004, Antonucci 2012) and are distributed to the fetus at term. The resulting prostaglandin inhibition has

several effects on the fetus (Aker 2015, Florescu 2005), depending on the type of agent, the dose and duration of therapy, the period of gestation, and the time elapsed between maternal NSAID administration and delivery (Antonucci 2012).

The use of COX inhibitors has recently been associated with infertility and miscarriage. The ability of NSAIDs to compromise reproductive function by inhibition of ovulation and as causative agents for miscarriage is still under debate (Ostensen 2004). Their use during the first trimester of gestation has been linked to a significant increase in the risk of miscarriage (Nielsen 2001, Li 2003; Nielsen 2004), although more studies are needed to confirm this association.

Also, cardiac malformations and midline defects have been described (Kozer 2002, Werler 1992, Ericson 2001) but cohort and case-control studies have found no statistically significant association between exposure and congenital malformations. The classical nonselective COX inhibitors, do not increase the risk of congenital malformations in humans but, if administered in the latter part of gestation, they can affect pregnancy and the fetus. Intrauterine fetal growth restriction and premature delivery are possible side effects of high doses (Ostensen 2004).

The risks associated with the use of diclofenac during the third trimester of pregnancy are widely described; maternal complications include prolonged pregnancy and labor (Ostensen 1998, Ostensen 1998), increased postpartum hemorrhage and gastric abnormalities (Risser 2009). Fetal complications include alteration of renal perfusion with the consequent oligohydramnios and premature closure of the ductus arteriosus that may lead to the production of persistent pulmonary hypertension and tricuspid regurgitation (Antonucci 2012, Momma 1983, Mas 1999, Moise 1993, Rein 1999, Zenker 1998). The most widely described drug that can lead to ductal constriction is indomethacin. When ingested by the mother, indomethacin will cause constriction and even complete and irreversible closure of the ductus.

All NSAIDs cross the human placenta and are distributed to the fetus at term (Siu 2004, Antonucci 2012). The main problems with administration of NSAIDs during pregnancy are adverse effects on the ductus arteriosus

and fetal renal function (Aker 2015). However, other adverse neonatal effects are also described, such as intraventricular haemorrhage, necrotising enterocolitis, periventricular leucomalacia, hyperbilirubinemia, respiratory distress syndrome and bronchopulmonary dysplasia (Aker 2015, Florescu 2005, Koren 2006). Fetal and neonatal adverse effects affecting the brain, kidney, lung, skeleton, gastrointestinal tract and cardiovascular system have also been reported after prenatal exposure to NSAIDs (Antonucci 2012).

For all these reasons, the Food and Drug Administration (FDA) has advocated cautious use of this drug during pregnancy. It has been listed in Category C before 30 weeks of gestation and Category D after 30 weeks (Cassina 2010, Phadke 2012).

NSAIDs should be given in pregnancy only if the maternal benefits outweigh the potential fetal risks, at the lowest effective dose and for the shortest duration possible (Antonucci 2012).

In conclusion, diclofenac should be used with caution during pregnancy due to possible adverse effects. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac during pregnancy, the possible adverse effects and safety.

2. Fetal and Neonatal Effects

2.1. Premature Constriction or Closure of the Fetal Ductus Arteriosus

In the fetal circulation, the ductus arteriosus (DA) is a blood vessel, which acts like a shunt connecting the pulmonary artery at the junction of the main pulmonary artery and the origin of the left pulmonary artery to the proximal descending aorta just after the origin of the left subclavian artery. It allows most of the oxygenated right ventricular output to bypass the high-resistance pulmonary vascular system to the systemic circulation (Enzensberger 2012), and is essential for normal fetal development (Schneider 2012). Typically, only about 10% of the right ventricular output passes through the pulmonary vascular bed.

Table 1. Premature constriction or closure of fetal ductus arteriosus

ETIOLOGY	Medication, structural heart disease, idiopathic.
INCIDENCE	Might be underestimated.
COMPLICATIONS	Pulmonary hypertension, right heart dysfunction, tricuspid and pulmonary regurgitation, congestive heart failure, fetal hydrops, intrauterine death, persistent neonatal pulmonary hypertension.
DIAGNOSIS: ECHOCARDIOGRAPHIC FINDINGS	Hypertrophy of the right ventricle, right atrial and ventricular dilatation, tricuspid and pulmonary regurgitation, decreased right ventricular fractional shortening or ejection force, pulsatility index <1.9 in the DA, peak systolic velocity >1.4 m/s and peak diastolic velocity >0.35 m/s.
DOSE OF DICLOFENAC	Independent.
GESTATIONAL AGE	The risk increases with advanced gestational age.
REVERSIBLE	DA constriction usually disappears after discontinuation of diclofenac.

Metabolites of arachidonic acid, which can be found in high concentrations in the fetal blood circulation, regulate the blood circulation in the umbilical cord, and ensure that the DA stays open during fetal life (Parer 1991). The DA needs to stay open during fetal life for the fetal tissues to receive oxygen (Gökçimen 2001).

The DA, also called the ductus Botalli, is a remnant of the distal sixth aortic arch and is a normal structure during fetal life. As a result of an intricately intertwined network of both physiological and biochemical changes, this vessel constricts rapidly after birth. Deoxygenated blood is diverted away from the placenta and through the lungs, which are now vital for gas exchange (Gökçimen 2001). In a healthy full-term neonate, the DA closes up 10–15 hours following birth, usually within the first 24 hours postpartum (Rein 1999, Enzensberger 2012).

Premature constriction of the DA is a rare condition that can occur during fetal life secondary to medication, structural heart disease or as idiopathic constriction (Karadeniz 1013). The true incidence might be underestimated because some cases can show a subclinical or mild course (Enzensberger 2012). Effects on the fetal DA have been shown for most non

selective COX inhibitors (Momma 1983). They may be less pronounced with the selective COX-2 inhibitors (Macones 2001). Unknown causes or other new substances were also described, such as naphazoline, fluoxetine, isoxsuprine, caffeine, pesticides, and maternal consumption of polyphenolrich beverages (Table 1).

The closure of DA in utero can lead to life-threatening intrauterine complications, as all the blood is ejected into the pulmonary circulation. This can lead to hypertrophy of the medial layer of the pulmonary arteries, and pulmonary hypertension. This can have a serious impact on the fetus, such as progressive right heart dysfunction with tricuspid and pulmonary regurgitation, congestive heart failure, fetal hydrops, and may even lead to intrauterine death and persistent neonatal pulmonary hypertension (Aker 2015, Enzensberger 2012, Menahem 1991).

Constriction of the DA is inevitable due to the treatment with tocolytic cyclooxygenase inhibitors to prevent premature birth (Momma 1983, Rein 1999, Zenker 1998). The constricted DA may, therefore, induce increased pulmonary vascular resistance, and dilatation of the right ventricle and tricuspid deficiency can also occur. Right ventricle insufficiency affects the liver, kidney, spleen and brain causing hyperemia and/or congestion in these organs due to stasis. Congestive heart failure dominated by right ventricular failure leads to congestion of the liver (Gökçimen 2001).

Fetal echocardiography is the most important diagnostic tool for assessment closure or constriction of the DA (Karadeniz 2013). The most frequent echocardiographic findings are hypertrophy of the right ventricle, right atrial and ventricular dilatation, and moderate to severe tricuspid and pulmonary regurgitation (Enzensberger 2012). Different sonographic criteria for detection are: right and left ventricular enlargement, tricuspid valve regurgitation, decreased right ventricular fractional shortening or ejection force, pulsatility index <1.9 in the DA, peak systolic velocity >1.4 m/s and peak diastolic velocity >0.35 m/s (Enzensberger 2012).

The placenta is known to be selectively permeable up to a certain level. However, NSAIDs inhibit the biosynthesis of prostanoids and can cross the placental barrier, into the fetal circulation, causing teratogenic effects on the fetus (Ostensen 1998, Zenker 1998).

There is less information about diclofenac and its adverse effects on the DA. It however shares the same pharmacological properties as the other prostaglandin synthesase inhibitors (Mas 1999). Diclofenac has a similar effect on the fetal DA as indomethacin (Karadeniz 2013). Though most reports deal with indomethacin, there is some evidence that links diclofenac to a similar effect on the fetal DA (Auer 2004). Diclofenac, indomethacin and other prostaglandin synthetase inhibitors have been implicated in many published cases with constriction of the DA (Aker 2015, Auer 2004, Karadeniz 2013, Mas 1999, Enzensberger 2012, Porta 2003).

Even a single maternal exposure to NSAIDs can lead to clinically significant ductal constriction. In previous reports the closure of the fetal DA following diclofenac treatment is very rare and also with repeated doses, but it was also seen after a single dose of diclofenac ingestion (Karadeniz 2013). Ductal closure has been described both after a single dose and after long term use of NSAIDs in pregnancy (Aker 2015). A case report demonstrated that even topical administration of diclofenac can induce constriction of DA in late pregnancy (Torloni 2006).

The risk of this complication increases with advanced gestational age and is known to cause dose independent premature constriction which is reversible following discontinuation of the medication (Enzensberger 2012). There is an increased risk of premature closure of the DA if NSAIDs are used after 28 weeks of gestation, and the risk increases with advancing gestational age (Aker 2015). Fetal echocardiography has shown that constriction of the ductus is independent of the fetal serum concentration of NSAID. It is, however, related to gestational age, rare before week 27, and increasingly affecting about 10–50% of the fetuses after week 31 (Ostensen 2004).

Thus, the proportion of fetuses developing ductal constriction after indomethacin exposure increases with advancing of gestational age, with 100% experiencing constriction at >34 weeks' gestation (Moise 1993). However, this effect is usually reversible when the drug is stopped (Musu 2011, Florescu 2005, Rein 1999). Doppler evidence of DA constriction usually disappears within 24 to 48 hours after discontinuation of medication (Enzensberger 2012). Fetal or neonatal side effects of COX inhibitors are

most often observed when administered shortly before delivery or to children born prematurely (Ostensen 2004).

The severity of the changes caused by premature closure of DA is related to several factors, including the treatment duration, dose, drug administration route, gestational age and fetal individual factors (Porta 2003).

The association between DA closure and diclofenac was first shown experimentally by Momma et al. They demonstrated that the constricting action of diclofenac was as severe as that of indomethacin (Momma 1983).

Momma et al. demonstrated that the application of NSAIDs, interrupts the active process of maintaining the DA patency in utero could while promoting its constriction and/or closure. In experimental studies with rats, diclofenac has been shown as one of the most potent NSAID to constrict the fetal DA (Momma 1983).

A PubMed search from 1998 until the present, revealed several case reports describing constriction or closure of the DA following maternal ingestion of diclofenac (Aker 2015, Karadeniz 2013, Siu 2004, Mas 1999, Rein 1999, Zenker 1998, Shastri 2013). It has also been described after maternal use of topical diclofenac in two case reports (Auer 2004, Torloni 2006).

The first case, published by Zenker in 1998, involved persistent pulmonary hypertension of the newborn in association with premature closure of the DA following maternal diclofenac treatment. It represents a more severe form of persistent pulmonary hypertension of the newborn induced by NSAID (Zenker 1998). This report described a term newborn with severe pulmonary hypertension due to premature closure of the DA following a 5 day maternal treatment with diclofenac two weeks before delivery. Pulmonary hypertension only responded to unusually high doses of inhaled NO. The treatment was necessary for 22 days suggesting a structural alteration of pulmonary vasculature. The child recovered, but tricuspid regurgitation persisted, presumably due to irreversible ischemic damage of one papillary muscle (Zenker 1998).

Shastri et al. described a case of premature closure of the DA in utero, diagnosed postnatally in a baby with hydrops and cardiac failure (Shastri

2013). An echocardiogram 6 hours postnatally showed marked dilation of the right atrium and hypertrophy of right ventricle with impaired function, elevated pulmonary pressures, a small pericardial effusion, and no flow through the DA. The mother was unaware of her pregnancy until she presented in labor, and she had taken diclofenac medication in the preceding months.

Siu and Lee described a case of severe pulmonary hypertension and transient right-sided hypertrophic cardiomyopathy in a neonate, caused by premature closure of DA after short-term maternal use of diclofenac sodium (Siu 2004). This suggests that maternal diclofenac ingestion may be suspected if a newborn develops severe pulmonary hypertension and right-sided hypertrophic cardiomyoptathy with closed DA.

Ishida et al. attempted to clarify the prognostic factors of intrauterine ductal closure, including maternal, fetal, and neonatal clinical information and their prognoses (Ishida 2017). They analyzed the data of 116 patients from 39 articles. Of these, 12 (10.3%) died after birth or in utero. Fetal or neonatal death was significantly correlated with fetal hydrops (OR 39.6, 95% CI 4.6-47.8) and complete closure of the DA (OR 5.5, 95% CI 1.2-15.1). Persistent pulmonary hypertension was observed in 33 cases (28.4%), and was also correlated with fetal hydrops (OR 4.2, 95% CI 1.3-4.6) and complete closure of the DA (OR 5.5, 95% CI 1.6-6.0). In summary, fetal hydrops and complete ductal closure were significant risk factors for both death and persistent pulmonary hypertension in this study. Cardiac or neurological prognoses was favorable if the patients overcame right heart failure during the perinatal period.

Following a similar approach, Lopes et al. analyzed the causes and perinatal outcome related to fetal DA constriction or closure at a single center over a 26-year period. They included 45 consecutive cases of constriction (n=41) or closure (n=4), of which 29 were related to maternal use of NSAIDs. Among the 29 cases of NSAIDs, 27.5% had consumed a single dose while 75% had taken multiple doses over several days. Right ventricular dilatation was present in 82.2% of the fetuses, tricuspid insufficiency in 86.6%, and heart failure in 22.2%. Neonatal persistent pulmonary hypertension occurred in 17.7% of the patients. Late follow-up

showed 43 survivors alive and healthy with two deaths from unrelated causes (Lopes 2016).

In conclusion, all pregnant women should be informed by an obstetrician or general practitioner about the side effects of NSAIDs on the fetus, especially in third-trimester pregnancies where such repeated or single-dose drugs should be avoided and assessed carefully by fetal echocardiography in terms of preterm constriction of the DA (Karadeniz 2013). Scientific evidence suggests that the fetal ductus state and velocities should be monitored by fetal echocardiography in women treated with diclofenac (Rein 1999).

Close monitoring is mandatory (Porta 2003) to rule out the development of right heart failure and to determine the intervention time. Most fetuses, with ductal constriction and a widely patent foramen ovale and normal venous Doppler findings, can be treated expectantly. These fetuses must not be delivered early. However, an abnormal cardiovascular profile is an indication for close surveillance. To date, there is no cutoff value for immediate delivery (Enzensberger 2012).

The administration of diclofenac for pain relief, like other NSAIDs, should be avoided during late gestation whenever possible. With their easy transplacental transfer, their unpredictable pharmacodynamics in the fetus, and their often profound effect on ductal constriction late in pregnancy, this group of drugs can have highly detrimental effects on the fetus and the neonate (Auer 2004).

Premature closure of the DA should be considered in any pregnancy where there is ingestion of NSAIDs, evidence of hydrops of unknown etiology, or signs of right ventricular dysfunction in the fetus (Mas 1999). If the clinician needs to prescribe diclofenac, particularly during third trimester, serial cross-sectional echocardiography and Doppler monitoring of the fetal heart and in particular ductal flow should be carried out to determine if narrowing and premature closure is occurring. Cessation of NSAIDs may lead to reopening of the ductus (Mas 1999).

2.2. Oligohydramnios

NSAIDs can also affect fetal and neonatal renal function. They can cause reduced renal perfusion, which leads to reduced urine production and oligohydramnios (Aker 2015).

The mechanism leading to oligohydramnios is poorly understood. Two major assumptions are based on direct renal effects: NSAID influence kidney development which leads to irreversible structural alterations, and NSAID induced inhibition of prostaglandin synthesis in the fetal kidney lowers renal blood flow and tubular function, resulting in a reduction of fetal urine production (Figure 1) (Boubred 2006).

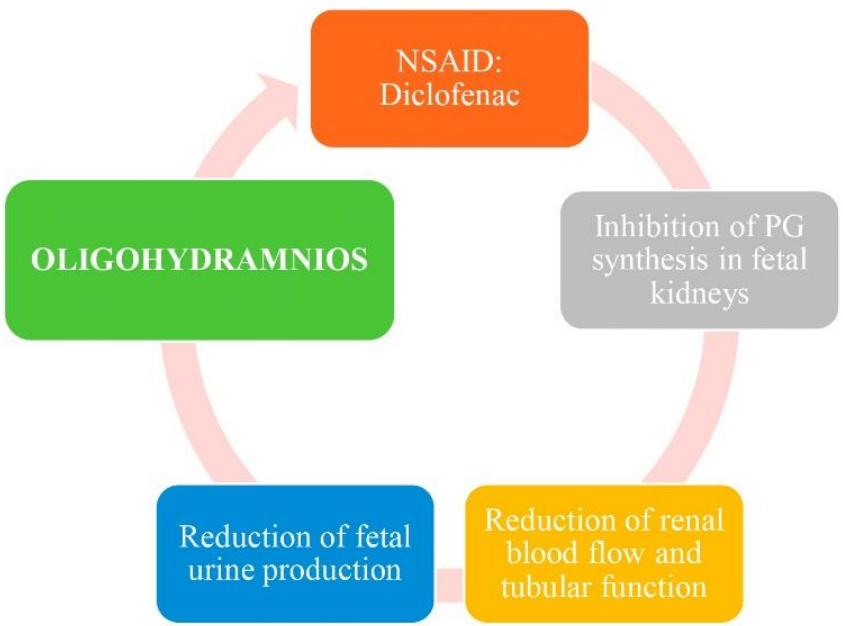

Figure 1. Mechanism leading to oligohydramnios.

The underlying mechanisms leading to renal dysfunction in the fetus are probably the same as those in postnatal and adult life. The major adverse effects associated with NSAID prenatal exposure are oliguria, anuria, oligohydramnios, renal failure, intracranial haemorrhage, necrotising

enterocolitis, augmentation of serum creatinine levels and metabolic acidosis (Musu 2011).

Oligohydramnios is a condition in pregnancy characterized by a deficiency of amniotic fluid. It occurs in 3-5% of pregnancies and can have multiple causes, such as placental insufficiency, premature rupture of membranes, renal or urinary tract malformations, maternal treatments, fetal chromosomal anomalies, or intrauterine infections.

Oligohydramnios has been described for several NSAIDs, including diclofenac, ibuprofen, indomethacin, ketoprofen, naproxen, niflumic acid, nimesulide, and piroxicam.

Fetuses exposed to NSAIDs often have decreased urinary output, but as with ductal closure, the amniotic fluid usually returns to normal after therapy is stopped (Florescu 2005).

The effect can be transient, but there are cases describing end-stage renal failure (Phadke 1012, Benini 2004). Adverse renal effects reported include oliguria, fatal anuria, renal failure, and oligohydramnios (Cuzzolin 2001).

With advancing pregnancy, diclofenac-mediated prostaglandin inhibition can cause severe adverse effects in the fetus, including acute renal failure resulting in oligohydramnios. For this reason it is generally accepted that NSAID-treatment be avoided after gestational week 28 (Scherneck 2015).

The main finding of published cases was early onset of oligohydramnios during the second trimester of pregnancy after maternal diclofenac therapy. The drug was administered over a prolonged period of time at high doses. Development of oligohydramnios was shown to be dose dependent. After discontinuation of the therapy, amniotic fluid volumes normalized within a few days, suggesting causality. There is evidence that renal function in the fetus recovers quickly after drug withdrawal (Ostensen 2004).

Our group published the case of a woman at 27 weeks of pregnancy with nephritic colic who developed acute oligohydramnios after receiving anti-inflammatory treatment with diclofenac. The oligohydramnios resolved after discontinuation of diclofenac (López del Cerro 2012).

2.3. Nephrotoxicity

Long-term treatment with NSAID, however, can even lead to acute or chronic nephrotoxicity (Musu 2011). There has been a case report of neonatal transient renal failure following combined use of diclofenac, paracetamol, and nimesulide (Benini 2004).

The use of NSAIDs during pregnancy has been reported to cause nephrotoxicity in the fetus. Phadke et al. reported a spectrum of three cases of neonatal renal failure following antenatal exposure to diclofenac alone, varying from reversible renal insufficiency to severe renal failure. One of the twins had severe oliguric renal failure requiring peritoneal dialysis. The other twin had nonoliguric renal failure which did improve, but renal function did not normalize by the time he was discharged. The third baby had oliguric renal failure which completely improved, with normal renal function at the 1-year follow-up. Thus, all three neonates were affected to a varying extent (Phadke 2012).

It has been shown that diclofenac-induced oligohydramnios in late pregnancy is followed by neonatal renal impairment (Phadke 2012). Similar observations were made for other NSAIDs, in particular for indomethacin which has been frequently used for the treatment of preterm labor since the 1970s.

The occurrence of renal side effects following prenatal exposure to NSAIDs seems to be rare considering the large number of pregnant women treated with them (Cuzzolin 2001). NSAID-related nephrotoxicity remains an important clinical problem in the newborns, in whom the functionally immature kidney may exert a significant effect on the disposition of the drugs (Musu 2011).

NSAIDs are generally considered to be safe and well tolerated, but current studies indicate that these pharmacological agents account for 7% of reported cases of acute renal failure and 35% of drug-induced acute renal failure in the general population (Musu 2011).

So far, several cases of severe and sometimes irreversible renal insufficiency have been described in neonates exposed to NSAID during fetal life, as discussed below. In a case-control study, 57 babies exposed

prenatally to indomethacin, had a reduction in urine output along with a moderate increase in serum creatinine levels during the first three days of life. Finally, babies born at less than 30 weeks' gestation, to mothers exposed to indomethacin for preterm labour, presented significantly higher frequency of oliguria in the first 24 hours of life compared to those exposed to placebo in utero (Musu 2011).

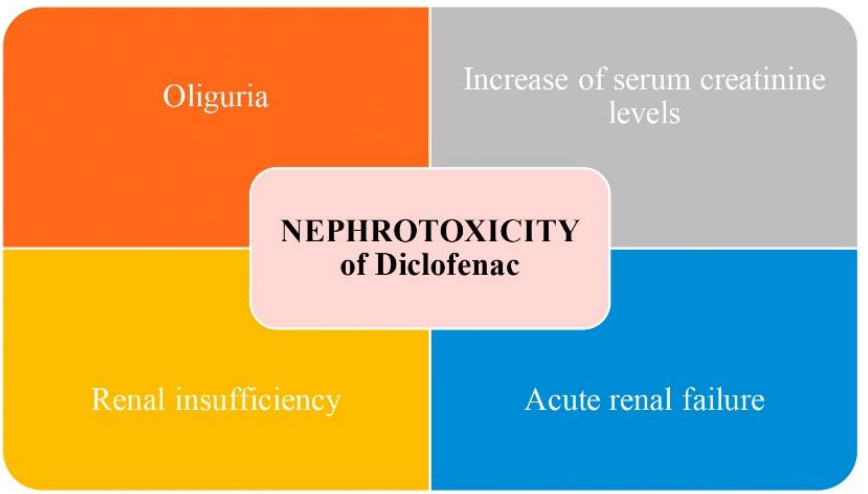

Figure 2. Spectrum of nephrotoxicity.

Exposure to NSAIDs in the neonatal period for the treatment of patent DA, through the inhibition of prostaglandin synthesis, is indicated before left to right ductal shunting occurs. However, during the neonatal period, this type of therapy may itself cause adverse effects such as oliguria, gastrointestinal haemorrhage, renal failure, decrease in cerebral blood flow, and increase of serum creatinine levels. Reduction of urinary volume and glomerular filtration rate are usually reversible within 48 hours after discontinuation of therapy, although oliguria may persist for 2 weeks. Strategies to minimize the adverse renal effects, such as the use of prolonged low doses of this drug, or the coadministration with other pharmacological agents (furosemide or dopamine), have not been successful (Musu 2011).

NSAID-related nephrotoxicity in the newborn may result from the mechanism of action of these drugs combined with renal immaturity. By blunting the effect of prostaglandins on the afferent arteriole, NSAIDs can impair glomerular filtration rate in both the fetus and the newborn (Figure 2) (Musu 2011).

On the basis of the specific mechanism involved in the activation, NSAID-induced nephrotoxicity should be considered as a significant adverse effect, particularly in high risk patients. Similar to many cases of drug-induced renal damage, acute renal failure is often initially non-oliguric. Thus, monitoring of renal function is mandatory during therapy (Musu 2011).

No effort should be spared in attempting to prevent unnecessary administration of NSAIDs, especially in high risk patients. These efforts should include the choice of compounds having a lower nephrotoxic potential, and identification of the most appropriate dosage. Further, concomitant administration of other nephrotoxic drugs should be avoided, the duration of the treatment should be limited and procedures should be put in place for early diagnosis of nephrotoxicity (Musu 2011).

In summary, in utero exposure to diclofenac may be associated with oligohydramnios, oliguria, fetal and neonatal renal failure that may be transient or irreversible. Therefore, it is recommended that the use of diclofenac during pregnancy be avoided.

2.4. First Trimester Exposure

NSAIDs are in common use, both for rheumatic and degenerative joint diseases, sports injuries, and temporary pain, (e.g., dysmenorrhea, migraine). First trimester exposures are, therefore, relatively common. As we have already seen, NSAIDs have a documented effect on the fetus toward the end of the pregnancy due to a premature closure of the DA, which in turn lead to pulmonary hypertension and respiratory problems (Ericson 2001). An effect on kidney function also has been observed leading to oligohydramnios (Ostensen 2004). These effects make NSAID use

unsuitable during the third trimester and notably just before expected delivery.

Relatively little is known about possible teratogenic effects, and first trimester use is usually regarded as safe (Ostensen 1998, Ostensen 1994). However, the possible teratogenic effects of NSAIDs used in early pregnancy include gastroschisis, cardiac malformations and orofacial clefts.

2.4.1. Congenital Malformations

There is no clear evidence of the teratogenicity of any NSAID in humans. Few specific studies regarding diclofenac teratogenicity in humans have been conducted.

The use of diclofenac during first trimester of pregnancy is not suspected to cause congenital malformations, but the substance has been shown to cross the placenta both in the first and in the third trimester (Siu 2004).

Some epidemiological studies have observed an association between NSAID exposure during the first trimester of pregnancy and congenital cardiac abnormalities, like ventricular and atrial septal defects, and gastroschisis (Kozer 2002, Werler 1992, Ericson 2001, Martínez-Frías 1997).

A prospective study on congenital malformations in infants whose mothers used NSAIDs in early pregnancy was carried out in Sweden in the late 1990s (n = 2557). The OR (after consideration of maternal age, parity, and smoking habits) for any congenital malformation was 1.04 (95%CI 0.84–1.29), but the OR for cardiac defects was 1.86 (1.32–2.62), and for orofacial clefts 2.61 (1.01–6.78). Although the exact exposure time or dosage was not recorded, however, in majority of cases, exposure occurred during the first trimester. Another source of error is the fact that some women may have used NSAIDs without reporting it or without it being recorded. Pregnancy outcome after the first trimester use of NSAIDs was essentially normal in this study except for two exceptions. One was an excess of rather mild cardiac defects. No drug specificity in the association between NSAID and cardiac defects was found. This may indicate that the effect was either due to a nonspecific NSAID effect (prostaglandin inhibition) or due to confounding by an underlying disease. Cardiac defects

described in patients treated with diclofenac: include ventricular septal defects, double outlet right ventricle, and atrial septal defects. In contrast, the association between NSAID use and orofacial clefts is specifically associated with one drug, naproxen. These observations may be useful for the understanding of the pathogenesis of the relevant defects, for example if prostaglandin inhibition in early pregnancy can affect cardiogenesis (Ericson 2001).

To assess the safety of diclofenac during pregnancy, a prospective observational cohort study was done, evaluating follow-up data of 145 pregnant women who were exposed to diclofenac between the fifth and the fourteenth gestational week. The rate of major malformations was 5.6% in the study group, and 2.4% in the control group, but it was not significantly different. The rate of cardiac malformations in the diclofenac cohort was 1.6% (OR 3.8, CI 0.5-28.1). The small number of cases in the study group does not allow firm conclusions to be made about the possible role of diclofenac in abnormal heart development. This study suggests that the use of diclofenac is relatively safe during first trimester of pregnancy and the studied sample size makes it possible to exclude a risk of congenital malformation higher than 3.3 with a power of 80% (Cassina 2010). However, the direction of effect points against diclofenac, so it should be used with caution.

Recently a study was published based on 1106 women who had received a prescription for an NSAID 30 days before pregnancy or during the first trimester of pregnancy (Nielsen 2001). The risk for a congenital malformation was 1.27 (95% CI 0.93–1.75).

Exposure to NSAIDs during pregnancy increases the incidence of midline defects, diaphragmatic hernias and ventricular septal defects in rats in rabbits. The incidence of these defects is also higher in aspirin-treated animals (Cook 2003). Experimental animal studies, however, have found these effects in association with very high dose levels. Only a limited number of animal studies are available concerning diclofenac's safety in pregnancy, and most of these failed to detect a significant increase in major malformations in the offspring of pregnant mice, rats and rabbits treated with doses up to five times higher than those used in humans (Cappon 2003,

Russell 1986). Only one study reported an increased risk of cleft palate in mice treated with doses similar to those used in humans (Montenegro 1990).

Ostensen and Ostensen prospectively studied pregnant women with rheumatic disease to compare the incidence of adverse fetal effects between those with and without exposure to NSAIDs. They found no increased risk of teratogenicity or neonatal adverse outcomes in the group exposed to NSAIDs, however, no fetal echocardiographic studies were conducted, so risk of transient ductal closure and reduced amniotic fluid volume could not be evaluated (Ostensen 1996).

Their recommendation is that NSAIDs can be used in the first half of gestation since there is no indication for teratogenic effects of salicylates, phenylbutazone, indomethacin, fenoprofen, ibuprofen, ketoprofen, naproxen, diclofenac, mefenamic acid and piroxicam (Ostensen 1996). However, both selective and nonselective inhibitors of cyclooxygenase can alter fetal circulation by inhibition of prostaglandin synthesis.

The only two NSAIDs extensively studied in pregnant women are acetylsalicylic acid (ASA) and indomethacin. The studies concluded that, overall, there was no increased risk of congenital malformations among fetuses exposed to ASA in utero (Kozer 2002). Case-control studies, however, showed an association between ASA and gastroschisis.

Indomethacin has been linked to a variety of adverse fetal effects, documented in several trials, like pulmonary hypertension, respiratory distress syndrome, bronchopulmonary dysplasia, and necrotizing enterocolitis. A meta-analysis found a 15-fold increased risk of ductal constriction (Koren 2006).

These data suggest a cautious approach, as it is likely that chronic use of NSAIDs in high doses, such as is necessary in the treatment of rheumatoid arthritis, is associated with an increased risk of adverse fetal effects (Florescu 2005).

One study reviews maternal and fetal side effects of NSAIDs and immunosuppressive agents in pregnant patients. The severity of the disease under treatment decides if continuation of one of these drugs is justified. Prepregnancy counselling and careful monitoring during pregnancy help to

tailor necessary drug treatment for the benefit of mother and child (Ostensen 2004).

Pregnancy occurring in women with rheumatic diseases may result in clinical and therapeutic problems. Some rheumatic diseases like ankylosing spondylitis and systemic lupus erythematosus remain active or even flare up during pregnancy. Furthermore, a rheumatic disease may negatively influence pregnancy outcome and neonatal health. Indications for treatment during pregnancy are control of disease activity in the mother, prevention of a flare and assuring a good pregnancy outcome (Ostensen 2004). Unfortunately, the number of controlled studies of drugs performed in pregnant women is small and experience with therapy often derives from other than rheumatic diseases. The main concerns of drug therapy in pregnancy are possible teratogenicity of drugs, adverse effects on fetal growth and development, a negative effect on the progress of pregnancy and harmful effects on the neonate. Late effects in offspring after in utero exposure to drugs are largely unknown (Ostensen 2004).

Counselling of the prepregnancy patient differs according to the disease present. In cases where drug treatment has to be continued, maternal and fetal tolerability of the drugs in question are required. When fetal effects of drugs are not or insufficiently known, the decision for or against therapy has to consider the risk imposed by treatment and the risk induced by a disease flare. Ideally, drug treatment during pregnancy should control the mother's disease activity, not harm the fetus and ensure a healthy pregnancy for mother and child (Ostensen 2004).

2.4.2. Miscarriages

Some studies in humans have observed an association of prenatal NSAID exposure with an increased risk of miscarriage (Nielsen 2001, Li 2003, Nielsen 2004). These results are limited because they do not consider confounders such as other drugs assumed by the study population and because of the small number of pregnancies evaluated.

A population-based retrospective study on the use of NSAIDs showed that they did not increase adverse birth outcomes, but did increase risk of miscarriage (Nielsen 2001).

Cassina et al. reported that the rate of spontaneous abortions was within the range of the general population both in the diclofenac-exposed and control group, but it was higher in the former (6.2% vs. 5.4%); nonetheless the difference was not statistically significant. The main limitations of the study were selection and response biases and the dimension of the study cohort (Cassina 2010).

Another retrospective study identified 166 women who were exposed to diclofenac/misoprostol in early pregnancy, and of which 28.3% (n 47) ended up in a miscarriage compared with 10.6% among unexposed. The adjusted hazard ratio of having a miscarriage after exposure to diclofenac/misoprostol in the first trimester was 3.6 (CI 95% 2.6-4.9). They found an increased risk of miscarriage after exposure to diclofenac/misoprostol during the early pregnancy and concluded that women in the fertile age should not be treated with the combination of diclofenac/misoprostol if other options were available (Andersen 2016). However, this may be due to a bias of the study since misoprostol induced uterine contractions could be responsible for the first trimester miscarriages. In fact, misoprostol is one of the first line tools used in pharmacological miscarriage treatment.

Further studies are warranted to confirm that the use of diclofenac is safe during first trimester of pregnancy (Cassina 2010).

3. Experiments in Animals

Gokçimen et al. investigated the possible postnatal effects on the liver, kidney and testicular tissues of the offspring of rats that were given diclofenac sodium (DS) during pregnancy. The gestation period was significantly prolonged with DS-treated rats ($p < 0.001$). A significant enlargement in the periportal area ($p < 0.05$), sinusoidal dilatation ($p < 0.001$), bile duct proliferation ($p < 0.001$), pyknosis in the nucleus of hepatocytes, and vacuolar degeneration in parenchymal cells ($p < 0.001$) were observed in DS-treated rats. Under light microscopy a similar morphological structure was observed in the kidney and testicular tissues of both the DS-treated and control rats (Gökçimen 2001).

They concluded that significant morphological changes were observed in the livers of the offspring whose parents had been treated with DS. No significant differences were observed in liver morphology between the female and male offspring. There were no significant effects of DS on the morphology of the kidney and testis in all offspring (Gökçimen 2001).

The most pronounced effect of DS could be the constriction of the fetal DA induced by DS. This can initially cause a decrease in the amount of oxygen-poor blood reaching the liver tissue, leading to a possible degeneration of liver parenchymal cells. The histopathological changes observed in this study cannot be directly attributed to constriction of the DA, the adverse effects of DS metabolites deregulating blood circulation in the umbilical cord and the possible combined effects of DS and its metabolites (Gökçimen 2001).

In conclusion, significant changes in liver histopathology were observed in the offspring of DS-treated rats compared to those of control rats. The difference in the number of bile ducts and sinusoidal width was found to be more significant than the difference in the diameter of the portal area. There were no hepatocytic degenerations in the control group, but there were small to moderate degenerations in the DS group (Gökçimen 2001).

Prostaglandin may play an important role in the normal differentiation of the developing palatine region. In the study conducted by Montenegro and Palomino, five different NSAIDs (naproxen, sulindac, indomethacin, diclofenac and mefenamic acid) were injected into pregnant mice. These drugs induced different frequencies of cleft plate in the offspring. The most teratogenic drug seemed to be sulindac, and indomethacin was almost ineffective (Montenegro 1990).

NSAIDs may be responsible for some cases of infertility. Carp et al. reported that rat blastocyst implantation was retarded by about 30% with the administration of DS to pregnant rats (Carp 1988). Needs and Brooks also found deformities in the ribs of fetuses of pregnant rats treated with DS (Needs 1985). Additionally, DS-injected rabbits expressed no teratogenic effects (Russell 1986).

Some adverse effects, such as prolonged pregnancy and labor, are possibly due to the shared property of inhibited prostaglandin synthesis

(Ostensen 1996, Ostensen 2004). Cervical maturation requires lower levels of prostaglandins than myometrial activity. The same effects were paradoxically observed after the inhibition of prostaglandin synthesis using diclofenac. It has been shown that PGE2 administration in the rat increases the cervical heparan sulfate concentration (Cabrol 1991). In this series, DS again provoked a decrease in the heparan sulfate concentration which was probably due to inhibition of the PGE2-induced increased synthesis. Cervical maturation in late pregnancy is controlled by prostaglandins, phospholipid metabolites and/or other factors which are probably due to the inhibition of PGE2 (Cabrol 1991). As reported in previous studies the gestation period of pregnant rats treated with DS was significantly increased compared with control rats (Gökçimen 2001).

Momma et al. studied the transplacental effects of NSAIDs on the fetal DA of full-term pregnant rats. In total, 58 NSAID were evaluated, and their potency in usual clinical doses was classified into 4 grades. Diclofenac and 15 other NSAIDs caused strong fetal ductal constriction. Consistent dose-dependent constriction of the fetal DA occurred with the vast majority of NSAIDs. Although animal studies cannot directly extend to the clinical situation, the results in this study present gradings of possible risks of administration of each NSAID to pregnant women (Momma 1984).

Arslan et al. studied the effects of DS exposure during gestation on the testes of rat pups to investigate the safety of its use during the prenatal period. Pregnant rats were separated into control, saline, low dose, medium dose and high dose groups. DS was given between weeks 15 and 21 of gestation. By the end of the study, the total number of Sertoli cells was decreased significantly in a dose dependent manner in the medium and high dose groups compared to controls. Similarly, the total number of spermatogonia was reduced in the medium and high dose groups while no significant differences were found in the control, saline and low dose DS groups. They suggest that prenatal administration of DS can cause deleterious effects on the testis development, especially in high doses (Arslan 2016).

CONCLUSION

- NSAIDs are commonly used among the general population. Their use during pregnancy has also increased, in spite of the emerging findings.
- In view of the presented evidence, diclofenac should be used with caution during all trimesters of pregnancy due to the possible adverse effects, including miscarriages, congenital malformations, impaired renal function and premature constriction of the DA.
- The use of NSAIDs during the first trimester of gestation has been linked to a significant increase in the risk of miscarriage, although more studies are needed to confirm that association.
- Also, cardiac malformations and midline defects have been described but cohort and case-control studies have found no statistically significant association between exposure and congenital malformations.
- Future research in this field should be undertaken to focus on the actual role of NSAIDs in clinical practice for each age group, concentrating particularly on pregnant women.
- Diclofenac exposure in utero could be associated with oligohydramnios, oliguria, fetal and neonatal renal failure that may be transient or irreversible. We therefore suggest that diclofenac should definitely not be considered as firstline treatment for non-obstetric reasons in pregnant women, whenever possible.
- With their easy transplacental transfer, their unpredictable pharmacodynamics in the fetus, and their often very profound effect on ductal constriction late in pregnancy, NSAIDs can have highly detrimental effects on the fetus and the neonate.
- All pregnant women should be informed by an obstetrician or general practitioner about the side effects of diclofenac on the fetus, especially in third-trimester pregnancies where such repeated or single-dose drugs should be avoided and assessed carefully by fetal echocardiography in terms of preterm constriction of the DA.

Scientific evidence suggests monitoring of the fetal ductus state and velocities by fetal echocardiography in women treated with diclofenac. Echocardiographic diagnosis of ductal constriction has led to an active medical approach resulting in low morbidity of this group of patients.

- No effort should be spared in attempting to prevent unnecessary administration of diclofenac, especially in high risk patients. Instead, compounds with lower nephrotoxic potential should be explored and the appropriate dosage and treatment duration identified. Simultaneous administration of other nephrotoxic drugs should be avoided and, where required, procedures should be put in place for early diagnosis of nephrotoxicity. In conclusion, it is recommended that the use of diclofenac be avoided during pregnancy.

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Chapter 6

THE USE OF DICLOFENAC IN OBSTETRICS AND GYNECOLOGY: INDICATIONS, EFFICACY AND ADVERSE EFFECTS

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Keywords: diclofenac, non-steroidal anti-inflammatory drugs (NSAIDs), pregnancy, pain, analgesia

ABSTRACT

Nowadays non-steroidal anti-inflammatory drugs (NSAIDs) represent the most widely used drug in medical practice in the treatment of a wide variety of inflammatory and painful processes. Indeed, some of these drugs can be bought without prescription. Their use during pregnancy has also increased, in spite of the recent findings. Indications of use of these agents are gynecological disorders like ectopic pregnancy, acute pelvic inflammatory disease, complications of ovarian cysts, endometriosis, dysmenorrhea and fibroids. Abdominal pain during pregnancy is also a relatively common symptom and may require treatment with analgesia. It may reflect anatomical and physiological changes of the pregnant state. Various obstetric conditions such as placental abruption, clinical chorioamnionitis, threatened preterm labour and uterine rupture present with acute abdominal pain. Pregnancy may also increase predisposition to certain clinical conditions like urinary tract infection, which in turn may present with abdominal pain. In conclusion, diclofenac is used in the treatment of a wide variety of gynecological and obstetric conditions. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac, possible adverse effects and its safety. For this purpose, we have conducted a literature research of clinical trials and other recent prospective studies on diclofenac in different areas.

1. INTRODUCTION

Diclofenac is a non-esteroidal anti-inflammatory drug (NSAID) which acts primarily by inhibiting prostaglandin synthesis through its activity on cyclooxygenase (COX) enzymes (Cassina 2010). Diclofenac is an antiphlogistic drug, derived from non-steroidal phenylacetic acid, closely related to indomethacin.

Over 50 years ago, indomethacin emerged as a potent non-steroidal anti-inflammatory drug during a concerted effort to find effective anti-inflammatory and analgesic medications. The 1960s saw acetic acid

derivatives developed into indomethacin, diclofenac and sulindac, and propionic derivatives into ibuprofen, naproxen and ketoprofen (Lucas 2016).

Diclofenac is an NSAID that is widely used; it is commonly prescribed to treat various diseases and symptoms such as inflammation and mild-to-moderate pain. Diclofenac is frequently used as a painkiller, and it is commonly used among the general population for the treatment of a wide variety of inflammatory, painful, febrile and rheumatic processes.

Nowadays NSAIDs represent the most widely used drug in medical practice. Indeed, some of these drugs can be bought without prescription. Their use during pregnancy has also increased, in spite of the recent findings; even short-term administration of NSAIDs during the late pregnancy period is correlated with a significant increase in adverse effects. Therefore, it is important to inform pregnant women of the potential side effects, and that these drugs should be avoided as self-medication especially in the third trimester (Aker 2015). Maternal side effects include the prolongation of pregnancy and labor (Ostensen 1998, Ostensen 1998).

In addition to the desired effect of reducing inflammation, non-selective COX inhibitors also inhibit gastric, platelet, and renal production of prostaglandin. In non pregnant adults, prostaglandin synthetase inhibitors have been reported to cause a number of side-effects, including: 1) gastrointestinal ulceration and perforation, 2) acute renal failure, 3) interference with renal excretion of water, sodium, and potassium, 4) interference with antihypertensive and diuretic therapy, 5) acute interstitial nephritis, 6) nephrotic syndrome, 7) and chronic renal injury. These renal complications are generally reversible, and can be attributed to interference with the physiological actions of prostaglandins on the kidney (Schlondorff 1993).

Indications of chronic use of these agents during pregnancy are inflammatory bowel or chronic rheumatic diseases. Additionally, as we said previously, diclofenac is commonly used among the general population in the treatment of a wide variety of inflammatory and painful processes.

While some gynecological disorders cause cyclic pain, others may be associated with discrete painful events unrelated to menstrual cycles. Gynecological causes of abdominal pain may arise from conditions

associated with pregnancy as well as the non-pregnant state. In the former, the presentation is usually as an emergency, and the most important diagnosis is ectopic pregnancy. In women who are not pregnant, the most common emergencies associated with abdominal pain are acute pelvic inflammatory disease and complications of ovarian cysts. Less acute causes of abdominal pain include endometriosis, dysmenorrhea, fibroids and chronic pelvic inflammatory disease. The most important cause of abdominal pain due to a gynecological problem is ovarian cancer. This rarely presents acutely, but often gives rise to vague abdominal symptoms (Hammond 2008).

Abdominal pain during pregnancy is a relatively common symptom. It may reflect anatomical and physiological changes of the pregnant state. Various obstetric conditions such as placental abruption, clinical chorioamnionitis, threatened preterm labour and uterine rupture present with acute abdominal pain. Pregnancy may also increase predisposition to certain clinical conditions like urinary tract infection, which in turn may present with abdominal pain. In addition, the presence of a fetus may impact on diagnosis and management. Timely diagnosis and appropriate treatment of conditions contributing to abdominal pain and acute abdomen during pregnancy, are likely to improve maternal and perinatal outcome (Devarajan 2014).

Acute abdomen refers to an intra-abdominal process that is characterized by abdominal pain, tenderness and muscular rigidity, for which an emergency surgery must be considered. Life-threatening conditions such as acute appendicitis, acute pancreatitis and intraperitoneal infection or haemorrhage may result in such an ‘emergency’ (Devarajan 2014). The use of diclofenac in all these situations is frequent.

Until now, there has been little evidence concerning the side effects of diclofenac ingestion during pregnancy. The only two NSAIDs extensively studied in pregnant women are acetylsalicylic acid and indomethacin (Florescu 2005). In addition to the treatment of non-obstetric conditions (e.g., painful and inflammatory conditions), NSAIDs have previously been widely used for the treatment of pregnancy related conditions, such as

polyhydramnios and the prevention of preterm labour, because of its tocolytic properties (Ostensen 1996).

In conclusion, diclofenac is used in the treatment of a wide variety of gynecological and obstetric conditions. The objective of this chapter is to review the available scientific evidence on the clinical uses of diclofenac, possible adverse effects and its safety. For this purpose, we have conducted a literature research of clinical trials and other recent prospective studies on diclofenac in different areas.

2. MATERNAL DISEASES

Women should be reassured that pain can be treated during pregnancy and lactation and that they need not suffer unnecessarily. Overall, appropriate therapeutic doses of the commonly used analgesics including paracetamol, aspirin and opioids have not been associated with an increased incidence of birth defects. However, the use of NSAIDs in the third trimester is not recommended. Untreated persistent pain can have adverse effects for the mother and her pregnancy and women with persistent pain should ideally have optimize their pain management before pregnancy (Kennedy 2011).

Rheumatic diseases occur frequently in women of childbearing years, necessitating drug treatment. A concurrent pregnancy may require modification of the treatment to control maternal disease activity and to ensure a successful pregnancy outcome (Ostensen 2004).

Pregnancy occurring in women with rheumatic diseases may result in clinical and therapeutic problems. Some rheumatic diseases like ankylosing spondylitis and systemic lupus erythematosus remain active or even flare during pregnancy. Furthermore, a rheumatic disease may negatively influence pregnancy outcome and neonatal health (Skomsvoll 1994). Indications for successful treatment during pregnancy are the control of disease activity in the mother, prevention of a flare and assurance of a good pregnancy outcome. Active rheumatic disease during pregnancy may require drug treatment to ensure that the mother's health is maintained and that there is a good outcome for the fetus.

Unfortunately, the number of controlled studies of drugs performed in pregnant women is small and experience with therapy often derives from causes other than rheumatic diseases. The main concerns of drug therapy in pregnancy include the possible teratogenicity of drugs, adverse effects on fetal growth and development, a negative effect on the progress of pregnancy and harmful effects on the neonate. Late effects in offspring after in utero exposure to drugs are largely unknown (Ostensen 2004).

The lack of knowledge on the use of anti-rheumatic drugs during pregnancy, causes difficulty in the prescription of these drugs for the patients. Most studies have concentrated on acetylsalicylic acid and indomethacin among NSAIDs in the treatment of rheumatoid arthritis. There is very little information on the use of ibuprofen, sulindac, ketoprofen, fenomates, oxicams and diclofenac during pregnancy (Gökçimen 2001).

Therapeutic decisions in nonpregnant patients with active rheumatoid arthritis, psoriatic arthritis or ankylosing spondylitis require that a desire to have children be taken into account. Control of progressive erosive joint disease needs effective drug treatment. The question is which suitable monotherapies or combination therapies are compatible with first trimester exposure and which must be prophylactically withdrawn before a planned pregnancy (Ostensen 2004).

Counselling of the prepregnancy patient differs according to the disease present. In cases where drug treatment has to be continued, maternal and fetal tolerability of the drugs in question are required. When fetal effects of drugs are not or insufficiently known, the decision for or against therapy has to consider the risk imposed by treatment and the risk induced by a disease flare. Ideally, the drug treatment during pregnancy should control the mother's disease activity, do no harm to the fetus and ensure a healthy pregnancy for mother and child (Ostensen 2004).

On the basis of the specific mechanism involved in the activation, NSAID nephrotoxicity should be considered as a significant adverse effect, in particular in high risk patients of all ages (Musu 2011). Similar to many cases of drug-induced renal damage, acute renal failure is often initially non-oliguric. Thus, monitoring of renal function and serum creatinine levels is

mandatory during therapy, along with assessment of electrolytes and blood pressure (Schetz 2005).

No effort should be spared in attempting to prevent the unnecessary administration of NSAIDs, especially in high risk patients such as patients with hypovolemia and/or pre-existing renal problems. These efforts should include the choice of compounds with a lower nephrotoxic potential, identification of the most appropriate dosage, avoidance of concomitant administration of other nephrotoxic drugs, provision for early diagnosis of nephrotoxicity, and limitation of the treatment duration. Future research in this field should be undertaken to focus on the actual role of NSAIDs in clinical practice for each age group, concentrating particularly on pregnant women (Musu 2011).

To conclude, NSAIDs should be given in pregnancy only if the maternal benefits outweigh the potential fetal risks, at the lowest effective dose and for the shortest duration possible (Antonucci 2012).

3. Analgesic Effects

3.1. Pregnancy Termination

Abortions, especially cervical dilation and suction aspiration, are associated with pain despite various methods of pain control. Pain is a predictable feature of medical abortion in both the first trimester and the second trimester. The following publications of clinical trials have evaluated optimal analgesia regimens during medical abortion.

- A prospective randomized study, carried out by Velipasaoglu et al., compared the analgesic effects of acetaminophen, diclofenac and hyoscine-N-butylbromide in cases of second trimester pregnancy termination. In 60 women with indications for second trimester pregnancy termination, three analgesic agents were randomized into three groups. The mean visual analog scale (VAS) score, last VAS scores before termination and the need for parenteral analgesia did

not differ among the groups. This study did not demonstrate a difference in pain perception among second trimester pregnancy termination cases using acetaminophen, diclofenac and hyoscine-N-butylbromide (Velipasaoglu 2016).

- Owolabi et al., carried out a prospective trial to evaluate and compare paracervical block with diclofenac for pain relief during manual vacuum aspiration for surgical termination of pregnancy. Participants were randomized into three groups: 1) diclofenac 75 mg intramuscularly 30 min before the procedure; 2) diclofenac 75 mg intramuscularly 30 min before the procedure, together with local infiltration of the cervix with lignocaine 1% (10 mL); 3) diclofenac 75 mg intramuscularly 30 min before the procedure together with local infiltration of the cervix with lignocaine 1% (10 mL) and paracervical block with lignocaine 1% (5mL). There was a significant difference in pain scores during the procedure between groups 1 and 2 ($p < 0.001$), and between groups 1 and 3 ($p < 0.001$). There was no difference in pain score between groups 2 and 3 ($p = 0.144$). The local anaesthetic infiltration of the cervix in combination with diclofenac and paracervical block provided better pain relief during and after the vacuum aspiration (Owolabi 2005).
- López et al. conducted a similar randomized study to estimate the effectiveness of different methods of analgesia among women treated with manual vacuum aspiration for spontaneous abortion. The 113 patients diagnosed with incomplete abortion were randomly assigned to 3 groups of analgesic administration: diclofenac plus paracervical block, meperidine plus diclofenac and meperidine alone. There was no significant difference between the analgesics used among the 3 groups (López 2007).
- A systematical review conducted by Renner et al., concluded that conscious sedation, general anesthesia involving premedication with diclofenac (and other COX inhibitors) and some nonpharmacological interventions decreased procedural and postoperative pain, while being safe and satisfactory to patients (Renner 2009, Renner 2010).

To conclude, few studies have examined pain management during medical abortion, and the heterogeneity of existing data limits comparison. Further research is needed to determine the optimal analgesia regimens for first-trimester and second-trimester medical termination of pregnancy (Jackson 2011).

- In addition to the above studies, which examine the efficacy of pain management during medical abortion, a randomized study was conducted to evaluate whether diclofenac given during medical abortion with mifepristone/misoprostol in the second trimester has a negative effect on the efficacy of the abortifacient by prolonging the induction-to-abortion interval.
  Co-treatment of diclofenac with misoprostol did not attenuate the efficacy of mifepristone and misoprostol. There was no significant difference between the NSAID and the non-NSAID group in the induction-to-abortion interval or the total doses of misoprostol needed and women in the group treated with diclofenac required significantly less opiates ($p = 0.042$) (Fiala 2005).

- Another study found that the co-treatment of diclofenac sodium with misoprostol did not attenuate the cervical ripening efficacy of misoprostol (Li 2003).

3.2. Episiotomy

Perineal pain after episiotomy is a common problem following vaginal birth. The pain affects either physical and mental function negatively. The treatment of pain from episiotomy or from tearing of perineal tissues during childbirth is often unapplied, although discomfort may be severe. There are many methods in perineal pain relief such as local ice pack and a bath, oral anesthesia and intravenous anesthesia. Analgesic rectal suppository is one of the various methods in pain relief, especially when oral preparation causes gastric discomfort, nausea or vomiting (Achariyapota 2008).

To assess the effectiveness of diclofenac rectal suppositories for relieving perineal pain after perineorrhaphy, a randomized double-blinded placebo controlled trial was conducted.

Seventy-two term, singleton, pregnant women who gave vaginal birth with second to third degree episiotomy tears were randomized to either diclofenac or placebo rectal suppositories group. No differences were found in the median pain scores before administration of medications and at 30 min, 1 hour and 2 hour after administration ($p > 0.05$), while the median pain scores were significantly reduced in the diclofenac group at 12 and 24 hours after administration compared to the control group.

This study, therefore, suggested that diclofenac suppository was effective on reducing perineal pain after episiotomy, especially at 12 and 24 hours after administration (Achariyapota 2008).

Dodd et al. carried out a similar randomized, double-blind trial to evaluate the efficacy of rectal diclofenac in the relief of perineal pain after trauma during childbirth was conducted.

A total of 133 women with a second-degree or greater perineal tear or episiotomy were randomly allocated to either diclofenac or placebo suppositories.

Women in the diclofenac group were significantly less likely to experience pain at 24 hours compared with the women who received placebo. The authors, therefore, concluded that the use of rectal NSAIDs suppositories is a simple, effective and safe method of reducing the pain experienced by women following perineal trauma within the first 24 hours after childbirth (Dodd 2004).

Searles at al. carried out a trial evaluating diclofenac suppositories administered prophylactically to produce effective and lasting analgesia following perineal injury.

A group of 100 women sustaining objective perineal injury (second degree tear or episiotomy) during spontaneous vaginal delivery at term participated in the randomized double blind placebo controlled trial. The mean pain score was significantly reduced in the diclofenac group at 24, 48 and 72 hours after delivery compared with the control group. In addition there was less supplementary analgesia required (Searles 1998).

Lim et al. carried out a study to compare oral celecoxib with oral diclofenac as pain relief after perineal repair, following normal vaginal birth.

A group of 329 women were randomized to 200 mg celecoxib or to 100 mg diclofenac orally, 12 hourly, for 24 hours after perineal repair.

Repeated measures analysis of variance showed a larger reduction of VAS pain score at rest with celecoxib compared to diclofenac ($p = 0.044$). The difference in pain score when mobilising was not significant ($p = 0.75$). Randomization to celecoxib was associated with less upper gastrointestinal symptoms reported: 23.3% versus 34.5% (relative risk 0.67 95% CI 0.48-0.96: $p = 0.029$) but additional analgesia for breakthrough pain was not significantly different (Lim 2008).

A randomized double-blind controlled trial was carried out by Facchinetti et al. to compare the effectiveness and side-effects of two analgesics in the management of postpartum perineal pain.

A total of 261 women were randomly assigned to receive either 100mg diclofenac or 100mg ketoprofen, both given orally every 12 hours up to 48 hours, as necessary.

Diclofenac and ketoprofen had similar analgesic properties in the first 24 hours postpartum. Significantly fewer subjects in the diclofenac group than in the ketoprofen group experienced side-effects (6.8% versus 15.6%; $p = 0.038$). There were no significant differences in overall patient satisfaction between the two groups. No main differences were found concerning pain relief between the two treatments, however diclofenac could be the preferred choice because it is associated with less adverse reactions, together with a faster action in the relief of pain (Fachinetti 2005).

Rezaei et al. carried out a study comparing the prophylactic efficacy of a diclofenac suppository and an indomethacin suppository on reducing post-episiotomy pain. A total of 90 women with second degree episiotomy were assigned to receive a single dose of diclofenac suppository, indomethacin suppository or placebo, according to randomized blocks. This study showed that in the group given diclofenac or indomethacin, at all the assessed hours, the pain measured was considerably less than in the suppository-free group ($p < 0.05$). Comparing the diclofenac and indomethacin groups, there were only significant differences in the 4 and 12 hour measurements: diclofenac

was more effective than the indomethacin (4th hour), but due to a shorter half-life, diclofenac group in the 12th hour had more pain ($p < 0.05$). For this reason, diclofenac suppository was recommended at 4-hour intervals for all patients, without internal disorders, to decrease episiotomy pain (Rezaei 2014).

Altungül et al. carried out a similar study comparing the analgesic effect of diclofenac sodium and indomethacin suppositories for management of right mediolateral episiotomy repair.

A total of 70 patients who gave birth vaginally with right mediolateral episiotomy were randomly assigned to receive 100 mg diclofenac sodium suppositories or 100 mg indomethacin suppositories per day after episiotomy repair and postpartum for three days. Diclofenac sodium was a more effective analgesic than indomethacin suppositories for right mediolateral episiotomy pain. The two analgesics were effective after episiotomy repair, however diclofenac sodium suppositories could be the preferred choice because they were more effective (Altungül 2012).

3.3. Gynecological and Obstetric Surgery

Patients frequently experience pain of moderate to severe degree during gynecological procedures. The pain after surgery is frequent and in urgent gynecological and obstetric processes it appears in a persistent way.

Acmaz et al. conducted a prospective, randomized, placebo-controlled trial investigating the analgesic efficacy of preoperative oral dexketoprofen trometamol, intravenous paracetamol, lidocaine spray, pethidine and diclofenac sodium on fractional curettage procedure.

A total of 144 multiparous women were randomly allocated to one of the six groups. The first group (control group) consisted of 22 participants who did not receive any treatment.

Pethidine was the best choice for reducing pain score during curettage procedure. All analgesic procedures were significantly effective in reducing pain during postoperative period but this study showed that lidocaine puffs provided the best pain relief than the other analgesics used (Acmaz 2015).

Vasallo et al. conducted a prospective cohort study to assess the effectiveness of postoperative multimodal analgesia using opioids and NSAIDs in gynecological and obstetric surgery on a group of 50 patients who were operated on due to gynecological and obstetric emergency. These patients were divided in a study group of 25 tramadol/diclofenac and including control group of 25 pethidine/dipyrone.

Although they had a similar analgesia in the immediate postoperative period, in time it was better combined with tramadol/diclofenac than with pethidine and dipyrone. The combination of tramadol and sodium diclofenac offered a better analgesia after surgical procedure in study patients being a therapeutic alternative to take into account (Vasallo 2011).

Chapa et al. investigated the effectiveness of a NSAID administered in combination with a local anesthetic as a deep paracervical block for in-office endometrial ablations. No statistically significant difference was noted in overall intraoperative VAS score ($p = 0.81$), however there was a significant reduction in postoperative VAS score ($p = 0.01$) (Chapa 2010).

3.4. Primary Dysmenorrhea

Primary dysmenorrhea is a syndrome characterized by painful uterine contractility caused by a hypersecretion of endometrial prostaglandins. NSAIDs are the first choice for its treatment. In vivo and in vitro studies have demonstrated that myometrial cells are also targets of the relaxant effects of nitric oxide (NO) (Facchinetti 2002).

In addition to being the first choice for primary dysmenorrhea treatment, NSAIDs are also the treatment of choice for intrauterine contraceptive device-induced dysmenorrhea and menorrhagia. NSAIDs can be used for effective control of menorrhagia and dysmenorrhea (Dawood 1993).

Facchinetti et al. carried out a study to determine the efficacy of glyceryl trinitrate, an NO donor, in the resolution of primary dysmenorrhea in comparison with diclofenac. A total of 24 patients with the diagnosis of severe primary dysmenorrhea were studied during two consecutive menstrual cycles. In an open, cross-over, controlled design, patients were

randomized to receive either diclofenac or glyceryl trinitrate patches the first days of menses.

Both treatments significantly reduced pain intensity score by the 30th minute. While diclofenac continued to be effective in reducing pelvic pain for two hours, glyceryl trinitrate scores remained more or less stable after 30 minutes and significantly higher than those for diclofenac. Low back pain was also relieved by both drugs. Headache was significantly increased by glyceryl trinitrate but not by diclofenac. These findings indicated that glyceryl trinitrate had a reduced efficacy and tolerability by comparison with diclofenac in the treatment of primary dysmenorrhea (Facchinetti 2002).

4. Assisted Reproduction Treatment

Insufficient information is available on the safety and efficacy of the potent analgesic diclofenac sodium administered following oocyte retrieval. The following studies aim to address this issue.

Kailasam et al. carried out a randomized prospective double-blind study of 381 assisted conception cycles. Patients included were <40 years old with early follicular FSH <10 IU/l and no medical contraindications to receiving NSAIDs. Patients were randomized to either receive diclofenac sodium suppository 100 mg at the end of oocyte retrieval or nothing. Effect of diclofenac sodium on outcome was assessed.

A total of 187 IVF/intracytoplasmic sperm injection cycles were randomized to receive diclofenac sodium at the end of oocyte retrieval and 194 cycles that did not receive diclofenac sodium. The number reaching embryo transfer in the two groups was 185 and 190 respectively. The implantation and pregnancy rates per embryo transfer were 25.3% and 38.9% in the diclofenac group and 21.6% and 32.6% in the control group. The use of diclofenac sodium, therefore, did not significantly compromise the implantation and pregnancy rates. Patients randomized to receive diclofenac sodium had statistically significantly reduced pain scores prior to discharge ($p = 0.030$) without compromising the treatment outcome (Kailasam 2008).

Akande et al. carried out a prospective study on infertile women undergoing IVF treatment to assess the effect of diclofenac on implantation rates, when administered as analgesia following transvaginal oocyte recovery.

Subjects were divided in two groups. Group A (n = 38) received 1 g paracetamol and 100 mg diclofenac and group B (n = 36) received 1 g paracetamol only. All medication was administered rectally immediately after the oocyte retrieval. Pregnancy and implantation rates were compared between group A and B using the chi2 test. In groups A and B, the implantation rates were 12.4% and 9.6% (p = 0.5) and the pregnancy rates were 28.9% and 19.4%, respectively (p = 0.67). Neither pregnancy nor implantation rates differed significantly between the two groups. The administration of diclofenac to patients at the time of egg collection did not appear to affect implantation or pregnancy rates, while being effective in reducing discomfort and pain associated with oocyte retrieval (Akande 2006).

5. GASTROINTESTINAL SIDE EFFECTS

The toxicity of NSAIDs related to the upper gastrointestinal tract is well established. However, they may cause injury distal to the duodenum as well, for example to the small and large intestines and to other organs of the digestive system.

NSAIDs can induce small intestinal perforations, ulcers or strictures requiring surgery and inflammation with blood and protein loss called NSAID enteropathy. These drugs can exacerbate pre-existing large bowel disease (e.g., ulcerative colitis, diverticular disease) and precipitate relapse of inactive disease or the new onset of inflammatory bowel disease with rapid resolution of symptoms on their withdrawal. They have been implicated in the development of microscopic colitis. NSAIDS-associated toxicity of the small and large bowel is increasingly recognized in clinical practice, as enteroscopic procedures become more frequently used (Kasztelan-Szczerbinska 2010).

Liver injury is an uncommon, but potentially lethal complication. It can occur with all NSAIDs, but diclofenac and sulindac seem to be most commonly associated with the problem. These drugs may contribute to acute fatty liver of pregnancy. Hepatotoxicity is likely due to an idiosyncratic reaction resulting from an immunological response or altered metabolic pathways. The benefits of NSAIDs relate to reports of possible prevention, delay or regression of progress towards cancers of the colon, oesophagus, stomach as well as that of cancers of the breast, lung, prostate and skin. Despite their promise, NSAIDs are not yet recommended for prevention or treatment of any cancer (Kasztelan-Szczerbińska 2010).

6. FINAL STATEMENTS

- Diclofenac is a potent non-steroidal anti-inflammatory drug that is used in a wide variety of gynecological and obstetric processes for the treatment of a variety of inflammatory and painful diseases.
- In all areas analyzed, diclofenac was an optimal analgesic regimen.
- The administration of diclofenac for pain relief, like other NSAIDs, should be avoided during late gestation whenever possible. With their easy transplacental transfer, their unpredictable pharmacodynamics in the fetus, and their profound effect on ductal constriction, this group of drugs can have highly detrimental effects on the fetus and the neonate (Auer 2004).
- The main concerns of drug therapy in pregnancy are possible teratogenicity of drugs, adverse effects on fetal growth and development, a negative effect on the progress of pregnancy and harmful effects on the neonate (Ostensen 2004).
- No effort should be spared in attempting to prevent the unnecessary administration of NSAIDs, especially in high risk patients such as patients with hypovolemia or pre-existing renal problems. These efforts should include the choice of compounds with a lower nephrotoxic potential, identification of the most appropriate dosage,

avoidance of concomitant administration of other nephrotoxic drugs, provision for early diagnosis of nephrotoxicity, and limitation of the duration of the treatment. Future research in this field should be undertaken to focus on the actual role of NSAIDs in clinical practice for each age group, concentrating particularly on pregnant women (Musu 2011).

- NSAIDs should be given in pregnancy only if the maternal benefits outweigh the potential fetal risks, at the lowest effective dose and for the shortest duration possible (Antonucci 2012).
- Co-treatment with diclofenac and misoprostol does not interfere with the action of mifepristone and misoprostol to induce uterine contractions and pregnancy expulsion in medical abortion. Prophylactic diclofenac administration reduces the need for supplementary analgesia. Co-treatment of diclofenac with misoprostol did not attenuate the efficacy of mifepristone and misoprostol given during medical abortion (Fiala 2005).
- Administration of diclofenac did not appear to affect implantation or pregnancy rates, did not compromise treatment outcome, while being effective in reducing discomfort and pain associated with oocyte retrieval (Akande 2006, Kasztelan-Szczerbińska 2010).
- The toxicity of NSAIDs related to the upper gastrointestinal tract is well established.

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Chapter 7

NOVEL APPROACHES TO MANAGE TOXICITY OF DICLOFENAC IN THE GASTROINTESTINAL TRACT

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ABSTRACT

The cyclooxygenase (COX) inhibitor diclofenac (DCF) is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) for managing pain and inflammation. The COX enzymes are key enzymes responsible for the conversion of arachidonic acid to prostaglandin E_2 (PGE_2) and other chemical mediators which enhance pain and inflammation but generally protect gastrointestinal (GI) tract. Reduction in the level of PGE_2 by DCF makes the GI tract vulnerable to ulceration. Interestingly, cytochrome P450 (P450) enzymes generate analgesic and anti-inflammatory epoxyeicosatrienoic acids (EETs) from cell membrane-derived arachidonic acids, ω-6 fatty acids. Similarly, P450s also generate anti-inflammatory epoxydocosapentaenoic acid (EDPs) from docosahexaenoic acid (DHA) and other anti-inflammatory epoxy fatty acids from related ω-3 lipids. Inducers of P450s including anti-ulcer medicine omeprazole increase the level of epoxy fatty acids and can reduce inflammation. The epoxy fatty acids are labile to metabolism by soluble epoxide hydrolase (sEH). Inhibition of the sEH alleviates pain and inflammation associated with GI ulcers. We have focused our discussion on anti-ulcer effects of a soluble epoxide hydrolase (sEH) inhibitor (sEHI) N-[1-(1-oxopropyl)-4-piperidinyl]-N′-[4-(trifluoromethoxy) phenyl]-urea (TPPU) in DCF-induced gastric and intestinal ulcers and on the anti-ulcer effects of the proton pump inhibitor omeprazole (OME). The sEHIs can also decrease pain, potentiate anti-inflammatory effects of NSAIDs and decrease the ulcerative potential of NSAIDs. Co-formulation of TPPU with low dose DCF can reduce the GI side effects. These side effects of NSAIDs act to counter the very benefits offered by these drugs. Possibly, by blocking these side effects of NSAIDs, we can make NSAIDs even more effective and be able to use them at lower doses. We also discussed about anti-ulcer effects of many other pharmacological agents. The Δ^9-tetrahydrocannabinol (THC), steroid (dexamethasone), and inhibitors of fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), JNK, β-glucuronidase, and cyclophilin D exhibit anti-ulcer effect against NSAIDs including DCF.

Keywords: diclofenac, soluble epoxide hydrolase inhibitor, gastrointestinal ulcer, FAAH inhibitor, JNK inhibitor, MAG lipase inhibitor

1. INTRODUCTION

DCF is one of the most widely used NSAIDs due to its efficacy to reduce pain, inflammation, and fever. DCF is known to be effective in reducing acute pain including pain due to tooth extraction, ankle sprain, postepisiotomy, dysmenorrhea, bunionectomy, etc. However, DCF is not the preferred drug for acute pain, and often opioid analgesics are prescribed to manage acute pain, particularly, in the case where NSAIDs fail to alleviate pain. As a relatively common NSAID, DCF is used to manage pain and inflammation associated with osteoarthritis and rheumatoid arthritis but has poor efficacy and can be counterproductive with neuropathic pain. As an aside, DCF also is used to treat actinic keratoses (Werner et al., 2015). Please, refer to the excellent review article by Altman et al. to learn more detail about the use and adverse effects of DCF (Altman et al., 2015).

The availability of various dosage forms such as injectables, tablet, capsule, and topical preparations including gel, patch, and solution is also responsible for the popularity of DCF. The Cmax for the DCF 50 mg tablet is approximately 2μg/mL, and extended-release tablets have been developed to minimize the use of multiple dosing and adverse effects. Even with the availability of many other NSAIDs and cyclooxygenase-2 selective inhibitors (coxibs) in the USA, more than 10 million prescriptions containing DCF were dispensed in 2012 (Altman et al., 2015). The wide use of DCF is also a cause of concern considering the adverse effects associated with its widespread and long-term use (Altman et al., 2015).

The adverse effects of DCF, including GI toxicity, due to inhibition of COX enzymes and the reduction in the level of prostaglandin are discussed. We have recently shown that DCF-mediated toxicity results in dysfunction of mitochondria and multiple downstream effects including inflammation (Ghosh et al., 2016; Goswami et al., 2016; Goswami et al., 2017). We suggest the hypothesis that the efficacy of NSAIDs is limited by the toxicity resulting from mitochondrial dysfunction and ER stress. By blocking these side effects with epoxy fatty acids-stabilized by sEHI or their mimics, we might rescue the primary beneficial roles of NSAIDs including DCF as anti-inflammatory and analgesic drugs.

2. THE PATHOLOGICAL AND BIOCHEMICAL MANIFESTATION OF DCF TOXICITY IN GI TRACT

Ulcers are evaluated by ulcer numbers, ulcerated area and/or ulcer index. A decrease in the levels of protein in serum including albumin and hemoglobin in blood is observed along with an increase in inflammatory markers in DCF-induced ulcers. Like other NSAIDs, the gastrointestinal ulcers created by DCF are associated with a decrease in the levels of PGE_2 and PGD_2 in the stomach and intestine (Kinsey et al., 2011; Fukui et al., 1988; Atchison et al. 2000a; Naidu et al., 2009; Ramirez-Alcantara et al., 2009; Goswami et al., 2016, Goswami et al., 2017).

The intestine is more vulnerable to the ulcerative effect of DCF when compared with stomach likely due to prolonged exposure of intestine to the DCF. After absorption from GI tract, DCF is biotransformed by the Phase II metabolic enzyme (UDP-glucuronosyltransferase (UGT)) in hepatocyte to generate the DCF acyl-β-D-glucuronide which then across the hepatocanalicular membrane through the conjugate export pump into bile (Zhu and Zhang, 2012). The DCF conjugates reach the intestine with bile. In the intestine, higher local pH favors cleavage of glucuronides by glucuronidases of the intestinal bacteria, and DCF again enters the hepatic circulation to complete another round of entero-hepatic circulation. Atchison et al. (2000a) through staining showed the formation of the DCF adduct in the cytoplasm of enterocytes after one h of DCF administration in the rats. By 3h, the DCF adduct concentrates in brush border/microvilli and ulceration starts in enterocytes by 6h when the concentration of adduct is higher. Some medicines including the antibacterial chloramphenicol is a competitive inhibitor/alternate substrate of the glucuronidase in the intestine and decrease the ulcerative potential of DCF (Atchison et al. 2000a; A Boelsterli and Ramirez-Alcantara, 2011; LoGuidice et al., 2012; Zhong et al., 2016; Saitta et al., 2014). Hepatocanalicular conjugate export pump-deficient (TR^-) Wistar rats were resistant to the ulcerative effect of DCF administered orally and parenterally (Seitz and Boelsterli, 1998). Phase I drug-metabolizing enzymes including CYP2C and CYP3A metabolize DCF

to 4- hydroxylated DCF (4-OH-DCF) and 5-hydroxylated DCF (5-OH-DCF), respectively. These derivatives are further metabolized to generate highly reactive and unstable quinoneimines which react with thiols including the endogenous antioxidant molecule glutathione (GSH). Alternatively, they can react with thiols on other proteins resulting in the further development of ulcerations. Silencing of the cytochrome P450 reductase (CPR), an enzyme that transfers the electron from NADPH to cytochrome P450 in the endoplasmic reticulum reduces intestinal ulceration by DCF (50 mg/kg, p.o.). Administration of grapefruit juice (20 ml/kg; 2x concentrate, p.o.) which interferes with the activity of many P450 enzymes and particularly P4503A4 2h before administration of DCF also decreases the ulcerative capacity of DCF in the intestine (Zhu and Zhang, 2012). Interestingly, low dose (10 mg/kg, 28 days) treatment with DCF does not result in gastric ulceration or enteropathy but results in dysfunction of cardiac mitochondria and proteasome (Ghosh et al., 2016). Age-dependent effects on the ulcerative potential of DCF have been demonstrated. Atchison et al. demonstrated that rats of 22 months age were susceptible to gastric ulceration but were resistant to intestinal ulcers after exposure to DCF (Atchison et al., 2000b). This is an important observation considering the large percentage of the aged population suffer from arthritis and have to rely on supplementation of DCF. Imaging is an integral part of the evaluation of ulceration and development of an effective treatment plan.

Endoscopy data are a reliable source for detection of ulceration with wireless capsule endoscopy advancing rapidly. In animal models, invasive procedures are followed to quantify ulceration. Generally, for histopathological evaluation of gastrointestinal ulcers, hematoxylin and eosin staining (H&E) is performed (Goswami et al., 2016, Goswami et al., 2017; Ramirez-Alcantara et al., 2009). During the anatomical evaluation of ulcers, a reflection of light from gastrointestinal tissue prevents proper imaging *in vitro*. Imaging under natural light and use of 4-nitro blue tetrazolium chloride staining which stains tissue around ulcers provide a good contrast for imaging (Ramirez-Alcantara et al., 2009). Intravenous administration of Evans blue dye (100 µL) before 30 min of ulcer evaluation also provides excellent contrast for imaging (Zhu and Zhang, 2012).

3. Strategies to Manage Gastrointestinal Toxicity of Diclofenac

Inhibition of COX-1 facilitates the gastrointestinal adverse events while COX-2 inhibition is likely to be responsible for the development of cardiovascular adverse events. Strategies to prepare slow-release formulations, reduce dose dumping, increase solubility and augment bioavailability are shown to be effective in reducing GI toxicity. Dose dumping is a situation where the body is exposed to large amount of drug due to the premature and a sudden release of drug in the GI tract due to environmental factors including food and additives associated with the formulation or other medications. Also, administration of other medicines along with DCF to decrease the generation of acid such as proton pump inhibitors, increase the production of mucus such as misoprostol or protect existing ulcers such as sucralfate also is reported in the management of GI ulcers.

3.1. Pharmaceutical and Formulation Strategy

Developing extended-release/slow release dosage forms which release small amount DCF over periods of time in the GI tract is intended to minimize the dose-dependent toxicity (Fowler et al., 1986; Chan et al., 2010). There is a concern that extended-release tablets may increase the GI and CV adverse events due to persistent inhibition of both isoforms of COX. The anti-ulcer agent misoprostol, a synthetic analog of prostaglandin was also formulated along with DCF as a sequential release tablet to minimize the ulcerative potential of DCF (Altman et al., 2015). Functionally modified poly(acrylamide-grafted-ghatti gum)-based pH-sensitive hydrogel beads are also being developed in an aim to minimize dose dumping which favors ulceration of a local area and the formulation is prepared to release DCF over a period of time (Moin et al., 2017). Although far more expensive, the powder form of DCF is slightly more effective in decreasing migraine faster

than a tablet (Diener et al., 2006; Lipton et al., 2010). Preparation of different formulations of DCF including spray gel, gel, solution and patch for topical use to manage pain including osteoarthritis pain is aimed at decreasing systemic exposure (Brunner et al., 2005; Barthel 2009; Baraf et al., 2011; Baer et al., 2005; Roth et al., 2004; Fuller et al., 2011; Altman et al., 2009; Kuehl et al., 2011), though the use of potentially toxic dimethyl sulfoxide (DMSO) to increase solubility and absorption of DCF has been a cause for concern (Shainhouse et al., 2010; Simon et al., 2009). The elopamine salt of DCF formulated as a topical patch can alleviate soft tissue injury and ankle sprain (McCarberg et al., 2010; Lionberger et al., 2011). Capsules containing sub-micron particles of DCF (35 mg, TID) are reported to relieve pain faster than celecoxib (200 mg, TID) (Gibofsky et al., 2013). DCF is poorly soluble in water, so different solvents/excipients have been tried to increase the solubility of DCF in an injectable formulation. Polyethylene glycol and benzyl alcohol (PG-BA) containing DCF injection are available in Europe, but this formulation is not available in North America (Leeson et al., 2007). Hydroxypropyl-β-cyclodextrin (HPβCD), as an excipient, increases the solubility of DCF in intravenous (i.v.) and intramuscular (i.m.) preparations (Mermelstein et al., 2013). A HPβCD-DCF injectable preparation has shown efficacy in reducing pain arising due to dental extraction and orthopedic surgery (Christensen et al., 2011; Leeson et al., 2007; Daniels et al., 2013). Guhmann et al. reported that DCF as a free acid form is less likely to cause dose dumping with varied diets as seen with sodium and potassium salts (Guhmann et al., 2013). Sub-micron size DCF acid at low doses (18 and 35 mg) successfully decreased pain due to bunionectomy and osteoarthritis (Gibofsky et al., 2013; Gibofsky et al., 2014).

3.2. Pharmacological Strategy

Alternatively, medicines which either reduce the formation of acid by parietal cells of the stomach including omeprazole or promote the secretion of mucus and bicarbonate such as misoprostol are prescribed along with DCF to reduce gastrointestinal side effects. Though the degree and number

of ulcerations caused by DCF decrease with co-administration of omeprazole and misoprostol, these agents also exhibit mechanism-based adverse effects (Altman et al., 2015). Misoprostol administration is associated with diarrhea and abdominal cramps, especially with higher doses (Raskin et al., 1995; Silverstein et al., 1995). Proton pump inhibitors including omeprazole are the mainline drugs to manage DCF-induced gastric ulcers. Though omeprazole is a better option as an anti-ulcer agent, adverse events including diarrhea and dry mouth are reported, and these are cited reasons for discontinuation of treatment (Altman et al., 2015; Massimo et al., 1998). Omeprazole administration is associated with changes in the number and types of microbial flora in the intestine (dysbiosis) due to alteration in the pH of the intestine (Wallace et al., 2011). Similar dysbiosis may be expected with acid-suppressing medicines including histamine H_2 receptor blocker ranitidine.

Researchers in the field of pain and inflammation have been persistently evaluating new chemicals to develop novel medicines which act via other targets to manage DCF-induced gastrointestinal ulcers. Inhibition of the c-Jun-N-terminal kinase (JNK) (Ramirez-Alcantara et al., 2009), fatty acid amide hydrolase (FAAH) (Naidu et al., 2009), monoacylglycerol lipase (MAGL) (Kinsey et al., 2011), and sEH (Goswami et al., 2016; Goswami et al., 2017) protects tissues from the ulcerative effects of DCF. Here, we discuss the anti-ulcer effect of sEH inhibitor TPPU and P450 mediated the anti-ulcer effect of omeprazole. It should be noted that recently FAAH and sEH inhibitors (Kodani et al., 2018) as well as COX and sEH inhibitors (Hwang et al., 2013) are incorporated into single molecules with a plan of reducing GI erosion and developing synergistic control of pain and inflammation. The sEHI TPPU is effective by blocking the metabolism of epoxy fatty acids by sEH, thus increasing these beneficial chemical mediators, whereas the efficacy of omeprazole is, at least, partly mediated via induction of various P450s and formation of anti-inflammatory fatty acids which block endoplasmic reticulum stress and protect mitochondrial and proteasomal function.

4. Methods to Conduct Ulceration Study, Detect Ulceration, and Quantify Ulcers

Generally, the animals including mice are kept on a special cage with wire mesh on the bottom of the cage to prevent coprophagy which may interfere with the assay results. Animals are also kept fasted before the study so that quantification of the ulcers are easy, and it helps in collection of tissue for biochemical estimation later. Time taken to wash the stomach and intestine are shorter when the animals fasted (Goswami et al., 2016 and Goswami et al., 2017). Animals also should be accustomed to restraint cages if the cages would be used to withdraw blood to quantify different markers and concentration of study drugs.

Animals are anesthetized, and gastrointestinal tracts are collected. Stomachs are opened at the greater curvature while dipped in cold phosphate saline buffer. Immediately, the images of the stomachs are taken with a digital camera attached to a microscope because the detection of ulceration in the stomach with naked eye could be difficult. The stomachs are then stored at -80 ˚C for biochemical assay and a portion of the stomachs also stored for Hematoxylin and Eosin (H&E) staining to visualize ulceration at tissue/ cellular level. The pH of the stomachs is also measured when one of the study drugs is expected to decrease the production of acid including omeprazole and ranitidine. Similarly, intestines are cut longitudinally at the mesentery. The ulceration of the intestines is more prominent in comparison to stomachs. The number of ulcers can be counted without the aid of a microscope, but images with a camera help in measuring the areas of ulceration by planimetry. Intestines are also stored at -80 ˚C for biochemical assay and a portion of the intestines also stored for H&E staining (Goswami et al., 2016, Goswami et al. 2017). Some researchers use stains i.e., 4-nitro blue tetrazolium chloride *in vitro* (Ramirez-Alcantara et al., 2009) or Evans blue *in vivo* (Zhu and Zhang, 2012) for visualizing the ulcers better. Blood samples are also collected to quantify inflammatory markers, prostaglandins and epoxy fatty acids in plasma. The tissue samples are also processed to quantify inflammatory markers and level of prostaglandins (Goswami et al.,

2016, Goswami et al., 2017). Tissue samples are also processed for studying the expression of important proteins and levels of RNAs.

5. A NOVEL MECHANISM TO ALLEVIATE GI SIDE EFFECT OF DCF

5.1. Arachidonic Acid/P450s/sEH Branch Can Be Modulated for Anti-Inflammatory Effect

Arachidonic acid is a ω-6 fatty acid released from the cell membrane. Unlike the cyclooxygenase and lipoxygenase branches, the P450 branch of the arachidonic acid cascade is important in biology as a largely pro-resolving, anti-inflammatory and analgesic pathway. Epoxy fatty acids generated from both ω-3 and ω-6 fatty acids reduce pain and inflammation and resolve both. The ω-3 fatty acid epoxides appear to be more potent. As expected, the sEH inhibitors which stabilize the epoxy fatty acids reduce pain and inflammation. Relevant to this chapter, the sEHIs have an additive to synergistic effect in potentiating analgesic effects of NSAIDs and synergize anti-inflammatory effect of NSAIDs and COXIBs. The synergism is due to multiple mechanisms, but certainly part of it is the combination of analgesic and anti-inflammatory compounds working by very different mechanisms. For example, NSAIDs cannot block PGE_2-induced pain but sEHI can, and sEHI transcriptionally down regulates induced COX-2 and other inflammatory mediators. One would expect an enzyme inhibitor to synergize with a transcriptional down-regulator of COX. TPPU decreases PGE_2-induced pain and omeprazole potentiates the action of TPPU by elevating the generation of EETs via induction of P450 1A and 2B and possibly other isoforms. This implies that omeprazole and TPPU should have additive or synergistic effect in reducing inflammation including that associated with ulcers. The sEHI *t*-AUCB protects from piroxicam and DSS-induced inflammatory bowel disease. The sEHIs protect from NSAID-induced GI erosion, and also protect from cardiovascular effects and platelet

instability associated with COX-2 inhibition. Additionally, epoxy fatty acids and sEHIs alleviate mitochondrial dysfunction and ER stress. Therefore, sEHIs can decrease toxicity of NSAIDs mediated via mitochondrial and ER stress (Schmelzer et al., 2006; Fer et al., 2008; Morisseau et al., 2010; Liu et al., 2010; Inceoglu et al., 2011; Zhang et al., 2012; Batchu et al., 2012; Zhang et al., 2013; Hwang et al., 2013; Inceoglu et al., 2015; Harris et al., 2015; Akhnokh et al., 2016; Inceoglu et al., 2017). In the following paragraphs, we will discuss the anti-ulcer effect of sEHI TPPU and proton pump blocker OME in more detail.

5.2. Inhibitors of sEH Alleviate DCF-Induced Gastrointestinal Ulcers

The COX mediated metabolites of arachidonic acids are chemical mediators that generally increase pain and inflammation. Interestingly, cytochrome P450 (P450) enzyme-mediated metabolites of arachidonic acid possess analgesic and anti-inflammatory properties (Goswami et al., 2015; Goswami et al., 2017; Goswami et al., 2017). Arachidonic acid is metabolized to 4 regioisomers of epoxyeicosatrienoic acids (EETs) by P450s. Ironically, the EETs are oxidized, esterified or hydrolyzed to less active metabolites (Revermann M, 2010). Hydrolysis largely by the sEH is a key step in the metabolism of EETs to generate less active dihydroxy derivatives called dihydroxyeicosatrienoic acids (DHETs). Chemical inhibitors of sEH which stabilize the levels of EETs or decrease the formation of DHETs dramatically reduce pain and intestinal inflammation in different animal models and alleviate ulcers generated by DCF (Zhang et al., 2012; Morisseau and Hammock, 2013; Kodani and Hammock BD, 2014; Inceoglu et al., 2015; Goswami et al., 2015; Goswami et al., 2016, Goswami et al., 2017).

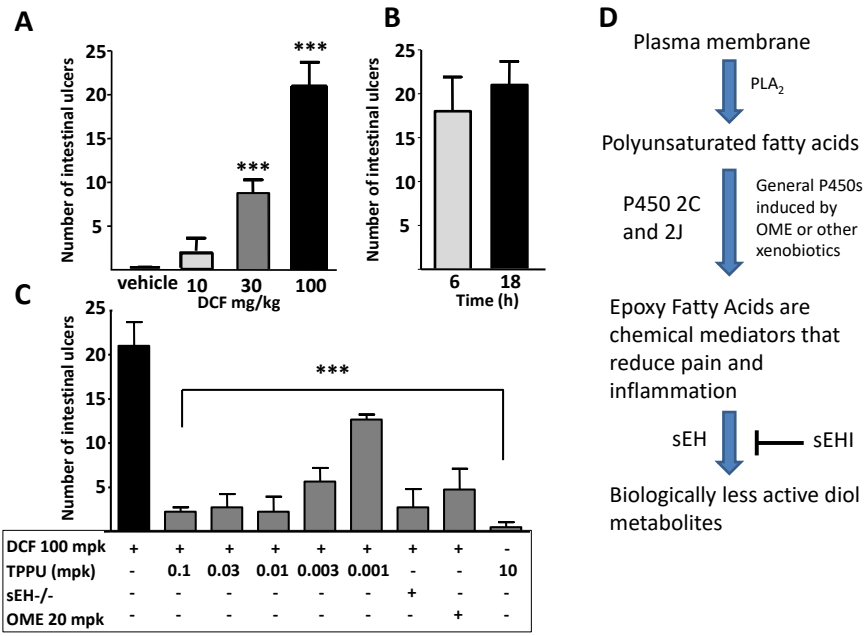

Figure 1. DCF generates ulcers in a dose-dependent fashion, and inhibition of sEH dose-dependently ameliorates ulcers. (A) 30 and 100 mg/kg dose of DCF dose-dependently generated ulcers after 6 h of oral administration in Swiss-Webster mice. *** $p < 0.001$, DCF vs vehicle. (B) The ulcerative potential of DCF was active up to 18 h after administration of DCF. (C) Pre-treatment with sEH inhibitor TPPU in drinking water containing 1% polyethylene glycol 400 for 1 week before administration of DCF decreased intestinal ulcers. A dose-dependent effect was observed from 0.001 mg/kg to 0.1 mg/kg dose of TPPU. Genetic deletion of sEH also alleviated DCF-led ulcers. Pre-treatment of a single oral dose of OME (20 mg/kg) before administration also decreased small intestinal ulceration. Administration with 10 mg/kg dose of TPPU did not cause intestinal ulceration. *** $p < 0.001$, DCF vs DCF + TPPU or sEH gene deletion or OME. Values are presented as mean ± SEM of 4 observations. One-way ANOVA followed by Tukey's multiple comparison test was used for calculating statistical significance. D) Phospholipase A_2 (PLA_2) generates polyunsaturated fatty acids from plasma membrane. Polyunsaturated fatty acids are epoxigenated by cytochrome P450 2C and 2J with high efficiency. Other P450s including 1A, 2B and 3A can also significantly generate epoxy fatty acids when induced by OME or other xenobiotics. Epoxy fatty acids are analgesic and anti-inflammatory, and are metabolized by sEH to less active diol. Inhibition of sEH stabilizes epoxy fatty acids to exert its analgesic and anti-inflammatory properties. The figure (A, B and C) is reproduced with permission. Refer Goswami SK, Wan D, Yang J, Trindade da Silva CA, Morisseau C, Kodani SD, Yang G-Y, Inceoglu B, Hammock BD. Anti-ulcer efficacy of soluble epoxide hydrolase inhibitor TPPU on DCF-induced intestinal ulcers, *J Pharmacol Exp Ther*, 2016;357(3):529-536.

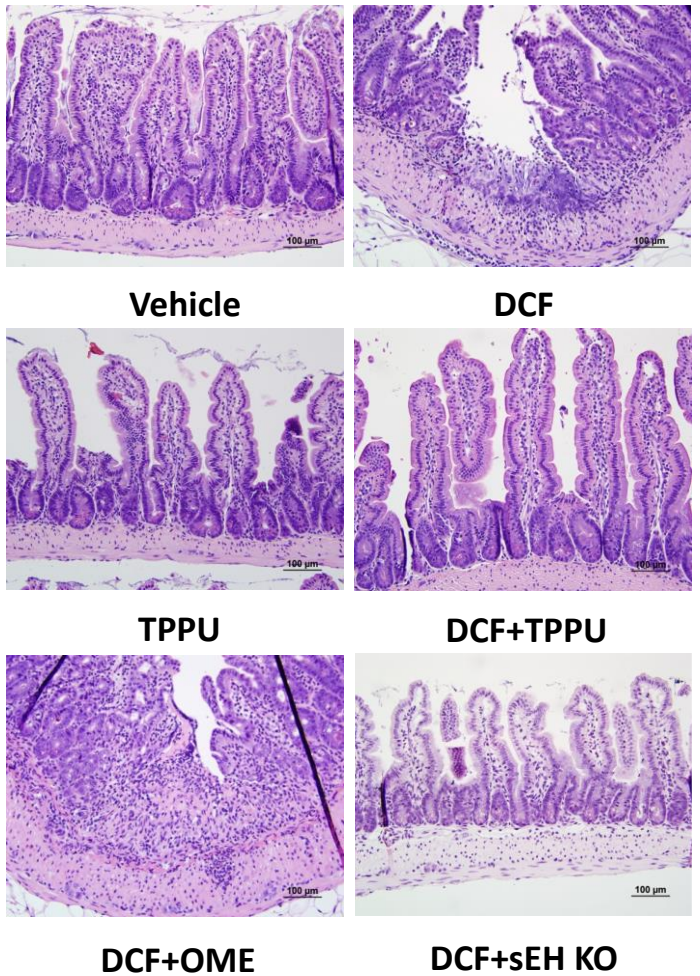

Figure 2. Histopathological observation of intestine with the help of hematoxylin and eosin staining reveals damage to the entire mucosal layer of the intestine of mice receiving DCF. A clear and healthy mucosa having distinct crypts were observed in the intestine of mice receiving the vehicle. Treatment with only TPPU did not damage intestinal mucosa. Pre-treatment with TPPU or OME decreased the ulcerative potential of DCF. Similarly, genetic deletion of sEH also protected the intestine from the ulcerative effect of DCF. Figures of mice intestine are shown at 100X. The figure is reproduced with permission. Refer Goswami SK, Wan D, Yang J, Trindade da Silva CA, Morisseau C, Kodani SD, Yang G-Y, Inceoglu B, Hammock BD. Anti-ulcer efficacy of soluble epoxide hydrolase inhibitor TPPU on DCF-induced intestinal ulcers, *J Pharmacol Exp Ther*, 2016;357(3):529-536.

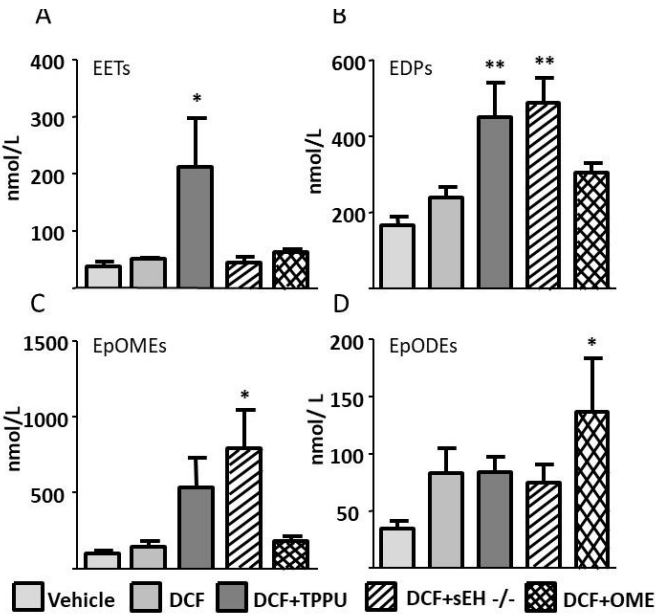

Figure 3. Anti-ulcer effect of TPPU, OME or sEH deletion was associated with an increase in the epoxy fatty acids in the plasma of treated mice. (A, B) Anti-ulcer effect of TPPU was associated with an increase in the level of anti-inflammatory epoxyeicosatrienoic acids (EETs), and epoxy docosapentaenoic acids (EDPs) derived from arachidonic acid and docosahexaenoic acid (DHA), respectively. (B, C) The anti-ulcer effect observed with deletion of sEH was associated with an elevation in the levels of EDPs, and linoleic acid-derived epoxy octadecenoic acids (EpOMEs) in plasma. (D) The anti-ulcer effect of OME was associated with an increase in the level of epoxy octadecadienoic acids (EpODE) derived from α-linolenic acid. Values are presented as mean ± SEM of 4 observations. One-way ANOVA followed by Dunnet's test was used for calculating statistical significance. *P < 0.05, **P < 0.01 vs vehicle. The figure is reproduced with permission. Refer Goswami SK, Wan D, Yang J, Trindade da Silva CA, Morisseau C, Kodani SD, Yang G-Y, Inceoglu B, Hammock BD. Anti-ulcer efficacy of soluble epoxide hydrolase inhibitor TPPU on DCF-induced intestinal ulcers, *J Pharmacol Exp Ther*, 2016;357(3):529-536.

Oral administration of DCF dose-dependently (30 and 100 mg/kg) increased ulceration of the intestine in comparison to animals treated with either vehicle or the sEH inhibitor TPPU (0.001-0.1 mg/kg). TPPU dose-dependently decreased DCF-induced (100 mg/kg) intestinal ulcers. Mice treated with omeprazole one hour before DCF administration or lacking sEH were resistant to the ulcerative effect of DCF (Figure 1). For administration,

TPPU was dissolved in PEG400 and subsequently diluted in distilled water being careful to avoid precipitation. The histological study, through H&E staining, revealed intestinal ulceration in the epithelial cells and lamina propria of the mucosal layer of mice treated with DCF. Pre-treatment with TPPU and omeprazole (20 mg/kg) or genetic deletion of the sEH protected the mucosal layer from the ulcerative effects of DCF (Figure 2).

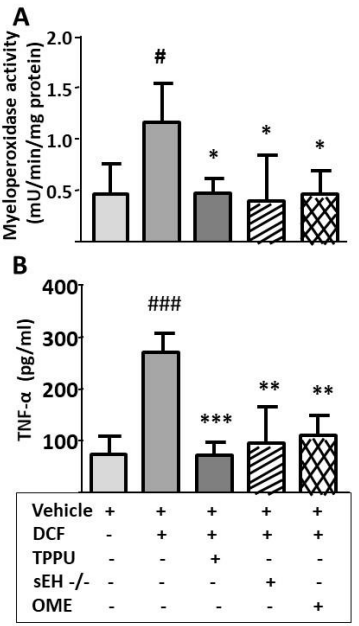

Figure 4. TPPU and OME minimize intestinal ulcer associated oxidative stress and inflammation. (A) The activity of neutrophil-derived myeloperoxidase in the intestine of mice receiving DCF was increased, in comparison to mice receiving the vehicle, $^{\#}$ $p < 0.05$. Neutrophil infiltration decreased in the intestine of mice receiving TPPU or OME before DCF treatment compared to DCF-treated mice. The sEH knockout mice were also protected from neutrophil infiltration with respect to wildtype mice treated with DCF, * $p < 0.05$. (B) An increase in the level of inflammation marker, TNF-α, was observed in the serum of mice treated with DCF, in comparison to the mice treated with vehicle, $^{\#\#\#}$ $p < 0.001$. Pre-treatment with TPPU or OME alleviated DCF-induced inflammation, in comparison to mice receiving only DCF. Knockdown of sEH also protected mice from the DCF-led intestinal ulcer, ** $p < 0.01$, *** $p < 0.001$. The figure is reproduced with Permission. Refer Goswami SK, Wan D, Yang J, Trindade da Silva CA, Morisseau C, Kodani SD, Yang G-Y, Inceoglu B, Hammock BD. Anti-ulcer efficacy of soluble epoxide hydrolase inhibitor TPPU on DCF-induced intestinal ulcers, *J Pharmacol Exp Ther*, 2016;357(3):529-536.

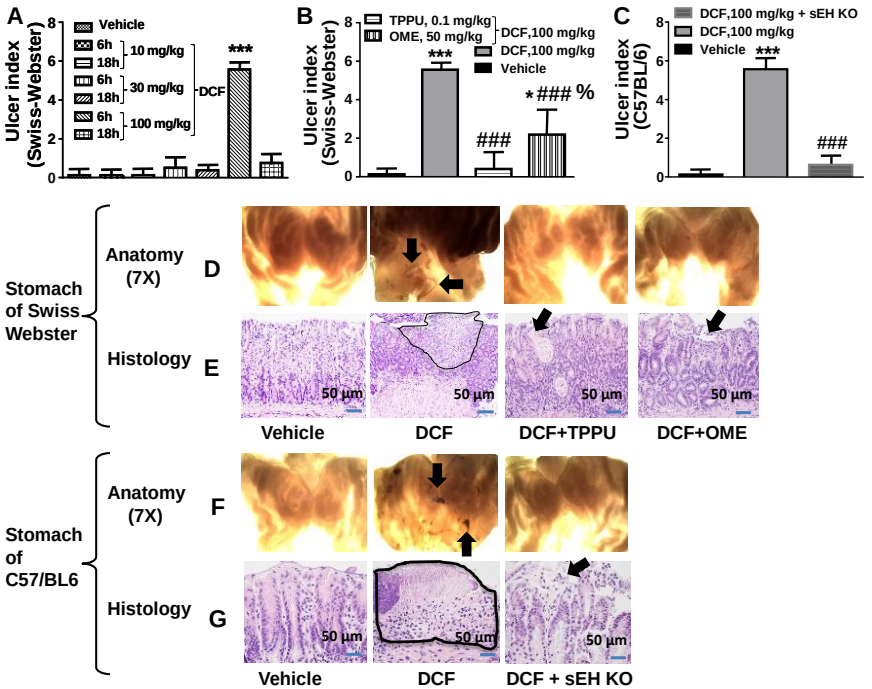

Figure 5. Inhibition or deletion of sEH protects the stomach from the ulcerative potential of DCF. (A) A dose-response study was performed to evaluate the ulcerative potential of ulcer in the stomach. Oral administration of DCF (100 mg/kg) to Swiss-Webster mice leads to the generation of ulcers after 6 h, in comparison to mice treated with vehicle (*** $p < 0.001$) and the ulcers are resolved by 18 h. (B) Pre-treatment with sEH inhibitor TPPU (0.1 mg/kg, p.o., 7 days) and proton pump inhibitor OME (50 mg/kg, p.o., 5 days) to Swiss-Webster mice prevented DCF-driven gastric ulcers. *** $p < 0.001$, DCF vs vehicle; * $p < 0.05$, DCF + OME vs vehicle; ### $p < 0.001$, DCF vs DCF+TPPU or DCF+OME; TPPU was more effective in reducing stomach ulcers than OME. $^{\%}p < 0.05$, DCF+TPPU vs DCF+OME. (C) Administration of DCF to C57BL/6 mice also resulted in gastric ulceration (*** $p < 0.001$, DCF vs Vehicle). Deletion of sEH also protected the stomach from the development of ulcers. (D and F) Anatomical study of the stomach was useful in studying the ulcerative potential of DCF and anti-ulcer potential of TPPU, OME or genetic deletion of sEH. (E and G) The histopathological study supported the finding of anatomical observation. Ulcers are either marked with closed lines or pointed with arrows. Values are presented as mean ± SEM of 3-5 observations. One-way ANOVA followed by Tukey's multiple comparison was used for calculating statistical significance. The figure is reproduced with permission. Refer Goswami SK, Rand AA, Wan D, Yang J, Inceoglu B, Thomas M, Morisseau C, Yang GY, Hammock BD. Pharmacological inhibition of soluble epoxide hydrolase or genetic deletion reduces DCF-induced gastric ulcers. *Life Sci*. 2017;180:114-22. doi: 10.1016/j.lfs.2017.05.018.

The anti-ulcer effect of TPPU (0.1 mg/kg), omeprazole or the sEH knockout was associated with a significant increase in the epoxide containing metabolites of arachidonic acid (ω-6 fatty acid), docosahexaenoic acid (DHA, ω-3 fatty acid), linoleic acid (ω-6 fatty acid) or α-linolenic acid (ALA, ω-3 fatty acid) in the plasma of mice (Figure 3). Loss of blood hemoglobin was associated with DCF-induced enteropathy. Pre-treatment with either TPPU or omeprazole minimized a decrease in the level of hemoglobin in the blood. A significant increase in the activity of myeloperoxidase was increased in the intestine of mice treated with DCF indicating neutrophil infiltration to the area of ulceration. Also, a significant increase in the level of TNF-α was observed in the serum of DCF treated mice indicating inflammation. Pre-treatment with TPPU or omeprazole or silencing of the sEH gene significantly decreased the ulcerative effect of DCF (Figure 4).

Oral administration of DCF at 100 mg/kg induced gastric ulcers in Swiss Webster and C57BL/6 mice. Pre-treatment with TPPU (0.1 mg/kg), omeprazole (50 mg/kg) or genetic deletion of sEH protected mice from the ulcerative effect of DCF which was evident from a decrease in the ulcer index and mucosal erosion (Figure 5). Anti-ulcer effect of TPPU was not associated with alteration of pH but, as expected, omeprazole increased the pH of the stomach to exert its anti-ulcer effect presumably due to its effect as a proton pump inhibitor. Ulcerative effects of DCF were associated with a two-fold increase in the level of TNF-α and IL-6 in serum. Pre-treatment of TPPU or omeprazole, as well as the sEH knockout, minimized the increase in the level of these inflammatory markers in serum. The level of apoptosis on epithelial cells of the stomach was twice as high in mice exposed to DCF than mice treated with vehicle. The degree of apoptosis was comparable in mice which received DCF and were pre-treated with TPPU, omeprazole or that lacked sEH (Figure 5).

The sEHIs including TPPU decrease pain and inflammation (Schmelzer et al., 2006; Inceoglu et al., 2015). These compounds also potentiate analgesic and anti-inflammatory effect of NSAIDs and COXIBs (Guedes et al., 2013; Schmelzer et al., 2006). Patrignani et al. (2011) discussed lowering the dose of NSAIDs as one of the ways to minimize the adverse effects of

NSAIDs. In this regard, co-administration of sEH inhibitors with NSAIDs can reduce the dose of NSAIDs needed to reduce pain and inflammation, and ameliorate gastrointestinal side effects. To treat or prevent ulceration, if sEH inhibitors are included in low release formulation with DCF, they would be released at the site of action where DCF causes side effects. The analgesic effects of TPPU are not through inhibition of COX. At a dose higher than 1000 times the anti-ulcer dose does not create ulcers in mice (Goswami et al., 2016). The sEHIs are being evaluated clinically (Imig and Hammock, 2009; Lazaar et al., 2016; McReynolds et al., 2016) for their use as therapeutic agents for multiple diseases and may be used with NSAIDs including DCF to reduce the dose and increase the effectiveness of NSAIDs while blocking the formation of gastrointestinal ulcers. The positive additive effects of sEHI with NSAIDs may facilitate clinical trials by not having to remove patients from their standard therapy for pain and inflammation.

5.3. Anti-Ulcer Effects of Omeprazole Might Be Mediated through Multiple Mechanisms

The proton pump inhibitor omeprazole is prescribed clinically for gastric ulcers. Its anti-ulcer effect is thought to be due to decreasing the generation of acid in the stomach by inhibiting proton pumps. However, the efficacy of omeprazole in reducing intestinal ulcers where the pH is alkaline suggests that omeprazole might have additional mechanisms of action. Kinsey et al. demonstrated that the anti-ulcer effect of omeprazole is not mediated via cannabinoid receptors i.e., type 1 and 2 (Kinsey et al., 2011). Omeprazole also does not alter the level of prostaglandins (PG-6-keto PGF_1 alpha, PGF_2 alpha, and PGD_2) (Fukui et al., 1988), but it increases the level of thromboxane/PG ($TxB2/PGE_2$) when administered with DCF in comparison to DCF treatment alone (Goswami et al., 2017). We demonstrated that administration of omeprazole increased the level of epoxyoctadecadienoic acid (EpODEs) which are metabolites of ω-3 fatty acid α-LA, in plasma of mice without any supplementation of α-LA in the mice chow (Goswami et al., 2016). Because ω-3 Fatty acids exhibit analgesic and anti-inflammatory properties and their metabolites are

generally more potent than EETs in reducing pain and inflammation, we suggest that anti-ulcer property of omeprazole could be due to an increase in the level of anti-inflammatory epoxy fatty acids (Wagner et al., 2011; Wagner et al., 2014; Wang et al., 2014; Goswami et al., 2016). Earlier, we showed that 7 days of treatment with omeprazole induce cytochrome P450 (P450) 1A1, 1A2, 2B2 and 3A1 in the liver of rats and increases the level of EETs, EDPs and epoxy-octadecenoic acid (or EpOME, metabolites of linoleic acid) in plasma in the presence of TPPU (Goswami et al., 2015). We propose that omeprazole relieves ulcer, in part, by inducing selective P450s which generate anti-inflammatory epoxy fatty acids. We also suggest that a ω-3 enhanced with an ω-6 restricted diets should reduce DCF-induced ulceration.

In this case, the effects are beneficial, but more generally a variety of environmental chemicals and drugs could act to induce or inhibit P450s involved in the metabolism of lipophilic chemical mediators resulting in unexpected side effects. For example, other chemicals including 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) are known to induce multiple P450s and generate epoxy fatty acids (Yang et al., 2013; Hankinson O, 2016). Thus, an inducer of P450s may influence analgesic and anti-inflammatory effect of NSAIDs.

6. Recent Advances to Find New Anti-Ulcer Agents

Many recent studies reported the anti-ulcer effect of many new chemicals.

6.1. Protective Effect of Cannabinoid System on DCF-Induced Ulceration

The Δ^9-tetrahydrocannabinol (THC), the primary psychoactive component of marijuana (Cannabis sativa) is shown to gastroprotective at 10 mg/kg when administered intraperitoneally to C57BL/6 mice challenged

with 100 mg/kg of DCF orally after 2 h of THC administration. The anti-ulcer effect of THC was mediated through cannabinoid receptor type 1 (CB1) but not 2 (CB2) because pre-treatment of rimonabant, a CB1 antagonist partially blocked gastroprotective effect of THC while SR144528, a CB2 antagonist failed to decrease anti-ulcer effect of THC (Kinsey et al., 2011). THC when administered intraperitoneally (0.01-100 mg/kg) and orally (1-100 mg/kg) before 1 h of DCF administration, it exerted a gastroprotective effect. A ceiling effect with respect to gastroprotective effect was observed at 10 mg/kg. THC at a dose more than 1 mg/kg exhibited analgesic effect and adverse CNS effects including immobility (≥ 5 mg/kg), hypothermia (≥ 5 mg/kg), tail withdrawal latency (≥ 10 mg/kg) and catalepsy (≥ 10 mg/kg) were also observed (Kinsey and Cole, 2013). Therefore, the THC at very low dose may be used as anti-ulcer agent, but additive property of this compound would be a cause of concern among physicians, health workers, public and regulators.

6.2. FAAH Inhibitors Alleviate Ulcer

N-arachidonoyl ethanolamine or anandamide is hydrolyzed to arachidonic acid by fatty acid amide hydrolase (FAAH) and inhibition of FAAH is reported to decrease pain and reduce ulcers created by NSAIDs including DCF (Naidu et al., 2009; Kinsey et al., 2011; Sasso et al., 2012; Grim et al., 2014). Administration of URB597 (10 mg/kg, s.c.) to 18-24h fasted mice and before 1 h of oral treatment with DCF (30 and 100 mg/kg) protected stomachs from the ulcerative effect of DCF when evaluated 6 h post the NSAID administration. The anti-ulcer potential of FAAH inhibitor was mediated through the cannabinoid receptor type 1 (CB1). Genetic knockdown of FAAH in the mice also protected the stomach from the ulcerative potential of DCF (Naidu et al., 2009). Another FAAH inhibitor PF-3845 (10 mg/kg) protected the stomach from ulcers when administered before 2 h of DCF (100 mg/kg). Acute and chronic dosing (5 days) of the FAAH inhibitor was effective in decreasing the ulceration assuring that tolerance does not develop to this property as opposed to

tolerance developed towards analgesic effect shown by opioids (Kinsey et al., 2011).

FAAH inhibitors have a synergistic effect with COX and sEH inhibitors in reducing pain and decrease the ulcerative potential of DCF and indomethacin (Naidu et al., 2009; Sasso et al., 2012; Grim et al., 2014; Sasso et al., 2015). FAAH inhibitors may reduce the dose of NSAIDs including DCF and decrease ulcerative potential in a clinical setting. Multiple pharmaceutical companies including Pfizer, Vernalis Ltd, Janssen Pharmaceutical Inc., Bial-Portela & Ca. SA are involved in the clinical development of FAAH inhibitor. Death and serious adverse effect of healthy volunteers administered with multiple high doses of FAAH inhibitor BIA 10-2474 of Portuguese pharmaceutical company Bial-Portela & Ca. SA. is a cause of concern (Kaur et al., 2016).

6.3. MAG Lipase Inhibitors Decrease Ulcer

The 2-arachidonoylglycerol (2-AG) is metabolized by monoacyl-glycerol lipase (MAGL) to arachidonic acid and inhibitors of this enzyme decrease pain without cannabimimetic effect and gastric toxicity of DCF (Kinsey et al., 2011; Kinsey et al., 2013; Ignatowska-Jankowskaet al., 2014; Crowe et al., 2015). MAGL inhibitor JZL184 (0.25 to 40 mg/kg, i.p., administered 2 h before DCF) decreased DCF-induced gastric ulcers. At 40 mg/kg dose a complete blockade of ulceration was observed. Gastroprotective property of this MAGL inhibitor is mediated through the CB_1 receptor. JZL184 increased the level of 2-AG but not the level of anandamide, arachidonic acid, PGE_2 and PGD_2 in the stomach. The 2-AG (50 mg/kg) did not generate ulcers and could not block the ulcers created by DCF suggesting the vital role of MAGL in maintaining the level of 2-AG. MAGL inhibitor JZL184 (4 mg/kg, i.p.) alleviated the ulcerative potential of DCF by minimizing the increase in the level of IL-1β, IL-6, TNF-α, G-CSF and IL-10 in the stomach. Tolerance did not develop to the anti-ulcer effect of JZL184 after chronic dosing with JZL184 (4 mg/kg) for 5 days (Kinsey et al., 2011). Kinsey et al., later demonstrated that acute and chronic

dosing with JZL184 (4 and 40 mg/kg, i.p.) increased the level of 2-AG and decreased the level of arachidonic acid in the brain. Acute and chronic administration of JZL184 (4 mg/kg) and acute administration of JZL184 (40 mg/kg) did not have any effect on the level of anandamide, oleoylethanolamide and palmitoylethanolamide but increased the level of these endogenous compounds when administration chronically at high dose (40 mg/kg). This suggests that JZL184 may exert some of its effects through different pathways when administered chronically at higher doses. Chronic intraperitoneal dosing of JZL184 beyond 8 mg/kg i.e., 16 and 40 mg/kg exhibited tolerance to its gastroprotective effect against DCF (Kinsey et al., 2013b). A highly selective monoacylglycerol lipase inhibitor KML29 also exhibited anti-nociceptive effect, decreased inflammation and protected from DCF's ulcerative effect. Administration of KML29 increased the level of 2-AG and decreased the level of arachidonic acid within 2 h but had no effect on the level of anandamide, oleoylethanolamide and palmitoylethanolamide in the brain. KML29 dose (5, 25 and 40 mg/kg) dependently decreased DCF (100 mg/kg)-induced hemorrhage of the stomach via CB_1 receptors (Ignatowska-Jankowska et al., 2014).

MAGL inhibitor synergistically ameliorates mechanical and cold allodynia when administered with non-specific COX inhibitor DCF (Crowe et al., 2015). Therefore, the MAGL inhibitor may reduce the NSAID's dose and gastrointestinal side effects. ABX-1431, a MAGL inhibitor is in clinical trials for different pathological conditions associated with pain and inflammation. Unfortunately, the clinical trials related to dyspepsia was terminated due to patient recruitment challenges (https:\\Clinicaltrials.gov).

6.4. Steroids as Anti-Ulcer Agents

Treatment of dexamethasone, a synthetic glucocorticoid (2 mg/kg, i.p.) 2 h prior to DCF (100 mg/kg, p.o.) administration alleviated stomach ulcer created by DCF in C57BL/6 mice 6 h post-dosing with the NSAID (Kinsey et al., 2011). Adverse effect of steroids, in general, would prevent the use of dexamethasone as an anti-ulcer agent, but when the use of this drug is

required to potentiate the analgesic and anti-inflammatory effect of DCF (Bamgbose et al., 2005), the chances of ulcer would decrease drastically.

6.5. The Inhibitor of JNK Reduces Diclofenac-Induced Ulcer

JNKs are a member of mitogen-activated protein kinases (MAPK) which are responsive to stress stimuli including cytokines and activates transcription factor activator protein 1 (AP-1) upon phosphorylation of c-JUN. Activation of AP1 is associated with apoptosis of cells. An increase in the expression of JNK (p46) was observed in the mucosal cell of intestine 18h following DCF (60 mg/kg, i.p.) administration in C57BL/6 mice. Administration of JNK inhibitor SP600125 (30 mg/kg, i.p.) 1 h prior to DCF decreased the ulcerative effect of the NSAID. Anti-ulcer effect of SP600125 was associated with a decrease in ER stress (Ramirez-Alcantara et al., 2009). SP600125 also can decrease the level of inflammatory marker TNF-α (Assi et al., 2006). Another JNK inhibitor D-JNKI-1 also decreased the inflammation of the large intestine (Kersting et al., 2013). The ability of JNK inhibitors to attenuate pain hypersensitivity (Gao et al., 2009) and mechanical allodynia (Zhuang et al., 2006) suggest that these pharmacological agents may have an additive effect to decrease pain when administered with DCF and decrease the ulcerative potential of DCF.

6.6. Inhibition of β-Glucuronidase to Decrease Ulcer Generated by Diclofenac

Role of β-glucuronidase in mediating intestinal ulceration by NSAIDs including DCF is discussed earlier. Inhibition of intestinal β-glucuronidase and bacterial β-glucuronidase is shown to decrease ulceration by DCF (A Boelsterli and Ramirez-Alcantara, 2011; LoGuidice et al., 2012; Saitta et al., 2014; Zhong et al., 2016). Bacterial β-glucuronidase inhibitor [1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)-3-(4- ethoxyphenyl)-1-(2-hydroxyethyl)thiourea]) (10 µg/mouse, p.o.) administered 3 h after DCF (60

mg/kg, i.p.) administration significantly decreased the number of intestinal ulcers but not the total ulcer area. Pre-treatment with this compound (10 µg/mouse, p.o., *t.i.d*) also blocked intestinal ulcers created by indomethacin (10 mg/kg, i.p.) and ketoprofen (100 mg/kg, i.p.) (Saitta et al., 2014).

6.7. Mitochondrial Cyclophilin D Modulator as an Anti-Ulcer Agent

NSAIDs including the DCF is known to cause dysfunction of mitochondria which leads to the death of cells (LoGuidice et al., 2010; Ghosh et al., 2016). Opening of mitochondrial permeability transition pore (mPTP) by DCF is one of the key steps for the death of mitochondria and cell. Enteropathy was reduced in C57BL/6 mice when the animals were treated with CypD inhibitor, cyclosporin A (CsA, 10 mg/kg, i.p.) after 1 h of administration of DCF (60 mg/kg, i.p.). Non-immunosuppressive cyclosporin analog, D-MeAla(3)-EtVal(4)-cyclosporin (Debio 025) also decreased DCF-induced enteropathy. Mice genetically deficient in mitochondrial CypD (peptidyl-prolyl cis-trans isomerase F [Ppif(-/-)]) were also resistant to enteropathy induced by DCF (LoGuidice et al., 2010).

CONCLUSION

DCF is widely used due to its ability to alleviate the pain and inflammation associated with many pathological conditions. Mechanism-based adverse effects on several tissues limit the use of DCF in certain conditions. The sEH is one of the novel targets which ameliorates DCF-induced GI erosion. The sEH inhibitors synergistically decrease inflammation when administered with NSAIDs. Therefore, it is possible that sEH inhibitors may be formulated with DCF to reduce both the dose needed for efficacy and the side effects of DCF and other NSAIDs.

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Chapter 8

MITOCHONDRIAL AND PROTEASOMAL DYSFUNCTION AS A POSSIBLE MECHANISM OF CARDIOVASCULAR TOXICITY OF DICLOFENAC

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ABSTRACT

Diclofenac, like many other non-steroidal anti-inflammatory drugs (NSAIDs), is associated with a cardiotoxic effect. Although the reduction in the level of thromboxane/prostaglandin A2 has been considered as a risk factor, the participation of other factors is involved. Recent studies suggest that chronic treatment of diclofenac to mice at a dose of 10 mg/kg for 28 days induced proteasomal dysfunction with a parallel increase in the level of polyubiquitinated protein and oxidized proteins in the heart. Similarly, acute dosing of diclofenac to mice at the dose of 100 mg/kg for 18 h also caused proteasomal dysfunction in the heart. Incubating cardiac (H9c2) cells with diclofenac (5 μM) also resulted in proteasomal dysfunction. Diclofenac dose-dependently increased the generation of reactive oxygen species (ROS) and induced cell death in cardiac cells. Diclofenac also decreased mitochondrial complex III activity and mitochondrial membrane potential. Overall, current experimental data suggest that diclofenac causes significant increases in ROS as well as mitochondria and proteasome dysfunction, which are likely to be associated with the cardiotoxic effect of diclofenac.

Keywords: diclofenac, cyclooxygenase, non-steroidal anti-inflammatory drugs (NSAID), mitochondrial dysfunction, cardiotoxicity

1. INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one of the most prescribed drug classes worldwide (Grosser, Ricciotti et al. 2017). More than 30 billion over-the-counter NSAID tablets are used annually around the world, due to their anti-inflammatory, analgesic and antipyretic effects (Schmeltzer, Kosinski et al. 2016). NSAID's anti-inflammatory action is mediated through the inhibition of cyclooxygenase (COX) activity, which reduces pain and inflammation through the inhibition of prostaglandin (PG) synthesis (Yamazaki, Muramoto et al. 2006). Based on mechanism of action, NSAIDs are classified as selective NSAIDs (celecoxib and rofecoxib) that selectively inhibit COX-2 activity (Yamazaki, Muramoto et al. 2006, Ghosh, Alajbegovic et al. 2015), non-selective NSAIDs (ibuprofen and naproxen) that inhibits both COX-1 and COX-2 activity, or semi-

selective NSAIDs (indomethacin, meloxicam, and diclofenac) that have high affinity for COX-2 but can inhibit COX-1 activity as well (Yamazaki, Muramoto et al. 2006, Ghosh, Alajbegovic et al. 2015). It is well documented that NSAIDs cause adverse effects including gastrointestinal, cardiovascular and renal injuries (Tatematsu, Fujita et al. 2018). Treatment with the NSAIDs indomethacin and diclofenac (DCF) in animal models were reported to develop gastrointestinal lesions (Yamazaki, Muramoto et al. 2006). Urinary proteomic profiling showed that DCF treatment to mice induces liver and kidney injury (Ghosh, Alajbegovic et al. 2015). In another study, the use of indomethacin was shown to enhance oxidative stress and mitochondrial dysfunction in the kidney (Nagappan, Varghese et al. 2015). NSAIDs such as nimesulide, celecoxib, and lumiracoxib were reported to cause mitochondrial dysfunction and thereby impaired cellular function (Berson, Cazanave et al. 2006, Syed, Skonberg et al. 2016a). Due to severe liver toxicity, lumiracoxib (which structurally resembles diclofenac) was withdrawn from the market (Singer, Lewitzky et al. 2010). Several studies have documented that NSAIDs-induced reactive oxygen species (ROS) generation leads to increased apoptosis (Li, Hortmann et al. 2008, van Leeuwen, Unlu et al. 2012, Ghosh, Goswami et al. 2016). A recent report showed that non-selective NSAIDs and selective COX-2 inhibitors can increase adverse cardiovascular outcomes (Tacconelli, Bruno et al. 2017). A study done with healthy volunteers first showed that inhibition of prostaglandin (PG) 2 by selective COX-2 NSAIDs (celecoxib and rofecoxib) is associated with a risk for cardiovascular toxicity (Grosser, Ricciotti et al. 2017). The selective COX-2 inhibitor rofecoxib was withdrawn from the market in 2004 due to risk for adverse cardiovascular outcomes (Nissen, Yeomans et al. 2016).

1.1. Mechanism of Action of NSAIDs

The mechanism of action of NSAIDs is through the inhibition of COX enzymes (COX-1 and COX-2) that metabolize arachidonic acid to prostaglandins (Figure 1). In plasma membrane, arachidonic acid exists in

the form of esterified phosphatidylcholine and phosphatidylethanolamine phospholipid and is released from the cell membrane with the help of the enzyme phospholipase A2 (Gunaydin and Bilge 2018). COX-1 and COX-2 convert arachidonic acid to the unstable precursor, PGG2 then PGH2. PGH2, in turn, is used as a substrate by tissue-specific PG isomerases to form various isoforms, such as PGE2, PGD2, PGF2α, prostacyclin (PGI2), and thromboxanes (Grosser, Ricciotti et al. 2017, Gunaydin and Bilge 2018). PGs and thromboxanes play an important role in fever, pain, and inflammation (Grosser, Ricciotti et al. 2017, Gunaydin and Bilge 2018). In addition, arachidonic acid is metabolized to leukotrienes by lipoxygenases via another pathway. It is reported that NSAIDs play a role in the synthesis and action of different inflammatory mediators such as PGs, interleukin-2, interleukin-6 and tumor necrosis factor (Grosser, Ricciotti et al. 2017, Gunaydin and Bilge 2018).

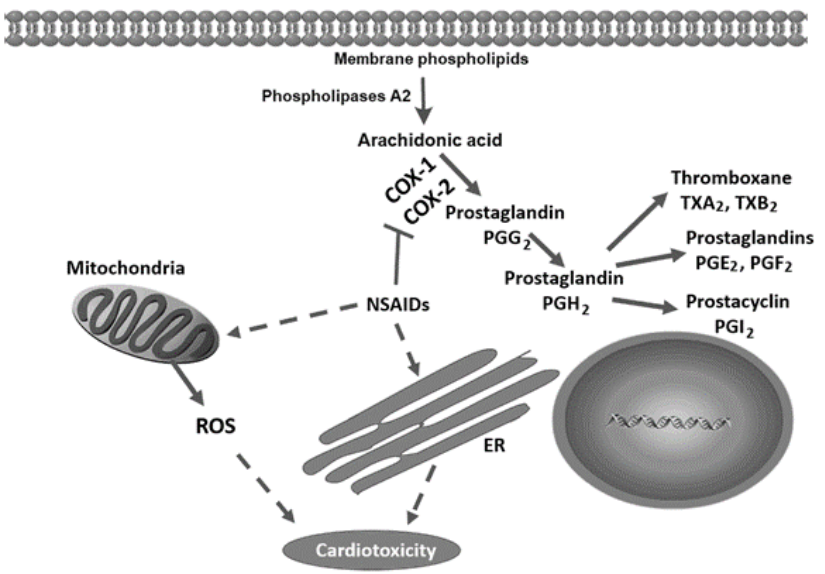

Figure 1. Schematic representation of the mechanism of action of NSAIDs. NSAIDs inhibit cyclooxygenases (COX) 1 and 2 that metabolize arachidonic acid to prostaglandins and thromboxanes and have effects on the mitochondria function and ROS generation. Dashed lines indicate that the mechanism associated with these effects are not well understood. ER, endoplasmic reticulum; PG, prostaglandin; ROS, reactive oxygen species; TX, thromboxane.

Mitochondria play an important role in regulating protein homeostasis and are a potential target for drug-induced adverse reactions (Miyake, Takasuka et al. 1991). Various reports suggest that mitochondrial dysfunction is one of the possible mechanisms for the NSAIDs mediated gastrointestinal, liver and cardiovascular toxicities (Miyake, Takasuka et al. 1991, Moreno-Sanchez, Bravo et al. 1999, Somasundaram, Sigthorsson et al. 2000, Krause, Brand et al. 2003, Labbe, Pessayre et al. 2008). These mechanisms include reduced mitochondrial membrane potential, increased ROS formation, reduced ATP synthesis, and inhibition of oxidative phosphorylation. By disrupting mitochondrial energy metabolism and proteasome function, pharmacological agents may induce apoptosis that can eventually lead to organ failure (Ghosh, Goswami et al. 2016).

2. DCF MECHANISM OF ACTION

Like other NSAIDs, the mechanism of action of DCF is not completely understood. DCF is a phenylacetic acid derivative that inhibits PG synthesis through the inhibition of COX-1 and COX-2. NSAIDs, except for aspirin, can increase the risk for cardiovascular toxicity including stroke, myocardial infarction, and heart attack. However, DCF increases the risk for myocardial infarction and stroke at a lower concentration (< 150mg/day) than other NSAIDs (Ray, Varas-Lorenzo et al. 2009). It has been suggested that the effects of DCF may be due to greater COX-2 inhibition associated with this drug (Bhala, Emberson et al. 2013). Aspirin (acetylsalicylic acid) in low-doses is used as a therapy in high cardiovascular risk patients. This cardio-protective effect is suggested to be due to the *irreversible* inhibition of thromboxane, which is unique to aspirin, as other nonselective NSAIDs are *reversible* inhibitors of thromboxane (Salpeter, Gregor et al. 2006).

3. CARDIOVASCULAR TOXICITY OF DCF

Several clinical reports suggest that NSAIDs may cause adverse cardiovascular effects (Pawlosky 2013, Ghosh, Alajbegovic et al. 2015, Kontogiorgis, Valikeserlis et al. 2016). A study on specific serum markers of cardiac damage, cardiac troponin I, and creatine kinase-MB, as well as nonspecific markers aspartate aminotransferase and lactate dehydrogenase, showed that rams injected with a single dose of DCF (2.5 mg/kg) had increased cardiac troponin I and lactate dehydrogenase after just 6 hours (Er, Dik et al. 2013). A study using Wistar male albino rats showed that vitamin B complex (a mixture of B1, B6, and B12) co-administered daily with 1.5 mg or 3 mg/kg body weight of DCF sodium resulted in reduced levels of serum markers of cardiac damage (Abdulmajeed, Alnahdi et al. 2015). Aspartate aminotransferase and creatine kinase-MB levels in serum, as well as phosphoglucoisomerase, glutathione reductase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase activities in cardiac tissue, were decreased in DCF and vitamin B treated rats compared with only DCF treated rats.

DCF administration (13.5 mg/kg, intraperitoneally) to rats daily for 1 week resulted in elevated levels of troponin I, lactate dehydrogenase and aspartate aminotransferase levels (Erdal T and L. 2017). DCF is often used by persons involved in sports (Paoloni, Milne et al. 2009). Rats treated with DCF that were exercised by swimming for 30 minutes each day for one week showed similar lactate dehydrogenase activity to non-exercised DCF treated rats (Paoloni, Milne et al. 2009). Exercised and DCF treated rats showed lower cardiac troponin I and aspartate aminotransferase compared to non-exercised DCF treated rats, suggesting that exercise did not worsen the effects of DCF on the heart but may also be partly beneficial in reducing some of the side effects of DCF.

To determine the molecular mechanism behind DCF cardiotoxicity, hearts, livers, and kidneys from Swiss Webster mice were acutely and chronically treated with DCF (100mg/kg for 18h, acute treatment or 10mg/kg for 28 days, chronic treatment) (Ghosh, Goswami et al. 2016). The 100mg/kg treatment resulted in plasma DCF concentration of 331 ng/ml

(Ghosh, Goswami et al. 2016). The main proteolytic system in cells, responsible for 60-80% of the degradation of intracellular proteins, the ubiquitin-proteasome system (UPS), was investigated (Gomes, 2013). The proteolytic component of the UPS, the proteasome, was found to be impaired in heart, liver, and kidneys from DCF treated mice. Proteasome dysfunction has been shown to be associated with several cardiovascular diseases, including cardiomyopathies (Gilda, Lai et al. 2016). Cultured cardiac H9c2 cells and neonatal cardiomyocytes showed proteasome dysfunction due to DCF (5 and 10 μM) treatment. Interestingly, DCF increased ROS produced in H9c2 cells and caused a DCF concentration-dependent cell death. Mitochondria isolated from mouse hearts showed that DCF treatment reduced the mitochondrial complex III activity, suggesting that DCF treatment causes proteasome and mitochondrial dysfunction and increases ROS production. Low dose DCF (5 and 10 μM) also resulted in reduced mitochondrial membrane potential (Ghosh, Goswami et al. 2016). Hence, it is likely that DCF induced cardiotoxicity occurs via a ROS-dependent mechanism that involves both mitochondrial and proteasome dysfunction.

DCF at 50 μM inhibited mitochondrial oxidative phosphorylation, resulting in the inhibition of ATP synthesis in rat liver mitochondria (Syed, Skonberg et al, 2016b). Incubation of mitochondria with DCF and reduced glutathione diminished the negative effect of DCF on ATP synthesis. DCF (150 μM) was found to cause apoptosis in a neuroblastoma cell line SH-SY5Y (Cecere, Iuliano, et al., 2010). This apoptosis was found to be associated with mitochondrial dysfunction, including impaired mitochondrial membrane potential, cytochrome c increase in the cytoplasm, and a decrease in mitochondrial superoxide dismutase which is an important antioxidant defense against ROS.

DCF (350 μM) was found to induce apoptosis in rat hepatocytes (Gómez-Lechón, Ponsoda, et al. 2003). The evidence suggests that DCF opened the mitochondrial permeability transition pore. Use of antioxidants prevented caspase activation, suggesting that increased mitochondrial oxidative stress resulted in mitochondrial permeability transition pore opening. DCF (500 μM) was also shown to induce proteasome dysfunction in Cos-7 and A549 cells (Amanullah, Upadhyay et al. 2017). The

proteasome dysfunction resulted in the accumulation of many ubiquitylated proteasome substrates. DCF (500 μM) also caused mitochondrial membrane depolarization, release of cytochrome c into the cytosol, and apoptosis in these cells.

A concern with some of the studies is that after typical doses used by patients (50-150mg/kg), the amount of DCF in the blood ranges from 10-30 μM (Mukherjee, Mahapatra et al. 2006). Although it has been suggested that the concentration of DCF in the blood could increase with long term use, diseases which result in slower clearance of the drug, and overdosing (Tang 2003), the amounts of DCF used in several publications that show physiological effects are relatively high, and may not be physiologically relevant.

CONCLUSION

Diclofenac at clinical doses can be toxic to the heart via augmenting mitochondrial and proteasomal dysfunction. Managing the mitochondrial and proteasomal toxicity could be a therapeutic target to deal with the cardiotoxic effect of diclofenac.

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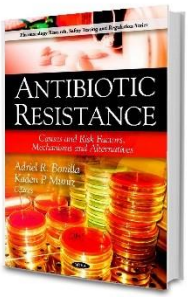

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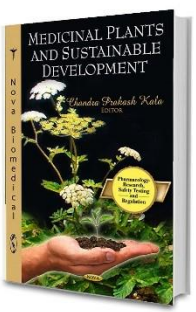

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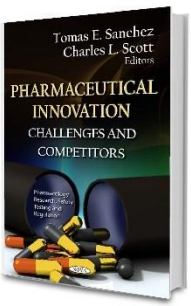

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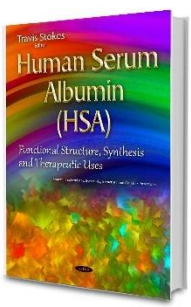

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