UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

PHARMACOLOGICAL MODULATION OF INFLAMMATION ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE
- STUDY IN AN ANIMAL MODEL -

Vanessa Alexandra Pinho Mateus

Orientadores: Professor Doutor Rui Manuel Amaro Pinto
Professor Doutor Bruno Miguel Sepodes

Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, especialidade Farmacologia e Farmacoterapia.

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2016

“Aprender é a única coisa de que a mente nunca se cansa,
nunca tem medo e nunca se arrepende.”
(Leonardo da Vinci)

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ABSTRACT

Inflammatory bowel disease is a common gastro-intestinal disorder marked with chronic inflammation of intestinal epithelium, damaging mucosal tissue and manifests into several intestinal and extra-intestinal symptoms, mainly related to oxidative stress, inflammation and autoimmune type [Mowat, 2011; Pawar, 2011]. Currently used medical therapy of inflammatory bowel disease aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism [Engel, 2010; Pithadia, 2011]. Furthermore, their use may result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases [Engel, 2010]. The main objective of the study is to evaluate the influence of a set of new drugs in inflammatory bowel disease, like erythropoietin, thiadiazolidinone-8 and hemin, through of an experimental colitis model induced by TNBS in rodents, contributing to facilitate a more effective and selective treatment than the currently known. Experimental colitis is induced by intracolonic administration of TNBS as described by Morris method [Morris, 1989]. The mice with colitis are treated with and without daily doses of erythropoietin, thiadiazolidinone-8 and hemin. The evaluated parameters are clinical symptoms/signs, colon length, fecal hemoglobin, ALP, urea, creatinine, ALT, MPO, TNF-α, IL-1β, IL-10 and histopathological score. TNBS-induced colitis was developed in 4 days, providing an acute intestinal inflammation model. These mice presented an increase of MPO, TNF-α, IL-1β and fecal hemoglobin. Erythropoietin treatment had a positive influence in the development of experimental colitis in all evaluated parameters, thus reducing its severity and extension. Thiadiazolidinone-8 derivate and hemin treatments had also a positive influence in the development of experimental colitis, but not in all evaluated parameters. All tested drugs significantly inhibit acute inflammatory response associated to the TNBS-induced colitis model.

KEY-WORDS: TNBS-induced colitis, Inflammation, Erythropoietin, Thiadiazolidinone-8, Hemin.

RESUMO

A doença inflamatória intestinal é um distúrbio gastrointestinal caracterizado por inflamação crónica do epitélio intestinal com ulceração recorrente, manifestando inúmeros sintomas intestinais e extraintestinais, principalmente relacionados com stress oxidativo, inflamação e do tipo autoimune [Mowat, 2011; Pawar, 2011]. O tratamento farmacológico atualmente utilizado tem por objetivo induzir e/ou manter o doente em remissão e melhorar os efeitos secundários da doença, no entanto não modifica ou reverte o mecanismo patogénico subjacente [Engel, 2010; Pithadia de 2011]. A sua utilização pode resultar em efeitos colaterais graves e complicações, tais como um aumento da taxa de malignidades ou doenças infeciosas [Engel, 2010]. Assim, a pesquisa de novas abordagens farmacológicas pode promover avanços importantes no tratamento da doença inflamatória intestinal. Neste sentido, um número cada vez maior de modelos animais de doença têm sido utilizados para estudos pré-clínicos, os quais apresentam manifestações clínicas semelhantes às observadas em humanos. No caso da doença inflamatória intestinal, o modelo de colite induzida por TNBS é um método eficiente, uma vez que mimetiza o padrão de inflamação característica da doença em humanos, produzindo a doença de forma rápida, fiável e reprodutível. Assim, o objetivo principal deste estudo é avaliar o efeito de um conjunto de novas moléculas na doença inflamatória intestinal, como eritropoietina, tiadiazolidinona-8 e hemina, por meio de um modelo de colite experimental induzida por TNBS em roedores, contribuindo para um tratamento mais eficaz e seletivo do que o atualmente conhecido.

Para tal, utilizaram-se ratos machos CD-1, com 30-40 g de peso e de 6-10 semanas de idade. A colite experimental é induzida por uma administração única intra-rectal de TNBS, de acordo com o método de Morris [Morris, 1989]. Os grupos experimentais foram estruturados com base nos principais objetivos do estudo. Os murganhos com colite são tratados com e sem doses diárias de eritropoietina, tiadiazolidinona-8 e hemina. Foram ainda formados grupos de referência para servirem de controlo na comparação dos resultados com os grupos de tratamento. No final do período experimental, os ratos foram anestesiados e foram recolhidas amostras de sangue por punção cardíaca. Em seguida, os murganhos foram sacrificados por deslocação cervical e necropsiados. Foram avaliados alguns parâmetros, como a hemoglobina fecal, fosfatase alcalina, ureia, creatinina e alanina aminotransferase através do sistema automático ADVIA® Chemistry XTP. A mieloperoxidase, TNF-α, IL-1β e IL-10

são determinados por ELISA. O *score* histopatológico foi atribuído com base em critérios adaptados de Corazza e colaboradores (1999) e Seamons e colaboradores (2013). A pontuação histopatológica das lesões foi parcialmente atribuída (0-4 gravidade crescente) de acordo com: (1) presença de perda de tecido / necrose, (2) gravidade da lesão da mucosa epitelial, (3) inflamação, (4) extensão 1 - a percentagem de intestino afetado em qualquer maneira e (5) extensão 2 - a percentagem de intestino afetado pela lesão mais grave. A gravidade da colite é finalmente calculada pela soma das pontuações parciais das lesões, promovendo um *score* final (pontuação máxima = 20). Foram ainda avaliados a manifestação de sintomas/sinais clínicos e comprimento do cólon.

Como primeiro objetivo a atingir tínhamos o desenvolvimento e padronização do modelo de colite induzida por TNBS, uma vez que o aparecimento da lesão intestinal mais grave causada pelo TNBS pode variar entre 3 dias a uma semana após a indução. Dois grupos independentes de colite induzida por TNBS foram então monitorizados sob as mesmas condições específicas, sendo que um grupo de murganhos foi sacrificado no dia 4 e o outro no dia 6, após a indução. O objetivo era identificar o dia em que a lesão intestinal aguda máxima teria sido alcançada pelo método de indução usada no presente estudo. Relativamente aos resultados obtidos nesta experiência, verificou-se que a colite induzida por TNBS foi então desenvolvida em 4 dias, proporcionando um modelo de inflamação intestinal agudo. O grupo TNBS apresentou uma alteração da motilidade intestinal caracterizada por diarreia, edema grave do ânus e morbilidade moderada, enquanto os grupos controlo permaneceram sem alterações. Estes murganhos apresentaram ainda uma diminuição progressiva do peso corporal, um comprimento do cólon reduzido e um aumento da concentração de hemoglobina fecal. A indução de colite por TNBS promoveu, ao fim de 4 dias, um aumento dos mediadores inflamatórios, tais como a mieloperoxidase, TNF-α e IL-1β, bem como a diminuição de uma citocina anti-inflamatória como a IL-10. A análise histopatológica revelou uma necrose transmural difusa com hemorragia grave, envolvendo a submucosa, camada muscular da mucosa e serosa e, muitas vezes, associada a peritonite. Estas lesões eram consistentes com uma indução correta de colite experimental por TNBS.

A eritropoietina foi testada neste modelo de colite induzida por TNBS. Após 4 dias de tratamento diário com eritropoietina, os nossos resultados demonstram que a eritropoietina exerce efeitos anti-inflamatórios sobre este modelo de colite experimental específica. O tratamento com eritropoietina apresentou um efeito

benéfico no desenvolvimento de colite experimental em todos os parâmetros avaliados, reduzindo assim a gravidade e extensão das lesões. Mais precisamente, a eritropoietina promoveu uma diminuição da perda de peso, hemoglobina fecal, fosfatase alcalina, mieloperoxidase, citocinas pró-inflamatórias (TNF-α e IL-1β) e *score* histopatológico. Por outro lado, a eritropoietina também induziu um aumento do comprimento do cólon e de citocinas anti-inflamatórias como a IL-10, bem como regulou as funções renal e hepática. No nosso estudo, foram utilizadas duas doses diferentes de eritropoietina, nomeadamente, 500 UI/kg e 1000 UI/kg, que são consideravelmente mais baixas do que normalmente é utilizado nestes estudos experimentais. Esta opção justifica-se, uma vez que o tratamento com eritropoietina pode afetar o nível de hematócrito e promover assim efeitos adversos cardiovasculares. Logo, optou-se por utilizar as doses mais baixas possíveis para produzir efeito no modelo, para além do que as doses administradas são relevantes para a prática clínica num contexto de farmacologia translacional. Ainda assim, o nível de hematócrito após o tratamento com eritropoietina foi avaliado e os resultados foram normais. Estes dados indicam que a eritropoietina inibe significativamente a resposta inflamatória aguda na colite experimental, sem eventos adversos relacionados com o aumento da viscosidade sanguínea.

A tiadiazolidinona-8 é outra molécula que foi testada no modelo de colite induzida por TNBS. O tratamento com tiadiazolidinona-8 foi capaz de modular o desenvolvimento de colite experimental. Particularmente, promoveu uma redução da hemoglobina fecal, fosfatase alcalina, mieloperoxidase e citocinas pró-inflamatórias (TNF-α e IL-1β). Além disso, também foi capaz de aumentar a expressão de citocinas anti-inflamatórias (IL-10), bem como regulou as funções renal e hepática. De acordo com a análise histopatológica, o tratamento com tiadiazolidinona-8 só produziu uma ligeira diminuição da extensão da doença. No entanto, os resultados obtidos com a concentração de citocinas sugerem e confirmam o seu efeito benéfico na redução da gravidade da doença. Estes dados indicam que a tiadiazolidinona-8 inibe significativamente a resposta inflamatória aguda na colite experimental.

A hemina também foi testada no modelo de colite induzida por TNBS. No final do período experimental, o tratamento com hemina apresentou uma influência benéfica no desenvolvimento da colite experimental, diminuindo a sua gravidade e extensão. A hemina promoveu uma redução da hemoglobina fecal, fostase alcalina, mieloperoxidase e citoquinas pró-inflamatórias (TNF-α e IL-1β). Foi também capaz de aumentar a concentração de uma citocina anti-inflamatória (IL-10), bem como regular

as funções renal e hepática. Além disso, o tratamento com hemina produziu uma diminuição estatisticamente significativa da taxa de mortalidade reduzindo para 0% as mortes no grupo de tratamento, promovendo resultados semelhantes aos obtidos com os murganhos saudáveis. Estes resultados sugerem que a hemina inibe significativamente a resposta inflamatória aguda na colite experimental, reduzindo completamente a mortalidade associada à doença.

Em conclusão, todas as moléculas avaliadas neste estudo inibem significativamente a resposta inflamatória aguda no modelo de colite experimental induzido por TNBS. Este estudo permitiu ainda explorar o efeito destas novas moléculas no desenvolvimento da doença inflamatória intestinal, bem como a sua influência sobre os mecanismos de resposta à lesão intestinal. Além disso, representa uma contribuição inovadora para o tratamento farmacológico da doença inflamatória intestinal, permitindo enriquecer a investigação de outras possibilidades terapêuticas no âmbito desta doença.

PALAVRAS-CHAVE: Colite induzida por TNBS, inflamação, eritropoietina, tiadiazolidinona-8, hemina.

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ACRONYMS AND ABBREVIATIONS

5-ASA – 5-Aminosalicylic acid
ALA - Delta-aminolevulinic acid
ALP - Alkaline phosphatase
ALT - Alanine aminotransferase
anti-TNF-α - Monoclonal antibodies against TNF-α
AP-1 - Activator protein 1
βcR - Beta common receptors
BVR - Biliverdin reductase
CD - Crohn's disease
CDAI - Crohn's disease activity index
CO - Carbon monoxide
COX - Cyclooxygenase
DMSO - Dimethyl sulfoxide
DNBS - Dinitrobenzene sulfonic acid
DSS - Dextran sulfate sodium
DVL - Termed dishevelled
eNOS - Endothelial nitric oxide synthase
EPO - Erythropoietin
EPOR - Erythropoietin receptor
ERK - Extracellular signal-regulated kinase
ESA - Erythropoiesis-stimulating agents
FRAT - Frequently rearranged in advanced T-cell lymphomas
GSH - Glutathione
GSK - Glycogen synthase kinase
GSSG - Oxidized glutathione
HIF - Hypoxia inducible factors
HO – Heme oxygenase
Hsp70 - Heat shock protein 70
IBD - Inflammatory bowel diseases
ICAM-1 - Intercellular adhesion molecule-1
IFN-γ - Interferon-γ
IκB - Inhibitory κB
IKK - IκB kinase
IL - Interleukin
iNOS - Inducible nitric-oxide synthase
IP - Intraperitoneal injection
IRS - Insulin receptor substrate proteins
IV - Intravenous

JAK - Janus Kinase

JNK - c-Jun N-terminal kinases

LPS - Lipopolysaccharide

MAPK - Mitogen-activated protein kinases

MPO - Myeloperoxidase

NaOH - Sodium hydroxide

NF-κB - Nuclear transcription factor kappa B

NO - Nitric oxide

Nrf2 - NF-E2 related factor-2

NSAID - Non-steroidal anti-inflammatory drugs

PBG - Porphobilinogen

PG - Prostaglandin

PI3K - Phosphatidylinositol 3 kinase

PKB - Protein kinase B

PPAR - Peroxisome proliferator-activated receptor

PtdIns(3,4,5)P_3 - Phosphatidylinositol-3,4,5-trisphosphate

ROS - Reactive oxygen species

STAT - Signal transducer and activator of transcription protein

SC - Subcutaneous

Th - T helper

TCF - T-cell factor

TDZD-8 - Thiadiazolidinone-8

TDZ - Thiazolidinediones

TLR - Toll like receptor

TNBS - Trinitrobenzene sulfonic acid

TNF-α - Tumor necrosis factor α

UC - Ulcerative colitis

VHL - Von Hippel-Lindau tumor suppressor protein

ANNEXES

ANNEXE 1 - Crohn´s Disease Activity Index

INTRODUCTION

Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, are chronic inflammatory diseases characterized by recurrent ulceration of the bowels [Pithadia & Jain, 2011]. Specifically, inflammatory bowel disease is a common gastro-intestinal disorder marked with chronic inflammation of intestinal epithelium, damaging mucosal tissue and manifests into several intestinal and extra-intestinal symptoms, mainly related to oxidative stress, inflammation and autoimmune type [Mowat et al., 2011; Pawar et al., 2011].

Inflammatory bowel disease is a common gastrointestinal disorder affecting, statistically, 7–10% of people worldwide, mainly of Caucasian descent [Hanauer, 2006; Spiegel, 2009]. Ulcerative colitis incidence has been increasing around world, even in (previously) low-incidence areas like Eastern Europe, Asia and developing countries. The Crohn's disease incidence is variable with 1/100.000 in Asia, South America until 7/100.000 in the USA. One hypothesis for the difference in incidence between developed and developing nations is the "hygiene hypothesis," which suggests that persons less exposed to childhood infections or unsanitary conditions lose potentially "friendly" organisms or organisms that promote regulatory T cell development, or alternatively do not develop a sufficient immune repertoire as they do not encounter noxious organisms. Such individuals are associated with a higher incidence of chronic immune diseases, including inflammatory bowel disease [Bernstein et al., 2015].

Although the etiology don´t be completely clear, it is generally agreed that a complex interplay between genetic predisposition, an environmental trigger and an aberrant immune reaction contributes to disease initiation and its progression [Wirtz & Neurath, 2007; Mayer, 2010]. Regarding to genetic factors, to date four genes have been associated with Crohn's disease (eg. CARD15, SLC22A4/5, DLG5, PPARG) and one with ulcerative colitis (eg. MDR1). The common threads are that the implicated genes regulate several important biologic functions, including immuno regulation, mucosal barrier integrity and microbial clearance and/or homeostasis [Sartor, 2006; Wirtz & Neurath, 2007]. Studies have implicated several environmental factors in the pathogenesis of inflammatory bowel disease [Loftus, 2004]. These factors include smoking, diet, the use of antibiotics and non-steroidal anti-inflammatory drugs, stress and infection. Unfortunately, the mechanisms by which these factors initiate the onset of disease or reactivate quiescent inflammatory bowel disease are not well understood. From a broad perspective, these triggering factors alter mucosal barrier integrity,

immune responses, or the luminal microenvironment, each of which have an impact on susceptibility to inflammation [Sartor, 2006]. About to immunological factors, inflammatory bowel disease patients have activated innate (macrophage, neutrophil) and acquired (T and B cell) immune responses and loss of tolerance to enteric commensal bacteria. Particularly, Crohn's disease is a pre-dominantly Th_1- and Th_{17}-mediated process, while ulcerative colitis seems to be an atypical Th_2 disorder. Inflammatory bowel disease is characterized by enhanced recruitment and retention of effector macrophages, neutrophils and T cells into the inflamed intestine, where they are activated and release pro-inflammatory cytokines. Accumulation of effector cells in the inflamed intestine is a result of enhanced recruitment as well as prolonged survival caused by decreased cellular apoptosis [Sartor, 2006]. Briefly, pathogenesis of inflammatory bowel disease is not fully understood but, currently, it's well accepted that inflammatory bowel disease is indeed characterized by an abnormal mucosal immune response but that microbial factors and epithelial cell abnormalities can facilitate this response [Strober, Fuss & Mannon, 2007].

Nowadays, used medical therapy of inflammatory bowel disease consists in salicylates, corticosteroids, immunosuppressants and biological therapy. These drug treatments aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism [Engel & Neurath, 2010; Pithadia & Jain, 2011]. Actually, their use may result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases [Engel & Neurath, 2010]. Drug delivery to the appropriate site(s) along the gastrointestinal tract also has been a major challenge, and second-generation agents have been developed with improved drug delivery, increased efficacy, and decreased side effects [Pithadia & Jain, 2011]. For many years there have been numerous efforts to find a new effective method that would allow controlling specifically unwanted immune responses that occur during autoimmune reaction [Szczepanik et al., 2012].

Thus, there are some new drugs, like erythropoietin, thiadiazolidinone-8 and hemin, that can modulate some important metabolic pathways in the establishment and development of inflammation and, for this reason, the evaluation of its influence in inflammatory bowel disease is relevant. These drugs may inhibit or stimulate the expression of these inflammatory pathways, contributing perhaps to facilitate a more effective and selective treatment than the currently known.

Erythropoietin, the principal hormone promoting the survival and differentiation of erythroid progenitor cells, is currently being used in the therapy of patients with chronic renal failure suffering from anemia [Cody et al., 2001; Nairz et al., 2011]. However, erythropoietin also bears extrahematopoietic properties that are transduced by erythropoietin receptors expressed on various nonerythroid tissues [Brines & Cerami, 2005; Jelkmann, 2007]. In experimental colitis, erythropoietin-erythropoietin receptors interaction decreases the production of NF-κB inducible immune mediators, thus limiting tissue damage and ameliorating disease severity. These immune-modulatory effects of erythropoietin may be of therapeutic relevance in inflammatory disease [Nairz et al., 2011]. Erythropoietin benefits have been studied in cardiovascular, neurologic, retinal, auditory, pancreatic, renal and liver injuries [Sanchis-Gomar et al., 2014; Chatagner, Huppi, Ha-Vinh Leuchter & Sizonenko, 2010; Loeliger, Mackintosh, Matteo, Harding & Rees, 2011; Olgun et al., 2013; Ucan et al., 2009; Imamura, Isaka, Ichimaru, Takahara & Okuyama, 2007; Sepodes et al., 2006]. Thus, the evaluation of the influence of erythropoietin in the inflammatory bowel disease is relevant and current.

Thiadiazolidinone-8 is the first non-ATP competitive GSK-3β inhibitors with highly effectivity and selectivity [Martinez, Alonso, Castro, Perez & Moreno, 2002; Dugo, Collin & Thiemermann, 2007]. In vivo studies, thiadiazolidinone-8 already has demonstrated that can substantially reduce the renal dysfunction, hepatocellular and lung injury [Dugo et al., 2005]. They already were tested in inflammatory bowel disease and the results revealed that the thiadiazolidinone-8 administration promoted a reduction of the colonic inflammation, of tissue injury and a reduced decline in body weight [Whittle et al., 2006]. Although it has been found the existence of multiple beneficial effects of thiadiazolidinone-8, the clinical utility of these drugs awaits animal and human trials [Martinez, Castro, Dorronsoro & Alonso, 2002].

Hemin is well known as an inducer of heme-oxygenase-1 and many studies have reported its protective effect in various animal models [Guan, Wen, Zhang, Wang & Zhao, 2009; Naito, Takagi, Uchiyama & Yoshikawa, 2011; Hualin et al., 2012]. Heme-oxygenase-1 expression can also confer cytoprotective, antiapoptotic and anti-inflammatory properties, suggesting thus that heme-oxygenase-1 can be a possible therapeutic target in several kinds of gastrointestinal diseases [Naito et al., 2011]. In inflammatory bowel disease, it is known that heme-oxygenase activity and heme-oxygenase-1 gene expression increase markedly after TNBS induction and the administration of tin mesoporphyrin, an heme-oxygenase inhibitor, potentiate the colonic damage as well as decrease heme-oxygenase-1 activity. These results indicate

that heme-oxygenase-1 plays a protective role in the colonic damage induced by TNBS enema [Wang et al., 2001]. However, more studies are needed to evaluate the influence of hemin in the inflammatory bowel disease [Naito et al., 2011].

The main objective of this study is the evaluation of the influence of a set of new drugs in inflammatory bowel disease, like erythropoietin, thiadiazolidinone-8 and hemin, through of an experimental colitis model induced by TNBS in rodents, contributing to facilitate a more effective and selective treatment than the currently known.

Thus, we developed the work that is presented in this thesis. The exhibition of the thesis was divided into three chapters of literature review, one chapter of methodology, four chapters of presentation and discussion of results related to the development of the model and the effect of each molecule under evaluated, one chapter of the final discussion and main conclusions and, finally, one last chapter of the references. Therefore:

- Chapter I: state of the art description of inflammatory bowel disease, including its classification, diagnosis and prognosis, as well as pharmacological treatment currently recommended;
- Chapter II: relevance of studies in animal models, presentation of existing animal models of experimental colitis and characterization of the chemically induced models most commonly used in experimental colitis;
- Chapter III: exhibition of new therapeutic approaches in inflammatory bowel disease, which their effect will be tested in this work;
- Chapter IV: description of the used methodology, such as the induction method, experimental groups, evaluated parameters and statistical tests;
- Chapter V: presentation of the results and respective discussion of the development of experimental colitis induced by TNBS;
- Chapter VI: presentation of the results and respective discussion of the erythropoietin effect in inflammatory bowel disease;
- Chapter VII: presentation of the results and respective discussion of the thiadiazolidinone-8 effect in inflammatory bowel disease;
- Chapter VIII: presentation of the results and respective discussion of the hemin effect in inflammatory bowel disease;
- Chapter IX: synthesis, discussion and the main conclusions of the work, as well as future prospects;
- Chapter X: all references used in the preparation of the thesis.

CHAPTER 1 - INFLAMMATORY BOWEL DISEASE

1. DEFINITION OF INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD) are chronic idiopathic gastrointestinal inflammatory disorders involving the recurrent ulceration of the bowels [Doherty & Cheifetz, 2009; Pithadia & Jain, 2011]. IBD, which include Crohn's disease (CD) and ulcerative colitis (UC), are common gastro-intestinal diseases marked with chronic inflammation of intestinal epithelium, damaging mucosal tissue and manifests into several intestinal and extra-intestinal symptoms, mainly related to oxidative stress, inflammation and autoimmune type [Mowat et al., 2011; Pawar et al., 2011].

The typical intestinal injuries of IBD reveal different characteristics and location, depending on whether CD or UC (FIGURE 1). Namely, CD is characterized by a chronic transmural inflammation of all or any part of the gastrointestinal tract involving mucosa, submucosa, muscular and connective tissue [Mowat et al., 2011]. Some patients with UC will have inflammation of the terminal ileum, which can make it difficult to distinguish from CD [Kornbluth & Sachar, 2010]. Nevertheless, UC is a chronic inflammatory condition causing continuous mucosal and sub-mucosal inflammation of the colon without granulomas on biopsy, affecting the rectum and a variable extent of the colon in continuity, which is characterised by a relapsing and remitting course [Silverberg et al., 2005; Mowat et al., 2011].

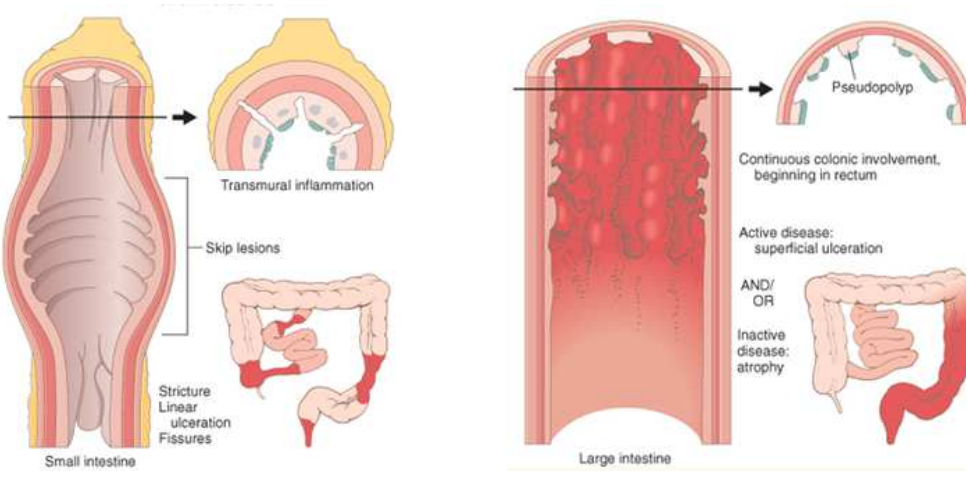

FIGURE 1. Differences between Crohn's disease and ulcerative colitis [adapted of Tresca, 2015].

The arbitrariness of some of the definitions is recognized, but the European Crohn´s and Colitis Organisation Consensus considers it useful to agree the terminology

[Dignass et al., 2012]. Other terms are used such as IBD unclassified and/or indeterminate colitis. IBD unclassified is the term best suited for the minority of cases where a definitive distinction between UC, CD or other cause of colitis cannot be made after the history, endoscopic appearances, histopathology of multiple mucosal biopsies and appropriate radiology have been taken into account [Silverberg et al., 2005; Satsangi, Silverberg, Vermeire & Colombel, 2006]. There are about 5% of patients with IBD unclassified after considering clinical, radiological, endoscopic and pathological criteria, because they have some features of both conditions [Silverberg et al., 2005]. On the other hand, indeterminate colitis is a term reserved for pathologists to describe a colectomy specimen which has overlapping features of UC and CD [Price, 1978; Satsangi et al., 2006]. It has distinct prognostic factors related to further surgery [Dignass et al., 2012].

1.1. CLASSIFICATION

The definitions of CD and UC acknowledge the revised Montreal classification which attempts to more accurately characterize the clinical patterns of IBD [Silverberg et al., 2005; Satsangi et al., 2006].

CD is classified according Montreal classification, which considers the age of onset (A), disease location (L) and disease behaviour (B) as the predominant phenotypic elements (TABLE 1) [Silverberg et al., 2005]. There is some evidence to suggest that patients stratified by age have different outcomes. Patients diagnosed before the age of 16 had a more aggressive initial course, while older age at diagnosis was found to be associated with a lower risk of colectomy [Barreiro-de-Acosta et al., 2010; Solberg et al., 2009].

TABLE 1. Montreal classification of Crohn's disease [adapted of Silverberg et al., 2005].

AGE AT DIAGNOSIS		LOCATION		BEHAVIOUR	
A1	< 16 years	L1	Ileal	B1	Non-stricturing, non-penetrating
A2	17 - 40 years	L2	Colonic	B2	Stricturing
A3	> 40 years	L3	Ileocolonic	B3	Penetrating
		L4	Isolated upper disease*	P	Perianal disease modifier**

Legend: *L4 is a modifier that can be added to L1-L3 when concomitant upper gastrointestinal disease is present; **P is added to B1-B3 when concomitant perianal disease is present.

The presentation of CD is highly variable. A single episode may not be followed by further episodes or the patient may experience continuous, unremitting disease. The time between the onset of complaints and the initial diagnosis may be as long as 3

years. For this reason, Crohn's disease activity index (CDAI) is an additional tool that should be considered in patients with CD (ANNEXE 1). The CDAI is not a rating of CD, but is a numerical estimate of the interpretation of the patient's symptoms, defining the severity of the disease activity. The calculation of CDAI is the sum of the products of a list of eight items, multiplied by weighting factors, the total of which defines the severity of disease activity [Freeman, 2008]. Since the classification of CD can be complex, the guidelines classify the severity of active CD by the presence of several signs and symptoms (TABLE 2) [Hanauer & Sandborn, 2001].

TABLE 2. Grading of disease activity in Crohn's disease [adapted of Dignass et al., 2012].

MILD	MODERATE	SEVERE
CDAI of 150 – 220 (eg. ambulatory, eating and drinking, < 10% weight loss)	CDAI of 220 – 450 (eg. intermittent vomiting or weight loss > 10%)	CDAI > 450 (eg. cachexia (BMI < 18 Kg m^{-2}) or evidence of obstruction or abscess)
No features of obstruction, fever, dehydration, abdominal mass or tenderness	Treatment for mild disease ineffective or tender mass. No overt obstruction	Persistent symptoms despite intensive treatment
CRP increased above the upper limit of normal	CRP elevated above the upper limit of normal	CRP increased

Legend: CDAI - Crohn's Disease Activity Index; BMI - Body mass index; CRP - C reactive protein.

UC is classified incorporating an assessment of disease extent and severity. The Montreal classification of disease extent of UC allows extent to be defined into three subgroups (TABLE 3), according to the maximal extent of inflammation observed at colonoscopy, because this is most clearly related to the risk of complications, including dilatation and cancer [Silverberg et al., 2005].

TABLE 3. Montreal classification for ulcerative colitis extent [adapted of Silverberg et al., 2005].

EXTENT	ANATOMY
Ulcerative proctitis	Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)
Left sided UC (distal UC)	Involvement limited to a proportion of the colorectum distal to the splenic flexure
Extensive UC (pancolitis)	Involvement extends proximal to the splenic flexure

Legend: UC – Ulcerative colitis.

Although a typical clinical picture of UC can be described, there is a wide range of presentation, from mild abdominal cramping with frequent small-volume bowel movements to profuse diarrhea. Most patients with UC experience intermittent bouts of

illness after varying intervals with no symptoms. Only a small percentage of patients have continuous unremitting symptoms or have a single acute attack with no subsequent symptoms. Complex disease classifications are generally not used in clinical practice for UC. The arbitrarily determined distinctions of mild, moderate and severe disease activity are generally used and these are largely determined by clinical signs and symptoms (TABLE 4) [Collins & Rhodes, 2006; Kornbluth & Sachar, 2010]. Thus, the Montreal classification also classified the UC severity [Silverberg et al., 2005].

TABLE 4. Montreal classification for ulcerative colitis severity [adapted of Dignass et al., 2012].

	MILD	MODERATE	SEVERE
BLOODY STOOLS/DAY	< 4	4 or more *if*	≥ 6 *and*
PULSE	< 90 bpm	≤ 90 bpm	> 90 bpm *or*
TEMPERATURE	< 37.5˚C	≤ 37.8˚C	> 37.8˚C *or*
HAEMOGLOBIN	> 11.5 g/dl	≥ 10.5 g/dl	< 10.5 g/dl *or*
ESR	< 20 mm/h	≤ 30 mm/h	> 30 mm/h *or*
CRP	Normal	≤ 30 mg/L	> 30 mg/L

Legend: ESR - Erythrocyte sedimentation rate; CRP - C reactive protein.

1.2. EPIDEMIOLOGY

IBD is a common gastrointestinal disorder affecting, statistically, 7–10% of people worldwide, mainly of Caucasian descent [Hanauer, 2006; Spiegel, 2009]. IBD can affect any age group, however there is a bimodal age-related incidence [Thoreson & Cullen, 2007; Lichtenstein, Hanauer & Sandborn, 2009], with the larger peak occurring between 15 to 30 years of age [Abraham & Cho, 2009] and a smaller peak occurring later in life [Thoreson & Cullen, 2007].

The incidence of IBD varies according to geographic location with higher rates occurring in more developed countries such as northern Europe, the United Kingdom, and North America and lower rates occurring in developing countries, Asia and southern Europe [Baumgart & Carding, 2007; Bernstein et al., 2015]. However, the incidence is rising in less-developed countries with the expansion of industrialization [Thoreson & Cullen, 2007].

One hypothesis for the difference in incidence between developed and developing nations is the “hygiene hypothesis,” which suggests that persons less exposed to childhood infections or unsanitary conditions lose potentially “friendly” organisms or

organisms that promote regulatory T cell development, or alternatively do not develop a sufficient immune repertoire as they do not encounter noxious organisms. Such individuals are associated with a higher incidence of chronic immune diseases, including IBD [Bernstein et al., 2015].

In Portugal, IBD prevalence has increased from 86 per 100 000 in 2003 to 146 in 2007 and this increase was consistent in all Portuguese districts. No north–south geographical distribution gradient is detected at the national level. The districts more affected by IBD are Lisbon and Oporto, with a prevalence that reached 173 and 163 per 100 000 in 2007, respectively. The estimated prevalence of UC increased from 42 per 100 000 in 2003 to 71 in 2007; and the prevalence of CD increased from 43 per 100 000 in 2003 to 73 in 2007. Furthermore, the prevalence of UC was higher in the 40–64 age range, whereas the prevalence of CD was higher in the 17–39 age range. Females had slightly higher prevalence than males [Azevedo et al., 2010].

There is an interesting discussion in the literature about the hypothesized 'north–south gradient' [Sonnenberg, McCarty & Jacobsen, 1991; Shivananda et al., 1996; Loftus & Sandborn, 2002; Loftus, 2004; Binder, 2004; Lakatos & Lakatos, 2006; Lakatos, 2006] in IBD epidemiology. However, the data contradicts this hypothesis because in Portugal, a southern European country, a lower prevalence would eventually be expected and because a geographical north–south gradient at a national level was not evident [Azevedo et al., 2010].

1.3. ETIOLOGY

The exact etiology of IBD is unknown. However, it is generally agreed that a complex interplay between genetic predisposition, an environmental trigger, an aberrant immune reaction and an infectious environment contributes to disease initiation and its progression [Wirtz & Neurath, 2007; Thoreson & Cullen, 2007; Mayer, 2010]. Evidence has suggested that IBD is the result of an abnormal immune response to intestinal bacteria in a genetically susceptible host [Abraham & Cho, 2009].

1.3.1. GENETIC FACTORS

Evidence for the contribution of genetic factors to IBD susceptibility was first identified through familial clustering of IBD cases and concordance studies in twins [Baumgart & Carding, 2007]; however, there is no evidence of classic Mendelian inheritance [Thoreson & Cullen, 2007]. Molecular studies have identified a number of different

genetic determinants of susceptibility for both the development of IBD and for variants that may determine disease phenotypes [Gaya, Russell, Nimmo & Satsangi, 2006]. These genetic abnormalities are thought to cause defects in the function of the epithelial barrier, immunoregulation, and clearance of bacteria [Abraham & Cho, 2009] that disturb the normal regulation of the intestinal immune system [Ponsky, Hindle & Sandler, 2007].

To date, four genes have been associated with CD and one with UC (TABLE 5); these data have been replicated. Strong associations with other chromosomal regions and genes (e.g. NF-κB1, TLR5) have yet to be replicated, but such associations make it highly likely that many additional genes will be implicated in the pathogenesis of IBD, while others will be associated with extra-intestinal disease (e.g. HLA-B27 and HLA-DR0103 human leukocyte antigen haplotypes) and with responses to pharmacologic treatment (e.g. pharmacogenomics) [Sartor, 2006; Wirtz & Neurath, 2007].

TABLE 5. Genes with functions associated with inflammatory bowel diseases and experimental colitis [adapted of Sartor, 2006].

GENE	CHROMOSOME	FUNCTION
CROHN'S DISEASE		
CARD15	16	NF-κB activation and/or regulation, killing of intracellular pathogens, paneth-cell function, α-defensin production
SLC22A4/5	5	Organic cation, carnitine transporters, possibly transport xenobiotic substances
DLG5	10	Epithelial scaffolding protein
PPARγ	3	Intracellular inhibitor of NF-κB and cellular activation
ULCERATIVE COLITIS		
MDR1	7	Efflux transporter for drugs and, possibly, xenobiotic compounds

Legend: CARD15 - Caspase recruitment domain family, member 15 (formerly NOD2); DLG5 - Discs large homolog 5 (Drosophila); MDR1 - Multidrug resistance 1; PPARγ - Peroxisome proliferative-activated receptor gamma; SLC22A4/5 - Solute carrier family 22 (organic cation transporter), members 4 and 5 (formerly OCTN1 and OCTN2).

1.3.2. ENVIRONMENTAL FACTORS

Epidemiological studies of worldwide trends have strongly supported a supporting role for environmental factors in the pathogenesis of IBD [Loftus, 2004; Lakatos, 2009]. These factors include diet, hygiene, smoking, the use of non-steroidal anti-inflammatory drugs (NSAID) and infection. Unfortunately, the mechanisms by which

these factors initiate the onset of disease or reactivate quiescent IBD are not well understood [Sartor, 2006]. From a broad perspective, these triggering factors alter mucosal barrier integrity, immune responses, or the luminal microenvironment, each of which have an impact on susceptibility to inflammation [Sartor, 2006].

A westernized diet (eg. low-fiber, high-sugar, high animal fat) has been proposed as a risk factor for the development of IBD. These theories are the result of an increasing incidence of IBD in areas such as Eastern Europe and Asia where westernized diets have been increasingly adopted. Dietary additives such as aluminum and iron have a well-described adjuvant activity and stimulate bacterial virulence [Perl, Fogarty, Harpaz & Sachar, 2004]. Other studies have reported links between various foods such as refined sugar, fast food, margarine, corn flakes, and some dairy products; however, there has been no conclusive evidence to suggest that any specific dietary factor or food directly contributes to the development of IBD [Lakatos, 2009].

Because IBD is more common in developed countries and urban areas, some evidence has suggested that improvements in hygiene and sanitation diminish exposure to environmental antigens, thereby impairing functional maturation of the mucosal immune system as well as disturbing normal development of immune tolerance. This may result in an inappropriate immune response when reexposure to these antigens occurs at a later time [Baumgart & Carding, 2007].

Smoking is perhaps the most thoroughly documented environmental contributor to IBD, but its opposite effect on CD and UC is difficult to understand, namely is detrimental in CD, but is protective in UC [Sartor, 2006]. Smoking has been shown to have a detrimental effect on CD [Lakatos, 2009]. CD patients who smoke tend to have a more severe course of disease with an increased incidence of fistulas, strictures and exacerbations [Baumgart & Carding, 2007]. Interestingly, the effect of smoking on UC appears to be protective and has been shown to postpone the onset of disease. Studies have shown that current smoking reduced the risk of UC [Thoreson & Cullen, 2007; Lakatos, 2009]. The risk of developing UC in smokers is approximately 40% of that in nonsmokers [Sandler & Eisen, 2000]. Nicotine, carbon monoxide (CO) and hypoxia have all been suggested to be mediators of the effects of smoking on IBD [Birrenbach & Bocker, 2004; Cosnes, 2004].

Infection and NSAID can transiently initiate nonspecific inflammation, break the mucosal barrier and activate innate immune responses. These events could then lead to enhanced uptake of commensal bacterial antigens and adjuvants that stimulate

protracted T-cell-mediated intestinal inflammation in the genetically susceptible host [Berg et al., 2002]. In the case of NSAID, the increased risk seems to be present for cyclooxygenase (COX)-2 inhibitors as well as COX-1 inhibitors, however it is unclear whether COX-2 inhibitors may be somewhat safer in select patients with IBD [Bonner, 2002; Mahadevan, Loftus, Tremaine & Sandborn, 2002].

Other environmental factors have been studied and have been implicated as playing a role in the pathogenesis of IBD including stress, oral contraceptive use and childhood immunizations; however, study results have been inconclusive [Lakatos, 2009].

1.3.3. IMMUNOLOGICAL FACTORS

The intestinal immune system is a complex system of humoral factors and a variety of lymphoid and nonlymphoid cell populations [Baumgart & Carding, 2007]. In persons with normal intestinal immune function, exposure to foreign antigens, bacteria and food is regulated through intestinal epithelial cells [Lakatos, 2009] that are covered by a mucus layer embedded with commensal microbes, protecting against disease. In IBD, the normal immune homeostasis of the intestine is disrupted at multiple levels starting with luminal antigens entering the underlying mucosa through a leaky barrier [Baumgart & Carding, 2007]. A cascade of events then causes an imbalance between the local mucosal production of proinflammatory cytokines and anti-inflammatory cytokines [Abraham & Cho, 2009], resulting in a cycle of uncontrolled inflammation [Thoreson & Cullen, 2007].

1.3.4. MICROBIAL FACTORS

Microorganisms and intestinal infections have been proposed as contributing factors to the pathogenesis of IBD by causing an inflammatory response to the infectious source. Bacteria that have been implicated include *Mycobacterium paratuberculosis*, *Pseudomonas* species, and *Listeria* species (in CD) and *Bacillus* species, adhesive *Escherichia coli* and *Fusobacterium varium* (in UC); however, evidence is inconclusive [Thoreson & Cullen, 2007].

Dysbiosis, a breakdown in the balance between normal enteric flora and harmful intestinal bacteria, has been theorized as a causal factor in the development of IBD. The beneficial effects seen with antibiotic use have supported this theory, but also symptomatic improvements that have been induced when the fecal stream is diverted away from inflamed bowel loops with recurrence of inflammation when intestinal continuity is restored. Other studies have found diminished bacterial diversity and a

greater number and concentration of bacteria in the bowel of patients with IBD [Lakatos, 2009].

1.4. PATHOGENESIS

Pathogenesis of IBD is not fully understood but two broad hypotheses have arisen regarding its fundamental nature. The first contends that primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microflora. The second suggests that changes in the composition of gut microflora and/or deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system. Currently, it's well accepted that IBD is indeed characterized by an abnormal mucosal immune response but that microbial factors and epithelial cell abnormalities can facilitate this response [Strober et al., 2007].

IBD patients have activated innate (macrophages, neutrophils) and acquired (T and B cells) immune responses and loss of tolerance to enteric commensal bacteria [Sartor, 2006]. Substantial progress has been made in characterizing immune cell populations and inflammatory mediators in patients with IBD (FIGURE 2), demonstrating that innate immune factors play an important role in the induction of mucosal inflammation [Strober et al., 2007].

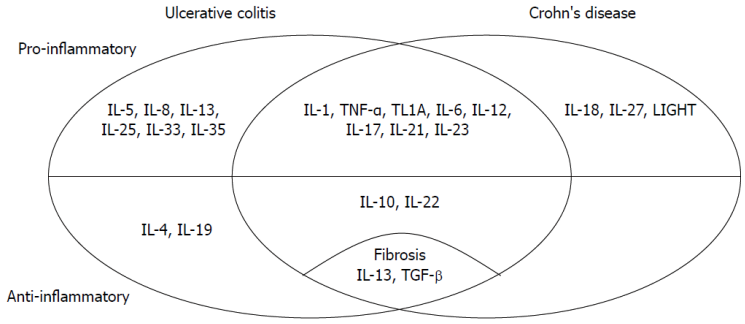

FIGURE 2. The disease-related pathogenic role of cytokines with pro-inflammatory, anti-inflammatory and pro-fibrogenic effects in ulcerative colitis and Crohn's disease [adapted of Műzes, Molnár, Tulassay & Sipos, 2012].

Legend: LIGHT - Lymphotoxins; IL - Interleukin; TNF-α - Tumor necrosis factor α; TL1A - TNF-like factor; TGF-β - Transforming growth factor-β.

In general, IBD are characterized by enhanced recruitment and retention of effector macrophages, neutrophils and T cells into the inflamed intestine, where they are activated and release pro-inflammatory cytokines. Accumulation of effector cells in the inflamed intestine is a result of enhanced recruitment as well as prolonged survival caused by decreased cellular apoptosis [Sartor, 2006]. However, CD and UC present

different cellular responses in the context of intestinal inflammation (FIGURE 3). CD is a dominantly T helper $(Th)_1$- and Th_{17}-mediated process, while UC seems to be an atypical Th_2 disorder [Sartor, 2006].

More precisely, CD is characterized by the generation of Th_1- and Th_{17}-polarized T cell responses [Fuss et al., 1996; Plevy et al., 1997; Cottrez, Hurst, Coffman & Groux, 2000]. Dendritic cells and macrophages are activated by interferon-γ (IFN-γ), which is produced by the activated Th_1 cells. Activated dendritic cells and macrophages produce cytokines such as interleukin (IL)-1β, IL-6, IL-12, IL-18, IL-23 and tumor necrosis factor- α (TNF-α). Th_{17}-polarized cells secrete IL-17 and IL-22 [Valatas, Vakas & Kolios, 2013]. The biological action of TNF-α and IL-6 is the main factor in the pathogenesis of CD and the regulation of this process is very important in controlling the disease [Desreumaux et al., 1997; Fiocchi, 1998; Sandborn & Hanauer, 1999]. TNF-α and IL-6 induce the expression of adhesion molecules in the vascular endothelium and invasion of inflammatory cells into the mucosal layer subsequently occurs. Selectins, intercellular adhesion molecule-1 (ICAM-1), and vasculocellular adhesion molecule-1, which are expressed at the surface of vascular endothelium, are involved in this process [Koizumi et al., 1992]. Thus, recruitment of inflammatory cells from the circulation is an important process in augmenting inflammatory response [Springer, 1994].

In contrast, the mucosa of patients with UC is characterized by an atypical Th_2-polarized T cell and natural killer T cells response mediated by IL-5 and IL-13 [Fiocchi, 1998]. Polarized T cell responses initiate an inflammatory cascade that involves endothelial activation, chemokine production and white blood cell (WBC) recruitment. Inappropriate triggering and maintenance of these pathogenic responses has been associated with innate immunity defects that include defective type I IFN production and lack of efficient control by anti-inflammatory cytokines such as IL-10 and transforming growth factor-β produced by regulatory T cells, macrophages, B cells, and stromal cells [Valatas et al., 2013].

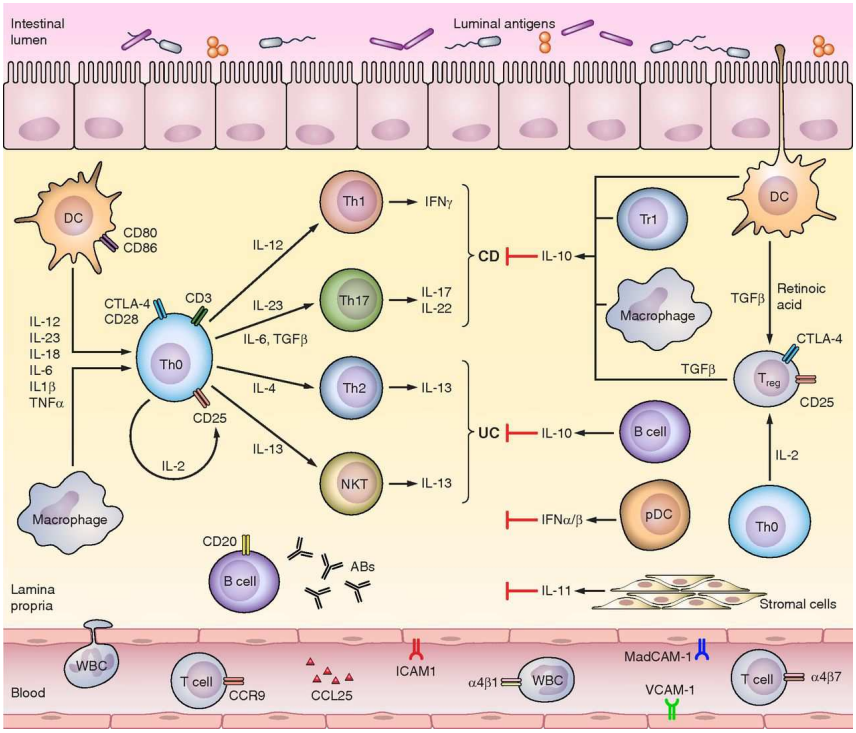

FIGURE 3. Inflammatory and regulatory pathways involved in inflammatory bowel disease [adapted of Valatas et al., 2013].

Legend: AB - Antibody; CD - Cluster of differentiation; CTLA-4 - Cytotoxic T lymphocyte antigen-4; ICAM-1 - Intercellular adhesion molecule-1; MAdCAM-1 - Mucosal addressin cell adhesion molecule-1; pDC - Plasmacytoid dendritic cell; VCAM-1 - Vascular cell adhesion molecule-1.

Moreover, it is well known that superoxide radical production and granular release are induced by the recruited leukocytes, especially granulocytes, and nonspecific inflammatory substances are subsequently produced. The main substances are arachidonic acid metabolites (such as thromboxane A2, leukotriene B4, and prostaglandin (PG) E2), reactive oxygen metabolites, free radicals, and nitric oxide (NO). These substances also play an important role in direct injury against the intestinal mucosa [Grisham, 1994; Cuzzocrea et al., 2001].

Thus, therapy can be directed towards blocking effector activation, blocking biologic activity of pro-inflammatory cytokines and their receptors, inhibiting T cell–APC interactions, selectively blocking effector cell entry, inducing apoptosis of activated effector cells or enhancing regulatory cell activity [Sartor, 2006].

2. DIAGNOSTIC ON INFLAMMATORY BOWEL DISEASE

Diagnosis of IBD is based on history and physical findings supported by characteristic endoscopic (sigmoidoscopy or colonoscopy) and histologic findings on biopsy once an infectious disorder has been ruled out as a potential cause [Kornbluth & Sachar, 2010]. The examination should include relevant questions regarding whether there is a family history of IBD, a history of blood or mucus in the stool, the frequency and consistency of the stool, nocturnal diarrhea, weight loss, extra-intestinal manifestations, foreign travel, nonsteroidal anti-inflammatory drug use and smoking [Collins & Rhodes, 2006; Kornbluth & Sachar, 2010].

Cardinal symptoms of IBD include bloody diarrhea, rectal urgency and tenesmus [Kornbluth & Sachar, 2010]. Lower abdominal pain also may be present when there is proximal extension of the disease [Thoreson & Cullen, 2007]. In mild disease, rectal bleeding may be absent and there are less than 4 stools a day. Severe disease involves more than 6 bloody stools per day along with systemic symptoms such as fever, tachycardia, anemia or hypoalbuminemia [Collins & Rhodes, 2006].

Associated extra-intestinal manifestations involve inflammation of the joints (sacroileitis, ankylosing spondylitis), eyes (anterior uveitis, episcleritis), skin (erythema nodosum, pyoderma gangrenosum) or bile ducts of the liver (primary sclerosing cholangitis) [Collins & Rhodes, 2006]. Toxic megacolon is a severe complication of IBD that can occur soon after diagnosis. Typical signs and symptoms include fever, abdominal tenderness and distension, hypotension, altered level of consciousness, anemia or leukocytosis [Thoreson & Cullen, 2007].

Laboratory tests for the evaluation of suspected IBD include a complete blood count with differential (to evaluate for anemia and infection), liver function tests, sedimentation rate and C-reactive protein [Carter, Lobo & Travis, 2004]. An elevated white blood cell count, platelet count and C-reactive protein level are consistent with extensive active intestinal inflammation, but these laboratory findings may be absent in left-sided UC [Nikolaus & Schreiber, 2007]. Other recommended laboratory testing includes microbiologic studies for bacterial infection, including specific assays for *Escherichia coli*, and parasitic infestation, along with serologic testing for ameba if indicated. Testing for *Clostridium difficile* toxin is indicated in patients who have been recently hospitalized or treated with antibiotics [Kornbluth & Sachar, 2010].

Proctosigmoidoscopy or colonoscopy with biopsy can diagnose the presence of colitis and help rule out other noninfectious or infectious causes [Kornbluth & Sachar, 2010]. When a complete colonoscopy cannot be performed, ultrasonography may be used in conjunction with flexible sigmoidoscopy to determine the extent of the disease, which can help guide treatment [Dietrich, 2009].

Alternative imaging modalities, such as magnetic resonance imaging and computed tomography may be beneficial when colonoscopy is not possible or the results are equivocal. In some cases, they can confirm the diagnosis, determine the extent of disease, evaluate complications and rule out other etiologies [Mackalski & Bernstein, 2006; Nikolaus & Schreiber, 2007].

3. CURRENT PHARMACOTHERAPY IN INFLAMMATORY BOWEL DISEASE

To treat IBD properly, the clinician must have a clear concept of realistic therapeutic goals for each patient. These goals may relate to resolution of acute inflammatory processes, resolution of attendant complications (e.g. fistulas and abscesses), alleviation of systemic manifestations (e.g. arthritis), maintenance of remission from acute inflammation, or surgical palliation or cure. The approach to the therapeutic regimen differs considerably with varying goals, so there is no evidence-based approach that applies to every clinical situation, as well as with the two diseases, UC and CD. Thus, treatment recommendations may differ for each patient and are individualized based on the patient's symptomatic response and tolerance to a specific therapy [Lichtenstein et al., 2009; Dignass et al., 2012].

In general, currently used medical therapy in IBD consists in salicylates, corticosteroids, immunosuppressants and biological therapy (FIGURE 4). These drug treatments have aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism [Engel & Neurath, 2010; Pithadia & Jain, 2011]. Actually, their use may result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases [Engel & Neurath, 2010]. Drug delivery to the appropriate site(s) along the gastrointestinal tract also has been a major challenge, and second-generation agents have been developed with improved drug delivery, increased efficacy and decreased side effects [Pithadia & Jain, 2011].

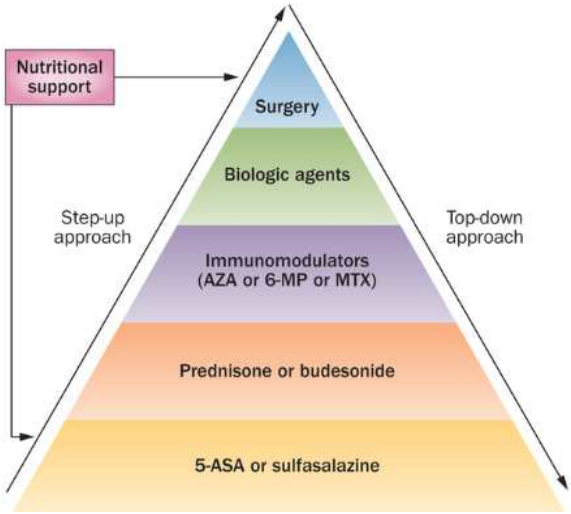

FIGURE 4. Therapeutic pyramid for the management of IBD
[adapted of Aloi, Nuti, Stronati & Cucchiara, 2014].
Legend: AZA – Azathioprine; 6-MP – 6-Mercaptopurine; MTX – Methotrexate; 5-ASA – 5-Aminosalicylic acid.

3.1. AMINOSALICYLATES

The archetype for this class of medications is sulfasalazine, which consists of an agent that combines sulfapyridine (sulfonamide antibiotic) and mesalamine (5-aminosalicylic acid – 5-ASA) in the same molecule linked by an azo bond. When it reaches the colon, the diazo bond is cleaved by bacterial azoreductase, liberating 5-ASA (which mostly remains in the colon and is excreted in stool) and sulfapyridine (which is mostly absorbed and excreted in the urine) [Sandborn & Hanauer, 2003; Buning & Lochs, 2006; Hanauer, 2006b].

5-ASA is the active therapeutic moiety, while sulfapyridine contributes little to the therapeutic effect. The mechanism of action of mesalamine is not well understood. COX or lipoxygenase inhibition alone does not entirely account for the agent's effects [Podolsky, 2002]; indeed, traditional NSAID may actually exacerbate IBD [Pithadia & Jain, 2011]. Many potential sites of action have been demonstrated *in vitro*, including inhibition of IL-1 and TNF-α production, inhibition of the lipoxygenase pathway, the scavenging of free radicals and oxidants, and inhibition of nuclear transcription factor kappa B (NF-κB), a transcription factor pivotal to the production of inflammatory mediators [Barnes & Karin, 1997; Podolsky, 2002]. Because the mechanism of action of sulfasalazine is not related to the sulfapyridine component, and because sulfapyridine is believed to be responsible for many of the adverse reactions to sulfasalazine, several second-generation 5-ASA compounds have been developed [Sandborn & Hanauer, 2003]. They are divided into two groups: pro-drugs and coated drugs [Pithadia & Jain, 2011].

Pro-drugs contain the same azo bond as sulfasalazine, but replace the linked sulfapyridine with either another 5-ASA (olsalazine) or an inert compound (balsalazide). These compounds act at similar sites along the gastrointestinal tract as sulfasalazine. The alternative approaches employ either a delayed-release formulation or a pH-sensitive coating, where mesalamine can be used alone. Delayed-release mesalamine is released throughout the small intestine and colon, whereas pH-sensitive mesalamine is released in the terminal ileum and colon. The different distributions of these drugs following delivery have potential therapeutic implications. In general, newer 5-ASA preparations have similar therapeutic efficacies with fewer side effects. 5-ASA can also be used in combination with steroids to induce and maintain remission in patients with IBD [Pithadia & Jain, 2011].

3.2. CORTICOSTEROIDS

Corticosteroids are potent anti-inflammatory agents for moderate to severe relapses of IBD. They act through inhibition of several inflammatory pathways like suppressing IL transcription, induction of inhibitory κB (IκB) that stabilises the NF-κB complex, suppression of arachidonic acid metabolism and stimulation of apoptosis of lymphocytes within the lamina propria of the gut [Mowat et al., 2011]. Thus, corticosteroids are believed to modulate the immune system and inhibit production of inflammatory cytokines and others inflammatory mediators [Hofer, 2003].

Corticosteroids have been widely used in IBD, given parenterally, orally or rectally [Friend, 1998]. The glucocorticoid properties of hydrocortisone and prednisolone are the mainstay of IBD treatment. The preferred steroid is prednisolone, administered in emergency situations. Hydrocortisone also can be given once or twice daily, delivering 80 mg hydrocortisone per application. This formulation can be useful in patients with very short areas of distal proctitis and difficulty retaining fluids [Pithadia & Jain, 2011]. Budesonide is a controlled release formulation designed to release in the terminal ileum [Greenberg et al., 1994; Hofer, 2003]. It is thought to deliver adequate steroid therapy to a specific portion of the inflamed gut while minimizing systemic side effects caused by extensive first-pass hepatic metabolism to inactive derivatives [Hofer, 2003].

Corticosteroids can be used either alone or in combination with a suitable mesalamine formulation to induce and maintain remission in IBD [Faubion, Loftus, Harmsen, Zinmeister & Sandborn, 2001; Pithadia & Jain, 2011]. Steroids are sometimes used for prolonged periods to control symptoms in steroid dependent patients. However, failure to respond to steroids with prolonged remission (i.e., a disease re-lapse) should prompt consideration of alternative therapies, including immunosuppressants and infliximab [Steinhart, Ewe, Griffiths, Modigliani & Thomsen, 2003]. Also, steroids are inappropriate for long term use, because of their side effects and inability to maintain remission in all patients [Steinhart et al., 2003; Pithadia & Jain, 2011]. Thus, their significant side effects have led to an increased emphasis on limiting the duration and cumulative dose of steroids in IBD. In this case, an alternate-day regimen can be helpful [Pithadia & Jain, 2011].

Adrenal insufficiency can be triggered in patients who have been recently tapered off of corticosteroids. Visual changes can occur because of steroid-induced hyperglycemia. Early cataract formation is another possible side effect. Aseptic joint necrosis, which is

the most dreaded side effect, usually occurs in patients receiving long-term, high-dose corticosteroid therapy [Pithadia & Jain, 2011].

3.3. IMMUNOSUPPRESSANTS

Immunosuppressant drugs can be an invaluable adjunct therapy for the treatment of patients with intractable IBD or complex, inoperable perianal disease. Although immunosuppressant agents have significant side effects, they are safer and better tolerated than long-term corticosteroid therapy. However, these agents should not be used in young patients who are candidates for surgery or in patients who are noncompliant and refuse to return for periodic monitoring. Before immunosuppressant therapy is initiated, side effects and other treatment alternatives should be discussed with the patient. At this stage, it is best to set a definable goal, such as closure of a fistula or tapering the patient off of corticosteroids, and a minimum three-month time frame should be set to reach that goal [Pithadia & Jain, 2011].

Azathioprine or mercaptopurine are widely used in IBD as adjunctive therapy and as corticosteroid-sparing therapies, although they are unlicensed therapies for IBD. Their slow onset of action precludes usage as sole therapy for active disease. Purine antimetabolites inhibit ribonucleotide synthesis, but the mechanism of immunomodulation is by inducing T cell apoptosis by modulating cell (Rac1) signalling. Azathioprine is non-enzymatically metabolised to mercaptopurine, which involves loss of a nitro-imidazole side chain; this is thought to explain some of the side effects seen with azathioprine and which may be less of a problem with mercaptopurine. Mercaptopurine is subsequently metabolised to 6-thioguanine nucleotides. 6-thioguanine nucleotides have been used for treatment of IBD, but caution is appropriate because of potential hepatotoxicity [Mowat et al., 2011].

Polyglutamated metabolites of methotrexate inhibit dihydrofolate reductase, but this cytotoxic effect does not explain its anti-inflammatory effect. Inhibition of cytokine and eicosanoid synthesis probably plays a role. At present methotrexate is positioned as a second-line immunosuppressive agent in patients resistant or intolerant of azathioprine or mercaptopurine, although it is currently unclear whether thiopurines are any more efficacious than methotrexate for induction or maintenance of remission in IBD [Mowat et al., 2011].

Cyclosporine is an inhibitor of calcineurin, which is a potent immunosuppressant drug used in organ transplantation. Since the mid-1980s, this drug has also been used to

treat patients with IBD [Pithadia & Jain, 2011]. It acts to prevent clonal expansion of T cell subsets. At this time, cyclosporine is effective in severe UC that has failed to respond adequately to glucocorticoid therapy. In such patients, intravenously administered cyclosporine is highly effective for rapid disease control, and it may allow patients to avoid surgery [Mowat et al., 2011]. However, after one year, 70 to 80% of these patients may still require surgery. Thus, in many patients, the role of cyclosporine is to change a risky emergency operation into a less urgent procedure. Cyclosporine is effective in specific clinical settings in IBD, but the high frequency of significant adverse effects limits its use as a first-line medication [Pithadia & Jain, 2011].

3.4. MONOCLONAL ANTIBODIES

Biological response modifiers are drugs that interfere with the inflammatory response. There are presently two biological agents licensed for the treatment of IBD; both are monoclonal antibodies against TNF-α (anti-TNF) [Mowat et al., 2011]. Although many pro- and anti-inflammatory cytokines are generated in the inflamed gut in IBD, there is some rationale for targeting TNF-α, because it is one of the principal cytokines mediating the Th_1 immune response characteristic of CD. The administration of humanized monoclonal antibodies is an entirely new and potentially highly successful concept for treating IBD [Pithadia & Jain, 2011].

The first such product, infliximab, is a chimeric immunoglobulin (75% human IgG and 25% murine) that binds to and neutralizes TNF-α, and it represents a new class of therapeutic agents for treating IBD [Targan et al., 1997]. Infliximab (infused intravenously) decreases the frequency of acute flare-ups in approximately two-thirds of patients with moderate to severe CD and facilitates the closing of enterocutaneous fistulas associated with CD [Present et al., 1999; Pithadia & Jain, 2011; Mowat et al., 2011].

Adalimumab is an anti-TNF agent similar to infliximab and decreases inflammation by blocking TNF-α. In contrast to infliximab, adalimumab is a fully humanized anti-TNF antibody (no mouse protein). Adalimumab is administered subcutaneously instead of intravenously, but is comparable to infliximab in effectiveness and safety for inducing and maintaining remission in patients suffering from CD that has failed to respond to standard immunosuppression (e.g, corticosteroids and thiopurine or methotrexate therapy). Adalimumab is also effective in healing anal fistulas in patients with CD. It is well tolerated and has been shown to be effective for patients who cannot tolerate infliximab [Mowat et al., 2011; Pithadia & Jain, 2011].

4. TREATMENT PROTOCOLS ON INFLAMMATORY BOWEL DISEASE

4.1. TREATMENT PROTOCOL FOR CROHN´S DISEASE

Management of CD often proves more difficult than management of UC, partly because of the greater complexity of presentation with CD (FIGURE 5). The disease may involve any segment of the gastrointestinal tract, from mouth to anus, and may involve other visceral structures and soft tissues through fistulization. There is a greater reliance on drug therapy with CD, because ressection of all involved intestine may not be possible. Unfortunately, recurrence of CD is common following surgery with reported rates of up to 64% recurrence following surgical resection of affected areas of bowel [Hancock, Windsor & Mortensen, 2006].

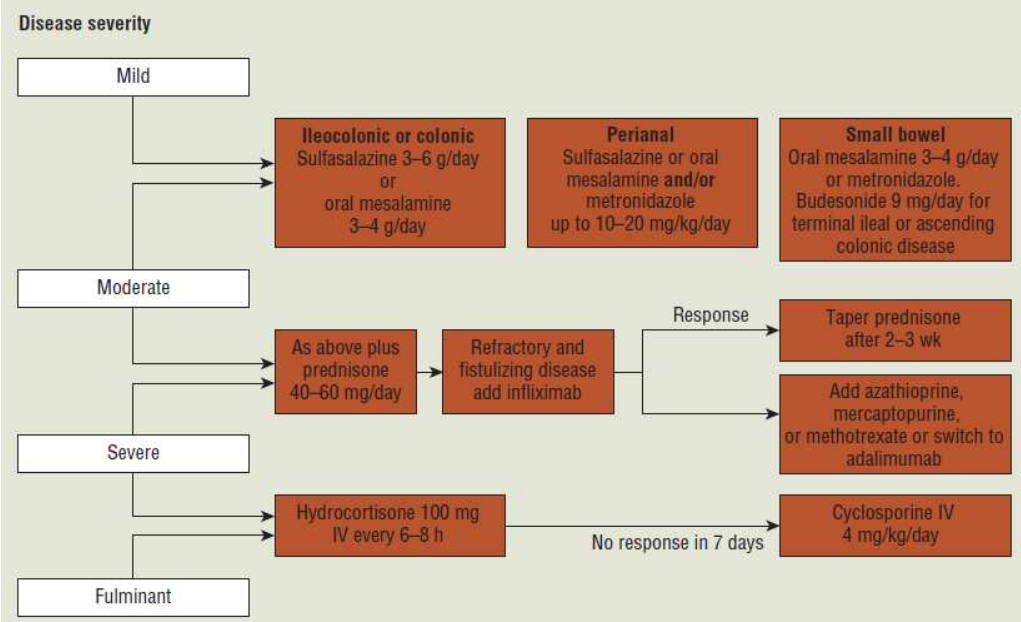

FIGURE 5. Treatment algorithm for the management of CD [adapted of Dipiro et al., 2008].

The goal of treatment for active CD is to achieve remission; however, in many patients, reduction of symptoms so the patient may carry out normal activities, or reduction of the steroid dose required for control, is a significant accomplishment. In the majority of patients, active CD is treated with sulfasalazine, mesalamine derivatives, or steroids, although azathioprine, mercaptopurine, methotrexate, infliximab, and metronidazole are frequently used [Pithadia & Jain, 2011].

Sulfasalazine is more effective when CD involves the colon [Hanauer & Sandborn, 2001]. In these circumstances, sulfasalazine is as effective as prednisone [Sandborn, 2003; Kornbluth & Sachar, 2004; Gionchetti, 2006]. Mesalamine formulations have the

ability to act in the small bowel, thus targeting ileal disease. However, these formulations have demonstrated variable results in patients with active CD [Sandborn, 2003; Gionchetti, 2006]. Despite variable effectiveness, the mesalamine derivatives may be better tolerated than sulfasalazine, particularly at higher doses. Thus, a trial of sulfasalazine or an oral mesalamine derivative is reasonable as initial therapy in patients with mild to moderate active CD with ileal, ileocolonic, or colonic involvement [Kornbluth & Sachar, 2004].

Steroids are frequently used for the treatment of active CD, particularly with more moderate to severe presentations, or in those patients unresponsive to aminosalicylates. Budesonide is a viable first-line option for patients with mild to moderate ileal or right-sided (ascending colonic) disease [Hofer, 2003; Otley & Steinhart, 2005; AGAI, 2006]. Budesonide is inferior to traditional systemic steroids for severe disease, although it carries a lower risk of adverse effects, and does not reach areas distal to the ascending colon. Oral systemic steroids are effective in inducing remission in up to 70% of patients and should be reserved for patients with moderate to severe disease who have failed aminosalicylates or budesonide [AGAI, 2006; Gionchetti, 2006]. Hospitalized patients with severe or fulminant disease, or those who are unable to tolerate oral therapy, are candidates for administration of parenteral steroids [Hofer, 2003; AGAI, 2006].

Metronidazole (given orally up to 20 mg/kg per day in divided doses) has demonstrated variable efficacy, but may possibly be useful in some patients with CD, particularly in patients with colonic or ileocolonic involvement, or in those patients with perineal disease [Sandborn, 2003; AGA, 2003; Guslandi, 2005; Gionchetti, 2006]. For these patients, metronidazole is added to a mesalamine product or steroids as adjunctive therapy, where satisfactory control of CD is not gained with first-line agents, or in attempts to reduce steroid dosage [Lawson, Thomas & Akobeng, 2006]. Ciprofloxacin has gained attention as an alternative to metronidazole [Sandborn, 2003; Gionchetti, 2006]. The combination of metronidazole and ciprofloxacin appears to be efficacious in some patients with perianal disease [AGA, 2003; Guslandi, 2005].

The immunosuppressive agents are effective, but are generally limited to use in patients who are not achieving adequate response to standard medical therapy, or to reduce steroid doses when high steroid doses are required [Sandborn et al., 2000; Derijks, Gilissen, Hooymans & Hommes, 2006; AGAI, 2006; Gionchetti, 2006]. Azathioprine and mercaptopurine have both demonstrated long-term benefits in

patients with CD [Derijks et al., 2006; Holtmann et al., 2006]. The usual dose of azathioprine is 2 to 3 mg/kg per day; the usual dose of mercaptopurine is 1 to 1.5 mg/kg per day [AGAI, 2006]. Starting doses are typically 50 mg/day and increased at 2-week intervals; complete blood counts with differential should be monitored every 2 weeks while doses are being titrated [AGAI, 2006]. The onset of therapeutic effects is delayed and a minimum of 3 to 4 months is often required to see clinical benefits [AGAI, 2006; Gionchetti, 2006].

Although mostly used in the setting of maintenance therapy, methotrexate is another option for use as induction therapy [Gionchetti, 2006]. Use of a weekly intramuscular injection of 25 mg has demonstrated efficacy for induction of remission in CD, and has also demonstrated corticosteroid-sparing effects [Alfadhli, McDonald & Feagan, 2004; AGAI, 2006; Gionchetti, 2006]. Although there are risks of bone marrow suppression, hepatotoxicity, and pulmonary toxicity, use of low-dose methotrexate appears relatively safe when continued as maintenance therapy if proper monitoring is implemented [AGAI, 2006].

Infliximab is used for treating moderate to severe active CD in patients failing immunosuppressive therapy, in those who are corticosteroid dependent, and for treatment of fistulizing disease [Rutgeerts, Van Assche & Vermeire, 2006; AGAI, 2006; Kamm, 2006]. Patients who receive infliximab often develop antibodies to infliximab, which can result in increases in the occurrence of serious infusion reactions and loss of response to the drug. Strategies to reduce formation of antibodies to infliximab include administration of a second dose within 8 weeks of the first dose, concurrent administration of steroids (hydrocortisone 200 mg intravenously on the day of the infusion or oral prednisone the day prior), and use of concomitant immunosuppressive agents [Rutgeerts et al., 2006; AGAI, 2006; Kamm, 2006]. Induction of tolerance using a dose escalation technique was also effective in administering infliximab to patients with previous severe infusion reactions [Duburque et al., 2006]. Treatment with adalimumab is recommended in patients with moderately to severe active CD, without effective response to infliximab. In these cases, the patients have demonstrated up to a 54% complete response [Konstantinos et al., 2005; Hanauer et al., 2006; Sandborn et al., 2007].

Cyclosporine is typically not recommended for treatment of CD, except for acute management of patients with severe fistulizing disease [McDonald et al., 2005; AGAI, 2006; Bressler & Sands, 2006]. Up to 83% of patients with refractory fistulas responded

to intravenous (IV) cyclosporine within a mean of 7.9 days [Gionchetti, 2006]. The dose of cyclosporine is important in determining efficacy. An oral dose of 5 mg/kg per day is ineffective, whereas 7.6 mg/kg per day has demonstrated effectiveness in some trials [McDonald et al., 2005; AGAI, 2006]. However, toxic effects limit the routine use of this higher dosage. At present, the therapeutic blood or plasma concentration range for cyclosporine has not been established for CD, but whole-blood trough concentrations of 300 to 500 ng/ml for IV therapy or 200 to 400 ng/ml for oral therapy are reasonable goals [AGAI, 2006; Gionchetti, 2006]. When using cyclosporine clinicians should recognize the accompanying long-term risk of renal toxicity and infection, as well as the potential for drug interactions [AGAI, 2006].

4.2. TREATMENT PROTOCOL FOR ULCERATIVE COLITIS

Prior to initiating therapy for UC, a patient must be evaluated for extent and severity of disease. Thenceforth, the management of UC can be applied (FIGURE 6). In general, if disease is distal to the splenic flexure, topical therapy such as suppositories and enemas may be the first choice. For more extensive disease, oral agents or a combination of oral and topical agents are indicated [Mahadevan, 2004].

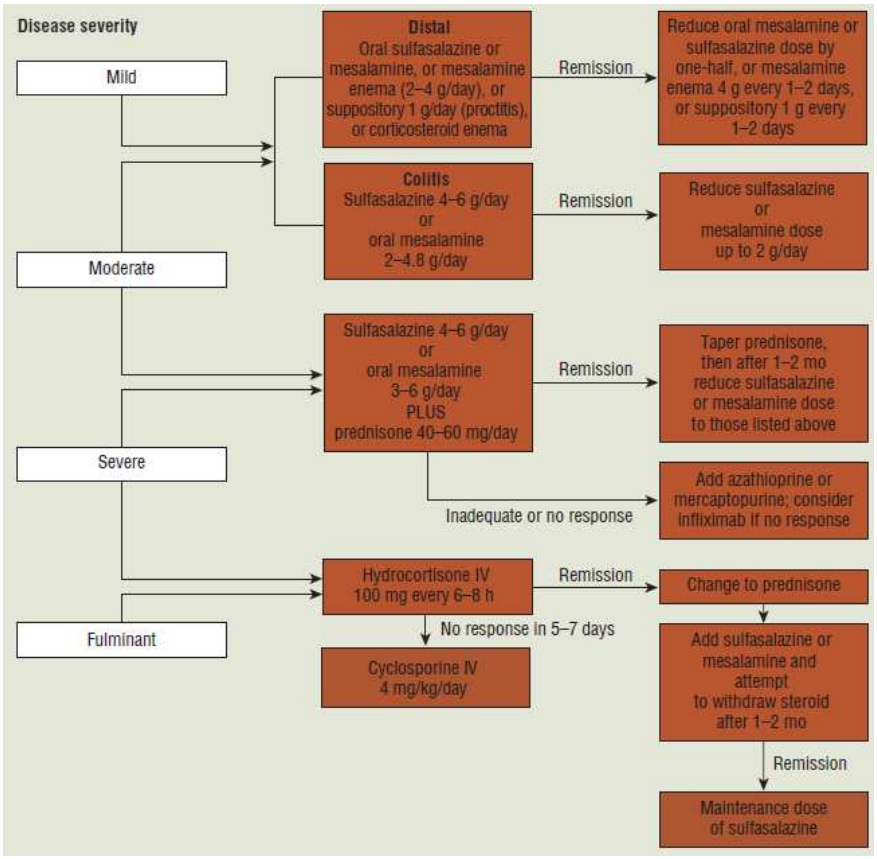

FIGURE 6. Treatment algorithm of management of UC [adapted of Dipiro et al., 2008].

Most patients with active UC have mild to moderate disease and do not require parenteral medications. The first line of drug therapy for patients with extensive disease is oral sulfasalazine or an oral mesalamine derivative [Kornbluth & Sachar, 2004]. Oral mesalamine derivatives are reasonable alternatives to sulfasalazine for treatment of UC. Oral mesalamine products are used for patients with extensive disease, while topical agents, such as enemas and suppositories, are used for distal disease. Mesalamine is not more effective than sulfasalazine for extensive disease [Hanauer, 2006a; Sutherland & MacDonald, 2006]. Topical mesalamine is more effective than oral mesalamine or topical steroids for distal disease [Kornbluth & Sachar, 2004]. Mesalamine preparations are typically better tolerated, and the majority of patients intolerant to sulfasalazine or topical steroids should tolerate one of the other oral mesalamine derivatives [Kornbluth & Sachar, 2004; Collins & Rhodes, 2006]. When used topically, mesalamine suppositories will only reach to approximately 10 to 20 cm and thus should be reserved for patients with proctitis. Because enema formulations reach to the splenic flexure, they can be used for distal disease [Regueiro, 2004; Kornbluth & Sachar, 2004]. Olsalazine is another 5-ASA that is given orally and is effective for treatment of mild to moderate UC. However, of patients taking olsalazine, 15% to 25% experience severe diarrhea, often necessitating discontinuation of the drug. This results from a direct osmotic effect of the drug to induce small-bowel fluid secretion. For this reason, it is not the drug of first choice [Kornbluth & Sachar, 2004].

Steroids have a place in the treatment of moderate to severe active UC regardless of disease location, or in those patients who are unresponsive to maximal doses of oral or topical mesalamine derivatives and/or sulfasalazine [Pruitt et al., 2002; Kornbluth & Sachar, 2004]. Overall, steroids and sulfasalazine appear to be equally efficacious; however, the response to steroids may be evident sooner. Oral steroids should not be used as initial therapy for mild to moderate UC, mainly because of the known risks of steroid use. If steroids are used to attain remission, tapered drug withdrawal should be accomplished to minimize long-term steroid exposure [Kornbluth & Sachar, 2004]. Rectally administered steroids given as suppositories, enemas, or foams can be used as initial therapy for patients with ulcerative proctitis, distal colitis or tenesmus. With these agents, local actions are believed to be responsible for drug effects. However, rectal mesalamine is more effective than rectal steroids for inducing remission [Regueiro, 2004; Kornbluth & Sachar, 2004; Sandborn, 2006; Gionchetto, 2006; AGAI, 2006].

Infliximab is another viable option for patients with moderate to severe active UC who are unresponsive to steroids or other immunosuppressive agents [Rutgeerts et al., 2006; Sandborn, 2006; AGAI, 2006; Lawson et al., 2006]. Outpatients with moderately active UC have response rates of up to 69% at 8 weeks following initial doses of 5 mg/kg [Rutgeerts et al., 2005]. Hospitalized patients have mixed results, with some demonstrating reduced rates of colectomy for patients receiving infliximab [Janerot et al., 2005; Lawson et al., 2006; Regueiro, Curtis & Plevy, 2006].

Nicotine has been proposed as a treatment for UC (but not as a treatment for CD) based on the observation of the onset of a flare of UC after smoking cessation in some individuals. While less effective than aminosalicylates, transdermal nicotine in daily doses of 15 to 25 mg may improve symptoms in patients with mild to moderate active UC and can be considered as an adjunctive therapy [Kornbluth & Sachar, 2004; McGrath, McDonald & MacDonald, 2004].

Patients with uncontrolled severe colitis or who have incapacitating symptoms require hospitalization for effective management. Under these conditions, patients generally receive nothing by mouth to put the bowel at rest. Most medication is administered by parenteral route. Sulfasalazine or mesalamine derivatives are not beneficial for treatment of severe colitis because of rapid elimination of these agents from the colon with diarrhea, thereby not allowing sufficient time for gut bacteria to cleave the molecules. Overall it is difficult to evaluate drugs in this setting, because patients with severe disease almost always receive additional medications including steroids [Kornbluth & Sachar, 2004].

Steroids have been valuable in the treatment of severe disease, because may allow some patients to avoid colectomy. Methylprednisolone is typically preferred, because of its lesser mineralocorticoid effects. A trial of steroids is warranted in most patients before proceeding to colectomy, unless the condition is severe or rapidly deteriorating. The length of the medical trial before consideration of surgery is open to debate. Steroids increase surgical risk, particularly infectious risk, if an operation is required later. After a colectomy is performed, steroids should no longer be required for the disease; however, they must be withdrawn gradually to avoid hypoadrenal crisis because of adrenal suppression [Kornbluth & Sachar, 2004; AGAI, 2006].

Patients who are unresponsive to parenteral corticosteroids after 7 to 10 days should receive cyclosporine by IV infusion [Durai & Hawthorne, 2005]. Continuous IV infusion

of cyclosporine is typically effective in steroid-resistant acute severe UC and may reduce the need for emergent colectomy [Durai & Hawthorne, 2005; Shibolet, Regushevskaya, Brezis & Soares-Weiser, 2005]. Patients who are controlled on IV cyclosporine can then be switched to an oral cyclosporine taper regimen with subsequent transition to azathioprine or 6-mercaptopurine [AGAI, 2006]. Infliximab is also an alternative to cyclosporine, and may also deter the need for colectomy in patients with severe disease not responsive to steroids [Rutgeerts et al., 2005; Janerot et al., 2005; AGAI, 2006; Lawson et al., 2006].

5. PROGNOSTIC OF INFLAMMATORY BOWEL DISEASE

The clinical course of IBD is relapsing and remitting; it can occur spontaneously or in response to concomitant illnesses or changes in treatment [Kornbluth & Sachar, 2010]. The symptoms can last for days, weeks, or months and then go into remission for months, years, or sometimes decades [Thoreson & Cullen, 2007]. The severity of disease varies but most patients have mild to moderate disease and will remain in remission for extended periods of time with maintenance medical therapy. However, at some time during the course of the disease, up to 15% of patients will develop a severe flare and up to 40% of patients will have a colectomy [Doherty & Cheifetz, 2009].

The natural history of IBD differs between adults and children. Pancolitis, proximal extension [Ponsky et al., 2007], and steroid dependency are more common in children [Levine, 2009]. Growth failure, which can occur in children with IBD, can negatively affect psychosocial development and self-image. Poor nutrition, inflammation of the gut, disruption of the growth hormone/insulin-like growth factor axis, and corticosteroid therapy may be contributing factors to the development of growth failure in these patients [Beattie, Croft, Fell, Afzal & Heuschkel, 2006].

The patients with IBD are also exposed to a risk of colorectal cancer, depending on the extent of disease and duration [Kornbluth & Sachar, 2010]. A meta-analysis found that colorectal cancer risk for any patient with UC was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease. This translates to a 5- to 10-fold increase in the relative risk for colorectal cancer compared with age-matched controls [Mpofu, Watson & Rhodes, 2004]. Nevertheless, UC that is limited to the rectum or rectosigmoid does not increase the risk [Itzkowitz & Present, 2005]. The relative lifetime risk of developing colon cancer for patients with either pancolitis or left-sided UC is much higher than in the general population [Eaden, Abrams & Mayberry, 2001]. However, a case-control study found that the risk was reduced by 75% with the regular use of aminosalicylate drugs (sulfasalazine and mesalamine) [Eaden, Abrams, Ekbom, Jackson & Mayberry, 2000].

Regarding mortality rate, a meta-analysis of 10 studies including 10,443 patients found that the overall risk of death was not significantly different between patients with IBD and the general population [Jess, Gamborg, Munkholm & Sorensen, 2007]. Due to more aggressive treatment and monitoring, mortality from acute severe disease has decreased by 25% [Doherty & Cheifetz, 2009].

CHAPTER 2 – ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

While the introduction of biologic inflammatory mediators such as TNF-α inhibitors has improved the IBD therapeutic landscape, there remains considerable unmet medical need particularly in patients with severe or complicated clinical manifestations and in pediatric populations [Yang, Alex & Catto-Smith, 2012a; Assaa et al., 2013; Mehta, Silver & Lindsay, 2013]. This need continues to drive IBD research efforts at universities, research foundations, and biotechnology and pharmaceutical companies spanning the spectrum from basic science to translational medicine. A common thread in most of these research efforts is the need for appropriate animal models. Animal models provide means of characterizing physiologic interactions when our understanding of such processes is insufficient to allow replacement with in vitro systems [Voss & Diehl, 2014].

Medical knowledge, treatment, and research that are involved in understanding the etiopathology of IBD have used laboratory animals for decades. The number of animal models used for preclinical studies and to understand the mechanisms of gastrointestinal inflammation is continually expanding. Currently, many animal models are being developed to focus on understanding the genetics and immunology of IBD. Many of these animal models are based on experimental needs; however, the positive impact of these animal models in advancing the medical knowledge of IBD cannot be underestimated [Murphy, 2006].

It is apparent that animal models do not entirely resemble human IBD, and also that one IBD patient does not entirely resemble another very well. Thus, the diversity of responses we observe in animal models is no different than what we observe in humans. Experience has been that even homogenously bred animals tend to respond differently to certain stimuli and treatments [Murphy, 2006]. Furthermore, although these models do not represent the complexity of human disease and do not replace studies with patient material, they are valuable tools for studying many important disease aspects that are difficult to address in humans, such as the pathophysiological mechanisms in early phases of colitis and the effect of emerging therapeutic strategies [Wirtz & Neurath, 2007; Szczepanik et al., 2012]. Indeed, animal models play pivotal role in the development of novel therapeutic drug to cure IBD and dissect the possible mechanism of action of a particular drug [Randhawa, Singh, Singh & Jaggi, 2014].

Clinically, there are several types of animal models of IBD (TABLE 6), who makes it difficult for the researcher to choose the most suitable animal model for its own study [Qin et al., 2011]. In general, these models can be divided into four major groups based upon how the disease is produced. These models include those that express intestinal inflammation spontaneously or naturally in their native or confined environment. The second category of model includes those models in which intestinal inflammation can be induced by specific immunological or chemical agents that physically induce an immune response and mucosal damage in the local bacterial environment in relation to the damaging agent used. The third category includes those models that are genetically engineered by gene knockout, gene knockin or transgenic methods, so that the genetic factors related to the disease could be well defined and drugs that target a specific gene or cytokine can be tested. The last category includes "adoptive transfer models" in which experimental inflammation is induced by transferring certain T cell populations into an immune-compromised host lacking lymphoid tissue. Thus, there is an assortment of animal models of inflammation available for pharmacological testing of anti-inflammatory agents specific to IBD [Murphy, 2006; Wirtz & Neurath, 2007; Randhawa et al., 2014].

Since so many experimental animal models are now available, investigators must take into consideration the species, strains, substrains, the microenvironment in which they live and the mediators involved in each of these models, for application to preclinical testing [Murphy, 2006]. The spontaneous models are not often used, because the sporadic nature of the appearance of the disease, self-limiting inflammation, expense in procuring some of these animals and their husbandry contribute to their limited use in preclinical trials [Murphy, 2006]. On the other hand, the use of genetically engineered models of IBD pretends to identify the genetic factors underlying experimental models of mucosal inflammation, followed by a candidate gene approach in humans, testing the relevance of the identified mouse gene to human IBD [Borm & Bouma, 2004]. However, these animal models are not appropriate for a preclinical efficacy testing. Genetically engineered models work with an inbred population, with total control of the environment and only one specific gene is modified, so all of these parameters don't mimetics the human IBD [Borm & Bouma, 2004]. Regarding immunological models, it requires a much more complex and labor-intensive protocol than many other IBD models, due to the extraction, isolation, purification, and injection of adoptive T cells, as well as the number and viability of T cells transferred and the presence of B cells in the recipient animals largely modified the obtained results [Ostanin et al., 2009].

Amongst the various models, chemically induced models are extensively used, as they offer reproducibility and ease of development [Randhawa et al., 2014]. Furthermore, one of the main goals of animal model use in IBD research is for preclinical efficacy testing. This is of considerable importance to pharmaceutical and biotechnology companies, and results of preclinical efficacy studies frequently serve as gating criteria that determine whether a therapeutic candidate will advance in the drug development pipeline. Preclinical efficacy studies most commonly utilize well-established chemically induced models of intestinal inflammation [Voss & Diehl, 2014].

Beyond the most suitable animal model, the animal specie used in the study is also important. Colitis models have been developed in rats, mice, pigs, rabbits, nonhuman primates, and dogs [Mansfield et al., 2001; Murthy, 2006; Holst, Kleinschmidt, Nolte & Hewicker-Trautwein, 2012; Wadie et al., 2012]. However, the use of mouse models enables relative ease in incorporating animals with specific genetic modifications, use of larger cohort sizes, and decreases the quantity of therapeutic reagents required [Voss & Diehl, 2014].

In sum, ideally, a disease model should closely parallel the human disease in clinical manifestations, pathophysiology, and response to existing therapeutic reagents. However, this is rarely possible with complex human diseases such as IBD where multiple genetic and environmental influences determine disease onset and clinical course. This creates ongoing challenges for scientists involved in IBD model development and analysis. Planning for success in IBD animal model development may require that we consider Voltaire's comment that ''the perfect is the enemy of the good'' (Voltaire, 1772) and focus our efforts on identifying the specific features we need to model rather than trying to recapitulate the entirety of human disease in an experimental animal [Voss & Diehl, 2014].

TABLE 6. Animal models of inflammatory bowel disease [adapted of Murthy, 2006].

MODEL	SPECIES	METHOD OF INDUCTION	TIME COURSE	DISEASE LOCATION	TYPE OF COLITIS
I. SPONTANEOUS MODELS					
1. C3H/HeJBir	Mice	Natural	4 - 6 weeks	Cecum and colon	Acute
2. Cotton top tamarins	Monkeys	Natural	< 1 year	Colon	Acute and chronic
3. SAMP1/YIT	Mice	Natural	30 weeks	Ileum	Chronic
II. IMMUNOLOGICAL MODELS					
1. Immune complex-mediated	Rabbits and rats	Injection albumin anti-albuin complex + a formalin enema	3h – 8 weeks	Colon	Acute
III. BACTERIA AND BACTERIAL PRODUCTS MODELS					
1. Helicobacter hepatics	Mice	Infection	2 - 90 days	Colon	Chronic
2. PGPS	Rats	Transmural injection of PGPS	2 - 90 days	Ileum	Chronic, relapsing
IV. CHEMICALLY INDUCED MODELS					
1. TNBS	Rats, rabbits and mice	TNBS enema (20-30mg in 30-50% EtOH)	3 days – 8 weeks	Small intestine or colon	Acute and chronic
2. DSS	Hamsters, mice and rats	2 - 10% DSS feeding	5 days – 15 weeks/longer	Colon	Acute and chronic
3. Acetic acid	Rats	1 – 10% acetic acid enema	1 day – 3 weeks	Colon	Acute
4. Carrageenan	Rats, guinea, pigs and rabbits	Variable oral dosing	1 – 4 weeks	Cecum and colon	Acute and chronic

MODEL	SPECIES	METHOD OF INDUCTION	TIME COURSE	DISEASE LOCATION	TYPE OF COLITIS
5. Indomethacin	Rats	Oral or SC once or twice	< 1 - 8 days	Small intestine	Acute
6. Oxazalone	Mice and rats	Intracolonic	Rapid	Colon	Acute
V. MISCELLANEOUS CHEMICAL MODELS					
1. Peroxinitrite	Rats	Enema	< 1 day – 3 weeks	Colon	Acute
2. Mitomycin C	Fischer rats	i.p. 2.75 mg/Kg	--	Colon	Acute
3. Iodoacetamide	Rats	3% enema	--	Colon	Acute and chronic
4. PMA	Rabbits	Enema	4 days	Colon	Acute
VI. GENETICALLY ENGINEERED MODELS					
1. HLA-B27/β2 microglobulin	Rats	Transgene	> 10 weeks	Intestines	Acute and chronic
2. STAT 4 transgenic	Mice	Systemic administration of DNP-KH limpet hemocyanin	--	--	Chronic
3. N-cadherin	--	Transgene	--	Colon	Chronic
4. IL-2 knockout	Mice	Gene deletion	> 5 weeks	Colon	Acute and chronic
5. IL-10 knockout	Mice	Gene deletion	> 6 weeks	Colon and small intestine	Acute and chronic
6. TCR knockout	Mice	Gene deletion	> 30 weeks	Colon	Acute and chronic
7. Gαi2 knockouts	Mice	Gene deletion	> 15 weeks	Colon	Acute and chronic
8. Mdr1a (multiple drug resistance gene)	Mice	Gene deletion	8 - 36 weeks	Colon	Acute and chronic

MODEL	SPECIES	METHOD OF INDUCTION	TIME COURSE	DISEASE LOCATION	TYPE OF COLITIS
9. TNF-α 3'(69 bp) deletion	Mice	Deletion 3'-AU-rich region 589	3 - 8 weeks	Ileum	Acute and chronic
10. Keratin 8	Mice	Gene deletion	--	Colon	Acute and chronic
11. TGF-β1 knockouts	Mice	Gene deletion	--	Colon	Multiorgan, early death
12. IL-7 transgenic	Mice	Transgene	> 8 weeks	Colon	Acute and chronic
13. A20	Mice	Gene deletion	< 1 week	Intestines	Acute, early death
14. STAT 3	Mice	Deletion of STAT 3 gene in bone marrow cells	< 2 months	Small intestine and colon	Acute and chronic
15. CD40	Mice	Transgene	8 – 15 weeks	Colon	Acute and chronic
VII. ADOPTIVE TRANSFER MODELS					
1. CD45RB	$Rag2^{-/-}$ and SCID mice	Adaptive transfer of CD45RB CD4+ T cells	--	Colon	Chronic
2. Tgε	Mice	Transfer of syngeneic bone marrow	--	Colon	Chronic

Legend: -- = Data not available; DSS - Dextran sulfate sodium; EtOH - Ethanol; IL - Interleukin; PGPS - Peptidoglycan polysaccharide; PMA - Phorbal-12-myristate-13-acetate; TGF - Transforming growth factor; TNBS - Trinitrobenzene sulfonic acid; TNF-α - Tumor necrosis factor-α.

1. CHEMICALLY INDUCED COLITIS MODELS

Chemically induced models to study IBD are the models studied in greatest detail so far and involve induction of colitis by administration of an exogenous colitogenic substance. In general, these models require the co-administration of a substance that temporarily disrupts the mucosal integrity, allowing the colitogenic components to gain access to the mucosal immune system [Borm & Bouma, 2004]. In one of the early models of IBD, rabbits were sensitized with ovalbumin. Then, the colonic epithelial barrier was disrupted by rectal administration of formalin, followed by re-administration of the antigen, leading to a transient inflammation with many similarities to UC [Kraft, Fitch & Kirsner, 1963]. An important conclusion from these and subsequent studies was that the perpetuating inflammation in IBD results from a break in 'tolerance' to antigens in the normal mucosal microflora [Borm & Bouma, 2004].

Among the various chemically induced colitis models, dextran sulfate sodium (DSS)-induced colitis and trinitrobenzene sulfonic acid (TNBS)-induced colitis models are the most widely used to induce IBD. These models symptomatically, morphologically and histopathologically resemble human IBD. These colitis models are appropriated to developing and testing novel therapeutic strategies for the treatment of IBD. Moreover, the plausible mechanism of action of a particular drug can be illustrated using a suitable colitis model [Randhawa et al., 2014]. Moreover, chemically induced models of colitis, such as DSS and TNBS, induce acute damage to the epithelium. These models are useful to explore pathways or therapeutics that are thought to protect intestinal epithelial cells from damage or stress response as well as pathways that maintain gut integrity in the presence of a biological insult [Voss & Diehl, 2014].

Differences between models may reflect the different subgroups of patients with IBD [Jurjus, Khoury & Reimund, 2004]. The scientific evidence suggests that TNBS-induced colitis model promotes a Th_1 response, resembling CD in humans [Hibi, Ogata & Sakuraba, 2002]. In turn, DSS-induced colitis model promotes a Th_2 response, resembling UC in humans [Randhawa et al., 2014]. In practice, this pattern of T-cell differentiation is associated with distinct functional activities: Th_1 T cells are the key players in delayed-type hypersensitivity reactions, whereas Th_2 T cells are potent inducers of antibody-mediated immunologic reactions [Abbas, Murphy & Sher, 1996].

1.1. INDUCTION BY DEXTRAN SULFATE SODIUM

In 1985, Ohkusa reported for the first time the animal model of colitis with DSS [Ohkusa, 1985]. Today, it is one of the most commonly used inducers of colitis in animal models, thanks largely to the ease of use and potentially short turnaround times for obtaining results [Bramhall, Flórez-Vargas, Stevens, Brass & Cruickshank, 2015]. The commonly used protocol for DSS-induced colitis in rats or mice is to add DSS to drinking water [Ohkusa, 1985; Okayasu et al., 1990; Bramhall et al., 2015]. DSS-induced colitis is a reproducible model that morphologically and symptomatically resembles UC in humans [Wirtz, Neufert, Weigmann & Neurath, 2007; Bramhall et al., 2015]. In the DSS model, the Th response may switch from Th_1 response in the acute phase to mixed Th_1/Th_2 response in the chronic phase of disease, mediated by IL-5 and IL-13 [Fiocchi, 1998; Dielemann et al., 1998].

DSS is commonly administered in a dose range of 2-10% for 5-10 days to induce an acute inflammation, after a single continuous exposure. By prolonging the DSS administration, acute colitis may be extrapolated to chronic colitis by repeated exposure administering in three to five cycles (e.g., 7 days DSS, 14 days water) punctuated with recovery periods [Wirtz & Neurath, 2007; Tran, Katsikeros & Abimosleh, 2012; Bramhall et al., 2015].

The severity of DSS-induced colitis model depends on dose, duration of administration and animal strain (C3H/HeJ and BALB/c mice strains are more susceptible) [Ohkusa, 1985; Okayasu et al., 1990; Bramhall et al., 2015]. Other factors that can influence the severity and susceptibility of exposure to DSS are the manufacturer and molecular weight of DSS (5kDa for mild and 40kDa for severe colitis), gender (male mice are more susceptible), and whether animals are raised in germ-free or specific pathogen-free environments [Perse & Cerar, 2012; Low, Nguyen & Mizoguchi, 2013]. Depending of these factors, DSS causes erosions with complete loss of surface epithelium because of its direct toxic effect on epithelial cells. It causes deformity in the epithelial integrity, thereby increases the colonic mucosal permeability allowing permeation of large molecules such as DSS with molecular weight upto 50,000 Da [Dharmani, Leung & Chadee, 2011; Dai et al., 2013].

DSS is a synthetic sulfated polysaccharide composed of dextran and sulfated anhydro-glucose unit [Tran et al., 2012]. The exact mechanism by which DSS induces colonic damage is beginning to emerge. Its primary mode of action seems to chemically

interfere with gut mucosa barrier integrity, allowing luminal antigens access to the lamina propria and the proinflammatory cells within [Low et al., 2013]. Moreover, since colonic bacteria can dissociate sulfate from DSS, the free sulfate present in the intestine could act as a substrate to produce hydrogen sulfide, which could significantly interfere with cellular metabolism to induce a toxic effect on the epithelium. Other possible mechanisms include alterations in luminal bacterial ecology and the activation of monocytes, macrophages and mast cells [Murthy, 2006; Wirtz & Neurath, 2007]. In the course of colonic inflammation, colonic mucosa shows increased expression of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and some adhesion molecules genes (ICAM-1) [Jurjus et al., 2004].

This model is one of the widely used models as it can be easily developed owing to the wide availability. It has been shown to be suitable to study epithelial repair mechanisms and the contribution of innate immune mechanisms of colitis [Williams et al., 2001]. Furthermore, it has been highly applicable for understanding the multistep neoplastic process involved in colitis-associated colon cancer and to test investigational drugs that are chemopreventive or therapeutic to combat colon cancer in high-risk IBD patients [Murphy, 2006].

The advantage of this model also resides in the ease of producing both “acute” and “chronic” disease by simple modification of the DSS feeding protocol. The disease is highly reproducible. Chronic inflammation in this model lasts for a longer period, permitting the evaluation of the efficacy of compounds without any inherent risk of self-healing. The functional end points are easy to measure, but labor intensive. However, the disadvantage of the model is that DSS is very expensive. Batch to batch variations in disease severity could occur due to small molecular weight DSS impurities in the DSS preparation. Furthermore, the disease is characterized by progressive crypt dropout, suggesting a direct effect of DSS on the epithelial cells as opposed to lamina propria cells as suggested in human IBD [Murphy, 2006].

1.2. INDUCTION BY TRINITROBENZENE SULFONIC ACID

Since Morris first described it in 1989, TNBS model has been very popular [Murphy, 2006]. Its popularity is well founded because a single application of TNBS in rats, mice, guinea pigs, dogs and rabbits produce rapid, reliable and reproducible disease. This animal model is an efficient method, since can mimic the pattern of inflammation with human IBD [Hibi et al., 2002; Linden, Chen, Gershon, Sharkey & Mawe, 2003; Randhawa et al., 2014]. Indeed, since TNBS is associated with predominant activation

of Th_1-mediated immune response, this model promotes chronic transmural colitis with severe diarrhea, weight loss, and rectal prolapse, an illness that mimics some characteristics of CD in humans [Morris et al., 1989; Elson, Sartor, Tennyson & Riddell, 1995; Neurath, Fuss, Kelsall, Stuber & Strober, 1995; Boirivant, Fuss, Chu, Strober, 1998; Yu et al., 2013].

The induction of colitis by TNBS is simple, requiring an introduction of 20–80 mg TNBS dissolved in 30–50% ethanol as an enema in the animal colon [Murphy, 2006]. Ethanol not only serves as a solvent or carrier, but also aids in inducing gut inflammation by breaking the mucosal barrier [Neurath et al., 1995; Ikeda et al., 2008]. The degree of disease and time required to produce the injury may vary between laboratories [Murphy, 2006]. In general, the acute transmural damage became maximal from 3 days to 1 week after instillation, which gradually progresses into chronic inflammation lasting for about 8 weeks [Morris et al., 1989; Linden et al., 2005; Lamb, Zhong, Gebhart & Bielefeldt, 2006; Murphy, 2006; Qin et al., 2011]. Concretly, TNBS presents an infiltration of inflammatory cells within 2 hrs after administration, but typical signs of chronic inflammation can develop after 48 hrs [Cheon, Cui, Yeon, Kwon & Park, 2012].

Protocols of the TNBS-induced colitis model are not standardized, such as the dose of TNBS, the depth of TNBS administration, the animal strain, and the time point for model evaluation [Wirtz et al., 2007; Qin et al., 2011]. Particularly, TNBS administered to rats at different doses gave an indication that TNBS induces acute inflammation and damage in a dose dependent manner with the highest doses being more effective. On varying alcohol concentration between 25% and 50% alcoholic concentration, it was found that the pathological score, like inflammation and visceral hypergelsia was more significant in TNBS-50% ethanol treated rats. Curiously, there are studies where instillation of a certain dose of TNBS at a depth of 4 cm or 8 cm produced similar pattern and severity of colonic inflammation [Qin et al., 2011]. In mice, TNBS colitis was initially described in SJL/J mice, which is a mouse strain with high susceptibility for the induction of colitis. But now-a-days, various other mouse strains are also frequently used for development of colitis, such as BALB/C and C57BL/6. Generally, it involves rectal application of low dose of TNBS for induction of colitis [Randhawa et al., 2014].

TNBS can induce colitis by intrarectal instillation of the haptenating substance dissolved in ethanol [Morris et al., 1989]. Regarding its mechanism of action (FIGURE 7), TNBS is a haptenating agent that couples trinitrophenyl groups to cell surface proteins (colonic autologous or microbiota proteins) more or less indiscriminately. Then, altered

self-proteins are presented to CD4+ T cells by antigen presenting cells throughout the colonic lamina propria (dendritic cells or macrophages), stimulating a local immunologic response. Consequently, CD4+ T cell immune response and cytokine production is induced, rendering them immunogenic to the host immune system [Allgayer, Deschryver & Stenson, 1989; Morris et al., 1989; Neurath et al., 1995; Cavani, Hackett, Wilson, Rothbard & Katz, 1995; Murphy, 2006; Wirtz et al., 2007; Qin et al., 2011]. In the absence of adequate counter-regulatory mechanisms, excessive secretion of IL-12 and, subsequent, induction of an unbalanced Th_1 T-cell response is produced, usually called a delayed hypersensitivity reaction with dense infiltration of lymphocytes/macrophages and thickening of the colon wall. Finally, the Th_1 cytokines, particularly IFN-γ, act on macrophages to induce the production of additional proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which then serve as the immediate causes of the inflammation. These findings probably indicate that a poorly regulated Th_1 T-cell response is the key mechanism underlying the pathogenesis of TNBS-induced colitis [Strober, Ludviksson & Fuss, 1998]. Furthermore, in TNBS-induced colitis, the injury is also associated with a substantial increase in myeloperoxidase (MPO) levels, suggesting that neutrophils play a significant role in the production of acute injury.

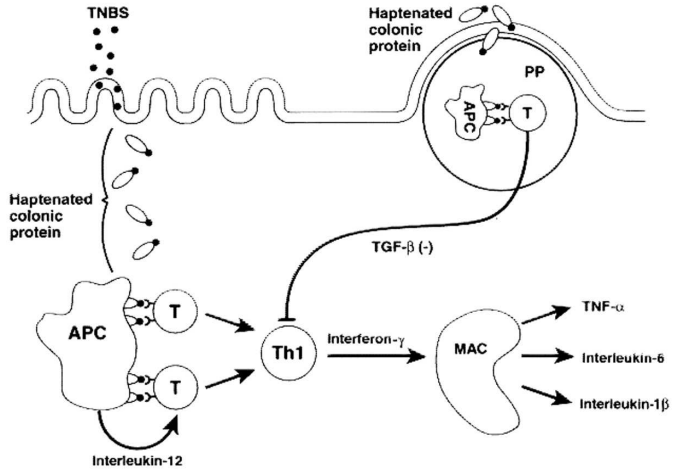

FIGURE 7. Mechanism of colitis induction and tolerance in the TNBS-induced colitis model [adapted of Strober et al., 1998].

Legend: TNBS - Trinitrobenzene sulfonic acid; APC – Antigen presenting cells; MAC - Membrane attack complex; TNF-α - Tumor necrosis factor α.

Over the last decade, this is by far one of the most popular models used for the preclinical evaluation of drugs that have been published. The success of these therapies provides valuable comparisons for future drugs to be tested [Murphy, 2006]. It is a very useful model and is frequently used in studying many aspects of gut

inflammation including cytokine secretion pattern, cell adhesion and immunotherapy [Wirtz & Neurath, 2007; Randhawa et al., 2014].

The advantages of this model are the simplicity and relative low cost of producing colitis. On the other hand, TNBS model of colitis suffers from some disadvantages, like its reproducibility, which is dependent of many factors as previously described [Murphy, 2006]. This handicap can be overcome with the padronization and validation of the animal model before the preclinical study is applied. Furthermore, the absence of spontaneous relapse which is the hallmark of human UC, as do many other methods [Stevenson, Marshall & Morgan, 2006]. However, recurrent UC model can also be induced by instilling TNBS into the colon through a cannula, but is followed by a second instillation with a lower dose of TNBS into the colon 14 days after the first induction of colitis leading to generation of recurrent model of UC [Yang et al., 2012b]. Recurrent colitis can be achieved by repeated enemas of TNBS, but never by oral feeding of TNBS, since will promote significant oral tolerance [Elson et al., 1996].

CHAPTER 3 – NEW PHARMACEUTICAL APPROACHES IN INFLAMMATORY BOWEL DISEASE

IBD are chronic relapsing inflammatory conditions of the intestine with a multifactorial pathogenesis that includes genetic susceptibility, impaired barrier function, altered gut flora and a dysregulated immune response [Neurath, 2014a]. In most cases, it is often required to treat patients over years and to adapt the therapy repeatedly with respect to flares, complications, personal circumstances or loss of effect of previous medication. This can be a quite challenging task but recent advances in understanding the nature of IBD have helped and will help to develop new and future therapies, which will enable us to improve our strategies regarding these needs. Collectively, our current therapeutic concepts for IBD will progressively evolve to become broader in general options but though more targeted and individualized in single patients [Zundler & Neurath, 2015].

Following the breakthrough of TNF-α blockade by neutralizing antibodies in IBD a magnitude of therapeutic agents inhibiting the activity of pro-inflammatory cytokines or supporting the action of anti-inflammatory cytokines was evaluated for IBD treatment. Unfortunately, many of them failed in clinical studies or had beneficial effects in subgroups of patients only [Neurath, 2014a], which under scores the notion that cytokine networks in human IBD are more complex than assumed before and may vary between different patients. This poses the question how further and profound progress can be made on the field of targeting cytokines in IBD. A possible answer is to aim at several cytokines at the same time, either through combination of different targeted antibodies, through interfering with overlapping intracellular signaling pathways or through additional enhancement of anti-inflammatory cytokines [Zundler & Neurath, 2015].

Hereupon, the therapy of IBD has recently been enriched by the successful launch of the anti-cytokine biologicals, such as Etanercept (TNF receptor-p75 Fc fusion protein), Infliximab (chimeric anti-human TNF-α monoclonal antibody) and Adalimumab (recombinant human anti-human TNF-α monoclonal antibody). The success of these novel treatments has impressively demonstrated the clinical benefit that can be gained from therapeutic intervention in cytokine signalling, highlighting the central role of proinflammatory cytokine systems like IL-1β and TNF-α to be validated targets. However, all of the anti-cytokine biologicals available to date are proteins, and

therefore suffering to a varying degree from the general disadvantages associated with protein drugs. Therefore, small molecular, orally active anti-cytokine agents, which target specific pathways of proinflammatory cytokines, would offer an attractive alternative to anti-cytokine biologicals [Peifer, Wagner & Laufer, 2006].

Ultimately, the therapeutic dogma in IBD has shifted away from the administration of nonspecific immunosuppressives toward a pathway-based approach. Availability of such diverse treatment modalities with specific pathway-based targets will increase the therapeutic options for patients with IBD [Bamias, Pizarro & Cominelli, 2016]. In the coming years, this trend is expected to continue. Yet, many challenges are still ahead. A strong collaborative effort by experts from different fields is encouraged and necessary to maximize our success in IBD drug targeting [Wolk, Epstein, Ioffe-Dahan, Ben-Shabat & Dahan, 2013].

In general terms, the aim of our work is to evaluate the influence of a set of drugs in the IBD. These drugs, such as erythropoietin (EPO), thiadiazolidinone-8 (TDZD)-8 and hemin, can modulate some important metabolic pathways in the establishment and development of inflammation. Indeed, they may inhibit or stimulate molecular pathways, contributing to facilitate a more effective and selective treatment than the currently known.

1. ERYTHROPOIETIN

More than a century ago, a plasmatic humoral factor was assumed to be essential for red blood cell production and was called hemopoietin. Hemopoietin was the initial name for EPO, which was later identified and described as a 34-kDa glycoprotein of 165 amino acids that plays the role of a hormone, cytokine and growth factor. The peritubular interstitial cells of the kidney are believed to be the main producers of EPO [Koury, Bondurant & Koury, 1988; Fisher, Koury, Ducey & Mendel, 1996]. Recently, EPO producing kidney cells were not clearly identified as reported in various publications [Suzuki, Obara & Yamamoto, 2007]. Indeed, the latest study from Frede *et al*., suggested a potential fibroblast-like/neuronal origin for the EPO-producing cells in the kidney [Frede, Freitag, Geuting, Konietzny & Fandrey, 2011]. The incontestable fact is that EPO gene is mainly expressed in the fetal liver, whereas it is expressed in the kidney after birth, and the kidney then becomes the predominant site of EPO production. Therefore, the liver is a secondary site of EPO production in the adult [Zanjani, Poster, Burlington, Mann & Wasserman, 1977; Lacombe et al., 1988; Koury, Bondurant, Koury & Semenza, 1991].

EPO is a multi-functional glycoprotein essential for hematopoietic system, regulating the bone marrow erythrocyte production by differentiation and inhibition of the apoptosis of erythroid progenitor cells [Chateauvieux, Grigorakaki, Morceau, Dicato & Diederich, 2011; Nairz et al., 2011]. Currently, EPO is commercialized to be used in the treatment of anemia related to chronic renal failure in dialysis patients, with an initial dose of 50 to 100 IU/kg IV or subcutaneous (SC) 3 times weekly, to increase the production of autologous blood in normal subjects or to reduce the duration of anemia in patients treated with chemotherapy [Willis, Cheung & Slifer, 2012; Jelkmann, 2012].

The induction of EPO expression is tightly dependent on the physiological conditions that control its production in the kidney. Hypoxia or anemias are the main events that are able to induce EPO gene expression [Chateauvieux et al., 2011]. Hypoxia inducible factors (HIF) are a family of transcription factors (FIGURE 8) that control the cellular response to hypoxia. In the presence of normoxia, HIFα is rapidly destroyed by a collaborative effect of oxygen, prolyl hydroxylase domain-containing enzymes and the von Hippel-Lindau tumor suppressor protein (VHL). Hydroxylated HIFα can bind to VHL, and the HIFα–VHL complex facilitates ubiquitin-mediated proteasomal degradation of HIFα. Under conditions of tissue hypoxia, the proteasomal degradation of HIFα is slowed, resulting in its cytoplasmic accumulation and subsequent

translocation to the nucleus, where it dimerizes with HIFβ and enhances the transcription of the EPO gene [Chateauvieux et al., 2011].

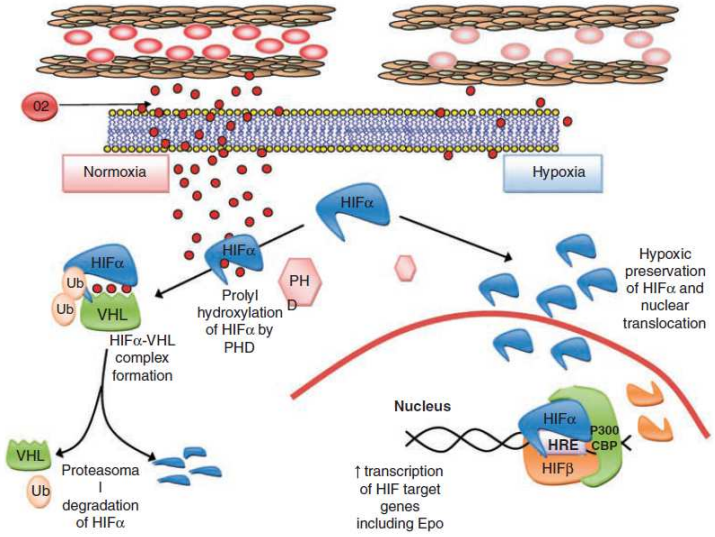

FIGURE 8. Intracellular oxygen sensing and erythropoietin production [adapted of Patnaik & Tefferi, 2009].

Legend: HIF - Hypoxia inducible factors; VHL - Von Hippel-Lindau tumor suppressor protein; EPO – Erythropoietin.

After induction of EPO expression, interaction between EPO and its homodimeric receptor (EPOR) function as the primary mediators of a general protective response to tissue hypoxia by acting to maintain adequate tissue oxygenation through adjustments of circulating red-cell mass by using a hormonal feedback control system involving the kidney and the bone marrow [Masuda, Nagao & Sasaki, 1999]. EPO physically interacts with EPOR that are expressed on the erythroid cell surface [Nairz et al., 2011], triggering conformational changes in the extracellular domain of the receptor with, subsequently, receptor homodimerization and activation of the EPOR-associated Janus Kinase (JAK)-2 by autophosphorylation (FIGURE 9) [Patnaik & Tefferi, 2009; Nairz et al., 2011].

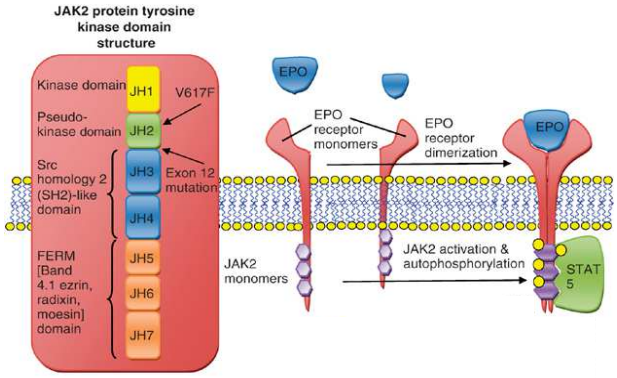

FIGURE 9. Scheme of the conformational changes in the EPO homodimeric receptor [adapted of Patnaik & Tefferi, 2009].

Legend: JAK - Janus Kinase; EPO - Erythropoietin; STAT 5 - Signal transducer and activator of transcription protein.

Activated JAK-2 in turn mediates the phosphorylation of key tyrosine residues on the distal cytoplasmic region of EPOR, which then serve as docking sites for downstream effectors, initiating various signaling pathways responsible for antiapoptotic effects (FIGURE 10) [Patnaik & Tefferi, 2009; Nairz et al., 2011]. First, activated signal transducer and activator of transcription protein 5 (STAT5) homodimerizes and translocates to the nucleus to affect gene transcription, including Bcl-X_L [Silva et al., 1999]. Second, phosphatidylinositol 3 kinase (PI3K) phosphorylates and activates protein kinase B (PKB)/Akt [Kashii et al., 2000]. Akt will phosphorylate and inactivate proapoptotic molecules, such as caspase 9, Bad or glycogen synthase kinase (GSK)-3β. It also phosphorylates IκB, which activates the transcription factor NF-kB, and induces cytoplasmic retention of FOXO transcription factors through their phosphorylation. FOXO proteins activate genes encoding proapoptotic molecules, such as Fas ligand or Bim. Third, EPO induces the phosphorylation of IκB (inhibitor of NF-κB) and, thus, activate the NF-κB in erythroid cells, which in turn enhances the transcriptional activity of target genes encoding antiapoptotic molecules, such as XIAP and c-IAP2 [Digicaylioglu & Lipton, 2001]. Fourth, EPO induces heat shock protein 70 (Hsp70), promoting antiapoptotic effects by inhibition of apoptosis protease-activating factor-1 and of apoptosis-inducing factor [Yang et al., 2003]. All these pathways play a crucial role in the regulation of erythro-specific genes to stimulate erythropoiesis by the control of cell proliferation, differentiation and apoptosis [Nairz et al., 2011].

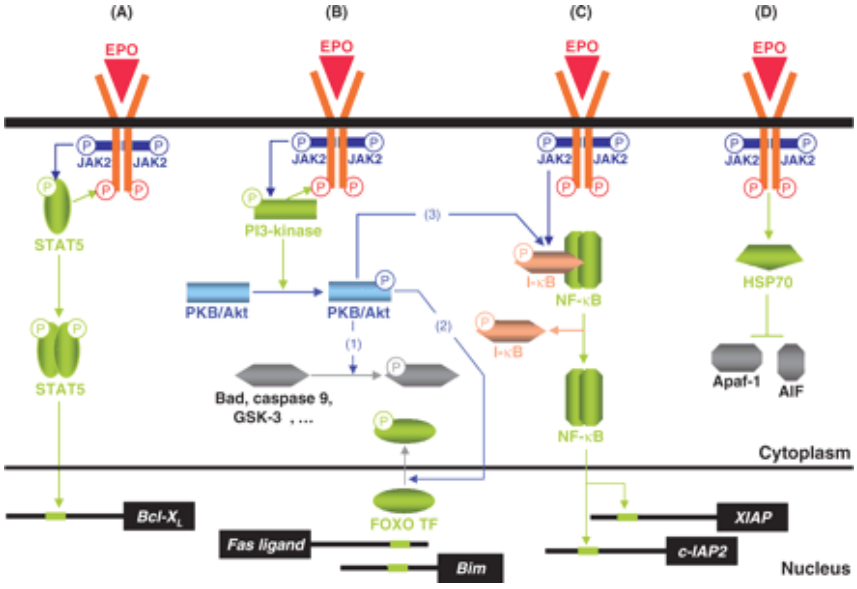

FIGURE 10. Schematic representation of the signaling pathways of EPO [adapted of Rossert & Eckardt, 2005].

Legend: (A) Phosphorylation of the STAT5 transcription factor; (B) Phosphorylation of PI3-K/Akt; (C) Phosphorylation of IκB; (D) Activation of Hsp70.

Interestingly, EPO also has non-hematopoietic properties through EPOR interaction that are expressed on various nonerythroid tissues [Brines & Cerami, 2005; Jelkmann, 2007], where it plays a role in the reduction in oxidative stress, apoptosis and inflammation due to hypoxia, toxicity or injury [Calo, Bertipaglia & Pagnin, 2006; Chateauvieux et al., 2011; Nairz, Sonnweber, Schroll, Theurl & Weiss, 2012; Stoyanoff, Todaro, Aguirre, Zimmermann & Brandan, 2014]. The presence of mRNA encoding the EPOR has been detected in brain, retina, heart, skeletal muscle, kidney and endothelial cells [Rossert & Eckardt, 2005]. The protective activity of EPO may be due, in some tissues, to the presence of a tissue-protective receptor that is a heterodimeric receptor composed of EPOR subunits disulfide linked with beta common receptors (βcR) (FIGURE 11) [Brines et al., 2004].

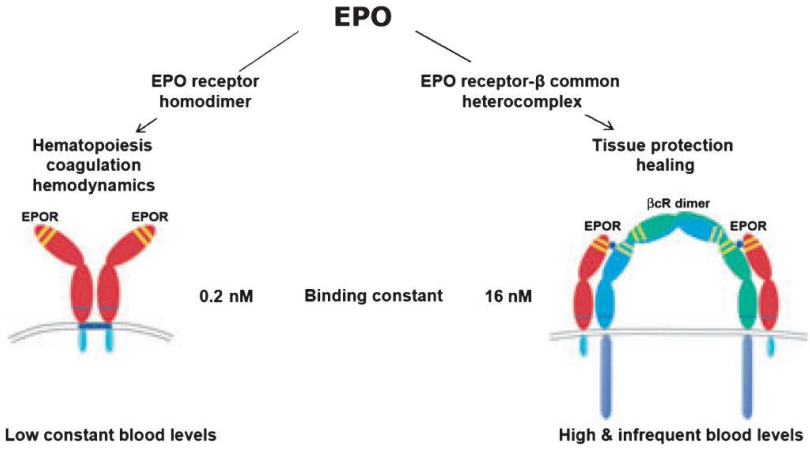

FIGURE 11. Erythropoietin signals via two distinct receptor isoforms [adapted of Cerami, 2011].

Legend: EPO - Erythropoietin; EPOR - Erythropoietin receptor; βcR - Beta common receptors.

The affinity of EPO for the EPOR/βcR receptor is low, but once it interacts with its ligand, the presence of the ligand is no longer required for bioactivity [Cerami, 2011; Hand & Brines, 2011]. So, the tissue-protective properties of EPO are reached with higher dosage than needed for its circulating hormonal effects and, concurrently, high doses of EPO are associated with side effects and abnormally increased erythropoiesis. To circumvent the side effects while preventing the cytoprotective activities of EPO, different non-erythropoietic EPO derivatives have been developed either by chemically modifying or mutating EPO [Chateauvieux et al., 2011].

Currently, there are several erythropoiesis-stimulating agents (ESA) available in the European Union, namely epoetin alfa, epoetin beta, epoetin zeta, epoetin theta, darbepoetin alfa, and continuous EPOR activator [Locatelli & Del Vecchio, 2011]. Epoetins alfa and beta are widely used and have relative short half-lives (6–8 hours IV

and 19–24 hours SC), and their optimal administration schedule is two or three times weekly IV or SC Epoetins zeta and theta are biosimilars of epoetin alfa, which are similar but not identical to their reference product, because their chemical characteristics directly depend on the manufacturing process, which cannot be precisely duplicated. The last two ESA have much longer half-lives than others of 25-48 hours or 130 hours, respectively, promoting an administration less frequent [Locatelli & Del Vecchio, 2011; Bernieh et al., 2014]. In general, all ESA are effective in correcting renal anemia and increasing hemoglobin levels, but the choice of which to use should also take into account their pharmacokinetics and pharmacodynamics, their administration route, and economic issues [Locatelli & Del Vecchio, 2011]. However, the expert opinion is that the likelihood of differences in clinical outcomes among ESA brands is low, although there is no robust evidence supporting this assumption [Willis et al., 2012].

Carbamylated EPO is a modified EPO with tecidual protective action devoid of erythropoietic activity, binds to these heterodimeric receptors and exerts tissue protective effects, while it does not bind to the classical EPOR and does not stimulate erythropoiesis [Brines et al., 2004; Leist et al., 2004; Fiordaliso et al., 2005]. This is the first evidence suggesting that EPOR expressed in different tissues are not identical [Rossert & Eckardt, 2005].

In fact, EPO benefits have been studied in several nonerythroid tissues, such as brain, heart and kidney injuries [Sakanaka et al., 1998; Parsa et al., 2003; Yang et al., 2003]. In 1998, Sasaki's group [Sakanaka et al., 1998] reported that intraventricular infusion of EPO might protect neurons against ischaemic injury. Following this pioneer work, experimental data have shown that systemic injections of large doses of EPO can decrease brain or spinal cord damage in various experimental models. EPO also has protective effects in experimental models of multiple sclerosis or status epilepticus [Brines et al., 2000; Agnello, 2002] and it can prevent or partially reverse experimental diabetic neuropathy [Bianchi et al., 2004]. These neuroprotective effects are associated with a strong inhibition of apoptosis [Siren et al., 2001; Celik et al., 2002; Junk et al., 2002] and with neurotrophic activity of EPO [Siren et al., 2001].

Regarding cardiovascular injuries, consistent with data from knockout mice, which show that the heart expresses functional EPOR, injections of large doses of EPO can protect against the consequences of transient or permanent coronary artery occlusion [Parsa et al., 2003; Moon et al., 2003; Calvillo et al., 2003; Wright et al., 2004]. After the initial phase, animals treated with EPO exhibited a reduction in myocardial damage,

as assessed by histological analyses and by measurement of haemodynamic parameters. These improvements were associated with decreased apoptotic cell death [Parsa et al., 2003; Moon et al., 2003; Calvillo et al., 2003].

In kidney injuries, since 2003, different groups have shown that pretreatment of animals with EPO can protect the kidney against ischaemia–reperfusion injury [Yang et al., 2003; Patel et al., 2004; Sharples et al., 2004; Gong et al., 2004; Vesey et al., 2004]. In these studies, injection of EPO at a dose ranging from 300 to 5000 IU/kg provided effective protection against renal dysfunction and reduced morphological damage. These protective effects were associated with a decrease in apoptotic cell death and in caspase activity, but not with increased cell proliferation [Yang et al., 2003; Sharples et al., 2004]. Furthermore, many studies have also been developed to evaluate the influence of EPO in other organ injuries, resulting in positive benefits for some diseases, such as liver, pancreatic, retinal and lung injuries [Sepodes et al., 2006; Ucan et al., 2009; Olgun 2013; Rocha et al., 2015].

In colon injuries, it has been studied the influence of EPO in experimental models of IBD with rodents and, in fact, there are several potential explanations for the protective effect of EPO requiring more research (FIGURE 12).

The inflammation and apoptosis are two distinct phenomena that contribute to the tissue deterioration and its regulation by EPO borrows a common part of the signaling pathway [Chateauvieux et al., 2011]. As already mentioned, primary investigations on the signaling pathways involved EPO demonstrated the phosphorylation and activation of JAK1/2, STAT3, STAT5a and PI3K [Chatagner et al., 2010]. Regarding antiapoptotic properties of EPO, Akt is able to induce the production of endothelial nitric oxide synthase (eNOS), NO, and Bcl2 [Dodd et al., 2000]; inhibition of caspase 3 and caspase 8 [Kim et al., 2000]; and is activated by EPOR through PI3K [Beleslin-Cokic et al., 2004]. EPO inhibits apoptosis through the activation of Akt, as well as the phosphorylation of GSK-3β to its inactive form [Fu & Arcasoy, 2007]. GSK-3β is known to be involved in the apoptosis by inhibiting Bcl2 and activating the proapoptotic protein BAX. In fact, the implication of Akt in tissue protection, angiogenese, inhibition of apoptosis and inflammation triggered by EPO appear to be involved in various signaling pathways [Chateauvieux et al., 2011].

The first studies linking EPO and inflammation showed an inhibition of inflammation by the induction of PI3K, activator protein 1 (AP-1) and eNOS via Akt activation [Rui et al., 2005], as well as the inhibition of the expression of IL-6, TNF-α, and IL-1β via STAT3/5

signaling [Li et al., 2006a]. Furthermore, pro-inflammatory protein COX-2 was also studied in the regulation of inflammation by EPO. On the one hand, the inhibition of COX-2 expression is coupled with the activation of the extracellular signal-regulated kinase (ERK)1/2 and is independent of Akt and STAT5 modulation [Li et al., 2006b]. On the other hand, EPO induces the expression and activity of COX-2 and subsequently the production of its products, such as PGE2 and PGF2α [Liu et al., 2006b]. Another study also reported the induction of IL-10 and the inhibition of NF-κB by EPO [Liu et al., 2006a]. NF-κB plays a key role in the pathophysiology of clinically important diseases in most organ systems, namely in the gut [Spehlmann & Eckmann, 2009].

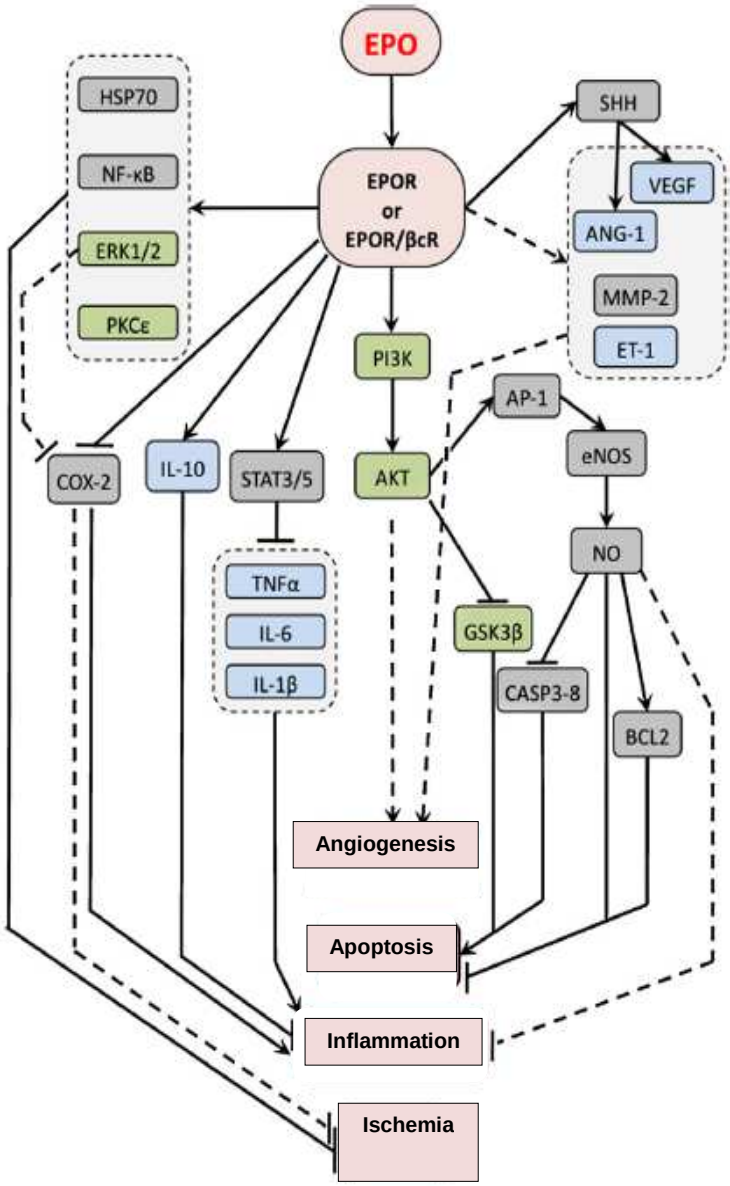

FIGURE 12. Overview of molecules and pathways implicated in tissues protection [adapted of Chateauvieux et al., 2011].

Legend: Solid lines - Established relationship; Dashed lines - Direct relationships not proved; Arrow - Positive regulation; Bar - Inhibition; Green proteins - Reperfusion injury salvage kinases; Blue proteins - Cytokines.

NF-κB is a key regulator of inducible expression of many genes involved in immune and inflammatory response (FIGURE 13) [Spehlmann & Eckmann, 2009]. The NF-κB family of proteins consists of five members in mammals, c-Rel, RelA (p65), RelB, NF-κB1 and NF-κB2 (p100), which all share a conserved Rel-homology domain responsible for DNA-binding activity, protein dimerization, and nuclear translocation. These subunits form homodimers or heterodimers, which constitute the transcriptionally active or suppressive forms of NF-κB. Inactive NF-κB is located in the cytosol complexed with an inhibitory protein, IκB. Ligand binding to particular membrane or cytosolic receptors leads to activation of the key enzyme, IκB kinase (IKK) [Spehlmann & Eckmann, 2009].

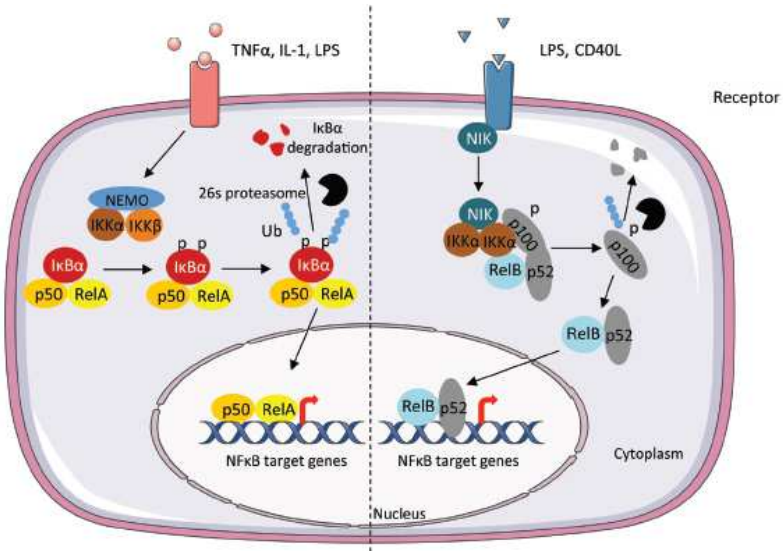

FIGURE 13. Regulation of NF-κB expression [adapted of Viennois, Chen & Merlin, 2012].

Legend: TNF-α - Tumor necrosis factor α; IL - Interleukin; LPS - Lipopolysaccharide; IκB - Inhibitory κB; IKK - IκB kinase; NF-κB - Nuclear transcription factor kappa B.

Pro-inflammatory cytokines and bacterial pathogens activate NF-κB, mostly through IKK-dependent phosphorylation and degradation of IκB proteins. NF-κB induced cytokines contribute to the stimulation, activation and differentiation of immune cells, thus perpetuating inflammation [Neurath, Pettersson, Meyer zum Buschenfelde & Strober, 1996].

Recent findings in gene-targeted mice indicate that NF-κB has more diverse functions than initially anticipated, governing both protective and destructive responses (FIGURE 14), which depend on the cell types involved and the specific pathophysiological condition. NF-κB predominantly acts in epithelial protection in homeostasis and certain acute inflammatory events by inducing the transcription of antimicrobial and antiapoptotic products. However, the cytokine-inducing and anti-apoptotic functions of

NF-κB in macrophages and T cells are predominant under chronic inflammatory conditions [Spehlmann & Eckmann, 2009].

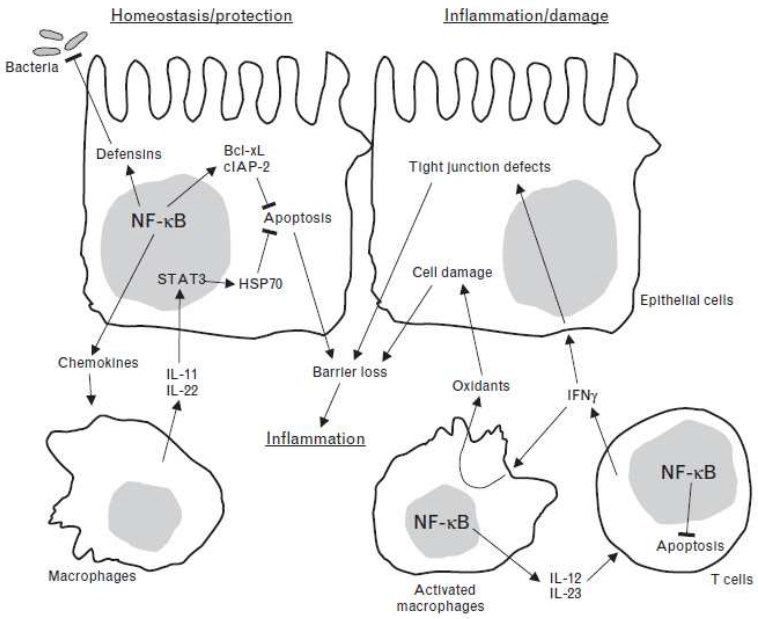

FIGURE 14. Intestinal protection and destruction induced by NF-κB [adapted of Spehlmann & Eckmann, 2009].

Legend: Hsp70 - Heat shock protein 70; IL - Interleukin; IFN-γ - Interferon-γ; STAT - Signal transducer and activator of transcription protein; NF-κB - Nuclear transcription factor kappa B.

Mucosal inflammation in patients with IBD and in experimental models of intestinal inflammation is accompanied by elevated levels of activated NF-κB [Spehlmann & Eckmann, 2009]. In parallel, expression of prototypic NF-κB target genes, including IL-1β, IL-6, TNF-α, and IL-12 p40 is increased [Sanchez-Munoz, Dominguez-Lopez & Yamamoto-Furusho, 2008]. Given that many of these gene products promote inflammation, it has been reasoned that inflammation in IBD and other conditions can be attenuated through inhibition of NF-κB activity [Spehlmann & Eckmann, 2009].

So, many established drugs are known to mediate, at least in part, anti-inflammatory effects of inflammation score via inhibition of NF-κB activity. NF-κB has been shown to be critically important in chronic inflammatory diseases, suggesting an anti-inflammatory strategy in IBD [Neurath et al., 1996]. Novel approaches that protect and salvage injured colon tissue would constitute important advances in the therapy of IBD [Cuzzocrea et al., 2004]. In this sense, EPO can be a possible new strategy of IBD [Cuzzocrea et al., 2004] and some studies have been developed to evaluate its efficacy (TABLE 7).

TABLE 7. Experimental studies with EPO in chemically induced colitis models.

CHEMICALLY INDUCED COLITIS	RODENT	EPO DOSE	TREATMENT	EVALUATED PARAMETERS	REFERENCE
DSS	Mice	5000 IU/Kg SC	Daily starting day 1 (12 days)	Body weight, histology, IFN-γ, TNF-α, e-selectin	Nakamura et al., 2015
DNBS	Rats	1000 IU/Kg SC	Daily starting day 2 (4 days)	$CD4^+$, $CD8^+$, caspase-3, histology	Tasdemir et al., 2013
TNBS (associated infection)	Mice	5000 IU/Kg IP	Daily starting day 2 (5 days)	Body weight , nitrite, TNF-α, IL-6, luciferase, NF-κB, histology, NO, IL-23	Nairz et al., 2011
DNBS	Mice	1000 IU/Kg IP	Daily starting day 2 (4 days)	Body and colon weight, MPO, TNF-α, IL-1β, PAR, ICAM-1, nitrotirosine, histology	Cuzzocrea et al., 2004

Legend: DSS - Dextran sulfate sodium; DNBS - Dinitrobenzene sulfonic acid; TNBS - Trinitrobenzene sulfonic acid; SC – Subcutaneous; IP – Intraperitoneal; IFN-γ - Interferon-γ; TNF-α - Tumor necrosis factor α; IL – Interleukin; NF-κB - Nuclear transcription factor kappa B; NO - Nitric oxide; MPO – Myeloperoxidase; PAR - Proteinase-activated receptors; ICAM-1 - Intercellular Adhesion Molecule 1.

Encouraged by the substantial body of evidence demonstrating the sizable beneficial effects of EPO in models of nervous system injury and ischemia/reperfusion, Cuzzocrea and colleagues (2004) designed a study to evaluate the possible beneficial effects of EPO treatment in rodent model of dinitrobenzene sulfonic acid (DNBS)-induced colitis. For the first time, demonstrated that EPO is protective to colon injuries and that inhibition of TNF-α formation (among other MPO effects that include inhibition of neutrophils infiltration) in the colon probably accounts for its beneficial effects. Since then, there were published two studies, evaluating the anti-inflammatory EPO effect on the experimental colitis. In fact, EPO inhibits the induction of pro-inflammatory genes including TNF-α and inducible NO synthase in activated macrophages, which is mechanistically attributable to blockage of NF-κB p65 activation [Cuzzocrea et al., 2004; Nairz et al., 2011]. Particularly, there is only one published study, where the influence of EPO in TNBS-induced colitis is evaluated, but mice are under an intestinal infection framework [Nairz et al., 2011]. So, future studies are needed to clarify the anti-inflammatory property of EPO treatment [Cuzzocrea et al., 2004].

2. THIADIAZOLIDINONE-8

The somehow chemical structure-related derivatives to TDZD, and already approved by FDA are the thiazolidinediones (TDZ), such as rosiglitazone, pioglitazone, and troglitazone, are known as agonists of the peroxisome proliferator-activated receptor (PPAR)γ [Lehmann et al., 1995; Willson, Brown, Sternbach & Henke, 2000]. PPAR are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (FIGURE 15), which includes retinoid, steroid, and thyroid hormone receptors that regulate transcription of distint genes through heterodimerization with the retinoid X receptors [Schoonjans, Staels & Auwerx, 1996]. Currently, TDZ are used as anti-diabetic agents and have been authorized in the European Union for combination with a glucose-lowering sulphonylurea (for patients in whom metformin is ineffective or poorly tolerated) or with metformin (for obese patients) [Krentz & Bailey, 2005]. The PPARγ receptor subtype seems to play a pivotal role in the regulation of cellular proliferation, differentiation, and inflammation. Furthermore, PPARγ agonists have demonstrated to have anti-inflammatory effects on astrocytes and microglial cells, inhibiting the production of proinflammatory and neurotoxic products. It has been recently shown that PPARγ ligands, including thiazolidinediones, have potent anti-inflammatory effects, such as the suppression of TNF-α and IL-1β, inducible nitric-oxide synthase (iNOS), and COX-2 [Jiang, Ting & Seed, 1998; Ricote, Huang, Welch & Glass, 1999; Maggi et al., 2000; Bernardo, Levi & Minghetti, 2000; Marx et al., 2000; Han et al., 2000; Subbaramaiah, Lin, Hart & Dannenberg, 2001].

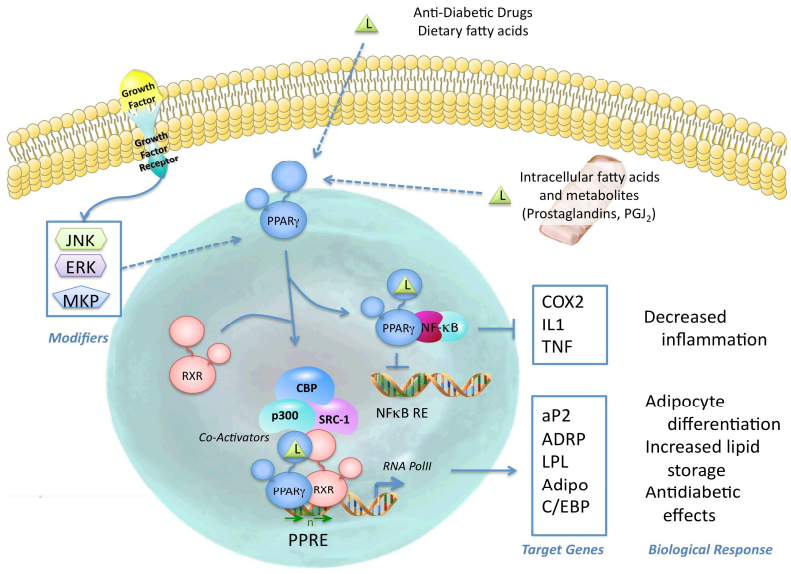

FIGURE 15. Regulation of PPARγ pathway by thiazolidinediones [adapted of Heuvel, 2009].

Many studies have been performed in order to study the influence of structural modifications in these molecules on its biological activity [Castro et al., 2008]. Thus, TDZD are small heterocyclic molecules, which were synthesized following a pathway that is based on the reactivity of *N*-alkyl-*S*-(*N´*-(chlorocarbonyl)amino) isothiocarbamoyl chlorides with isocyanates [Ottman & Hooks, 1966]. The inhibitory kinase activity of these compounds, considering as secondary products in the obstention of potassium channel openers [Martinez, Castro, Cardelus, Llenas & Palacios, 1997], muscarinic agonist [Martinez et al., 1999], or acetylcholinesterase inhibitors [Martinez et al., 2000], was discovered in a GSK-3 directed screening program initiated two years ago [Martinez et al., 2002b]. Currently, their structural key features have been established. Preliminary structure–activity relationship studies point out to the size and the nature of the substituents attached to both nitrogen atoms of the TDZD as crucial features for GSK-3 inhibition. Additionally, carbonyl or thiocarbonyl moieties in a 1,3-disposition on the heterocyclic framework may be critical for binding. Moreover, a hypothetical GSK-3 binding mode has been proposed in where the TDZD derivatives may bind to the primed phosphate substrate-binding site of the kinase. Interactions of TDZD with the main amino acids involved in this recognition site (Arg 96, Lys 205, Tyr 216) of GSK-3 explain the structure–activity relationships found in this family of compounds until now [Martinez et al., 2002b].

In 2002, Martinez and his group described the synthesis, structure-activity relationship and a hypothetical binding mode of new TDZD heterocyclic non-ATP competitive GSK-3β inhibitors [Martinez et al., 2002a; Dugo et al., 2007]. Of these, the 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) was considered the most effective (FIGURE 16) [Martinez et al., 2002a]. It is a small molecule with favorable pharmacokinetic properties, such as good oral bioavailability and blood-brain barrier penetration [Alonso et al., 2004]. The capacity of TDZD to suppress the expression of inflammatory cytokines and present tissue protective action by GSK-3β inhibiton suggests that this agent may be effective in the treatment of several inflammatory diseases [Luna-Medina et al., 2005].

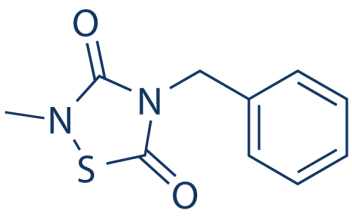

FIGURE 16. Chemical structure of TDZD [adapted of Martinez et al., 2002b].

Currently, there are several GSK-3β inhibitors, some already used for the treatment of various diseases, namely lithium, valproic acid, insulin and some purines derivatives (like SB216763 and SB415286) [Martinez et al., 2002b; Dugo et al., 2007]. However, the toxicity, associated side-effects and concerns regarding the absorption, distribution, metabolism and excretion of these inhibitors affect their clinical potential [Meijer, Flajolet & Greengard, 2004]. Generally, these GSK-3β inhibitors have some possible obstacles, since the chronic suppression of this kinase may lead to cardiac complications and increase the propensity for developing certain cancers. Furthermore, these compounds are ATP competitive, and therefore, block the phosphorylation of every substrate [Martinez et al., 2002b]. However, the discovery of the way in which ‘‘primed’’ substrates dock with GSK-3 might have opened a new opportunity to develop compounds that target only the site that binds the ‘‘priming phosphate’’ of substrates [Martinez et al., 2002b]. So, TDZD compounds, the only non ATP-competitve GSK-3 inhibitors known until the moment, might be less likely to be oncogenic in contrast to ATP competitive inhibitors. Clearly, the clinical utility of these drugs awaits animal and human trials [Martinez et al., 2002b].

GSK-3 is a serine/threonine protein kinase highly abundant in brain, involved in the regulation of glycogen by insulin [Martinez et al., 2002b]. In 1980, Embi and colleagues identified GSK-3 as one of the protein kinases able to phosphorylate glycogen synthase [Embi, Rylatt & Cohen, 1980]. The signalling pathway by which insulin inhibits GSK-3 and contributes to the stimulation of glycogen and protein synthesis is a fact well established now-a-days (FIGURE 17) [Martinez et al., 2002b]. The binding of insulin to its receptor activates the intrinsic protein tyrosine kinase activity of the receptor, allowing it to phosphorylate itself at several sites. One phosphotyrosine residue interacts with the phosphotyrosine-binding domain of the insulin receptor substrate proteins (IRS1 and IRS2), recruiting them to the plasma membrane, where they undergo phosphorylation by the insulin receptor. As a result, they interact with the Src-homology-2 domains of the p85 subunit of PI3K. This recruits PI3K to the plasma membrane, enabling the p110 catalytic subunit to catalyse the formation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) from PtdIns(4,5)P_2. PtdIns(3,4,5)P_3 binds to the pleckstrin homology domains of both PDK1 and PKB/Akt, co-localizing them at the membrane and allowing PDK1 to activate PKB/Akt. PKB/Akt, in turn, phosphorylates and inhibits GSK-3, resulting in the dephosphorylation of substrates of GSK-3, including glycogen synthase and eIF2B. This contributes to the insulin-induced stimulation of glycogen and protein synthesis [Cohen, 2001].

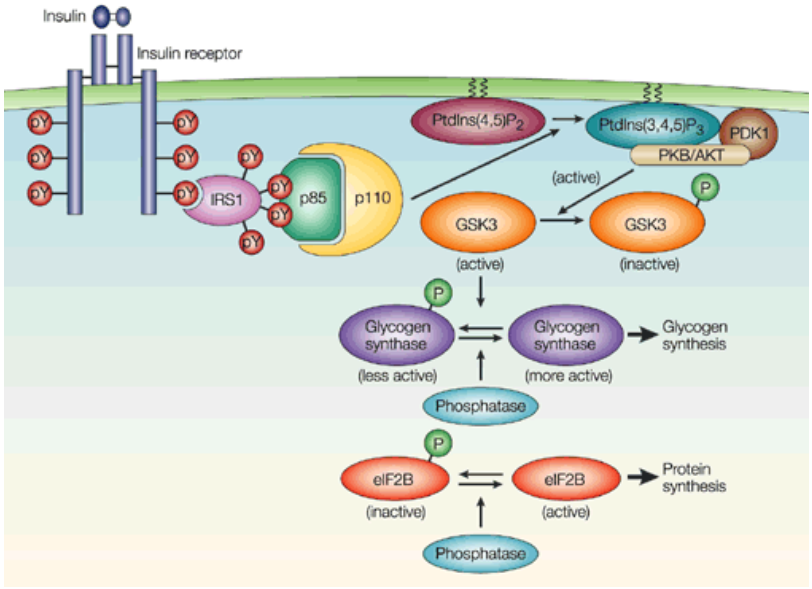

FIGURE 17. GSK-3 signalling pathway by insulin receptors [adapted of Cohen, 2001].

Legend: pY – phosphotyrosine residue; PDK1 - 3-Phosphoinositide-dependent protein kinase 1; PKB/Akt - Protein kinase B; eIF2B - Eukaryotic initiation factor 2B.

Initially, the functions of GSK-3 were thought to be limited to glycogen metabolism; however, 20 years after its discovery, we know that this kinase also has a major role in Wnt and Hedgehog signaling pathways and is involved in the regulation of many cell functions, including the specification of cell fate during embryonic development, cell division and apoptosis, and signaling by insulin, growth factors and nutrients (FIGURE 18) [Frame & Cohen, 2001; Meijer et al., 2004; Whittle et al., 2006]. Today, it is known that GSK-3 phosphorylates, and thereby, regulates the functions of many metabolic, signaling and structural proteins. Notable among the signaling proteins regulated by GSK-3 are many transcription factors, including AP-1, cyclic AMP response element binding protein, nuclear factor of activated T cells, heat shock factor-1, β-catenin and NF-κB [Martinez et al., 2002b]. More specifically, a proportion of GSK3 in cells is present in a multiprotein complex together with axin, the adenomatous polyposis coli protein and β-catenin. In the absence of secreted glycoproteins, called WNTs, the GSK3 in this complex is active and phosphorylates axin, adenomatous polyposis coli and beta-catenin. Axin is stabilized by phosphorylation, but phosphorylation of β-catenin targets it for ubiquitylation and subsequent proteolytic destruction. The role of adenomatous polyposis coli phosphorylation is less clear, but it seems to enhance its interaction with beta-catenin. After the binding of WNTs to their receptors, a signal-transduction pathway is triggered that has yet to be fully elucidated, but which seems to involve a protein, termed dishevelled (DVL), which, together with FRAT (frequently rearranged in advanced T-cell lymphomas), results in the displacement of axin (and

hence adenomatous polyposis coli and β-catenin). This leads to the dephosphorylation of axin, adenomatous polyposis coli and β-catenin. The dephosphorylation of β-catenin leads to its accumulation in cells and translocation to the nucleus, where it binds to members of the T-cell factor (TCF) family of transcription factors (also called LEF, for lymphoid-enhancer factor), and stimulates the transcription of genes that are required for embryogenesis. The same pathway is likely to be involved in regulating the expression of other genes in adult tissues.

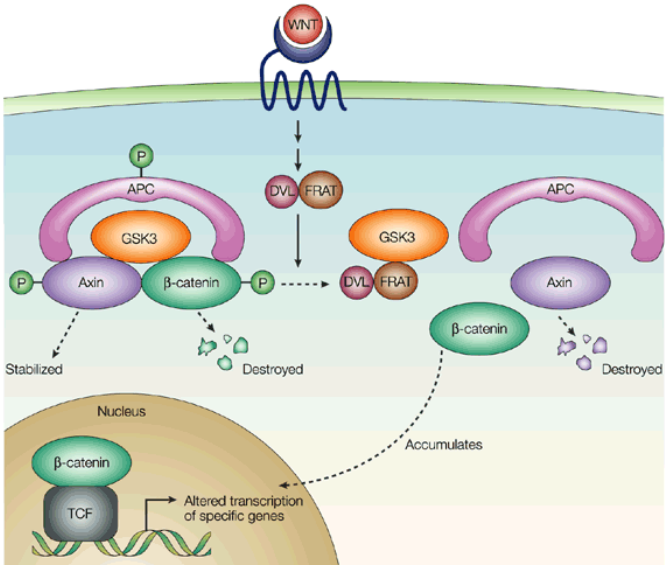

FIGURE 18. GSK-3 signalling pathway by insulin receptors [adapted of Cohen & Frame, 2001].

Legend: APC - adenomatous polyposis coli; GSK - Glycogen synthase kinase; TCF - T-cell factor; FRAT - Frequently rearranged in advanced T-cell lymphomas; DVL - Termed disheveled.

GSK-3 has two isoforms, GSK-3α and GSK-3β, encoded by distinct genes and the most common regulatory mechanism is inhibition by phosphorylation of Ser21 in GSK-3α or Ser9 in GSK-3β [Beurel, Michalek & Jope, 2009]. These isoforms are ubiquitously expressed, highly homologous and usually have equivalent actions. However, they can have distinct biological roles in some specific pathways. Currently, it is known that GSK-3β plays a fundamental role in the regulation of the activity of NF-κB and, for this reason, it has been investigated the effects of this protein kinase in the regulation of inflammatory process [Ali, Hoeflich & Woodgett, 2001; Frame & Cohen, 2001; Dugo et al., 2007]. NF-κB is a known key transcription factor for the genes involved in the production of pro-inflammatory mediators [Karin, Yamamoto & Wang, 2004]. This concept was first base on the findings that GSK-3β gene-deleted mice exhibit a phenotype comparable to that of mice in which the gene for NF-κB subunit p65, or the IKK 2 involved in the activation of NF-κB, had been deleted [Hoeflich et al., 2000]. Deletion of the GSK-3β gene had no effect on TNF-α-induced IκB-α degradation, but did prevent the activation of NF-κB [Hoeflich et al., 2000]. The few

findings suggest that the inhibition of GSK-3β promotes a reduction of activation of NF-κB. Probably, GSK-3β is able to affect NF-κB activity at different sites (FIGURE 19), namely the phosphorylation of IκB-α, the translocation of p50/p65 to the nucleus, its binding to DNA and/or phosphorylation of p65 [Schwabe & Brenner, 2002; Sanchez et al., 2003; Takada, Fang, Jamaluddin, Boyd & Aggarwal, 2004].

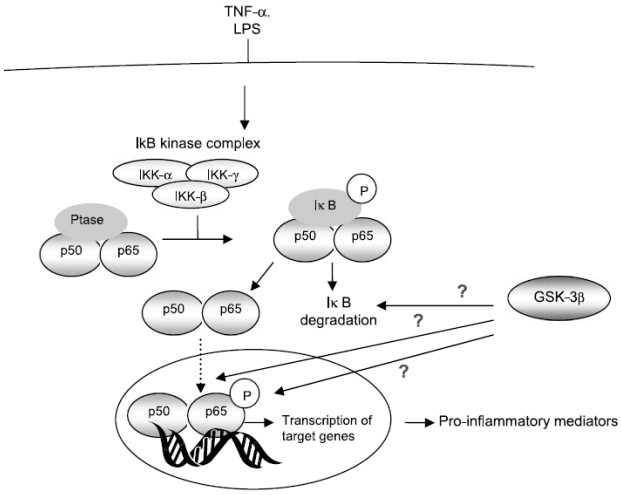

FIGURE 19. Possible sites for GSK-3β activity on NF-κB regulation [adapted of Dugo et al., 2007].

Legend: TNF-α - Tumor necrosis factor α; IκB - Inhibitory κB; GSK - Glycogen synthase kinase; IKK - IκB kinase; LPS – Lipopolysaccharide.

Furthermore, the inactivation of GSK-3β is also mediated by Akt (one of the most important physiological mediators of the PI3K survival pathway). Both studies in vitro and in vivo have demonstrated that the activation of PI3K-Akt pathway promotes the inactivation of GSK-3β by phosphorylation of Ser9, thereby providing an anti-apoptotic effect [Cross, Alessi, Cohen, Andjelkovich & Hemmings, 1995; Hurel et al., 1996; Moule et al., 1997; Dugo 2007].

Many recent studies suggest GSK-3β has become a novel and important therapeutic target in inflammatory and autoimmune diseases and, consequently, several pharmacological inhibitors of this kinase have been developed and studied in vitro and in preclinical studies in vivo [Cohen & Goedert, 2004; Beurel et al., 2009]. The inhibition of GSK-3β activity can be useful in several diseases, including the treatment of neurodegenerative diseases, diabetes type II, bipolar disorders, stroke, sepsis, cancer and chronic inflammatory disease [Martinez et al., 2002a; Meijer et al., 2004]. In the last few years, several potent and specific inhibitors of GSK-3β have been developed by the pharmaceutical industry and academic centers due to their therapeutical ability and, hence, their potential to be of benefit in a vast number of diseases [Martinez et al.,

2002b; Cohen & Goedert, 2004]. Althought previous studies in animal models have suggested that some GSK-3β inhibitors have anti-inflammatory effect in vivo, the full pharmacological profile and selectivity of these agents awaits further evaluation [Dugo et al., 2005; Whittle et al., 2006].

Briefly, many drugs are known to promote anti-inflammatory effects through inhibition of GSK-3β with consequently suppression of NF-κB signaling pathway, via reduction of phosphorylation of IκB-α [Buss et al., 2004; Takada et al., 2004; Puangpraphant et al., 2013]. In fact, GSK-3β inhibition has been shown to be critically important in chronic inflammatory diseases, suggesting an anti-inflammatory strategy in IBD [Whittle et al., 2006]. Since novel approaches in the management of injured colon tissue would constitute important advances in the therapy of IBD [Cuzzocrea et al., 2004], some studies have been developed to evaluate the efficacy of GSK-3β inhibitors in this disease (TABLE 8).

TABLE 8. Experimental studies with GSK-3β inhibitors in chemically induced colitis models.

CHEMICALLY INDUCED COLITIS	RODENT	DRUG USED	TREATMENT	EVALUATED PARAMETERS	REFERENCE
TNBS	Rats	Geraniol	Daily (11 days)	Body weight, MPO, caspase-3, NO, IL-1β, ICAM-1, NF-κB, MAPK, PGE2, PPARγ, MDA	Soubh et al., 2015
DSS	Mice	Carbon monoxide	Single inhalation at day 6	Body weight , colon lenght, iNOS, IL-10, TNF-α, GSK-3β, histology	Udin et al., 2013
DNBS	Rats	Yerba mate tea	Daily (8 days)	Body weight, colon length, histology, NF-κB, GSK-3β, IκB-α, iNOS, COX-2	Puangpraphant et al., 2013
TNBS	Rats	TDZD-8 0.1; 0.33; 1 mg/Kg SC	Twice a day (3 days)	Body and colon weight, TNF-α, MPO, NF-κB, protein levels, macroscopic score, % area inflamatory	Whittle et al., 2006

Legend: TNBS - Trinitrobenzene sulfonic acid; DSS - Dextran sulfate sodium; DNBS - Dinitrobenzene sulfonic acid; TDZD-8 - Thiadiazolidinone-8; MPO – Myeloperoxidase; NO - Nitric oxide; IL – Interleukin; MDA – Malondialdehyde; ICAM-1 - Intercellular adhesion molecule-1; NF-κB - Nuclear transcription factor kappa B; MAPK - Mitogen-activated protein kinases; PG – Prostaglandin; PPARγ - Peroxisome proliferator-activated receptor γ; iNOS - Inducible nitric-oxide synthase; TNF-α - Tumor necrosis factor α; GSK-3β - Glycogen synthase kinase 3β; IκB - Inhibitory κB; COX-2 – Cyclooxygenase-2.

Since TDZD-8 can be a possible new strategy of IBD, it is crucial to implement preclinical studies to evaluate the real efficacy of this compound in IBD [Whittle et al., 2006]. Curiously, the assessment of TDZD-8 effect in experimental colitis was tested

for the first time in 2006. Since that this was the unique study where the TDZD-8 effect was explored in IBD until nowadays. The findings of Whittle and colleagues revealed that the administration of TDZD-8 promotes a reduction of the colonic inflammation, of tissue injury and a reduced decline in body weight [Whittle et al., 2006]. Furthermore, the observed increase in the levels of the proinflammatory cytokine TNF-α in the inflamed colon was significantly reduced by TDZD-8. The mechanisms underlying this anti-inflammatory action may be related to downregulation of NF-κB activity (by GSK-3β inhibition), involved in the generation of proinflammatory mediators. Additionally, it was found the existence of multiple beneficial effects of TDZD-8, but lacks potential explanations of its mechanism of action [Whittle et al., 2006]. So, more studies are needed to evaluate the anti-inflammatory effect of TDZD-8 in IBD.

3. HEMIN

Hemin is an enzyme inhibitor that is derived from processed red blood cells and was formerly known as hematin. The term hematin describes the chemical reaction of hemin and sodium carbonate solution. Hemin, or ferriprotoporphyrin IX chloride, is an iron-containing metalloporphyrin and hematin is prepared from outdated HBAg negative human blood [Abbott Laboratories, 1997]. In 1971, hemin became the rationale for treatment of acute porphyria [Bonkowsky et al., 1971]. Given the rarity of acute attacks of porphyria, safety and efficacy information built slowly, case-by-case. By the late 1980s, however, a consensus existed, and a commercial formulation was developed (Panhematin®). It was the first therapeutic approved under the U.S. Orphan Drug Act of 1983 [Bissell & Wang, 2015]. Currently, hemin is used in the treatment of acute attacks of inducible porphyria (acute intermittent porphyria, variegate porphyria and hereditary coproporphyria). It is also used for the amelioration of recurrent attacks of acute intermittent porphyria that are temporally related to the menstrual cycle [Abbott Laboratories, 1997]. The clinical features of an acute attack include abdominal pain, mental symptoms, and a subacute polyneuropathy [Bosch, Pierach & Bossenmaier, 1977; Walsh, 1977; Lamon & Tschudy, 1978; Anon, 1978; Pierach, 1982].

The porphyria attack is accompanied by an induction of gamma-aminolevulinic acid synthetase, which is the rate limiting enzyme in heme biosynthesis. This induction is caused by the excessive production in the liver of porphyrin precursers alanine (delta-aminolevulinic acid - ALA) and porphobilinogen (PBG). The fundamental defect in acute intermittent porphyria is a decrease in uroporphyrinogen I synthetase which leads to a partial deficiency of heme production. This deficiency in heme inhibits the negative feedback cycle on gamma-aminolevulinic acid synthetase, which accounts for the acute attack. IV infusion of hemin decreases the concentration of PBG and ALA in the blood of an anuric patient with acute porphyria [Bonkowsky et al., 1971].

The regular pathway of heme synthesis consists in combine the building blocks of heme, like succinyl CoA and glycine, to form ALA, the first committed intermediate of the pathway (FIGURE 20). ALA synthase is encoded by two distinct genes: ALAS2 in the bone marrow and ALAS1 elsewhere, including the liver. The second step involves condensation of two molecules of ALA to form PBG, the pyrrole subunit of the heme ring. Four PBG are linked initially in a linear tetrapyrrole, hydroxymethylbilane, which cyclizes to form the initial porphyrin of the pathway, uroporphyrinogen. The sequential

conversion of uroporphyrinogen to coproporphyrinogen and finally to protoporphyrinogen involves successive removal of peripheral carboxyl groups. Heme is the end-product and exerts feedback regulation on the formation of ALA, the initial committed intermediate [Bissell & Wang, 2015].

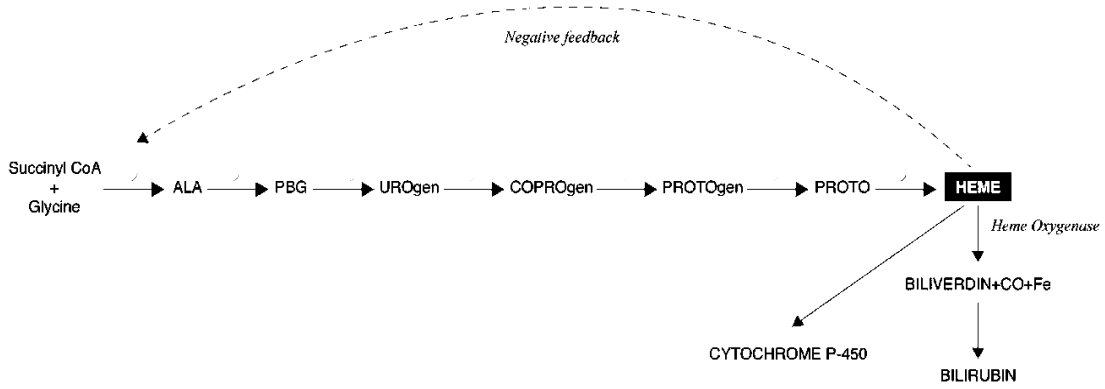

FIGURE 20. The pathway of heme synthesis and the enzymes mediating specific steps [adapted of Bissell & Wang, 2015].

Legend: ALA - Delta-aminolevulinic acid; PBG - Porphobilinogen; UROgen - Uroporphyrinogen; COPROgen - Coproporphyrinogen; PROTO – Protoporphyrin; PROTOgen – Protoporphyrinogen; CO – Carbon monoxide; Fe – Iron.

Hemin is also well known as an inducer of heme oxygenase (HO) [Guan et al., 2009; Hualin et al., 2012]. Many studies have reported the protective effect of hemin via HO-1 induction in various animal models [Guan et al., 2009; Hualin et al., 2012]. A wide variety of compounds with therapeutic properties have been recognized as inducers of HO-1. The induction of this enzyme, or its catalytic activity by either natural or synthetic compounds, may represent an effective strategy to intervene in several pathologic conditions [Ferrándiz & Devesa, 2008]. With few exceptions, the response to HO-1 inducers depends largely on transcriptional activation of the HO-1 gene and the synthesis of mRNA, regardless of cell type or inducing chemical [Ryter, Alam & Choi, 2006]. Several studies with HO-1 inducers have been confirmed its beneficial effects in many diseases, such as hippocampal injury, renal fibrosis, cardiac ischemia/reperfusion, lung injury and sepsis [Guan et al., 2009; Naito et al., 2011; Hualin et al., 2012]. Thus, targeting HO-1 to achieve pharmacologic and therapeutic benefits is becoming widely accepted [Ferrándiz & Devesa, 2008].

HO is the rate-limiting enzyme in heme catabolism, a process that leads to the generation of equimolar amounts of biliverdin, free iron and CO [Maines, 1997]. Three mammalian HO isozymes have been identified, namely HO-1, HO-2 and HO-3. HO-1 is a stress-responsive protein induced by various oxidative agents [Naito et al., 2011]. HO-1 occurs at a high level of expression in the spleen and other tissues that degrade

senescent red blood cells, including specialized reticuloendothelial cells of the liver and bone marrow. In most other tissues not directly involved in erythrocyte or hemoglobin metabolism, HO-1 typically occurs at low to undetectable levels under basal conditions but responds to rapid transcriptional activation by diverse chemical and physical stimuli [Ryter et al., 2006]. HO-2 is a constitutively expressed isozyme and is found abundantly and ubiquitously in several systemic tissues including, but not limited to, the brain and central nervous system, vasculature, liver, kidney, and gut [Ryter et al., 2006; Naito et al., 2011]. HO-3 is a newly identified isoform, which is constitutively expressed in the liver, spleen, brain and kidney in rats, but its biological role requires further elucidation [Naito, Takagi & Yoshikawa, 2004]. Although both HO-1 and HO-2 catalyze the identical biochemical reaction, there are some fundamental differences between the two in genetic origin, primary structure and molecular weight. HO-1, once expressed under various pathological conditions, has an ability to metabolize high amounts free heme to produce high concentrations of its enzymatic by-products that can influence various biological events, and has recently been the focus of considerable medical interest [Abraham & Kappas, 2008].

Indeed, HO-1 is highly inducible by a vast array of stimuli, including oxidative stress, heat shock, ultraviolet radiation, ischemia-reperfusion, heavy metals, bacterial lipopolysaccharide (LPS), cytokines, NO and elevated levels of its natural substrate, heme [Shibahara, 1988]. Accumulating evidence indicates that these inducers of HO-1 activate protein phosphorylation-dependent signaling cascades that ultimately converge on the transcription factors that regulate the HO-1 gene (FIGURE 21) [Ryter et al., 2006]. Recent studies have implicated a major role for the mitogen-activated protein kinases (MAPK) in HO-1 activation, though other kinases, including tyrosine kinases, PI3K and protein kinases A, G, and C have also emerged as potential contributing mechanisms [Salinas et al., 2004; Ryter et al., 2006; Lin, Chiang & Chau, 2008]. Particularly, increases of intracellular reactive oxygen species (ROS) lead to perturbation of intracellular thiol equilibrium, leading to reduction of glutathione/oxidized glutathione (GSH/GSSG) ratio, and redox regulation of Keap1. On the other hand, treatment with heavy metals and/or heme promotes NF-E2 related factor-2 (Nrf2) nuclear translocation and nuclear export of the transcriptional repressor Bach-1. Finally, activation of mitogen-activated protein kinase activities (MAPK) by environmental stresses and cytokines has also been implicated in HO-1 activation. The link between redox regulation and MAPK activation is not completely clear. Other nuclear factors involved in certain models include activation of Hif-1 (by hypoxia) and AP-1 (by hypoxia, and LPS) [Ryter et al., 2006; Naito et al., 2011].

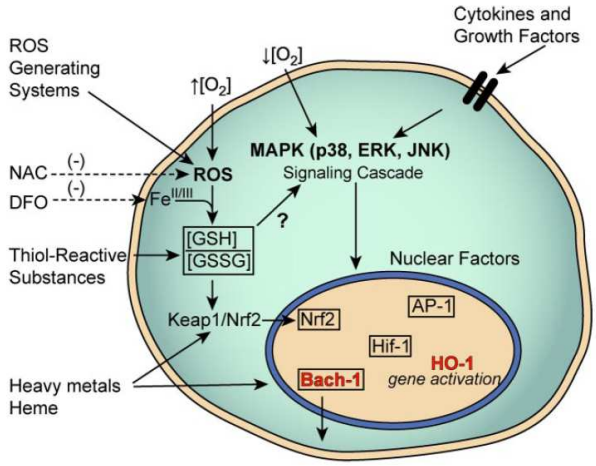

FIGURE 21. Signaling pathways leading to HO-1 activation [adapted of Ryter et al., 2006].

Legend: GSH - Reduced glutathione; GSSG - Oxidized glutathione; MAPK - Mitogen-activated protein kinase; Nrf2 - NF-E2 related factor-2; Hif-1 - Hypoxia inducible factor; AP-1 - Activator protein-1; Bach-1 - HO-1 transcriptional repressor; ROS - Reactive oxygen species; NAC - N-acetyl-L-cysteine; DFO - Desferrioxamine; HO-1 - Heme oxygenase-1.

The HO system, along with its catabolism products, is involved in a variety of crucial physiological functions, including cytoprotection, anti-inflammation, anti-oxidative effects, apoptosis, neuro-modulation, immune-modulation, angiogenesis and vascular regulation [Pittala, Salerno, Romeo, Modica & Siracusa, 2013]. Their beneficial properties may be probably attributed not only its own action, but also to other actions of three by-products of HO-1 activity (FIGURE 22). The degradation of the pro-oxidant heme by HO-1 itself, the signaling action of CO, the antioxidant properties of biliverdin/bilirubin and the sequestration of free iron by ferritin could all concertedly contribute to the anti-inflammatory effects observed with HO-1 [Naito et al., 2011].

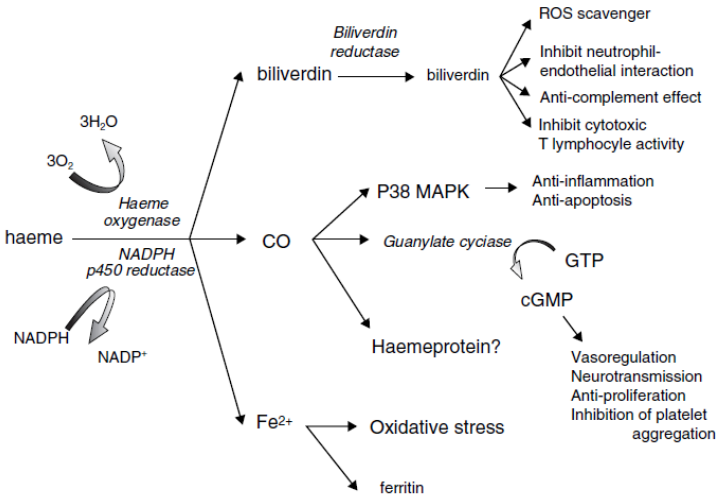

FIGURE 22. Mechanism underlying the biological actions of heme oxygenase [adapted of Naito et al., 2004].

Legend: ROS - Reactive oxygen species; CO - Carbon monoxide; MAPK - Mitogen-activated protein kinases.

The pharmacological application of CO mimics the HO-1 dependent cytoprotection and anti-inflammation in many injury models (FIGURE 23) [Naito et al., 2011]. CO is known to be an activator of soluble guanylate cyclase, akin to its classical regulator NO, promoting the increase of cGMP production and, consequently, smooth muscle relaxation by direct effects on the contractile machinery as well as by altering calcium homeostasis and voltage-gated ion channel activity [Carvajal, Germain, Huidobro-Toro & Weiner, 2000]. CO can also modulate MAPK, including the p38 MAPK, ERK, and c-Jun N-terminal kinases (JNK) pathways [Ryter et al., 2006]. Thus, CO is able to inhibit the production of proinflammatory cytokines, such as TNF-α, IL-1β, and macrophage inflammatory protein-1 [Otterbein et al., 2000; Ryter et al., 2006]. The heat shock factor 1 and Hsp70 may act as potential intermediates in this pathway [Ryter et al., 2006]. CO also inhibited the expression of other proinflammatory enzymes, such as iNOS and COX-2 via the regulation of NF-κB activation [Suh, Jin, Yi, Wang & Choi, 2006]. Furthermore, it has also been reported its anti-apoptotic potential due to inhibition of TNF-α in fibroblasts and endothelial cells by MAPK and NF-κB pathways [Petrache, Otterbein, Alam, Wiegand & Choi, 2000; Brovard et al., 2000; Zhang, 2003; Brovard et al., 2002]. Briefly, the potential physiological effects of CO include tissue specific protection throught its anti-inflammatory, anti-apoptotic, anti-proliferative, and anti-thrombotic properties [Ryter et al., 2006].

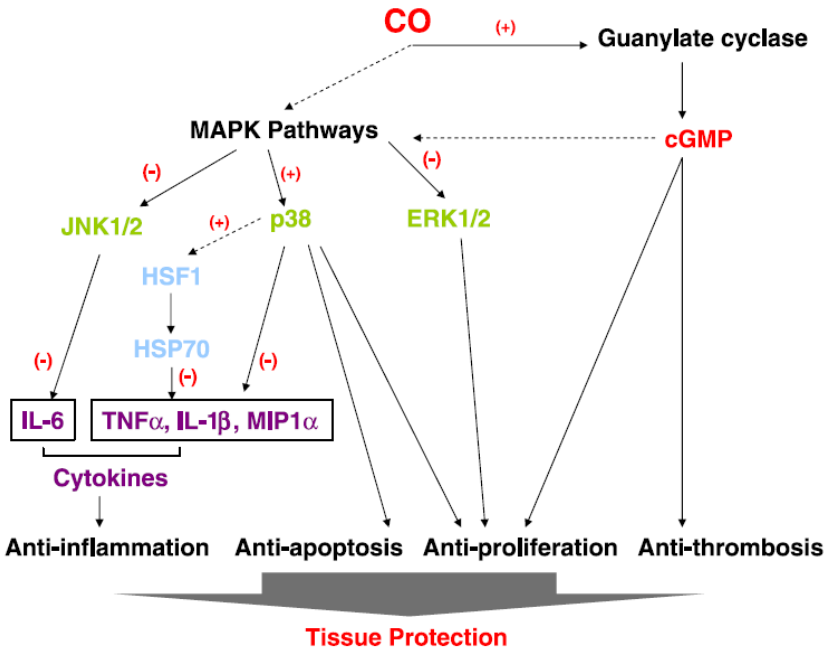

FIGURE 23. Potential signaling pathways activated by CO leading to tissue protection [adapted of Ryter et al., 2006].

Legend: CO - Carbon monoxide; MAPK - Mitogen-activated protein kinases; JNK - c-Jun N-terminal kinases; ERK - Extracellular signal-regulated kinase; Hsp70 - Heat shock protein 70; IL – Interleukin; TNF-α - Tumor necrosis factor α.

Biliverdin, the first product of HO-catalyzed heme cleavage, is a soluble greenish pigment. Its enzymatic reduction by biliverdin reductase (BVR) produces bilirubin, a hydrophobic yellowish pigment that partitions to the lipid phase. Like CO, bilirubin is formally considered as a metabolic waste product with potentially harmful effects. Under normal physiological conditions, biliverdin and bilirubin are processed for rapid elimination. Whether during the course of their elimination these substances provide intrinsic benefit to an organism remains controversial and has provided fuel for recent debate. Potential antioxidative effects of bilirubin were first noted as early as 1976 [Ryter 2006]. In normal physiological condition of oxidative stress, BVR catalyzes biliverdin to bilirubin and induces HO, which further provides more biliverdin from heme (FIGURE 24). And the resulting bilirubin may turn back to biliverdin partially by oxidants. This bicyclic nature of bilirubin system provides the efficient physiological antioxidative capacity. But in harsh conditions of high oxidative stress, BVR is catalytically inactivated and is blocked for nuclear translocation, which inhibits the induction of HO, resulting in inhibition of catalytic turnover of heme to biliverdin and also biliverdin to bilirubin, leading to oxidative damages of the biomolecules and finally to tissue injury [Kim & Park, 2012].

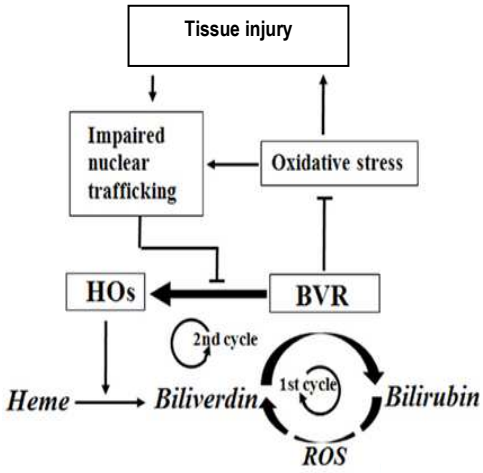

FIGURE 24. Physiological network of the bicyclic bilirubin system in tissue injury [adapted of Kim & Park, 2012].

Legend: HO – Heme oxygenase; BVR - Biliverdin reductase; ROS - Reactive oxygen species.

In fact, both molecules act as antioxidants in vitro and in vivo and their increased local concentrations after HO induction may be beneficial in protecting several types of cells from injury [Naito et al., 2011]. Bilirubin can scavenge peroxyl radicals in vitro as effectively as α-tocopherol, which is regarded as the most potent antioxidant against lipid peroxidation [Stocker, Yamamoto, McDonagh, Glazer & Ames, 1987]. Several

epidemiological studies indicate that mild to moderately elevated serum bilirubin levels are associated with a better outcome in diseases involving oxidative stress [Djousse, Rothman, Cupples, Levy & Ellison, 2003; Sedlak & Snyder, 2004]. Bilirubin also acts as an efficient scavenger of hypochlorous acid, a macrophage-derived oxidant [Stocker & Peterhans, 1989]. Finally, biliverdin and bilirubin also display in vitro reactivity toward various forms of reactive nitrogen species, including NO gas, NO donor compounds, and *S*-nitrosothiols, peroxynitrite, or nitroxyl anion, which is prevented by thiol antioxidants [Kaur et al., 2003; Mancuso, Bonsignore, Di Stasio, Mordente & Motterlini, 2003]. These findings suggest that biliverdin and bilirubin can promote antioxidative and antinitrosative effects [Ryter et al., 2006].

The iron released from heme by HO activity potentially enters such a pool of “labile” or “chelatable” iron, where it may be available for cellular processes that depend on iron [Ryter & Tyrrell, 2000]. One hypothesis states that the iron released from HO activity is transiently available for the promotion of intracellular ROS production. The potential reactions in which iron may play a catalytic role include but are not limited to the multiplication of lipid peroxidation chain reactions, the NO-dependent nitrosylation of thiols, and the Haber-Weiss reaction, which generates the reactive oxidant hydroxyl radical from the metal catalyzed decomposition of hydrogen peroxide [Halliwell & Gutteridge, 1999]. However, HO activity seems to have an antioxidative protection with respect to iron metabolism, facilitating further coupling with proteins that either promote the sequestration or export of the liberated iron. Cells that realize an excess of intracellular iron utilize these mechanisms to reduce intracellular iron levels [Eisenstein & Munro, 1990; Kuhn, 1998]. The enhancement of ferritin synthesis by high iron levels increases the iron storage capacity of the cell. Furthermore, ferritin synthesized as a consequence of HO activity has been proposed as a contributory mechanism underlying HO-dependent cytoprotection [Vile, Basu-Modak, Waltner & Tyrrell, 1994].

Indeed, HO-1 has clearly emerged as a therapeutic target that offers protection against several diseases. HO-1 induction using gene therapy or pharmacological modulation has shown promising results both in vitro and in vivo [Ferrándiz & Devesa, 2008]. Thus, a better understanding of the heme-HO system may result in novel therapeutic strategies for some important pathological disorders and in the development of novel inducers of HO-1. In the management of colitis, novel approaches would constitute important advances in the therapy of IBD [Cuzzocrea et al., 2004]. Some studies have been developed to evaluate the efficacy of HO-1 inducers in this disease (TABLE 9).

TABLE 9. Experimental studies with HO-1 inducers in chemically induced colitis models [adapted of Naito et al., 2011; Takagi et al., 2010].

CHEMICALLY INDUCED COLITIS	RODENT	DRUG USED	TREATMENT	EVALUATED PARAMETERS	REFERENCE
DSS	Mice	Hemin	Once a day (at day 0, day 1 and day 6)	Treg, IL-17, HO-1	Zhong et al., 2010
DSS	Mice	Tranilast	Once a day (at day 0, day 2 and day 4)	Body weight, colon lengh, TNF-α, IFN-γ, IL-17, IL-6, IL-10, HO-1, histology	Sun et al., 2010
DSS	Mice	CoPP	Once a day (at day 1 and day 3)	MPO, HO-1, histology	Paul et al., 2005
DSS	Mice	CoPP	3 times a day	Body weight, HO-1	Berberat et al., 2005
TNBS	Rats	5-ASA	Once daily (3 days)	MPO, TNF-α, HO-1	Horváth et al., 2008
TNBS	Rats	Heme ZnPP SnPP Cadmium chloride	Once daily (10 days)	HO-1, MPO	Varga et al., 2007
TNBS	Mice	Chalcone	Once a day (at day -1, day 3 and day 6)	Body weight, colonic damage, ICAM-1, IL-1β, TNF-α, histology	Lee et al., 2007
TNBS	Rats	Glutamine	Once a day (18 days)	MDA, GSH, caspase-3, HO-1, NF-κB	Giriş et al., 2007
TNBS	Rats	Octreotide	Once a day starting day -5 (20 days)	MDA, GSH, HO-1, NF-κB	Erbil et al., 2007
TNBS	Mice	Gliotoxin	Once a day (at day -1, day 3 and day 6)	Body weight, HO-1, MPO, NF-κB, IL-1β, TNF-α, IL-12, IL-8, I-κB, histology	Jun et al., 2006
TNBS	Mice	Bolinaquinone Petrosaspongiolide	Once a day (4 days)	Colonic length and weight , MPO, PGE2, IL-1β, NO, NF-κB	Busserolles et al., 2005
TNBS	Rats	Hemin SnMP	Single administration	HO-1, MPO, iNOS	Wang et al., 2001

Lengend: CoPP - Cobalt-protoporphyrin; 5-ASA - 5-Aminosalicylic acid; MDA – Malondialdehyde; GSH – Glutathione; SnMP - Tin mesoporphyrin IX chloride; TNBS - Trinitrobenzene sulfonic acid; DSS - Dextran sulfate sodium; HO – Heme oxygenase; MPO – Myeloperoxidase; NO - Nitric oxide; IL – Interleukin; GSH – Glutathione; ICAM-1 - Intercellular adhesion molecule-1; NF-κB - Nuclear transcription factor kappa B; PG – Prostaglandin; iNOS - Inducible nitric-oxide synthase; TNF-α - Tumor necrosis factor α; IκB - Inhibitory κB; IFN-γ - Interferon-γ.

Briefly, HO-1 expression can confer cytoprotective, antiapoptotic and anti-inflammatory properties, suggesting thus that HO-1 can be a possible therapeutic target in several kinds of gastrointestinal diseases [Naito et al., 2011]. About IBD, Wang and colleagues used a rat model of colitis induced by TNBS to investigate whether the expression of HO-1 is an endogenous mechanism responsible for host defense against inflammatory injury in colonic tissue. They demonstrated that HO activity and HO-1 gene expression increased markedly after TNBS induction, and that administration of tin mesoporphyrin, an HO inhibitor, potentiated the colonic damage as well as decreased HO-1 activity. These results indicate that HO-1 plays a protective role in the colonic damage induced by TNBS enema [Wang et al., 2001]. Since then, some studies have been developed in DSS and TNBS colitis models to evaluate the effect of HO-1 inducers on the management of IBD. However, the biological significance of HO-1 up-regulation in gastrointestinal inflammation remains to be fully elucidated as well as the mechanisms underlying HO-1 activity on gene expression [Naito et al., 2011].

AIM

The main objective of the study is to test a set of drugs that modulate some important metabolic pathways in the establishment and development of inflammation in IBD, through an experimental model of IBD in rodents. These drugs may inhibit or stimulate the expression of these pathways, contributing to facilitate a more effective and selective treatment than the currently known. Thus, the specific objectives are:

- Development of animal model of TNBS-induced colitis;
- Evaluation of the influence of EPO in the IBD;
- Evaluation of the influence of TDZD-8 in the IBD;
- Evaluation of the influence of hemin in the IBD.

CHAPTER 4 - MATERIALS AND METHODS

CHEMICALS / MATERIALS

2,4,6-Trinitrobenzene sulfonic acid (TNBS 5%), 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), ferriprotoporphyrin IX chloride (Hemin), dimethyl sulfoxide (DMSO) and sodium hydroxide (NaOH) were purchased from Sigma Chemical Co. Erythropoietin (Eprex® 10000 IU/ml) was purchased from Janssen-Cilag Farmacêutica. Ketamine (Imalgene® 1000) was purchased from Merial. Xilazine (Rompun® 2%) was purchased from Bayer. ADVIA® kit was purchased from Siemens Healthcare Diagnostics. ELISA assay kits for TNF-α, IL-1β and MPO measurements were obtained from Hycult Biotechnology.

ANIMALS

Male CD-1 mice, 30-40 g in weight and 6-10 weeks of age, obtained from Charles River. Animals were housed in standard polypropylene cages with *ad libitum* access to food and water, under uniform and controlled temperature, humidity and lighting conditions. Animal care was in strict accordance with the Declaration of Helsinki, EEC Directive of 24th November 1986 (nº 86/609/EEC), the relevant Portuguese laws D.R. nº 31/92, D.R. 153 I-A 67/92, and all subsequent legislation.

INDUCTION OF EXPERIMENTAL COLITIS

TNBS was instilled intracolonic single dose as previously described by Morris *et al.* [Morris et al., 1989]. Briefly, mice were left unfed during 24h. In the induction day (day 0), mice were anesthetized with around 40µl of ketamine 100mg/Kg + xilazine 10mg/Kg by intraperitoneal injection (IP). Then, 100µl of TNBS solution was administered through a catheter carefully inserted until 4 cm into the colon. Mice were kept for 1 min in a Tredelenburg position to avoid reflux [Mazzon et al., 2005; Wirtz et al., 2007].

On day 4 and 6, depending the experimental group, mice were anesthetized and blood samples were collected by cardiac puncture. Then, the mice were euthanized by cervical dislocation and necropsied. The abdomen was opened by a midline incision. The small intestine and colon were removed, freed from surrounding tissues and washed with phosphate buffered saline.

EXPERIMENTAL GROUPS

Groups were categorized based on the main objectives of this study, namely the development of the TNBS-induced colitis model and the evaluation of the influence of a set of drugs, as EPO, TDZD-8 and hemin in the IBD (FIGURE 25). Thus, the experimental groups were:

A. DEVELOPMENT OF ANIMAL MODEL OF TNBS-INDUCED COLITIS

The TNBS day 4 group (n = 35) and the TNBS day 6 group (n = 35) received both 100 µl intrarectal of 2.5% TNBS in 50% ethanol and its necropsy was made on day 4 and 6, respectively. The ethanol group (n = 20) received 100µl intrarectal of 50% ethanol (TNBS vehicle). The sham group (n = 20) received 100µl intrarectal of saline solution.
After data analysis, the TNBS day 4 group came to be called TNBS group. So, the TNBS, ethanol and sham groups were then used as a reference groups to compare and evaluate the influence of EPO, TDZD-8 and hemin in the treatment of IBD.

B. EVALUATION OF THE INFLUENCE OF ERYTHROPOIETIN IN THE IBD

The TNBS+EPO500 group (n = 35) and TNBS+EPO1000 group (n = 35) were a TNBS-induced colitis models treated daily with 500 IU/kg and 1000 IU/kg IP of EPO (dissolved in saline solution) since the induction day, respectively. As a control, the EPO1000 group (n = 20) only received 1000 IU/kg IP of EPO daily since the induction day.

C. EVALUATION OF THE INFLUENCE OF TDZD-8 IN THE IBD

The TNBS+TDZD-8 group (n = 35) was a TNBS-induced colitis model treated daily with 5 mg/kg IP of TDZD-8 (dissolved in DMSO) since the induction day. The TNBS+Vehicle group (n = 20) was a TNBS-induced colitis model treated daily with the TDZD-8 vehicle (10% IP of DMSO saline solution) since the induction day. As a control, the TDZD-8 group (n = 20) only received 5 mg/kg IP of TDZD-8 daily since the induction day.

D. EVALUATION OF THE INFLUENCE OF HEMIN IN THE IBD

The TNBS+Hemin5 group (n = 35) and the TNBS+Hemin10 group (n = 35) were a TNBS-induced colitis models treated daily with 5 mg/kg and 10 mg/Kg IP of hemin (dissolved in NaOH and PBS) since the induction day, respectively. The TNBS+Vehicle group (n = 20) was a TNBS-induced colitis model treated daily with the hemin vehicle (10 mg/Kg IP of NaOH aqueous solution) daily since the induction day. As a control, the Hemin10 group (n = 20) only received 10 mg/kg IP of hemin daily since the induction day.

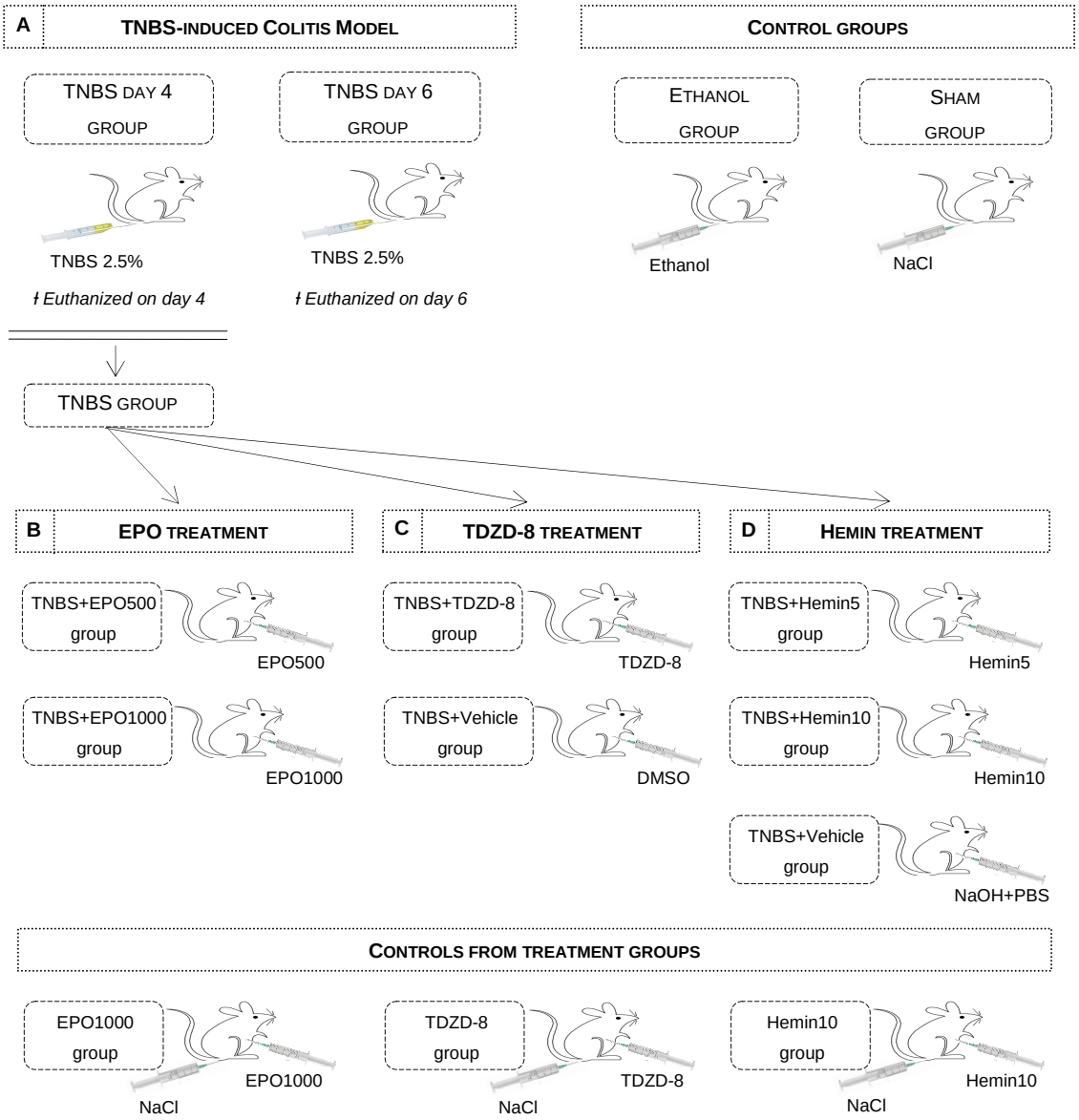

FIGURE 25. Scheme of study design with the involved experimental groups.

MONITORING OF CLINICAL SYMPTOMS/SIGNS

After induction, the animals were observed daily, monitoring body weight, morbidity, stool consistency and anus appearance.

BIOCHEMICAL MARKERS

The serum from collected blood samples was separated by centrifugation at 3600 rpm for 15 min and sent to a reference laboratory. Serum samples were analyzed by an automated clinical chemistry analyzer (ADVIA® Chemistry XPT).

Biochemical markers were evaluated to determine the severity of colitis, namely:

. Colon length, as a marker of tissue integrity, determined using a measuring scale;

. Fecal hemoglobin, as an index of hemorrhagic focus, measured using a quantitative method by immunoturbidimetry (Kroma Systems);

. Alkaline phosphatase (ALP) was determined as a marker of intestinal homeostasis. ALP in the sample catalyzes the hydrolysis of colorless *p*-nitrophenyl phosphate to give *p*-nitrophenol and inorganic phosphate. At the pH of the assay (10.3 e 10.4), the *p*-nitrophenol is in the yellow phenoxide form. The rate of absorbance increase at 410/478 nm is directly proportional to the ALP activity in the sample. Optimized concentrations of zinc and magnesium ions are present to activate the ALP in the sample;

. Urea was determined as a marker of renal function. Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia reacts with 2-oxoglutarate in the presence of glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide. The oxidation of nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide is measured as an inverse rate reaction at 340/410 nm;

. Creatinine was determined as a marker of renal function. Creatinine reacts with picric acid in an alkaline medium to produce a red-colored creatinine-picrate complex. The rate of complex formation is measured at 505/571 nm and is proportional to the creatinine concentration;

. Alanine aminotransferase (ALT) was determined as a marker of hepatic function. The reaction is initiated by the addition of α-ketoglutarate. The concentration of reduced nicotinamide adenine dinucleotide is measured by its absorbance at 340/410 nm and the rate of absorbance decrease is proportional to the ALT activity;

. Hematocrit was determined in the experimental groups from EPO treatment, in order to evaluate the chance to promote cardiovascular adverse effects. It was used a hematologic autoanalyzer (ADVIA® 2120 – SIEMENS®).

MYELOPEROXIDASE ACTIVITY

Neutrophil infiltration to the inflamed colon was indirectly quantitated using an MPO assay and it was expressed in ng/ml. The colon was weighed, homogenized (Ultra-turrax T25, 13.500rev/min, twice for 30s) in phosphate buffer and then centrifuged (at 15.000rpm for 15min at 4ºC). An aliquot of the supernatant was incubated in microtiter wells coated with biotinylated tracer antibody, recognizing mouse MPO. Streptavidin-peroxidase conjugate was added to bind the biotinylated tracer antibody and then was mixed with tetramethylbenzidine substrate. The reaction is stopped by the addition of oxalic acid and assayed spectrophotometrically at 450nm (ELISA 1kit HK210, Hycult Biotech).

MEASUREMENT OF CYTOKINES

The TNF-α and IL-1β (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine) were measured and expressed as pg/ml. The colonic tissue samples were weighed and homogenized (Ultra-turrax T25, 13.500rev/min, twice for 30s) in phosphate buffer and then centrifuged (at 15.000rpm for 15min at 4ºC). The aliquots of the supernatant were stored at -20ªC until use. The cytokine levels were measured spectrophotometrically at 450 nm (ELISA kit Quantikine, Hycult Biotechnology).

HISTOPATHOLOGICAL ANALYSIS

The intestine samples were fixed in 10% phosphate-buffered formalin, processed routinely for paraffin embedding, sectioned at 5 µm, and stained with hematoxylin and eosin. The morphological features of small intestine and colon were evaluated by the same evaluated criteria. However, in the small intestine were analyzed several sections along this intestinal portion due to its length and because no macroscopic lesions were identified. In the colon, there were an identified macroscopic lesions, so it was analyzed several sections of this specific location.

MICROSCOPIC ASSESSMENT OF COLITIS SEVERITY

Two blinded independent histopathologists to treatment groups from Faculty of Veterinary Medicine (FMV-ULHT) and Institute of Molecular Medicine (IMM) carried out the histopathological studies. Sections of distal colon were evaluated based on adapted criteria of Corazza and colleagues (1999) and Seamons and colleagues (2013) (TABLE 10). The histopathological score of lesions were partially scored (0–4 increasing severity) with some parameters, namely: (1) presence of tissue loss/necrosis, (2) severity of mucosal epithelial lesion, (3) inflammation, (4) extent 1 - the percentage of intestine affected in any manner and (5) extent 2 - the percentage of intestine affected by the most severe lesion. The colitis severity was calculated by summing the individual lesions and the extent scores, promoting a final colitis score (max score=20).

TABLE 10. Scoring system for histopathologic evaluation of TNBS-induced colitis.

POINTS	TISSUE LOSS	EPITHELIAL LESION	INFLAMMATION	EXTENT
0	None	None	None	None
1	Mucosa < 50%	Mild mucous cell depletion	Mild – mucosa only	< 5%
2	Mucosa > 50%	Moderate mucous cell depletion	Moderate – mural	6-30%
3	Mural*	Severe with aberhant crypts***	Transmural	31-60%
4	Transmural**	Severe with crypt loss	Extension to the mesentery	> 61%

Legend: * Mural - Involves mucosa and submucosa; ** Transmural - Involves mucosa, submucosa, muscle layer and subserosa; *** Aberhant crypts - Corresponds to dilated crypts.

The colonic lesions in the TNBS-induced colitis can be illustrated in some histological images that exemplify the observed lesional characteristics, corresponding to a progressive increase of injury severity from 0 (1st picture) to 20 (last picture) (FIGURE 26). Thus, the histological images can be characterized in:

A. In normalcy, the wall of the colon is composed of four layers: mucosa, submucosa, muscularis (or *muscularis propria*), and adventitia (or serosa). The mucosa is the innermost layer formed by glandular epithelium, lamina propria, and *muscularis mucosae*. The glandular epithelium forms cylindrical structures, called crypts. The *lamina propria*, which supports the epithelium, is a layer of reticular connective tissue with elastin, reticulin, and collagen fibers, lymphocytes, plasma cells, and eosinophilic granulocytes, as well as lymphatics and capillaries. The *muscularis mucosae* consist of a thin layer of smooth muscle at the boundary of the mucosa and submucosa. The submucosa, between the *muscularis mucosae* and the *muscularis propria*, is a fibrous connective tissue layer that contains fibroblasts, mast cells, blood and lymphatic vessels, and a nerve fiber plexus. The *muscularis propria*, mainly responsible for contractility, consists of two layers of smooth muscle: an inner circular coat and an outer longitudinal coat arranged in a helicoidal pattern. The serosa is the outermost layer of connective tissue, covered by a single layer of mesothelial cells;
B. Initial stages of the TNBS-induced colitis are characterized by mucous cell depletion, with loss of the large mucus-laden vacuoles within the epithelial cells of the mucosa, accompanied by mild inflammatory cell infiltration extending to the submucosa;
C. The inflammation then progresses in extent (involving the *muscularis propria*) and severity (with more cells and more severe edema);
D. Then the crypts become dilated and the epithelium eroded, to which follows mucosal loss (disappearance of the top of the crypts) and ulceration (disappearance of the whole crypt/epithelium);

E-F. In the most severe stages the necrosis extends firstly to the submucosa and muscle layer (E), and then to the serosa, often associated with peritonitis.

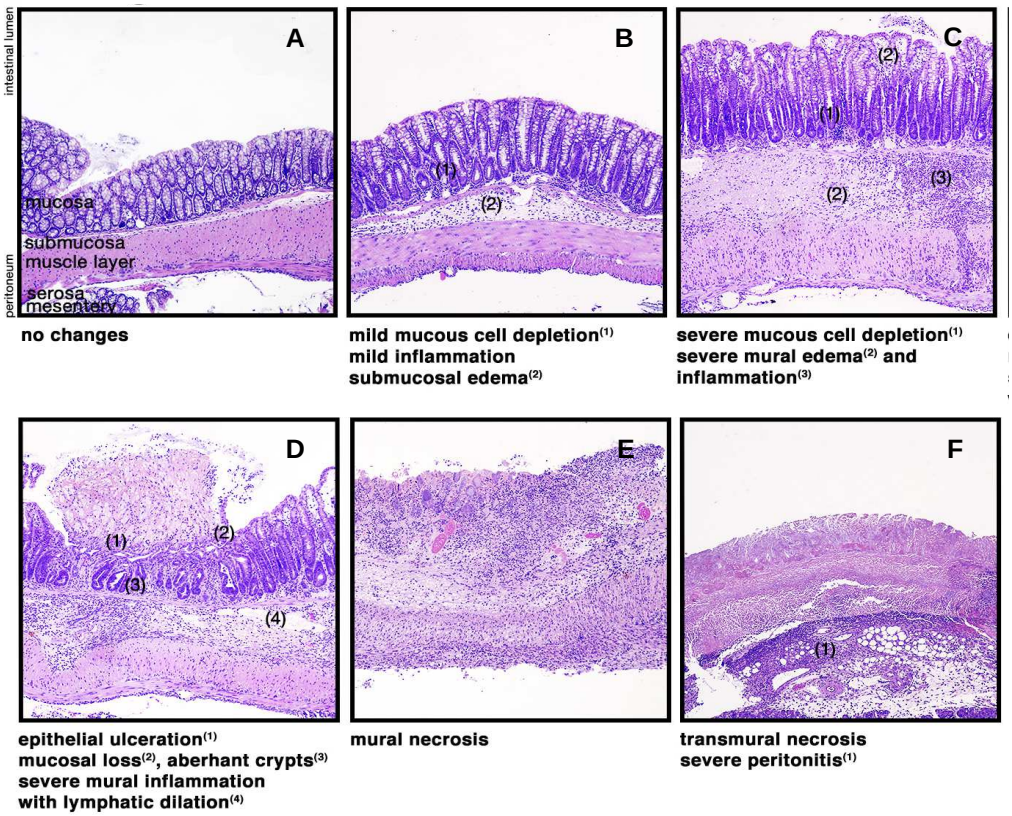

FIGURE 26. Characterization of the colonic lesions in TNBS-induced colitis.

STATISTICAL ANALYSIS

All results were expressed as mean ± SEM of *N* observations, where *n* represents the number of animals studied. Data analysis was performed by using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). The results were analyzed by one-way ANOVA to determine statistical significance between the experimental and control groups followed by Tukey's post hoc test for multiple comparisons or chi-square test depending on the variables under study. A *p*-value of less than 0.05 was considered significant.

CHAPTER 5 – DEVELOPMENT OF A TNBS-INDUCED COLITIS MODEL

The protocols of the TNBS-induced colitis model are not standardized, since the acute transmural damage became maximal from three days to one week after instillation [Qin et al., 2011]. Thus, two independent groups of TNBS-induced colitis were developed and monitored under the same specific conditions, however one group of mice were sacrificed on day 4 (TNBS day 4 group) and the other one on day 6 (TNBS day 6 group), after instillation. The main objective is to identify the maximal acute damage with the induction method used on this study.

1. RESULTS

MONITORING OF CLINICAL SYMPTOMS/SIGNS

During six days, the mice were observed daily for morbidity, stool consistency and anus appearance. On day 4, all mice presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity. But, on day 6, the mice presented an apparently recover, revealing the same clinical signs, but more lightly. In the control groups, namely ethanol and sham groups, were not identified any alteration, keeping these observed clinical symptoms/signs during all experimental period. Regarding body weight, both TNBS groups demonstrated a very similar curve in the register of body weight each day (FIGURE 27). For example, on day 4, the TNBS day 4 group revealed a weight loss of around -12 ± 1.4% of its initial weight, whereas the TNBS day 6 group lost -9.9 ± 3.3% of its initial weight. After day 4, the mice body weight began to increase gradually day by day until day 6, where the mice just had -7.6 ± 3.4% of its initial weight. On the other hand, the ethanol and sham groups presented a considerable increase in body weight, especially from day 0 to day 1, around 20 to 30% at day 1. At the end of experimental period, the ethanol and sham groups gained 15.2 ± 1.1% and 31.2 ± 1.6% ($p<0.0001$, compared with TNBS day 4 group) of its initial weight, respectively.

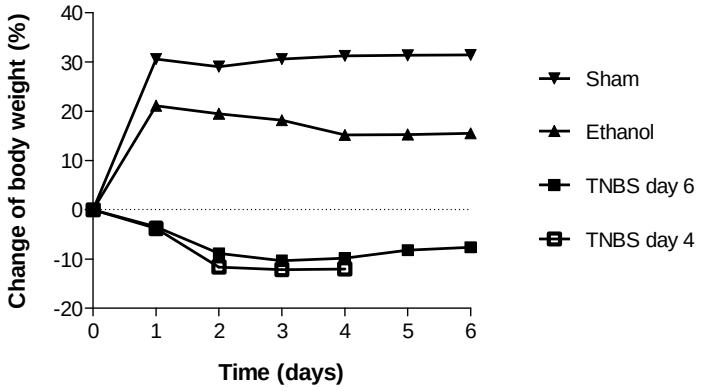

FIGURE 27. Change of body weight during the development of TNBS-induced colitis.

BIOCHEMICAL MARKER

The colon length was determined at the end of the treatment period, using a measuring scale (FIGURE 28 AND 29). Four days after intra-colonic administration of TNBS, the colon appeared flaccid and filled with liquid stool. Furthermore, the TNBS-induced colitis had an influence on colon length comparing with control groups, especially on day 4. More specifically, the TNBS day 4 group presented around 10.1 ± 0.2 cm of colon and, after two days, the TNBS day 6 group revealed a significantly increase of colon length with 11.1 ± 0.3 cm ($p<0.01$). On day 4, the colon length of TNBS group was considerably lower than control groups, as ethanol and sham groups, which presented 11.5 ± 0.4 cm and 11.5 ± 0.2 cm of colon, respectively ($p<0.001$).

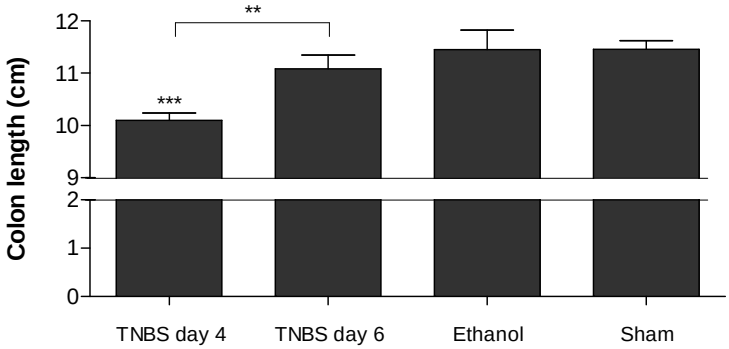

FIGURE 28. Effect of TNBS-induced colitis on colon length in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; ** $p<0.01$; *** $p<0.001$ compared with sham group or between groups.

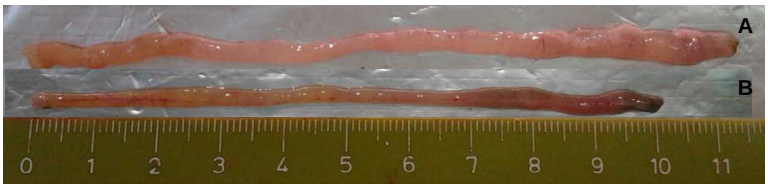

FIGURE 29. Appearance and length of colon in the TNBS and sham groups.

Legend: (A) Sham group; (B) TNBS day 4 group.

The fecal hemoglobin allows evaluating the intensity of hemorrhagic focus (FIGURE 30). Thus, the TNBS-induced colitis significantly increased the fecal hemoglobin concentration comparing with sham group ($p<0.001$). On day 4, the mice with colitis had 12.5 ± 0.3 µmol Hg/g feces and, passed 2 days, the fecal hemoglobin considerably decreased to 6.6 ± 0.6 µmol Hg/g feces ($p<0.001$). The control groups presented very low concentrations of fecal hemoglobin with 1.1 ± 0.1 µmol Hg/g feces on ethanol group and 0.7 ± 0.1 µmol Hg/g feces on sham group.

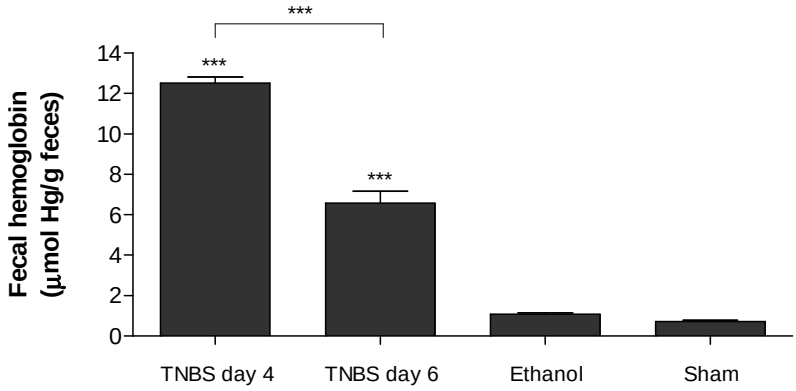

FIGURE 30. Effect of TNBS-induced colitis on fecal hemoglobin in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** $p<0.001$ compared with sham group or between groups.

It was evaluated the ALP concentration on blood, due to ALP plays an essential role in some systems, like intestinal homeostasis (FIGURE 31). The TNBS day 4 group presented the highest values with around 72.7 ± 2.3 IU/L of ALP ($p<0.001$, compared with the sham group). On day 6, the ALP significantly decreased to 51.6 ± 1.5 IU/L of ALP ($p<0.001$, compared with the TNBS day 4 group). Ethanol and sham groups presented lower concentrations of ALP with around 38.5 ± 0.6 IU/L and 19.2 ± 1.8 IU/L, respectively.

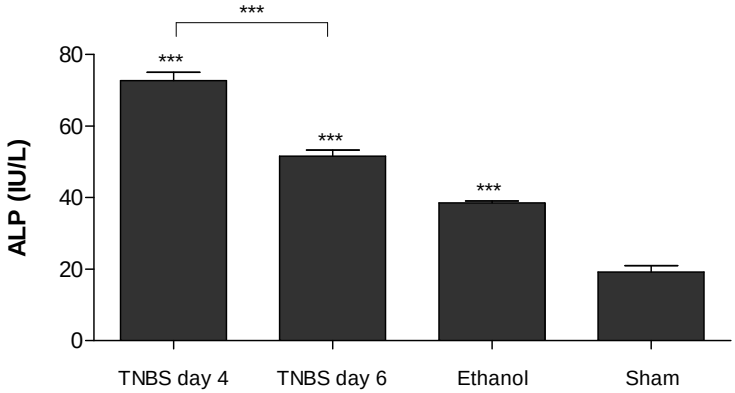

FIGURE 31. Effect of TNBS-induced colitis on serum total ALP concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** $p<0.001$ compared with sham group or between groups.

The renal damage was assessed based on urea and creatinine concentrations (FIGURE 32 AND 33). The TNBS day 4 group revealed an increase in urea level compared with sham group (62.4 ± 1.5 vs 49.3 ± 0.9 mg/dl, p<0.001), as well as the creatinine level compared with sham group (0.27 ± 0.01 vs 0.20 ± 0.02 mg/dl, p<0.001). The TNBS day 6 group presented a significant decrease of urea with around 54 ± 1.5 mg/dl, which is quite similar with sham group (p<0.001, compared with TNBS day 4 group). With respect to creatinine at day 6, the concentration slightly decreased to 0.24 ± 0.01 mg/dl (p<0.05, compared with sham group). There are no statistically significant differences in urea and creatinine levels between the control groups. Thus, the ethanol group presented 41.4 ± 0.5 mg/dl of urea and 0.21 ± 0.01 mg/dl of creatinine.

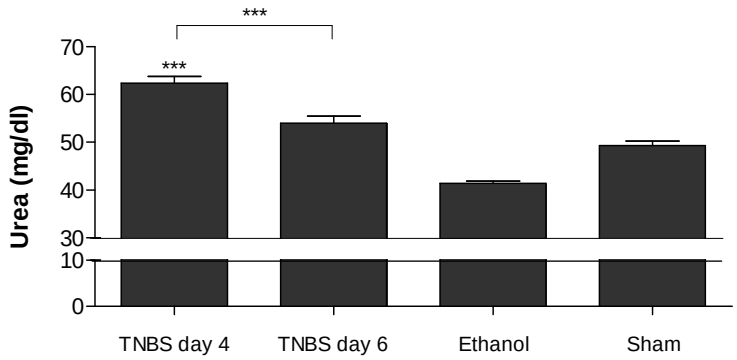

FIGURE 32. Effect of TNBS-induced colitis on serum urea concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

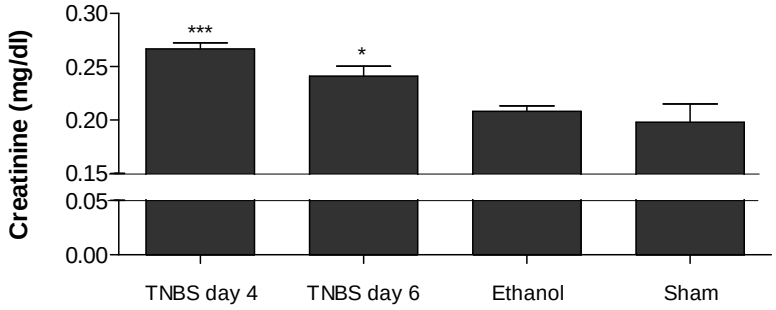

FIGURE 33. Effect of TNBS-induced colitis on serum creatinine concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; *** p<0.001 compared with sham group.

The hepatic function was evaluated according serum ALT concentration (FIGURE 34). The ALT concentration was significantly higher in the TNBS day 4 group compared with the sham group (38.7 ± 1.2 vs 17.5 ± 0.8 IU/L, p<0.001). On day 6, the ALT decreased to 31.7 ± 0.6 IU/L compared to TNBS day 4 group (p<0.001), but yet for higher values than sham group (p<0.001). The ALT concentration on ethanol group (32.3 ± 1.2 IU/L) was higher than the sham group (p<0.001).

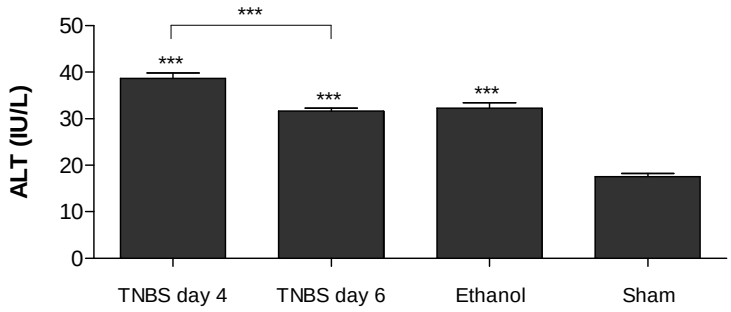

FIGURE 34. Effect of TNBS-induced colitis on serum ALT concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

MYELOPEROXIDASE ACTIVITY

The MPO was evaluated in the development of the experimental colitis as inflammation marker (FIGURE 35). The TNBS-induced colitis was characterized by an increase in MPO comparing with sham group (p<0.001). The mice with colitis showed 42 ± 2.8 ng/ml of MPO, 4 days TNBS after instillation. Thenceforth, the MPO tends to decrease, presenting 30.5 ± 2.3 ng/ml of MPO (p<0.01, compared with TNBS day 4 group). The control groups, as ethanol and sham groups revealed 5.8 ± 0.9 ng/ml and 2.3 ± 0.4 ng/ml of MPO, respectively.

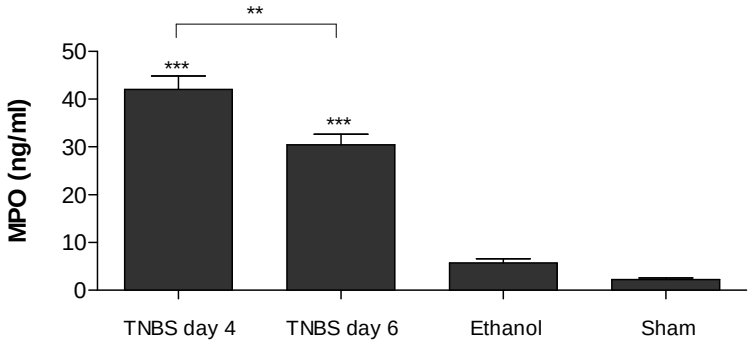

FIGURE 35. Effect of TNBS-induced colitis on MPO activity in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; ** p<0.01; *** p<0.001 compared with sham group or between groups.

MEASUREMENT OF CYTOKINES

The TNBS-induced colitis showed a significant production of pro-inflammatory cytokines, as TNF-α and IL-1β, after four days of TNBS administration (FIGURE 36 AND 37). However, these pro-inflammatory cytokines were significantly reduced on day 6 (p<0.001, compared with TNBS day 4 group). The TNBS day 4 group presented 253.2 ± 31.4 pg/ml of TNF-α and 263.9 ± 25 pg/ml of IL-1β, whereas the TNBS day 6 group presented 121.8 ± 8.54 pg/ml of TNF-α and 165.2 ± 13.2 pg/ml of IL-1β, passed only two days. Both TNBS groups presented higher levels of pro-inflammatory cytokines than sham group (p<0.001). Even, the ethanol and sham groups had very similar

concentrations of pro-inflammatory cytokines, without statistically significant differences. The ethanol group presented 14.9 ± 2 pg/ml of TNF-α and 16.2 ± 1.6 pg/ml of IL-1β and the sham group presented 11.2 ± 0.2 pg/ml of TNF-α and 12.7 ± 0.1 pg/ml of IL-1β.

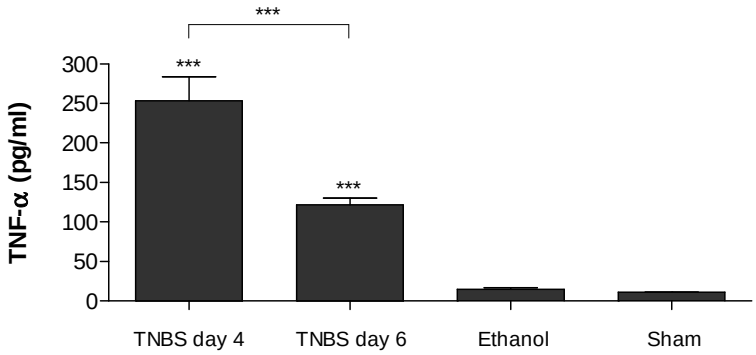

FIGURE 36. Effect of TNBS-induced colitis on TNF-α concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

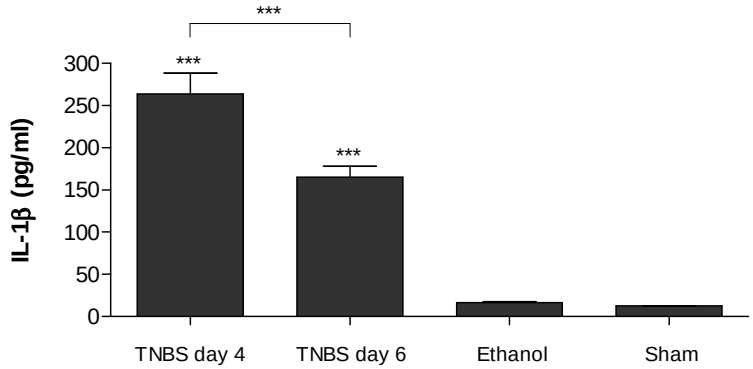

FIGURE 37. Effect of TNBS-induced colitis on IL-1β concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

Cytokines can also become dysregulated under in inflammation conditions with anti-inflammatory effect as IL-10 (FIGURE 38). Usually the IL-10 decrease when the TNF-α and IL-1β concentrations increase under inflammatory conditions. Curiously, these results of IL-10 did not confirm the obtained results in the TNF-α and IL-1β measurements. The TNBS day 4 group presented the highest concentrations of IL-10 with 31.4 ± 3.3 pg/ml ($p<0.001$, compared with sham group). However, the TNBS day 6 group presented a decrease of IL-10 concentration of around 25.5 ± 2.2 pg/ml, contrary to our expectations ($p<0.05$, compared with sham group). The control groups, as ethanol and sham groups, had similar results with 17.3 ± 1.2 pg/ml and 15.7 ± 0.1 pg/ml of IL-10, respectively.

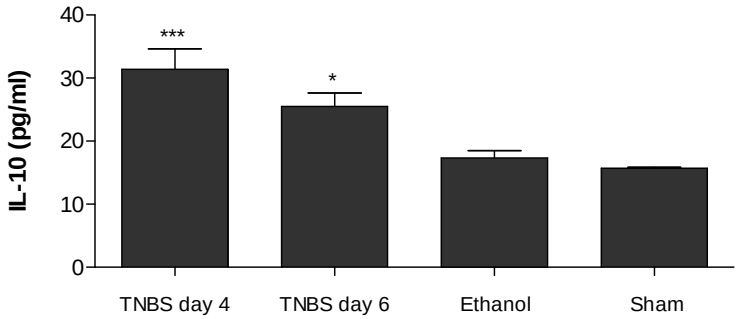

FIGURE 38. Effect of TNBS-induced colitis on IL-10 concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; *** p<0.001 compared with sham group.

ASSESSMENT OF SMALL INTESTINE LESIONS

Since colitis induction can eventually promote tissue injury in the small intestine [Murthy 2006], it was analyzed and showed similar histological results among evaluated groups and the number of days after induction. Apparently, no macroscopic lesions were observed, but microscopically, a slight lymphoplasmacytic infiltrate in the lamina propria was identified and it was similar to all studied groups independently of the day after induction (FIGURE 39).

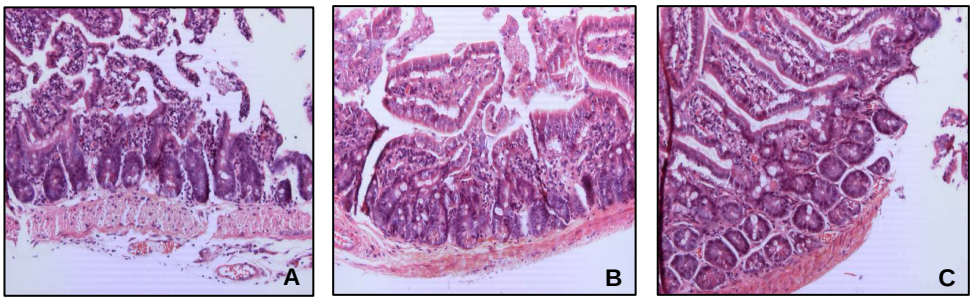

FIGURE 39. Histopathological features of small intestine sections.

Legend: Each image corresponding to a different experimental group,
namely (A) TNBS group, (B) Ethanol group, and (C) Sham group.

ASSESSMENT OF COLITIS SEVERITY

Regarding macroscopic evaluation of colon, there were observed several hemorrhagic focus and generalized edema, whereas no macroscopic alterations were observed in the control groups. Microscopically, the colon was analyzed and it showed different histological results, depending the evaluated experimental group. In relation to development of this animal model, the TNBS group presented severe lesions at day 4 and no substantial morphological changes were detected at day 6 compared with day 4. Furthermore, all biochemical markers and the concentrations of pro- and anti-inflammatory cytokines, under evaluation, corroborate that the damage became maximal at day 4 after the induction. For all these reasons, it will be used only the

obtained results of TNBS day 4 group to validate and compare this animal model of TNBS-induced colitis with the control groups, as ethanol and sham groups.

The histopathological score was calculated based on the presence of tissue loss/necrosis, severity of mucosal epithelial lesion, inflammation and extension of the lesions (TABLE 11). At day 4, the mice with TNBS-induced colitis revealed a significantly higher score in all evaluated parameters of severity and extension comparatively to the ethanol and sham groups. The control groups presented residual scores in all parameter under evaluation. The partial histopathological scores of the ethanol group were slightly higher than sham group.

TABLE 11. Average (± SEM) of partial histopathological score of TNBS-induced colitis.

	TNBS DAY 4	**ETHANOL**	**SHAM**
MUCOSAL LOSS	3.3 ± 0.2	0.2 ± 0.1	0 ± 0
EPITHELIAL LESIONS	3.4 ± 0.2	0.9 ± 0.5	0 ± 0
INFLAMMATION	3.8 ± 0.1	1.1 ± 0.2	0.1 ± 0.1
EXTENT 1*	3.7 ± 0.1	1.3 ± 0.3	0.1 ± 0.1
EXTENT 2**	3 ± 0.2	1 ± 0.2	0.1 ± 0.1

Legend: * Extent 1 - Percentage of intestine affected in any manner;
** Extent 2 - Percentage of intestine affected by the most severe lesion.

The final histopathological score ranges between 0 and 20 (FIGURE 40). With regard to mice with TNBS-induced colitis, the TNBS group had a final score of around 17 ± 0.6, presenting a score substantially higher than the sham group ($p<0.001$). The control groups, as the sham group, revealed a residual histopathological score with around 0.2 ± 0.2 of final score. However, the histopathological score of the ethanol group was slightly higher with 4.5 ± 1.2 ($p<0.01$ compared with sham group). These results are consistent with the histopathological images.

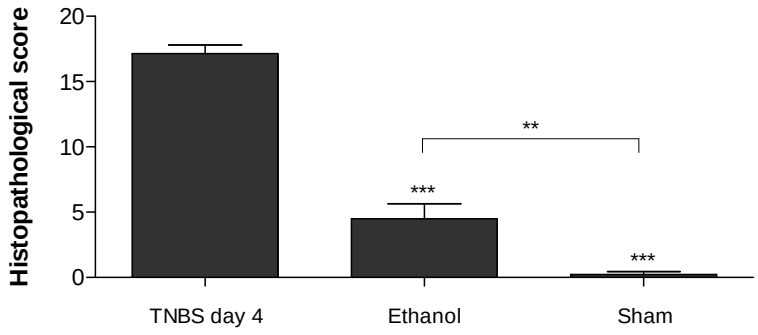

FIGURE 40. Effect of TNBS-induced colitis on histophatological score.

Legend: One-way ANOVA and Tukey's post hoc test; ** $p<0.01$; *** $p<0.001$ compared with TNBS group or between groups.

Representative images translating the histopatological score for the experimental groups analyzed are showed herein (FIGURE 41). The histopathology analysis of mice with TNBS-induced colitis displays diffuse transmural necrosis with severe hemorrhaging, involving the mucosa, submucosa, muscle layer and serosa, and often associated with peritonitis. The ethanol group showed only mild epithelial erosion, and no lesions were observed in the sham groups.

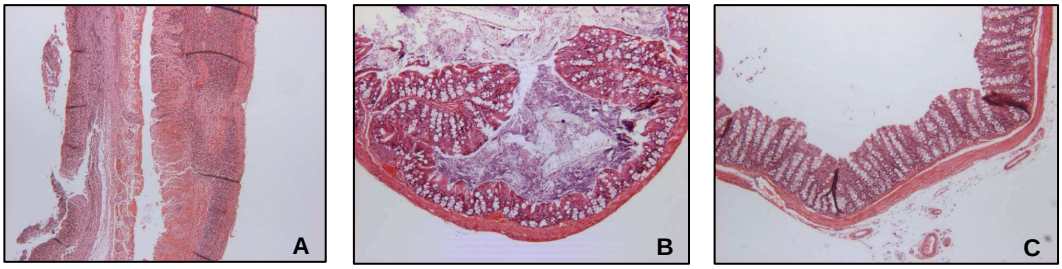

FIGURE 41. Effect of TNBS-induced colitis on histopathologic changes.

Legend: Each column corresponding with a different experimental group, namely (A) TNBS group, (B) Ethanol group, and (C) Sham group.

2. DISCUSSION OF RESULTS

IBD is a common gastro-intestinal disorder marked with chronic inflammation of intestinal epithelium, damaging mucosal tissue and manifests into several intestinal and extra-intestinal symptoms, mainly related to oxidative stress, inflammation and autoimmune type [Mowat et al., 2011; Pawar et al., 2011]. Animal models mimic the characteristics of disease in real life and play an important role in studies of action mechanisms. There are several chemical agent-induced colitis models and all of them couldn't fully reflect the complexity of the disease present in human and each method has its own disadvantages [Wirtz & Neurath, 2007]. However, between the TNBS-induced colitis and the human disease exist a similar pathogenesis of IBD [Pawar et al., 2011], and this is the reason why so many research groups are now using this model to investigate novel approaches for the treatment of IBD. Furthermore, the major advantages of this model include proposing a simple process and reproducible colonic damage, short duration of the experiment, long-lasting damage accompanied by inflammatory cell infiltration and ulcers [Zheng, Gao & Wang, 2000]. In addition, this model can mimic both acute and chronic phases of human colitis [Dohi & Fujihashi, 2006]. The TNBS can induce colitis by intrarectal instillation of the haptenating substance dissolved in ethanol, resulting in a dysregulation of the mucosal immune system [Morris et al., 1989; Li et al., 2005; Menozzi et al., 2006]. Therefore, the aim of

this study is to develop an acute experimental colitis model, by induction with TNBS, in order to evaluate the influence of EPO, TDZD-8 and hemin in the IBD.

The first step on this study was to develop and validate the TNBS-induced colitis model, since the protocols of this animal model are not standardized, such as the dosage of TNBS, the depth of TNBS administration, and the time point for model evaluation [Qin et al., 2011]. The TNBS-induced colitis model was applied in 1989 for the first time by Morris *et al*. and since that the acute transmural damage became maximal from 3 days to 1 week after instillation, and resolved within 2 weeks [Morris et al., 1989; Linden et al., 2005; Lamb et al., 2006; Liang et al., 2007; Qin et al., 2011]. Therefore, it was implemented a protocol to development of TNBS-induced colitis model based on Morris *et al*. procedure [Morris et al., 1989]. Briefly, two independent groups of TNBS-induced colitis were developed and monitored under the same specific conditions, however one group of mice were sacrificed on day 4 and the other one on day 6, after induction. During these 6 days, there were evaluated several parameters, like clinical symptoms/signs, biochemical markers, histopathological analysis and cytokines concentration. The main objective was to identify the day where the maximal acute damage was achieved by the induction method used on this study.

Regarding monitoring of clinical symptoms/signs, TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity, while ethanol and sham groups remained without any alterations. The manifestations became maximal at day 4 after induction and the effect began to decline starting that day. These clinical manifestations in the TNBS group were expected and compatible with a correct induction of CD [Cuzzocrea et al., 2004; Motavallian-Naeini et al., 2012].

The peak of clinical symptoms/signs was also confirmed by the progressive fall in body weight over the 4-day experimental period on TNBS with around -12 ± 1.4% of weight loss, consistent with other research groups [Cuzzocrea et al., 2004; Motavallian-Naeini et al., 2012; Szczepanik et al., 2012]. After day 4, the body weight of these mice began to increase slightly until day 6, presenting a weight recovery on day 6, with a gain of 2.3% of weight in just 2 days. Although the weight difference between day 4 and day 6 was not statistically significant, this change represents a weight gain trend. Regarding control groups, they presented a considerable increase in body weight with a gain of around 20 to 30% of its initial weight, especially from day 0 to day 1. In fact, they were fasted for 12 hours since the day before of the induction day. After induction, they woke

up from anesthesia, felt healthy and prepared to eat, unlike the other groups with experimental colitis, thus justifying the increase.

The colon length was also measured as a marker of tissue integrity. We found that TNBS-induced colitis significantly shortened colon length on day 4 and, passed only 2 more days, the colon length significantly recovered TNBS effects, suggesting that the maximal damage is manifested on day 4. On day 6, the colon length is similar with the control groups. In this study, mice without TNBS-induced colitis showed a normal colon length with around 11.5 cm, however we verified that TNBS-induced colitis promotes a reduction of 1cm in the colon length compared with sham group and, this is consistent with the findings from other research groups [Nayak, George & Mishra, 2012; Szczepanik et al., 2012].

The determination of fecal hemoglobin allows the diagnosis and evaluation of various colorectal diseases, as for example identify patients with IBD with active inflammation [Hirata et al., 2007; Mooiweer, Fidder, Siersema, Laheij & Oldenburg, 2014]. Thus, fecal hemoglobin can be useful in the detection of lesions accompanied by bleeding, since it determines the intensity of eventual hemorrhagic focus in the colonic tissue [Hirata et al., 2007; Jagtap, Niphadkar & Phadke, 2011]. In this regard, the fecal hemoglobin also confirmed the manifestation of maximal damage on day 4, since we verified a significantly reduction of around 6 µmol Hg/g feces on day 6, presenting thus a recovery of hemorrhagic ulcers. The control groups presented residual concentrations of fecal hemoglobin.

ALP is regularly measured in clinical practice and its changes in serum levels are observed in a number of clinical conditions of organs where it can be found like bone, liver, bowel, kidney and placenta [Moscandrew, Mahadevan & Kane, 2009; Tinnion & Embleton, 2012]. Concretely, the intestinal ALP is expressed on the apical surface of enterocytes and it has received increasing attention as a factor responsible for mucosal defense, promoting the interaction between toll like receptor (TLR)-4 in the intestinal mucosa and LPS derived from the bacterial flora [Malo et al., 2010; Nagalingam, Kao & Young, 2011]. Therefore, TNBS group presented the highest values of serum total ALP concentration and the peak of maximal damage was also achieved on day 4, since the mice revealed a decrease in around 29% of ALP on day 6. Thus, the low concentrations of APL in both control groups suggest that the origin of increased APL in the TNBS group is due to intestinal lesion inducted in this study. On the other hand, the ethanol group presented a slight increase of ALP compared with sham group, perhaps due to its ability to induce an intestinal permeability alteration promoting a

superficial and mild damage of colonic tissue with subsequently release of intestinal ALP to systemic circulation. These our results are consistent with other studies, which observed a higher serum ALP activity in the colitic animals comparing with non-colitic animals from acute intestinal inflammation model induced by TNBS [Cruz, Gálvez, Crespo, Ocete & Zarzuelo, 2001; Luchini et al., 2008].

MPO activity represents the degree of neutrophil infiltration in a tissue. Under normal physiological conditions, MPO is released from azurophillic storage granules [Krawisz, Sharon & Stenson, 1984]. When an inflammatory insult leads to the formation of reactive species, release of this enzyme increases MPO of colonic mucosa scraping which is a manifestation of increased infiltration [Patil, Kandhare & Bhise, 2012]. Indeed, neutrophils play a crucial role in the development and full manifestation of gastrointestinal inflammation, because they represent a major source of free radicals in the inflamed colonic mucosa, promoting the destruction of foreign antigens and in the breakdown and remodeling of injured tissue [Grisham, 1994; Cuzzocrea et al., 2001]. In this study, the TNBS-induced colitis was characterized by an increase of MPO activity, especially on day 4. Since that day, the MPO activity significantly decreased, suggesting a reduction of the colon inflammation. The control groups presented a residual MPO activity, compatible with the absence of an inflammatory process. These findings were fully confirmed by the measurements of pro-inflammatory and anti-inflammatory cytokine concentrations. Furthermore, these data are consistent with the findings of other research groups, since they registered the same MPO activity after TNBS-induced colitis [Ukil et al., 2003; Szczepanik et al., 2012].

Cytokines are crucial for fighting off infections and in other immune responses. However, they can become dysregulated and pathological in inflammation, trauma and sepsis [Műzes et al., 2012]. TNF-α and IL-1β are pro-inflammatory cytokines, released through the activation of white blood cells (including macrophages) via neutrophils [Paunovic et al., 2011]. These are clearly involved in the pathogenesis of colitis, since they are increased in inflamed colon tissue [Carty, De Branbander, Feakins & Rampton, 2000; Nairz et al., 2011]. This current study confirms that the used TNBS-induced colitis model promotes a significantly increase in the levels of these pro-inflammatory cytokines in the colon. Specifically, these cytokines registered the maximal peak of its concentration on day 4 and, passed only 2 more days, the cytokine levels decreased in average 44.6%, indicating an eventual onset of the chronic phase of this inflammatory disease.

Although the data of TNF-α and IL-1β levels have been enlightening, to confirmation the inflammatory process observed in the TNBS-induced colitis, culture supernatants were additionally tested for the presence of anti-inflammatory cytokines, as IL-10. The obtained data allows concluding that TBNS-induced colitis promotes a decreased production of IL-10 by mesenteric lymph nodes, as expected [Coquerelle et al., 2009; Yang, Meng, Jiang, Chen & Wu, 2010].

In the histopathological analysis of the small intestine, there was a slight lymphocytic inflammatory infiltrate in the lamina propria similar with all experimental groups. These lesions are consistent with a sub-acute to chronic process, suggesting no relationship with this study. Perhaps, the reason is that the mice used in the study are not Specific Pathogen Free, even because there are similar for all experimental groups. These should therefore be devalued, so no morphological alterations were detected in the small intestine.

Regarding to histopathological analysis of colon, the morphological features in the TNBS-induced colitis were evaluated and no substantial morphological changes were detected between day 4 and day 6. In general, the histopathological analysis revealed a diffuse transmural necrosis with severe hemorrhaging, involving the mucosa, submucosa, muscle layer and serosa, and often associated with peritonitis. These lesions are consistent with a correct induction of experimental colitis by TNBS [Alex et al., 2009; Pawar et al., 2011; Nairz et al., 2011; Motavallian-Naeini et al., 2012]. On the other hand, no histological alterations were observed in the control groups, except in the ethanol group where it was observed mild epithelial erosion due to its ability to induce an intestinal permeability alteration, promoting a superficial and mild damage of colonic tissue.

The prevalence of extraintestinal manifestations in IBD varies from 6% to 46% [Rojas-Feria, Castro, Suárez, Ampuero & Romero-Gómez, 2013; Corica & Romano, 2015]. These manifestations can be divided in to 3 groups: those that are seen in association with IBD, those that are due to metabolic and physiologic changes induced by the IBD and those that are secondary to the drugs used in the treatment of IBD [Yarur, Czul & Levy, 2014]. Regarding our findings, the TNBS-induced colitis presented a significantly alteration of renal and hepatic functions comparing with control groups. The peak of maximal damage was again detected on day 4, presenting a considerable decline of all evaluated parameters since then. This is consistent with the literature, since extraintestinal manifestations can involve almost every organ system and some of the most frequently involved organs are the liver and kidney [Oikonomou, Kapsoritakis,

Eleftheriadis, Stefanidis & Potamianos, 2011; Rojas-Feria et al., 2013; Corica & Romano, 2015].

Briefly, a TNBS-induced colitis model was monitored during 6 days, since we wanted to identify the day where the maximal acute damage was achieved. Indeed, all clinical symptoms/signs, biochemical markers, histopathological analysis and concentrations of pro- and anti-inflammatory cytokines, under evaluation, corroborate that the damage became maximal at day 4 after the induction. Possibly, because some mice died during the early days of the study, no resisting to aggravation of the disease in its acute phase, while the remaining mice resisted and progressed to chronic phase of the disease, showing the same symptoms but more lightly. These data allow concluding that TNBS-induced colitis was developed in 4 days, providing an acute intestinal inflammation model. This model exhibits many of the pathological, molecular and immunological features of human colitis [Boughton-Smith, Wallace & Whittle, 1988; Morris et al., 1989; Sun et al., 2001; Whittle, Cavicchi & Lamarque, 2003; Riviera, 2006]. Furthermore, the validation of this animal model is truly relevant to the scientific community, since there is no standard practice in the induction of colitis by TNBS [Tomasello et al., 2015].

Nowadays, no medical cure has been developed for IBD and treatment focuses on producing and maintaining remission [Podolsky, 2002; Forbes et al., 2004; Baumaqart & Sandborn, 2007]. Conventional pharmacotherapy for both types of IBD is treatment with aminosalicylates and corticosteroids [Sands, 2000; Langmead & Rampton, 2006]. Moreover, immuno-suppressive agents and biological response modifiers are considered as alternative therapies [Braus & Elliott, 2009]. Nonetheless, available medicines are not universally effective and result in marked deleterious effects [Dubinsky, 2004]. Researchers have shown an increased interest in investigating the effect of different drugs used for the treatment of IBD. In order to understand how drugs affect the colitis, animal models of colitis are normally applied [Motavallian-Naeini et al., 2012]. Therefore, this study evaluated the influence of a set of drugs in this experimental colitis model, as EPO, TDZD-8 and hemin, which are able to stimulate or inhibit some inflammatory pathways involved in IBD.

CHAPTER 6 – ERYTHROPOIETIN EFFECT IN INFLAMMATORY BOWEL DISEASE

1. RESULTS

MONITORING OF CLINICAL SYMPTOMS/SIGNS

The animals were observed daily for stool consistency, anus appearance, morbidity and body weight changes (FIGURE 42). Following experimental induction of colitis, TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity. Since day 0, it was found a progressive fall in body weight over the 4-day experimental period on TNBS group (-12 ± 1.4%). However, in both doses of EPO treatment, the same clinical signs were found, but lighter, like soft stools, moderate edema of the anus and mild morbidity. Furthermore, the fall in body weight determined 4 days following induction of experimental colitis with TNBS group was attenuated by EPO treatment and the effects were statistically significant with the higher dose. At the end of experimental period, the TNBS+EPO500 group lost -3.7 ± 3% of its initial weight and TNBS+EPO1000 group gained 7.3 ± 3.8% ($p<0.0001$) of its initial weight. The EPO1000, ethanol and sham groups remained without any alterations with respect to morbidity, stool consistency and anus appearance during the study. At the end of experimental period, they also gained 21 ± 2.7%, 15.2 ± 1.1% and 31.2 ± 1.6% ($p<0.0001$, compared with TNBS group) of its initial weight, respectively.

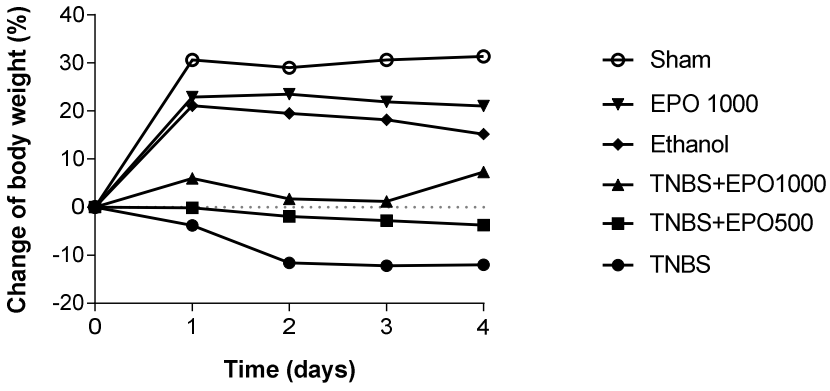

FIGURE 42. Change of body weight during EPO treatment in the IBD.

BIOCHEMICAL MARKERS

The observed inflammatory changes of the colon were associated with a reduction in the length of the colon (FIGURE 43). TNBS induced colitis (10.1 ± 0.2 cm) reduced significantly the colon length relatively with the sham group (11.5 ± 0.2 cm) as a comparable tissue ($p<0.001$). Furthermore, EPO treatment showed an influence on the colon length in a dependent dose manner, but without statistical significance. TNBS+EPO500 group presented an increase on the colon length with 10.4 ± 0.2 cm, as well as in the TNBS+EPO1000 group with 10.9 ± 0.2 cm of colon length. The EPO1000, ethanol and sham groups presented similar results between them, like 11.8 ± 0.1 cm, 11.5 ± 0.4 cm, 11.5 ± 0.2 cm, respectively.

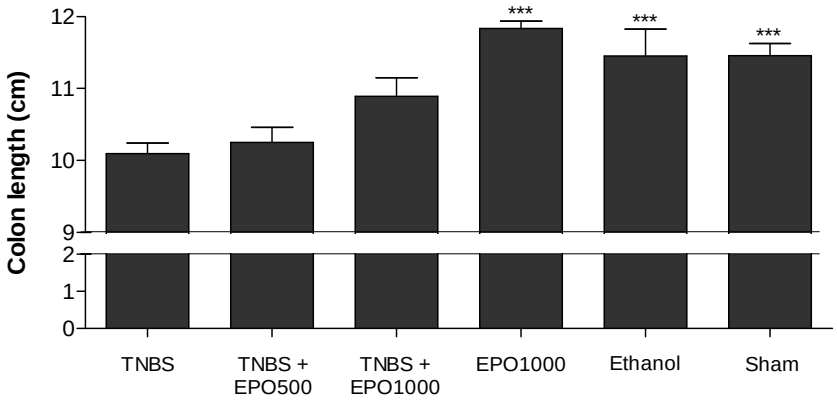

FIGURE 43. Effect of EPO treatment on colon length in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** $p<0.001$ compared with TNBS group.

The intensity of hemorrhagic focus was evaluated by the fecal hemoglobin determination (FIGURE 44). The findings in colons from the TNBS group included severe bloody stools compared to the sham group (12.5 ± 0.3 vs 0.7 ± 0.1 µmol Hg/g feces, $p<0.0001$). EPO treatment has an influence in the intensity of hemorrhagic focus, since a significant difference in fecal hemoglobin was also observed between the TNBS group and both EPO doses, namely TNBS+EPO500 group (6.6 ± 0.1 µmol Hg/g feces, $p<0.0001$) and TNBS+EPO1000 group (2.4 ± 0.3 µmol Hg/g feces, $p<0.0001$). The EPO dose has a positive influence in the intensity of hemorrhagic focus, since the fecal hemoglobin decreased when the EPO dose was increased ($p<0.0001$). Mice from EPO1000 (1.6 ± 0.1 µmol Hg/g feces) and ethanol groups (1.1 ± 0.1 µmol Hg/g feces) did not display significant changes compared to the sham group (0.7 ± 0.1 µmol Hg/g feces).

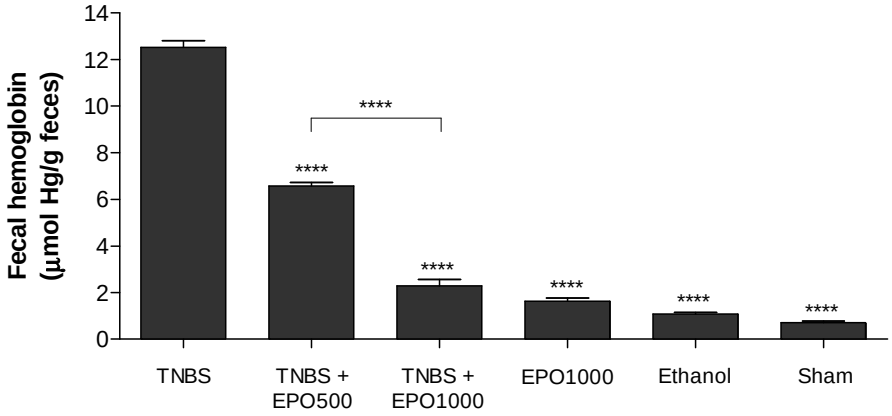

FIGURE 44. Effect of EPO treatment on fecal hemoglobin in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group or between groups.

ALP was identified in all experimental groups (FIGURE 45). The ALP concentration on the TNBS group was considerably higher compared with the sham group (72.7 ± 2.3 vs 19.2 ± 1.8 IU/L, p<0.0001). The EPO treatment was able to decrease the ALP concentration on blood at both EPO doses, but it was not achieved a dose dependent effect with statistical significance. The TNBS+EPO500 group presented 45 ± 4.8 IU/L, whereas the TNBS+EPO1000 group presented 39 ± 0.9 IU/L (p<0.0001, compared with TNBS group). The ALP in the EPO1000 group is increased by 53.3 ± 1.1 IU/L compared with the remaining control groups, like ethanol (38.5 ± 0.6 IU/L) and sham (19.2 ± 1.8 IU/L) groups (p<0.0001 compared with sham group). Even the ethanol group presents statistically significant differences with sham group (p<0.0001).

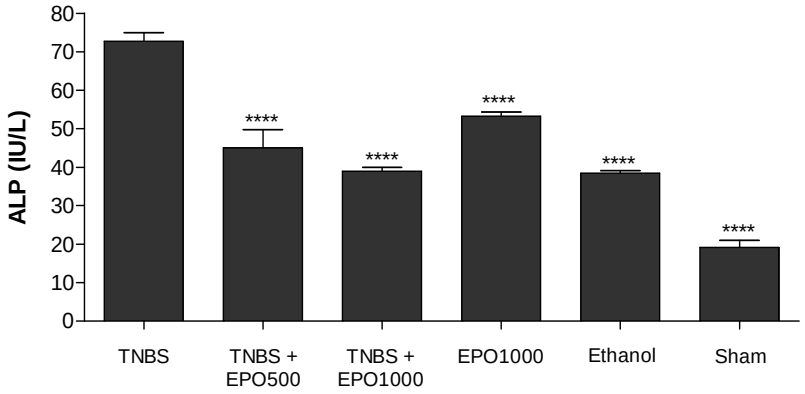

FIGURE 45. Effect of EPO treatment on serum total ALP concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group.

The EPO treatment can affect the hematocrit level and promote cardiovascular adverse effects, so it is important evaluate the range of hematocrit in some experimental groups (FIGURE 46). All evaluated experimental groups, like TNBS+EPO500 (46.7 ± 0.9 %),

TNBS+EPO1000 (47.2 ± 0.7 %) and EPO1000 (47.1 ± 0.5 %) groups, presented similar values of hematocrit with sham group (48.3 ± 0.7 %), without statistically significant differences.

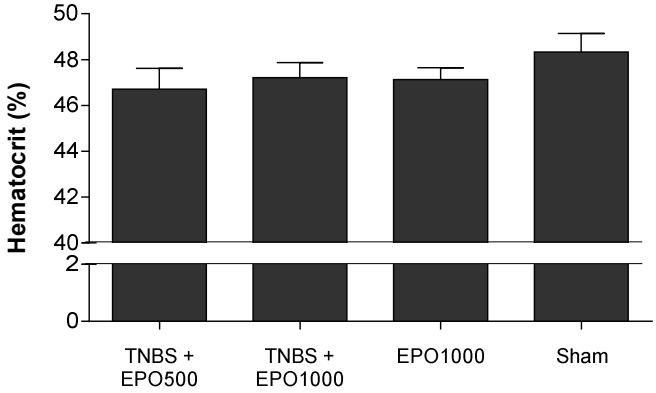

FIGURE 46. Effect of EPO treatment on hematocrit in the IBD.

It were analyzed sera from mice with TNBS induced colitis and mice treated with EPO to determine if there were alterations in the values for the renal damage markers, like urea and creatinine (FIGURE 47 AND 48). The TNBS group exhibited a significant increase in urea compared with sham group (62.4 ± 1.5 vs 49.3 ± 0.9 mg/dl, $p<0.01$), as well as the creatinine compared with sham group (0.27 ± 0.01 vs 0.20 ± 0.02 mg/dl, $p<0.0001$). The mice treated with EPO presented a significant decrease of urea and creatinine levels to values quite similar with sham group ($p<0.01$, compared with TNBS group), promoting thus a dose-dependent effect but without statistical significance between EPO doses. The renal biochemistries of TNBS+EPO500 group revealed 51 ± 5.3 mg/dl of urea and 0.22 ± 0.01 mg/dl creatinine, while the TNBS+EPO1000 group revealed 44.5 ± 1.1 mg/dl of urea and 0.20 ± 0.01 mg/dl of creatinine. There are no statistically significant differences in urea and creatinine levels between the control groups, like EPO1000, ethanol and sham. In the control groups, the creatinine concentration in EPO1000 group (0.26 ± 0 mg/dl) was the only parameter that was significantly higher than sham group ($p<0.0001$).

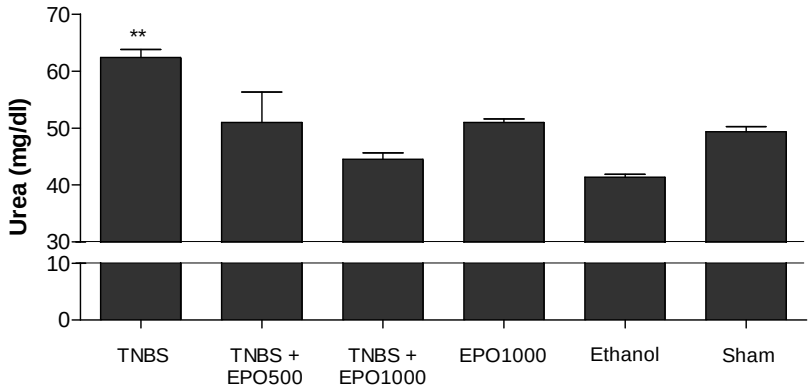

FIGURE 47. Effect of EPO treatment on serum urea concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; ** p<0.01 compared with sham group.

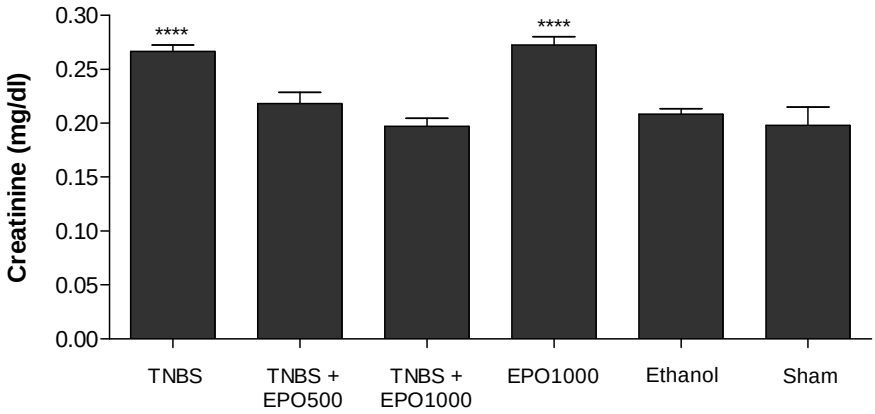

FIGURE 48. Effect of EPO treatment on serum creatinine concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with sham group.

The hepatic function was evaluated according serum ALT concentration from mice with TNBS induced colitis and mice treated with EPO (FIGURE 49). The ALT concentration in blood was significantly higher in the TNBS group compared with the sham group (38.7 ± 1.2 vs 17.5 ± 0.8 IU/L, p<0.0001). The EPO treatment at both doses promoted a decreased of ALT levels (27.4 ± 0.7 and 27.1 ± 0.9 IU/L, respectively) relatively to TNBS group (p<0.0001), but yet for higher values than sham group (p<0.001). The ALT levels on EPO1000 (27.5 ± 0.7 IU/L) and ethanol (32.3 ± 1.2 IU/L) groups were higher than the sham group (p<0.0001).

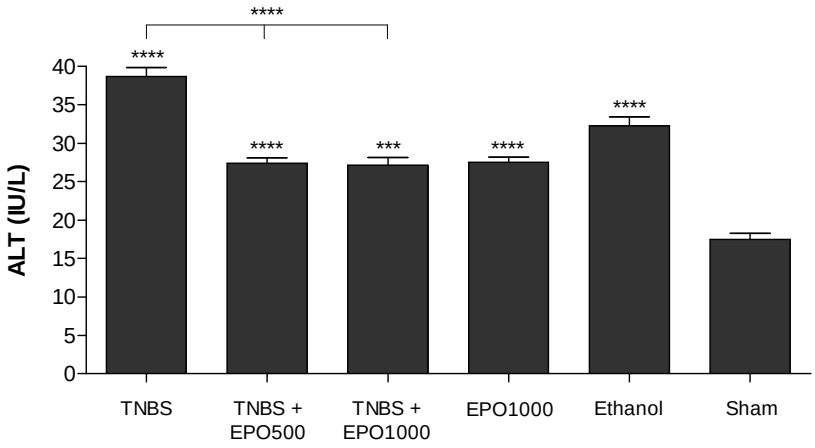

FIGURE 49. Effect of EPO treatment on serum ALT concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001; **** p<0.0001 compared with sham group or between groups.

MYELOPEROXIDASE ACTIVITY

The MPO activity was measured in all experimental groups as inflammation marker (FIGURE 50). The TNBS group presented an increased MPO concentration comparing with the sham group (42 ± 2.8 vs 2.3 ± 0.4 ng/ml, p<0.0001). Furthermore, the EPO treatment was able to decrease the MPO concentration with both EPO doses. In the TNBS+EPO500 group was registered 21.7 ± 4.6 ng/ml of MPO, whereas in the TNBS+EPO1000 group was registered 13.5 ± 2.3 ng/ml of MPO (p<0.0001, compared with TNBS group). A dose dependent effect was identified in the decrease of MPO after EPO treatment, however without statistical significance between doses. The EPO and ethanol groups presented a significantly decrease of MPO activity to around 2.4 ± 0.4 ng/ml and 5.8 ± 0.9 ng/ml of MPO, respectively.

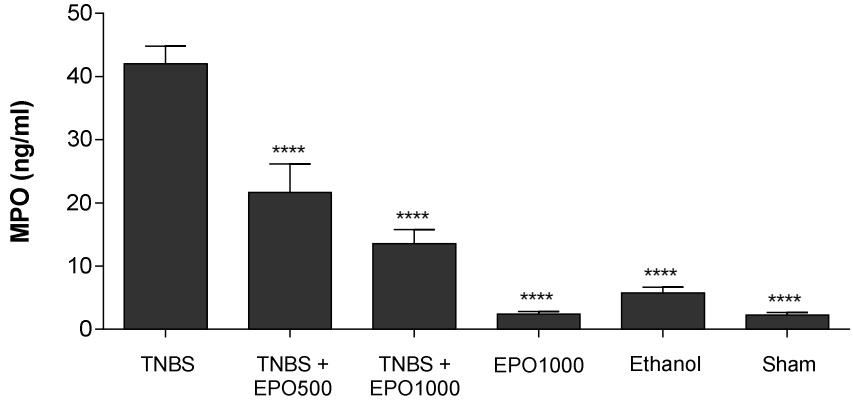

FIGURE 50. Effect of EPO treatment on MPO activity in the IBD.

Legend: One-way ANOVA and Tukey's post hoc- test; **** p<0.0001 compared with TNBS group.

MEASUREMENT OF CYTOKINES

Cytokines can become dysregulated in pathological conditions promoting inflammatory effect as TNF-α (FIGURE 51) and IL-1β (FIGURE 52). In this case, the TNBS group revealed a

significant increase of TNF-α compared with sham group (253.2 ± 31.4 vs 11.2 ± 0.2 pg/ml, p<0.0001), as well as IL-1β compared with sham group (263.9 ± 25 vs 12.7 ± 0.1 pg/ml, p<0.0001). Under treatment with 500 IU/Kg of EPO, the mice had 127.4 ± 6.6 pg/ml of TNF-α and 126.5 ± 13 pg/ml of IL-1β. When it was administrated 1000 IU/Kg of EPO, the mice presented 99.1 ± 10.2 pg/ml of TNF-α and 71.9 ± 10.6 pg/ml of IL-1β. Indeed, the mice under EPO treatment exhibited a significant decrease of these inflammatory cytokines (p<0.0001, compared with TNBS group), with a dose-dependent effect. In the IL-1β concentration, this effect had a statistical significance between EPO doses. The remaining control groups presented values of TNF-α and IL-1β quite similar with sham group. The EPO1000 group had 11.7 ± 0.5 pg/ml of TNF-α and 12.6 ± 0.1 pg/ml of IL-1β. And the ethanol group had 14.9 ± 2 pg/ml of TNF-α and 16.2 ± 1.6 pg/ml of IL-1β.

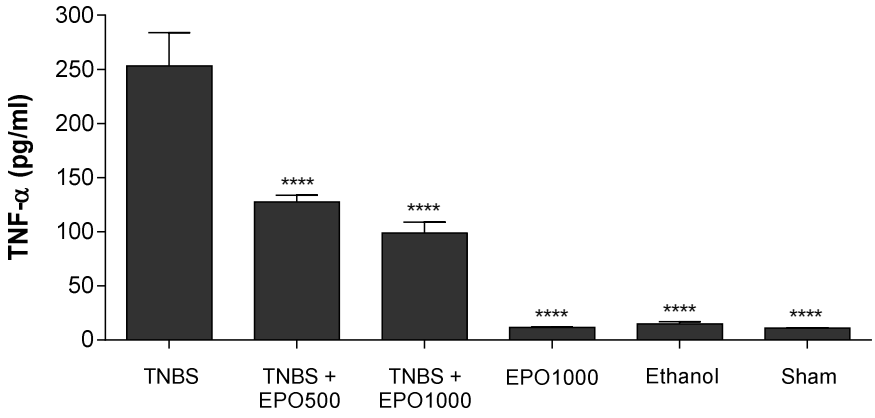

FIGURE 51. Effect of EPO treatment on TNF-α concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group.

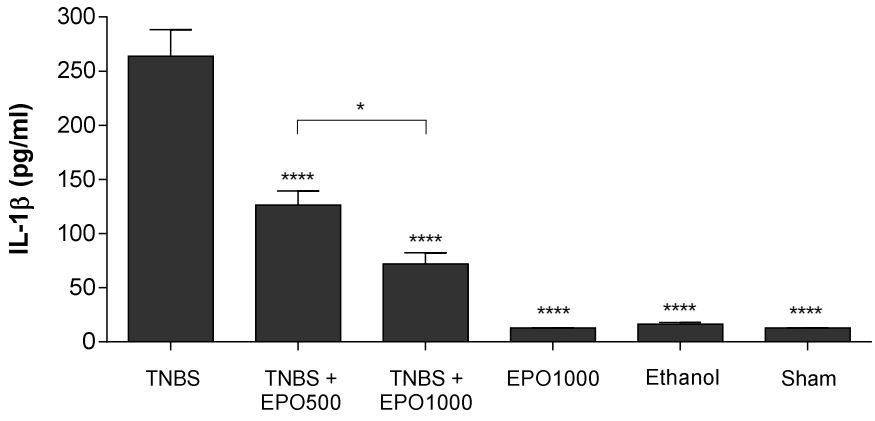

FIGURE 52. Effect of EPO treatment on IL-1β concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; **** p<0.0001 compared with TNBS group or between groups.

The results of IL-10 measurements, as anti-inflammatory cytokine, confirm the obtained results with TNF-α and IL-1β measurements, because usually the IL-10 decrease when

the TNF-α and IL-1β increase under inflammatory conditions (FIGURE 53). Therefore, the TNBS group presented a low IL-10 concentration of around 31.4 ± 3.3 pg/ml. The EPO treatment promoted an increase of IL-10, with a dose-dependent effect and statistical significance at the higher dose ($p<0.0001$, compared with TNBS group). Concretely, the TNBS+EPO500 and TNBS+EPO1000 groups had 63.8 ± 5.5 pg/ml and 151.1 ± 25.3 pg/ml, respectively ($p<0.0001$). The control groups, like EPO1000, ethanol and sham groups, had similar results with 21.6 ± 2.4 pg/ml, 17.3 ± 1.2 pg/ml and 15.7 ± 0.1 pg/ml of IL-10, respectively.

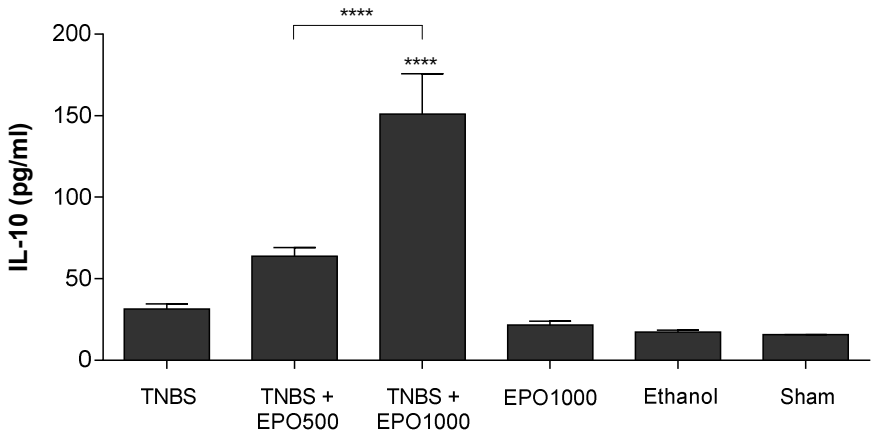

FIGURE 53. Effect of EPO treatment on IL-10 concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** $p<0.0001$ compared with TNBS group or between groups.

ASSESSMENT OF COLITIS SEVERITY

The macroscopic inspection of cecum, colon, and rectum in the TNBS group showed presence of mucosal congestion, hemorrhagic ulcerations and necrosis. After EPO treatment, the lesions appeared to have the same severity, but with less extension. No macroscopic alterations were observed in the control groups.

The histopathological score was calculated based on the presence of tissue loss/necrosis, severity of mucosal epithelial lesion, inflammation and extension of the lesions (TABLE 12). The analysis of partial scores allows evaluating the beneficial effect of EPO treatment and which parameters are affected. Daily treatment with EPO (500 and 1000 IU/kg) resulted in a decrease in the severity of colitis in each parameter under evaluation, but especially in the decrease of the mucosal loss. This treatment also allowed a decrease of extension of colitis, especially with a decrease of the percentage of intestine affected by the most severe lesion. The administration of the higher EPO dose promoted a slight decrease of inflammation and the percentage of intestine affected in any manner and intestine affected by the most severe lesion. On the other hand, the control groups revealed partial scores too low in all evaluated parameters.

TABLE 12. Average (± SEM) of partial scores of histopathological score of TNBS-induced colitis under EPO treatment.

	TNBS	TNBS+EPO 500	TNBS+EPO 1000	EPO1000	ETHANOL	SHAM
MUCOSAL LOSS	3.3 ± 0.2	1.9 ± 0.5	1.8 ± 0.5	0 ± 0	0.2 ± 0.1	0 ± 0
EPITHELIAL LESIONS	3.4 ± 0.2	2.7 ± 0.5	2.8 ± 0.4	0 ± 0	0.9 ± 0.5	0 ± 0
INFLAMMATION	3.8 ± 0.1	2.7 ± 0.4	2.3 ± 0.4	0.5 ± 0.2	1.1 ± 0.2	0.1 ± 0.1
EXTENT 1*	3.7 ± 0.1	3.2 ± 0.3	2.8 ± 0.3	0.5 ± 0.2	1.3 ± 0.3	0.1 ± 0.1
EXTENT 2**	3 ± 0.2	1.8 ± 0.3	1.6 ± 0.3	0.5 ± 0.2	1 ± 0.2	0.1 ± 0.1

Legend: * Extent 1 - Percentage of intestine affected in any manner;
** Extent 2 - Percentage of intestine affected by the most severe lesion.

The histopathological score ranges between 0 and 20 (FIGURE 54). With regard to mice with TNBS-induced colitis, the TNBS group had a final score of around 17 ± 0.6, presenting a score substantially higher than the sham group ($p<0.0001$). Under EPO treatment, the histopathological score decreased, such that the TNBS+EPO500 and TNBS+EPO1000 groups obtained a final score of 12.2 ± 1.6 and 11.3 ± 1.4, respectively ($p<0.05$ and $p<0.01$, compared with TNBS group). No statistically significant differences were found between EPO doses. The control groups revealed a residual histopathological score with EPO1000 and sham groups presenting around 1.5 ± 0.6 and 0.2 ± 0.2 of final score, respectively. The ethanol control group was the only group with a histopathological score slightly higher with 4.5 ± 1.2, but without statistically significant differences with sham group. This result is consistent with the histopathological images.

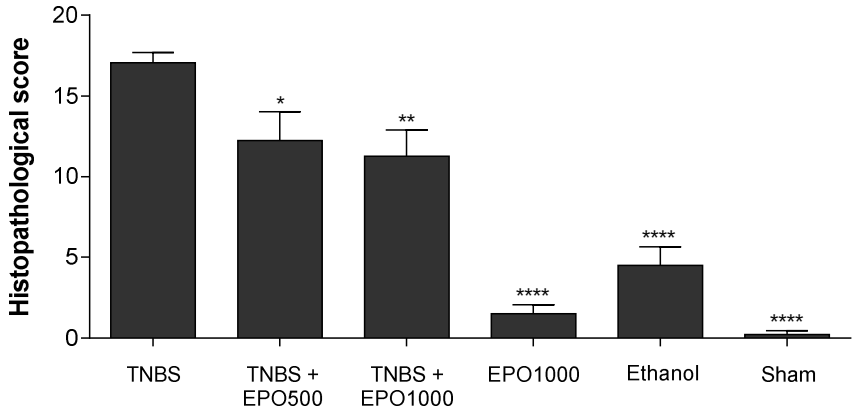

FIGURE 54. Effect of EPO treatment on histopathological score.

Legend: One-way ANOVA and Tukey's post hoc test; * $p<0.05$; ** $p<0.01$; **** $p<0.0001$ compared with TNBS group.

Representative images translating the histopatological score for the experimental groups analyzed are showed herein (FIGURE 55). Briefly, the TNBS group displays diffuse transmural necrosis with severe hemorrhaging, involving the mucosa, submucosa,

muscle layer and serosa, and often associated with peritonitis. Under EPO treatment, similar lesions are seen, namely the transmural necrosis, but with lesser extent and with a multifocal pattern, interspersed with areas in which the mucosal integrity is maintained. In these areas there is mild to moderate epithelial erosion and ulceration, and severe inflammatory cell infiltration, extending to the submucosa. The increase of EPO dose has a minor effect in the histopathological score, with lower extent of the lesions, but without statistical significance. The ethanol group showed only mild epithelial erosion, and no lesions were observed in the EPO and sham groups.

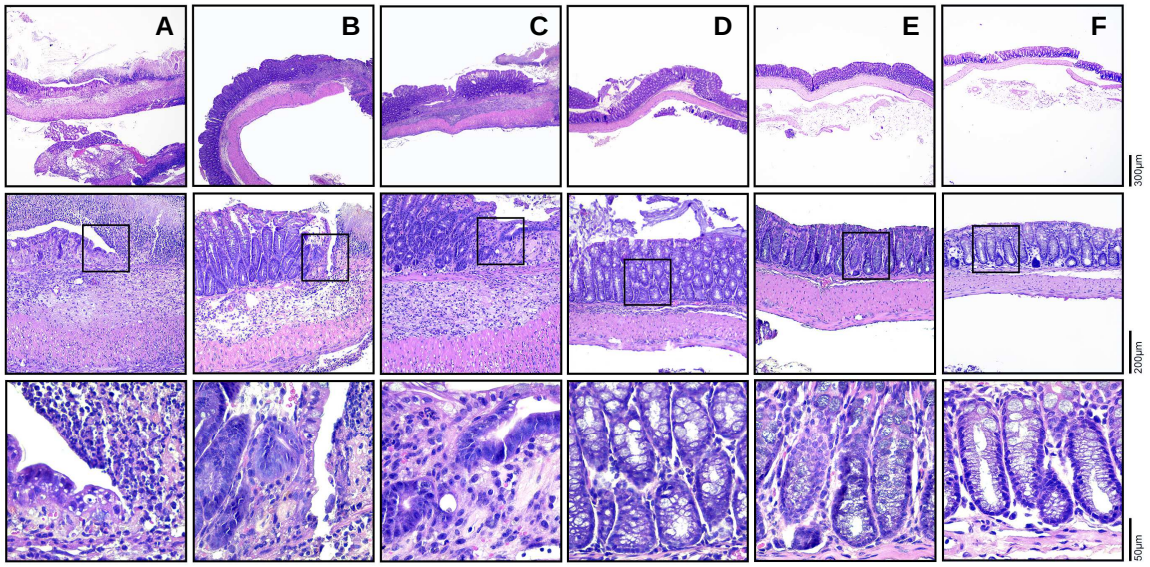

FIGURE 55. Effect of EPO treatment on histopathologic changes in the IBD.

Legend: Each column corresponding with a different experimental group, namely (A) TNBS group, (B) TNBS+EPO500 group, (C) TNBS+EPO1000 group, (D) EPO group, (E) Ethanol group and (F) Sham group.

MORTALITY RATE

At the end of the study, it was evaluated the mortality rate in the experimental groups as a sign of toxicity (TABLE 13). The TNBS group presented 31.4% of mortality rate 4 days after induction. The mortality rate decreased, when these mice were treated with a daily dose of EPO. The TNBS+EPO500 group presented 28.6%, while the TNBS+EPO1000 group presented 25.7% of mortality rate. Although it have not been verified a statistically significant differences between groups, the mortality rate decreased with the increase of EPO dose. In the sham group, all mice survived during the study.

TABLE 13. Effect of EPO treatment on mortality rate in the IBD.

		TOTAL	DEATHS	SURVIVORS	P-VALUE
		n (%)	n (%)	n (%)	
EXPERIMENTAL GROUPS	TNBS	35 (100%)	11 (31.4%)	24 (68.6%)	0.353 (ns)
	TNBS + EPO500	35 (100%)	10 (28.6%)	25 (71.4%)	
	TNBS + EPO1000	35 (100%)	9 (25.7%)	26 (74.3%)	
	Sham	20 (100%)	0 (0%)	20 (100%)	

Legend: Chi-square test; ns – no statistical significance.

2. DISCUSSION OF RESULTS

EPO, the principal hormone promoting the survival and differentiation of erythroid progenitor cells, is currently being used in the therapy of patients with chronic renal failure suffering from anemia [Cody et al., 2001; Nairz et al., 2011]. However, EPO also bears extrahematopoietic properties that are transduced by EPOR expressed on various nonerythroid tissues [Brines & Cerami, 2005; Jelkmann, 2007]. In the TNBS-induced colitis, there is some scientific evidence that EPO-EPOR interaction inhibits NF-κB pathway, decreasing the production of NF-κB inducible immune mediators and, subsequently, limiting tissue damage and ameliorating disease severity. These immune-modulatory effects of EPO may be of therapeutic relevance in inflammatory disease [Nairz et al., 2011]. EPO benefits have been studied in cardiovascular, neurologic, retinal, auditory, pancreatic, renal and liver injuries [Sanchis-Gomar et al., 2014; Chatagner et al., 2010; Loeliger et al., 2011; Olgun et al., 2013; Ucan et al., 2009; Imamura et al., 2007; Sepodes et al., 2006]. Until nowadays, some studies have evaluated the influence of EPO on the experimental colitis, but much more research is needed to understand and confirm the effect of this drug in IBD. Particularly, mice with TNBS-induced colitis (and without any other comorbidity) were never subjected to an EPO treatment. The results on this specific colitis model are very relevant, since TNBS-induced colitis is one of the most used as colitis model and promotes a typically colon injury compatible with CD. Thus, we proposed to assess the influence of EPO in the TNBS-induced colitis model. Furthermore, EPO doses used in this study will be lower than those which have been used in other studies, so are more approached of the doses usually used in the clinical practice in humans.

Mice with TNBS-induced colitis were daily followed, monitoring by some clinical symptoms/signs. We observed that EPO treatment was able to attenuate the diarrhea

and moderate the edema of the anus in these colitic mice. Furthermore, EPO also significantly reduced the percentage of weight loss of mice during the experimental period at both administrated doses. Curiously, the mice treated with the higher EPO dose presented a weight gain of around 7.3% of its initial weight. This data might suggest that similarly to other studies [Cuzzocrea et al., 2004; Nairz et al., 2011]

Variations in the length of any part of the colon are the developmental origin and may lead to variety of acute and chronic pathological conditions [Nayak et al., 2012]. TNBS-induced colitis is characterized by a shortened colon. EPO treatment was able to increase the colon length in a dose dependent manner but without statistical significance. This finding suggests a beneficial effect of EPO in this biomarker, eventually in a highest dose.

In regarding to fecal hemoglobin, its determination can be useful in the detection of lesions accompanied by bleeding [Hirata et al., 2007; Jagtap et al., 2011], however it had never been measured the influence of EPO treatment in this biological marker. Thus, we verified that EPO also has an influence in the intensity of hemorrhagic focus, since a significant reduction of fecal hemoglobin was observed after EPO treatment in a dose dependent manner. In the higher EPO dose, the mice presented fecal hemoglobin values similar with the control groups.

The intestinal APL is expressed on the enterocytes and it is responsible for mucosal defense [Malo et al., 2010; Nagalingam et al., 2011], so the influence of EPO treatment on ALP level must be evaluated. We observed that the elevated level of ALP was significantly attenuated by the EPO treatment by virtue of its anti-inflammatory potential. This data is consistent with other previous findings, where anti-inflammatory drugs are able to decrease ALP level [Kumar et al., 2014]. In both EPO doses, the mice presented serum ALP values similar with the EPO1000 and ethanol groups.

EPO treatment significantly reduced the level of MPO in inflamed colon in a dose dependent manner. The inhibition of neutrophil infiltration in colonic tissue by EPO is also evident in the histopathological observations. Thus, beneficial effect of EPO treatment may be due in part to the suppression of inflammatory response via the inhibition of leukocyte infiltration [Cuzzocrea et al., 2004; Karatepe et al., 2010].

To test whether EPO treatment may modulate the inflammatory process through the regulation of the secretion of some cytokines, we analyzed the colon levels of pro-inflammatory cytokines, as TNF-α and IL-1β, in mice treated with EPO. In this sense,

we observed that the levels of these cytokines were significantly reduced in the colon tissues collected from TNBS-treated mice after EPO administration in a dose dependent manner. These findings, therefore, suggest that EPO reduced the activation and the subsequent expression of pro-inflammatory cytokines, confirming again its anti-inflammatory activity, which is mechanistically attributable to blockage of NF-κB activation [Sandborn & Hanauer, 1999; Cuzzocrea et al., 2004; Nairz et al., 2011].

To confirmation the mechanism of observed amelioration of TNBS-induced colitis by EPO, culture supernatants were additionally tested for the presence of IL-10. IL-10 itself functions as an anti-inflammatory cytokine and limits excessive tissue disruption caused by inflammation [Coquerelle et al., 2009; Yang et al., 2010]. We observed that EPO also promoted a significantly increase of IL-10 levels with a dose dependent effect, constituting an important role in controlling inflammatory response in the TNBS-induced colitis. In the higher EPO dose, the IL-10 concentration increased around 80% comparing with TNBS-induced colitis. However, this data is not consistent with other studies, since their findings concluded that EPO treatment promoted a decrease of pro-inflammatory cytokine without affecting the expression of IL-10 [Nairz et al., 2011].

The histopatological score was significantly reduced in TNBS-treated mice receiving EPO treatment. EPO was able to decrease the severity and the extension of the colon injury, suggesting a remarkable recovery of the disease. These beneficial effects are coincident with other findings observed previously [Cuzzocrea et al., 2004; Karatepe et al., 2010; Nairz et al., 2011; Tasdemir et al., 2013].

The well-documented extra-intestinal manifestations and complications of IBD, as well as the possible renal and hepatic side effects of pharmacotherapy, emphasize the need for periodic evaluation of renal and hepatic functions [Larsen, Bendtzen & Nielsen, 2010; Oikonomou et al., 2011; Rojas-Feria et al., 2013]. The evaluation of the influence of EPO in the extra-intestinal manifestations of IBD is truly relevant, since had never been investigated. For the first time, we observed that a single daily dose of EPO is able to recover the renal and hepatic functions to normal levels, similarly to sham group. Our findings suggest that EPO has a beneficial effect in the extra-intestinal manifestations due to metabolic and physiologic changes induced by the IBD. Furthermore, we also can conclude that EPO does not promote renal and/or hepatic changes as adverse drug reaction, in this experimental colitis model. More studies are needed to understand the mechanism beside of the beneficial effect of EPO in the extra-intestinal manifestations of IBD. Probably, it is due to the interaction of EPO-

EPOR expressed on various nonerythroid tissues [Brines & Cerami, 2005; Jelkmann, 2007].

EPO stimulates erythropoiesis which leads to increased production of erythrocytes and, subsequently, an increase of hematocrit [Panjeta et al., 2015]. Moreover, some trials has raised safety concerns with the drug and suggested that potential benefits may be mitigated by prothrombotic effects. However, the small sample sizes produced underpowered results [Gao et al., 2012]. In fact, the influence of EPO treatment in the appearance of possible cardiovascular adverse events was never evaluated in other studies, previously. Therefore, although our acute colitis model has been developed in only four days, the hematocrit level was measured at the end of experimental period to evaluate the risk of related side effects. All experimental groups registered normal hematocrit levels, according to reference levels in CD-1 mice between 43.9 and 53.3%, as previously described [Prefontaine, 2013]. These data suggest that EPO does not increase the risk of adverse events related with blood viscosity.

Briefly, EPO treatment had a positive influence in the development of experimental colitis in all evaluated parameters, thus reducing its severity and extension. More precisely, EPO reduced the percentage of weight loss, fecal hemoglobin, ALP, MPO, pro-inflammatory cytokines (TNF-α and IL-1β) and histopathological score. On the other hand, EPO also increased colon length and anti-inflammatory cytokine (IL-10) and regulated the renal and hepatic functions. Furthermore, the mortality rate reduced around 5.7% with the higher EPO dose, as well as the hematocrit remained unchanged after EPO treatment. These data indicate that EPO significantly inhibits acute inflammatory response in the TNBS-induced colitis model, without adverse events related to blood viscosity. All of these findings also support the view that EPO is protective in experimental colitis and that inhibition of TNF-α and IL-1β formation (among other MPO effects that include inhibition of neutrophils infiltration) in the colon probably accounts for its beneficial effects.

CHAPTER 7 – THIADIAZOLIDINONE-8 EFFECT IN INFLAMMATORY BOWEL DISEASE

1. RESULTS

MONITORING OF CLINICAL SYMPTOMS/SIGNS

During the experimental study, the mice were observed daily for stool consistency, anus appearance and morbidity. After the induction of colitis, the TNBS group revealed an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity. Under TDZD-8 treatment, these mice maintained the alterations of intestinal motility, as diarrhea or soft stools, that it was observed in the TNBS group. However, the other clinical symptoms/signs showed an improvement with moderate edema of the anus and mild morbidity, while the control groups, like TDZD-8, ethanol and sham groups, didn't reveal any alteration in these clinical symptoms/signs. The change of body weight was also measured daily since the induction day until the end of the study (FIGURE 56). The TNBS group presented a progressive fall in body weight over experimental period, reaching a loss of around -12 ± 1.4% of its initial weight. The TNBS+TDZD-8 group also followed the same profile with a fall in body weight of -11.5 ± 3%. Curiously, the TNBS+Vehicle group had a better profile in the changes of body weight ending with -4.4 ± 9.9% of weight loss ($p<0.05$, compared with TNBS group). However, the control groups presented a considerable increase in body weight, especially from day 0 to day 1, around 10 to 30% at day 1. At the end of experimental period, the TDZD-8, ethanol and sham groups gained 10.6 ± 5.2%, 15.2 ± 1.1% and 31.2 ± 1.6% ($p<0.0001$, compared with TNBS group) of its initial weight, respectively.

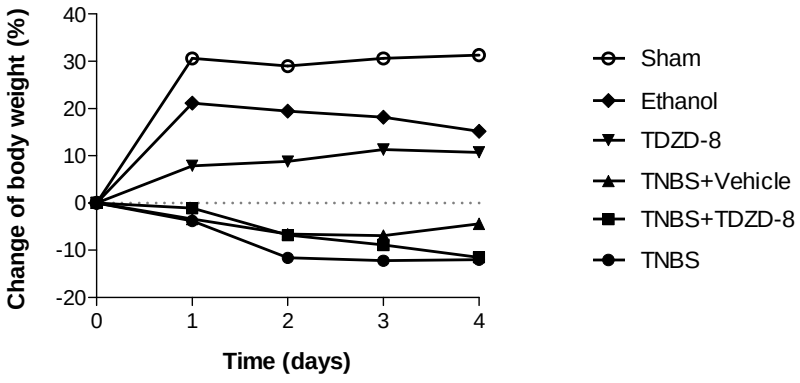

FIGURE 56. Change of body weight during TDZD-8 treatment in the IBD.

BIOCHEMICAL MARKERS

The colon length was evaluated and compared between all experimental groups, as a marker of tissue integrity (FIGURE 57). The mice with TNBS-induced colitis had a shortening of colon length (10.1 ± 0.2 cm) relatively to the sham group (11.5 ± 0.2 cm) as a comparable normal colonic tissue ($p<0.01$). However, the TDZD-8 treatment had no effect on the colon length, because the TNBS+TDZD-8 (10.3 ± 1.2 cm) and TNBS+Vehicle (10.3 ± 0.6 cm) groups presented similar results to TNBS group. The other control groups, as the TDZD-8 and ethanol groups, presented an increase of colon length coincident to the sham group, like 11.7 ± 0.9 cm and 11.5 ± 0.4 cm, respectively, without statistically significance differences between them.

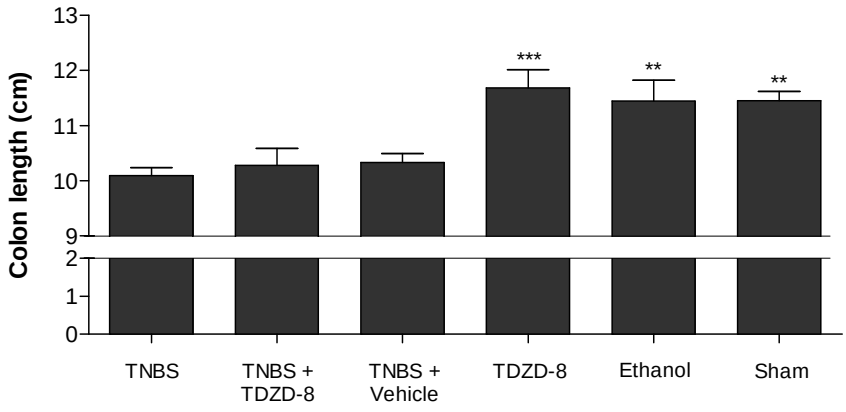

FIGURE 57. Effect of TDZD-8 treatment on colon length in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; ** $p<0.01$; *** $p<0.001$ compared with TNBS group.

The fecal hemoglobin was measured in all groups and it allowed evaluate the impact of TDZD-8 treatment in the experimental colitis (FIGURE 58). The concentration of fecal hemoglobin in the TNBS group was increased comparing to the sham group (12.5 ± 0.3 vs 0.7 ± 0.1 µmol Hg/g feces, $p<0.0001$). This biochemical marker was also higher than the other control groups, like TDZD-8 (1 ± 0 µmol Hg/g feces) and ethanol (1.1 ± 0.1 µmol Hg/g feces) ($p<0.0001$, compared with TNBS group). No statistically significant changes were found between control groups. The mice with TNBS-induced colitis revealed a decreased of fecal hemoglobin after TDZD-8 daily administration, with 4.4 ± 0.3 µmol Hg/g feces in the TNBS+TDZD-8 group ($p<0.0001$, compared with TNBS group). Curiously, the TNBS+Vehicle group also presented a decrease of fecal hemoglobin comparing with TNBS group (9.7 ± 0.2 µmol Hg/g feces, $p<0.0001$), but not so expressive as TNBS+TDZD-8 group.

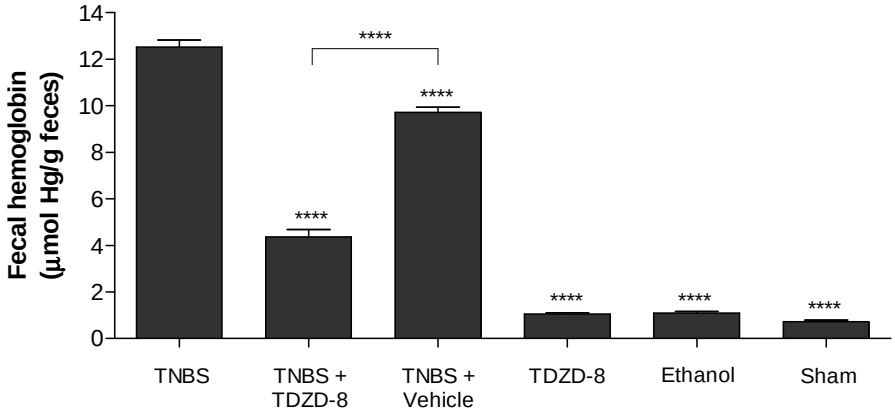

FIGURE 58. Effect of TDZD-8 treatment on fecal hemoglobin in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group or between groups.

The APL concentration was evaluated in all experimental groups, due to its protective effect on intestinal system (FIGURE 59). The TNBS group presented 72.7 ± 2.3 IU/L of ALP concentration on blood, as well as the TNBS+Vehicle group with 70.3 ± 1.7 IU/L. The TDZD-8 treatment had a protective effect with a decrease of ALP concentration to 44.9 ± 1.3 IU/L (p<0.0001, compared with TNBS group). The TDZD-8 and ethanol groups had quite similar results, like 42.3 ± 1 U/L and 38.5 ± 0.6 IU/L, respectively. The sham group presented 19.2 ± 1.8 IU/L of APL (p<0.0001, compared with TNBS group).

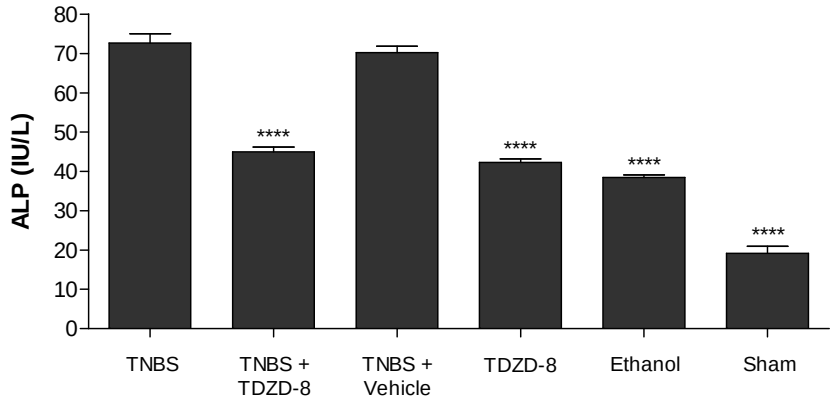

FIGURE 59. Effect of TDZD-8 treatment on serum total ALP concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group.

The renal function of mice with TNBS-induced colitis was evaluated based on serum urea and creatinine concentrations (FIGURE 60 AND 61). The TNBS group had 62.4 ± 1.5 mg/dl of urea concentration comparing to 49.3 ± 0.9 mg/dl in the sham group (p<0.0001). The mice under TDZD-8 treatment presented 44.3 ± 0.7 mg/dl of urea similar with the control groups, whereas the TNBS+Vehicle group presented 65.5 ± 1.2 mg/dl similar with TNBS group. The other control groups, like TDZD-8 and ethanol

groups, revealed 43 ± 1.1 mg/dl and 41.4 ± 0.5 mg/dl of urea, respectively. The creatinine concentration described the same tendency than urea concentrations. The TNBS and TNBS+Vehicle groups were those with the highest values, like 0.27 ± 0.01 mg/dl and 0.28 ± 0.01 mg/dl, respectively ($p<0.0001$, compared with sham group). The TNBS+TDZD-8 group had 0.19 ± 0.01 mg/dl of creatinine comparable with the control groups. The TDZD-8, ethanol and sham groups presented coincident results, namely 0.20 ± 0 mg/dl, 0.21 ± 0.01 mg/dl and 0.20 ± 0.02 mg/dl, respectively.

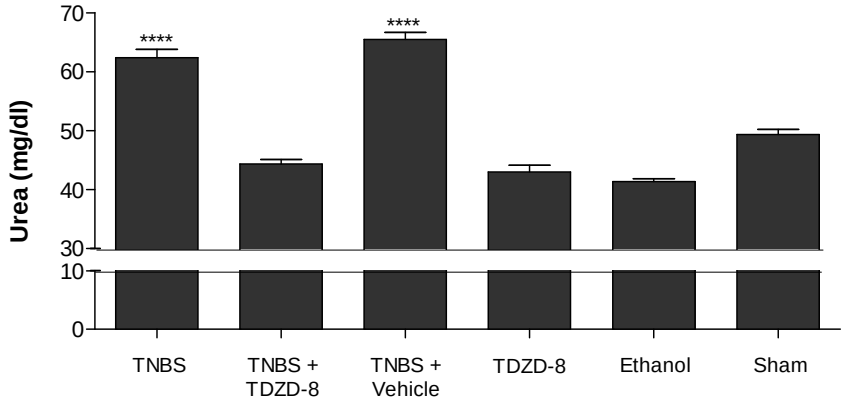

FIGURE 60. Effect of TDZD-8 treatment on serum urea concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** $p<0.0001$ compared with sham group.

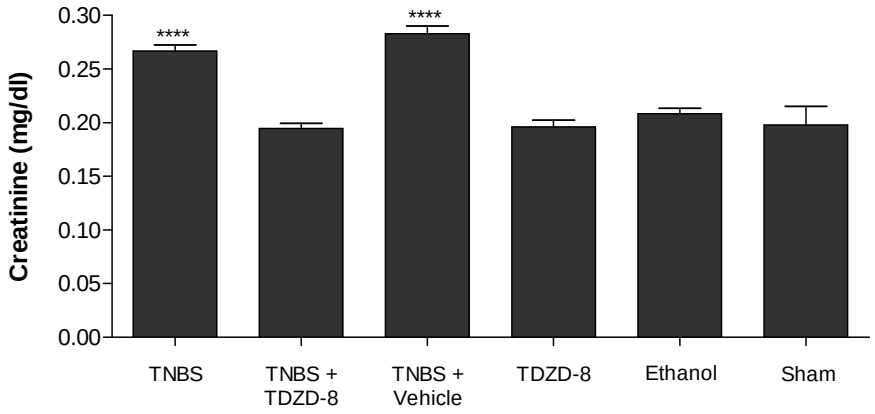

FIGURE 61. Effect of TDZD-8 treatment on serum creatinine concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** $p<0.0001$ compared with sham group.

The hepatic function was evaluated based on serum ALT concentration (FIGURE 62). The TNBS group exhibited an increase in ALT concentration compared with sham group (38.7 ± 1.2 vs 17.5 ± 0.8 IU/L, $p<0.0001$). The mice treated with TDZD-8 presented a significant decrease of ALT levels to around 28.3 ± 0.8 IU/L ($p<0.0001$, compared with TNBS group). The hepatic biochemistries of TNBS+Vehicle group revealed 38 ± 1.1 IU/L of ALT, without statistically significant differences with TNBS group. In the TDZD-8

and ethanol groups, the ALT concentration was slightly higher than the sham group (29.4 ± 0.8 IU/L and 32.3 ± 1.2 IU/L, respectively, p<0.0001).

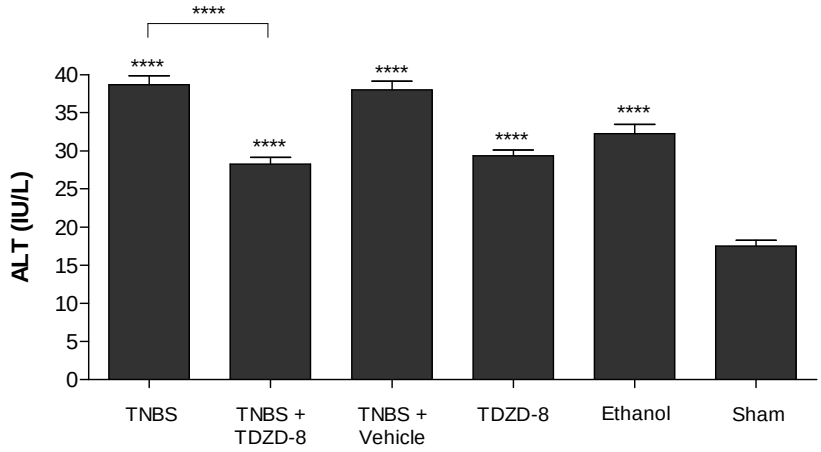

FIGURE 62. Effect of TDZD-8 treatment on serum ALT concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with sham group or between groups.

MYELOPEROXIDASE ACTIVITY

The MPO measurements allowed identifying differences in its activity between all experimental groups (FIGURE 63). The TNBS group presented an increase of MPO concentration comparing with the sham group (42 ± 2.8 vs 2.3 ± 0.4 ng/ml, p<0.001). As expected, in the TNBS+Vehicle group (41.5 ± 1.9 ng/ml) was registered a MPO activity quite similar with TNBS group. Furthermore, the TDZD-8 treatment was able to decrease the MPO activity to 27.6 ± 3.2 ng/ml (p<0.001, compared with TNBS group). The TDZD-8 and ethanol groups presented a significantly decrease of MPO activity to around 10.8 ± 3.8 ng/ml and 5.8 ± 0.9 ng/ml of MPO, respectively.

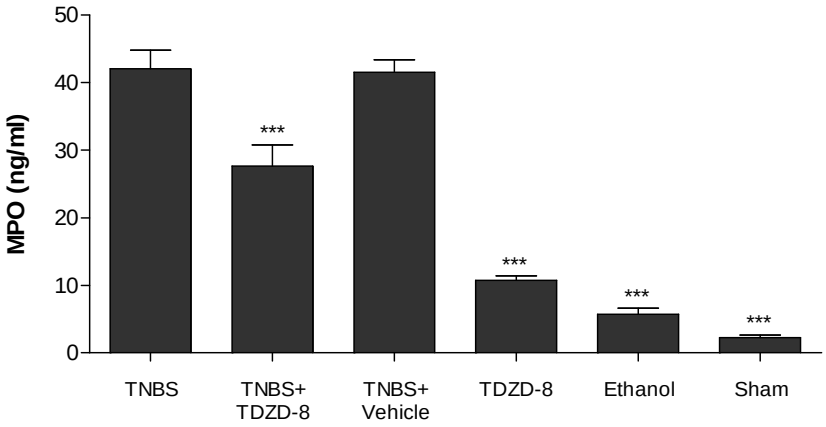

FIGURE 63. Effect of TDZD-8 treatment on MPO activity in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with TNBS group.

MEASUREMENT OF CYTOKINES

The TDZD-8 treatment revealed a significant decrease in the pro-inflammatory cytokines production, as TNF-α and IL-1β (FIGURE 64 AND 65). Concretely, the TNBS+TDZD-8 presented 153.3 ± 12.1 pg/ml of TNF-α and 129.1 ± 9.9 pg/ml of IL-1β. Indeed, the TDZD-8 treatment had a positive influence on the TNBS-induced colitis, since the mice from TNBS group presented a considerable increase of pro-inflammatory cytokines with 253.2 ± 31.4 pg/ml of TNF-α and 263.9 ± 25 pg/ml of IL-1β ($p<0.01$ and $p<0.001$, respectively, compared with TNBS+TDZD-8 group). The TNBS+Vehicle group also registered an increased TNF-α and IL-1β levels with 274.8 ± 21.9 pg/ml and 271.1 ± 22.6 pg/ml, respectively. However, all control groups presented the lowest levels of pro-inflammatory cytokines. The ethanol and sham groups had very similar concentrations of pro-inflammatory cytokines. The ethanol group presented 14.9 ± 2 pg/ml of TNF-α and 16.2 ± 1.6 pg/ml of IL-1β and the sham group presented 11.2 ± 0.2 pg/ml of TNF-α and 12.7 ± 0.1 pg/ml of IL-1β. Nevertheless, the TDZD-8 group was the only group with a TNF-α and IL-1β levels slightly higher with 71.2 ± 46.1 pg/ml and 92 ± 45.8 pg/ml, respectively, but without statistically significant differences with sham group.

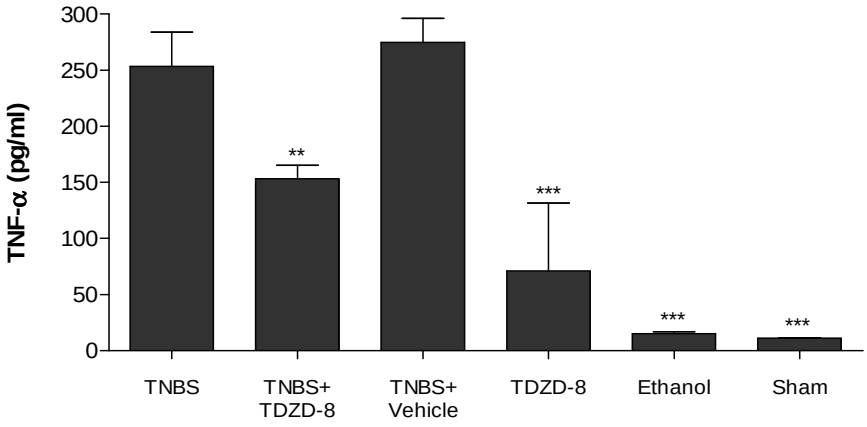

FIGURE 64. Effect of TDZD-8 treatment on TNF-α concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; ** $p<0.01$; *** $p<0.001$ compared with TNBS group.

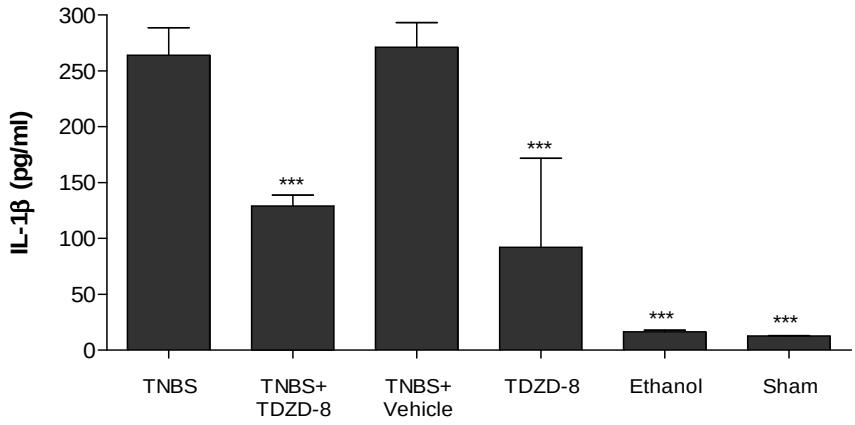

FIGURE 65. Effect of TDZD-8 treatment on IL-1β concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with TNBS group.

In return, the anti-inflammatory cytokines, as IL-10, are typically decreased in the TNBS-induced colitis (FIGURE 66). The assessment of IL-10 concentrations confirms the obtained results in the TNF-α and IL-1β measurements. Concretely, the TNBS group presented a low IL-10 concentration with 31.4 ± 3.3 pg/ml ($p<0.01$, compared with sham group), as well as the TNBS+Vehicle group with 32.1 ± 1.9 pg/ml of IL-10. However, after TDZD-8 treatment, the mice showed a significant increase of IL-10 with 75.1 ± 4.9 pg/ml ($p<0.001$, compared with TNBS group). The control groups, as TDZD-8, ethanol and sham groups, had similar results with 19.8 ± 0.9 pg/ml, 17.3 ± 1.2 pg/ml and 15.7 ± 0.1 pg/ml of IL-10, respectively.

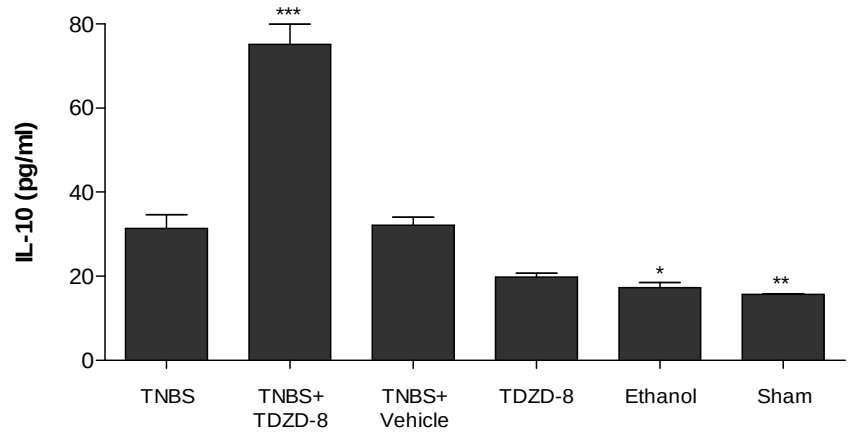

FIGURE 66. Effect of TDZD-8 treatment on IL-10 concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; ** p<0.01; *** p<0.001 compared with TNBS group.

ASSESSMENT OF COLITIS SEVERITY

The macroscopic inspection of cecum, colon, and rectum in the TNBS group showed presence of mucosal congestion, hemorrhagic ulcerations and necrosis. After TDZD-8

treatment, the macroscopic alterations were the same than were observed in the TNBS group. No macroscopic alterations were observed in the control groups.

The histopathological score was calculated based on severity (the presence of tissue loss/necrosis, severity of mucosal epithelial lesion and inflammation) and extension of the lesions (TABLE 14). The treatment with TDZD-8 has no effect on the severity of colitis comparing with TNBS group. However, it is possible to identify a slight decrease of the percentage of intestine affected by the most severe lesion from 3 ± 0.2 to 2.3 ± 0.2 after TDZD-8 treatment. The TNBS+Vehicle group presented results on each parameter very similar with the TNBS group. Finally, the control groups revealed partial scores too low in all evaluated parameters, except the ethanol group with a slight increase in values, consistent with the histopathological images.

TABLE 14. Average (± SEM) of partials scores of histopathological score of TNBS-induced colitis under TDZD-8 treatment.

	TNBS	TNBS + TDZD-8	TNBS + VEHICLE	TDZD-8	ETHANOL	SHAM
MUCOSAL LOSS	3.3 ± 0.2	3.9 ± 0.1	3.8 ± 0.5	0 ± 0	0.2 ± 0.1	0 ± 0
EPITHELIAL LESIONS	3.4 ± 0.2	4 ± 0	4 ± 0	0 ± 0	0.9 ± 0.5	0 ± 0
INFLAMMATION	3.8 ± 0.1	4 ± 0	4 ± 0	0.4 ± 0.2	1.1 ± 0.2	0.1 ± 0.1
EXTENT 1*	3.7 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	0.4 ± 0.2	1.3 ± 0.3	0 .1 ± 0.1
EXTENT 2**	3 ± 0.2	2.3 ± 0.2	2.8 ± 0.2	0.4 ± 0.2	1 ± 0.2	0.1 ± 0.1

Legend: * Extent 1 - Percentage of intestine affected in any manner;
** Extent 2 - Percentage of intestine affected by the most severe lesion.

The histopathological score was evaluated in all experimental groups (FIGURE 67). The TNBS group presented a final score of around 17 ± 0.6, substantially higher than the sham group ($p<0.0001$). Curiously, when the mice are treated with TDZD-8, the histopathological score keeps unchanged (18 ± 0.2). The results from TNBS+Vehicle are quite similar with the TNBS and TNBS+TDZD-8 groups (18.5 ± 0.3). The control groups revealed a histopathological score very low, namely 1.3 ± 0.6 for the TDZD-8 group, 4.5 ± 1.2 for the ethanol group and 0.2 ± 0.2 for the sham group. The ethanol control group was the only group with a histopathological score slightly higher.

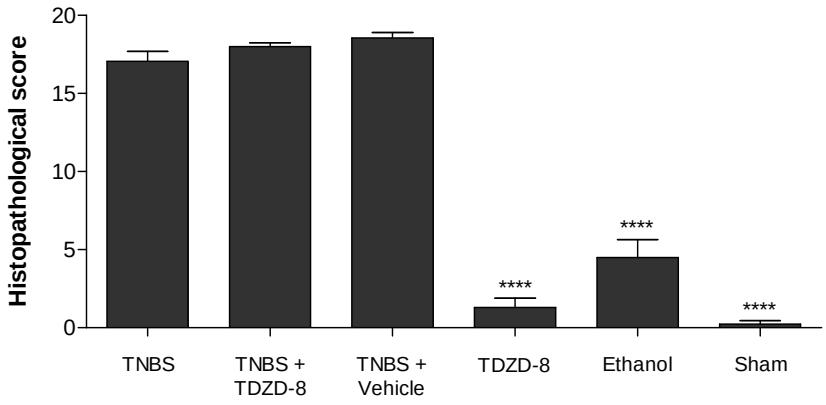

FIGURE 67. Effect of TDZD-8 treatment on histopathological score.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group.

The images reflect the histopatological score for these experimental groups (FIGURE 68). The TNBS group displays diffuse transmural necrosis with severe hemorrhaging, involving the mucosa, submucosa, muscle layer and serosa, and often associated with peritonitis. Under TDZD-8 treatment, similar lesions are seen, without any improvement in the severity and/or extension of the lesion. The histopathological images of TNBS+Vehicle group present the same lesions than TNBS group. The ethanol group showed only mild epithelial erosion, and no lesions were observed in the TDZD-8 and sham groups.

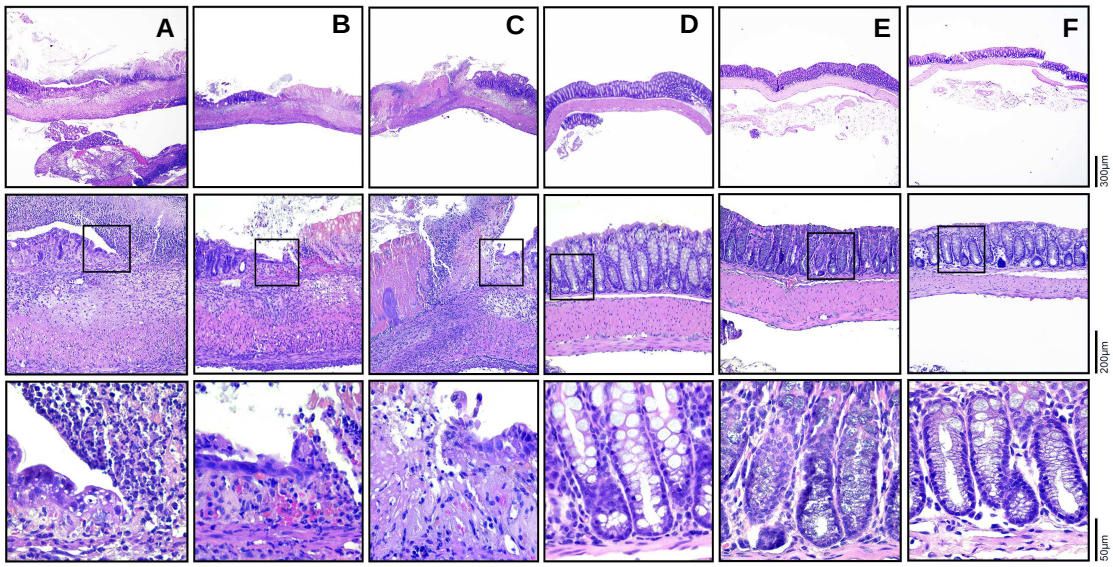

FIGURE 68. Effect of TDZD-8 treatment on histopathologic changes in the IBD.

Legend: Each column corresponding with a different experimental group, namely (A) TNBS group, (B) TNBS+TDZD-8 group, (C) TNBS+Vehicle group, (D) TDZD-8 group, (E) Ethanol group and (F) Sham group.

MORTALITY RATE

The mortality rate was evaluated as a sign of toxicity (TABLE 15). The TNBS group presented 31.4% of mortality rate in the end of the study, but this percentage decreased for 25.7% when these mice were treated with a daily dose of TDZD-8. The TNBS+Vehicle group presented 20% of mortality rate. Although it have not been verified a statistically significant differences between groups, the mortality rate decreased after TDZD-8 treatment. In the sham group, all mice survived during the study.

TABLE 15. Effect of TDZD-8 treatment on mortality rate in the IBD.

		TOTAL	DEATHS	SURVIVORS	P-VALUE
		n (%)	n (%)	n (%)	
EXPERIMENTAL GROUPS	**TNBS**	35 (100%)	11 (31.4%)	24 (68.6%)	0.499 (ns)
	TNBS + TDZD-8	35 (100%)	9 (25.7%)	26 (74.3%)	
	TNBS + Vehicle	20 (100%)	4 (20%)	16 (80%)	
	Sham	20 (100%)	0 (0%)	20 (100%)	

Legend: Chi-square test; ns – no statistical significance.

2. DISCUSSION OF RESULTS

This study also evaluated the influence of TDZD-8 in the TNBS-induced colitis model. TDZD-8 is the first non-ATP competitive GSK-3β inhibitors with highly effectiveness and selectivity [Martinez et al., 2002a; Dugo et al., 2007]. The few findings suggest that the inhibition of GSK-3β promotes a reduction of NF-κB, suggesting the influence of this protein kinase in the regulation of inflammatory process [Whittle et al., 2006]. Many recent studies suggest GSK-3β has become a novel and important therapeutic target in inflammatory and autoimmune diseases [Cohen & Goedert, 2004; Whittle et al., 2006; Beurel et al., 2009]. Indeed, GSK-3β emerged in a therapeutic potential for the treatment of several diseases, such as cancer, diabetes type II, chronic inflammatory processes, stroke, and neurological diseases [Castro & Martinez, 2000; Kim & Kimmel, 2000; Wagman & Nuss, 2001; Cohen, 2001; Sasaki et al., 2001]. Until nowadays, the influence of TDZD-8 on the experimental colitis was not well described, as well as the full pharmacological profile and selectivity of this agent awaits further evaluation [Dugo et al., 2005]. Only one study was published, describing the data from rats with TNBS-induced colitis treated with TDZD-8 and, furthermore, some inflammation parameters

were not evaluated yet. So, more studies are needed to understand the effect of this drug in IBD.

Regarding clinical symptoms/, TDZD-8 treatment showed an improvement with moderate edema of the anus and mild morbidity comparing with non-treated mice. In body weight, we observed that the fall in body weight determined 4 days following challenge with TNBS was not attenuated by TDZD-8 treatment. On the other hand, the mice with TNBS-induced colitis treated with DMSO (TDZD-8 vehicle) presented a reduction of the percentage of weight lost compared with non-treated mice. These findings are not consistent with other studies, where TDZD-8 or other drug is used as GSK-3 inhibitor [Whittle et al., 2006; Uddin et al., 2013]. Because TDZD-8 treatment usually promotes an increase of body weight and DMSO supposedly has no effect on the disease management [Whittle et al., 2006]. One possible explanation can be based on some recent studies, which demonstrated that DMSO can present anti-inflammatory and anti-oxidant effects, beyond its use as vehicle of several pharmaceuticals [Li et al., 2015]. However, DMSO is the most recommended vehicle for TDZD-8 and these properties only manifested when DMSO is used above of 10% of the administrated solution [Whittle et al., 2006; Li et al., 2015]. In our study, DMSO was diluted in the proportion of 1:10 in a saline solution, so it means that the dose was below those that promote anti-inflammatory and anti-oxidant effects.

In the evaluation of colon length, this study verified that the shortening of the colon due to TNBS-induced colitis was not also influenced by TDZD-8 treatment, as well as with DMSO administration. One more time, this finding is not consistent with other studies [Whittle et al., 2006]. Apparently, there is no explanation for this data, since the dose of TDZD-8 used during the treatment was above than the doses previously used in other studies. Curiously, these findings (eg. body weight and colon length) are not also consistent with the remaining evaluated parameters, where the TDZD-8 treatment had a significant influence in the reduction of inflammation associated to TNBS-induced colitis. In sum, data are not coherent among studies, so more research studies are needed to evaluate the influence of TDZD-8 treatment in specific parameters, as body weight and colon length.

Bloody diarrhea is the hallmark of IBD, but in mild disease, rectal bleeding may be absent [Kornbluth & Sachar, 2010]. Therefore, to assess whether TDZD-8 has influence in TNBS-induced colon damage, there was analyzed the fecal hemoglobin and quantified the intensity of hemorrhagic damage. Mice with TNBS-induced colitis

revealed an elevated concentration of fecal hemoglobin, however TDZD-8 was able to significantly attenuate its presence in the feces, after only 4 days of daily treatment. These data suggest that TDZD-8 promotes a decrease of intensity of hemorrhagic focus.

Regarding to ALP measurement, we observed that TNBS-induced colitis presented an elevated ALP concentration, however TDZD-8 treatment had a protective effect, decreasing its serum concentration on blood. This result suggests that TDZD-8 has an anti-inflammatory effect by GSK-3 inhibition, since the inhibition of inflammatory process promotes a decrease of ALP level [Kumar et al., 2014].

Neutrophils have an important involvement in these inflammatory bowel processes, therefore the TNBS-induced colitis is always accompanied by a substantial increase in the levels of MPO [Ukil et al., 2003; Szczepanik et al., 2012]. The MPO measurements revealed that the level of MPO activity in the colonic tissue was substantially increased after DMSO administration (TNBS+Vehicle group) following TNBS challenge, compared with that from the nonchallenged control group. This result confirms that DMSO has no effect on amelioration of colonic inflammation, as previously known [Whitlle, 2006]. On the other hand, treatment with TDZD-8 caused a significant reduction in the elevated MPO activity, confirming its potent anti-inflammatory effect in a TNBS-induced colitis model.

GSK-3β might be such a potential switch protein and was recently identified as a critical regulator in the modulation of TLR-induced inflammatory responses of blood monocytes, promoting the production of pro-inflammatory cytokines such as TNF-α and IL-1β [Carty et al., 2000; Hofmann, Dunger, Scholmerich, Falk & Obermeier, 2010]. Therefore, the effect of TDZD-8, as GSK-3β inhibitor, on the colonic levels of these pro-inflammatory cytokines was evaluated. In our study, the observed increase in the levels of TNF-α and IL-1β in the inflamed colon was significantly reduced by TDZD-8 treatment, suggesting a beneficial effect on TNBS-induced colitis where inhibition of GSK-3β is involved. This finding confirms the obtained data previously with TDZD-8 [Whittle et al., 2006], as well as with other GSK-3 inhibitors [Uddin et al., 2013; Soubh, Abdallah & El-Abhar, 2015]. Thus, the blockade of GSK-3β attenuates TLR-mediated excessive pro-inflammatory cytokines and constitutes a promising therapeutic option for reducing intestinal immune reactions toward the luminal flora in IBD [Hofmann et al., 2010].

GSK-3β promotes the production of pro-inflammatory cytokines, while simultaneously suppressing the secretion of anti-inflammatory cytokines as IL-10 [Carty et al., 2000; Hofmann et al., 2010]. In our study, we observed that assessment of IL-10 concentrations confirms the obtained results in the TNF-α and IL-1β measurements. After TDZD-8 treatment, the mice with TNBS-induced colitis showed a significant increase of IL-10, suggesting and confirming the mechanism of observed amelioration of TNBS-induced colitis by GSK-3β inhibition [Hofmann et al., 2010].

Regarding to histological score, we observed that colons of TDZD-8 treated mice were similar than those of non treated mice, suggesting that TDZD-8 treatment possibly has no effect on the severity and/or extension of the intestinal lesion inducted by disease. However, the histological features of the lesions allow identifying a slight decrease of the percentage of intestine affected by the most severe lesion after TDZD-8 treatment. So, these results suggest that TDZD-8 also reduced the extension of colon injury.

TNBS-induced colitis model presented an increase of renal and hepatic markers, as secondary extra-intestinal symptoms to colon injury. However, TDZD-8 treatment significantly suppressed the level of these markers, suggesting a beneficial effect in the extra-intestinal symptoms due to GSK-3β inhibition. Renal markers were even reduced to similar concentrations with sham group. Our findings also suggest that TDZD-8 does not promote renal and/or hepatic changes as adverse drug reaction, in this experimental colitis model.

As already mentioned, there is only one published paper relating the TDZD-8 treatment with IBD and, therefore, several parameters had never been evaluated in a TNBS-induced colitis model after TDZD-8 administration, such as IL-1β, IL-10, histological features and extra-intestinal symptoms. Thus, this study was innovative, since some new parameters were measured for the first time. Briefly, TDZD-8 had also a positive influence in the development of experimental colitis, but not in all evaluated parameters. This drug promoted a reduction of fecal hemoglobin, ALP, MPO and pro-inflammatory cytokines (TNF-α and IL-1β). TDZD-8 was also able to increase the anti-inflammatory cytokine (IL-10) and the renal and hepatic functions were regulated. Indeed, TDZD-8 treatment significantly improved several inflammation markers and, subsequently, promoted a reduction of severity of this TNBS-induced colitis model, suggesting an anti-inflammatory effect of TDZD-8 by GSK-3β inhibition. Furthermore, TDZD-8 treatment slightly decreased the extension of colon injury, which was identified in the histological images. In sum, these data indicate that TDZD-8 significantly inhibits acute inflammatory response in the experimental colitis.

CHAPTER 8 – HEMIN EFFECT IN INFLAMMATORY BOWEL DISEASE

1. RESULTS

MONITORING OF CLINICAL SYMPTOMS/SIGNS

The clinical symptoms/signs daily observed during the experimental period were stool consistency, anus appearance and morbidity. Following experimental induction of colitis, the TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity. Under hemin treatment, these mice maintained the alterations of intestinal motility, as diarrhea or soft stools, which were observed in the TNBS group. However, the other clinical symptoms/signs showed an improvement with moderate edema of the anus and mild morbidity. The control groups, as hemin10, ethanol and sham groups, remained without any alterations.

The body weight change was other clinical sign under evaluation (FIGURE 69). The TNBS group presented a progressive fall in body weight over the 4-day experimental period (-12 ± 1.4%). Curiously, the fall in body weight in mice with TNBS-induced colitis was attenuated by hemin treatment, but without a dose dependent effect. The TNBS+Hemin5 group decreased the body weight to around -5 ± 1.8% ($p<0.05$, compared with TNBS group), whereas the TNBS+Hemin10 group presented a body weight change coincident with the TNBS group with -10.2 ± 1.5% of weight loss. At the end of experimental period, the TNBS+Vehicle group also lost -10.4 ± 1.3% of its initial weight. The hemin10, ethanol and sham groups gained 7.5 ± 1.2%, 15.2 ± 1.1% and 31.2 ± 1.6% ($p<0.0001$, compared with TNBS group) of its initial weight, respectively. It was observed statistically significant differences between the body weights of the control groups. The considerable increase in body weight was observed in the control groups, especially from day 0 to day 1, around 7 to 30% at day 1.

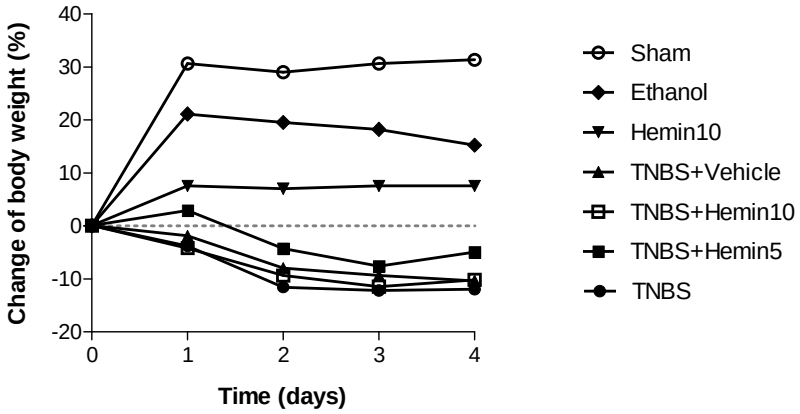

FIGURE 69. Change of body weight during hemin treatment in the IBD.

BIOCHEMICAL MARKERS

The colon length, used as an indirect marker of inflammation, was determined at the end of the treatment period (FIGURE 70). Briefly, the colon appearance of TNBS group was flaccid and filled with liquid stool. Furthermore, the TNBS-induced colitis (10.1 ± 0.2 cm) reduced significantly the colon length relatively with the sham group (11.5 ± 0.2 cm) as a comparable tissue ($p<0.0001$). The hemin treatment showed an influence on the colon length at both doses, but without statistical significance. The colon length in the TNBS+Hemin5 group was higher (10.9 ± 0.2 cm) than TNBS group, even as the TNBS+Hemin10 group with 10.8 ± 0.2 cm of colon length. The TNBS+Vehicle group presented intermediate values for colon length between colitis groups with and without hemin treatment (10.5 ± 0.2 cm). The hemin10, ethanol and sham groups presented similar results between them, like 11.1 ± 0.2 cm, 11.5 ± 0.4 cm, 11.5 ± 0.2 cm, respectively.

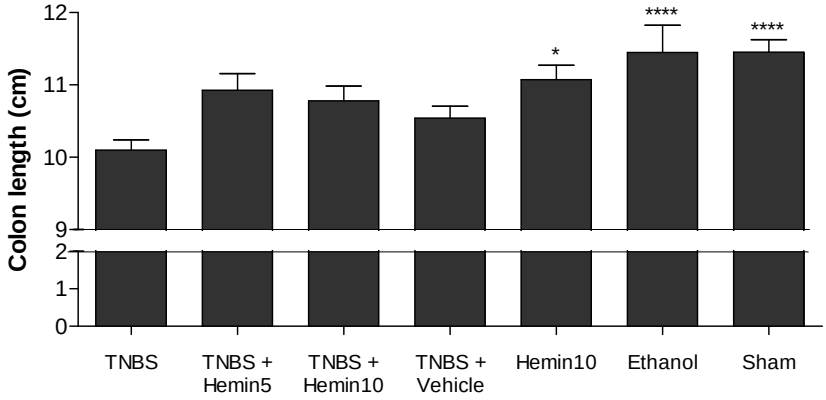

FIGURE 70. Effect of hemin treatment on colon length in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * $p<0.05$; **** $p<0.0001$ compared with TNBS group.

The intensity of hemorrhagic focus is evaluated by the fecal hemoglobin concentration (FIGURE 71). The fecal hemoglobin concentration in TNBS group was significantly higher than the sham group (12.5 ± 0.3 vs 0.7 ± 0.1 µmol Hg/g feces, p<0.0001). In the TNBS+Vehicle group, the data were similar with the TNBS group, presenting 13 ± 0.6 µmol Hg/g feces. Hemin treatment had a positive influence in the intensity of hemorrhagic focus, since the fecal hemoglobin concentration decreased when the hemin dose was increased, verifying a dose dependent effect (p<0.0001). Concretely, the TNBS+Hemin5 group presented 8.1 ± 0.4 µmol Hg/g feces and the TNBS+Hemin10 group presented 5.6 ± 0.5 µmol Hg/g feces (p<0.0001, compared with TNBS group). Mice from hemin10 (1.6 ± 0.1 µmol Hg/g feces) and ethanol groups (1.1 ± 0.1 µmol Hg/g feces) did not display significant changes compared to the sham group (0.7 ± 0.1 µmol Hg/g feces).

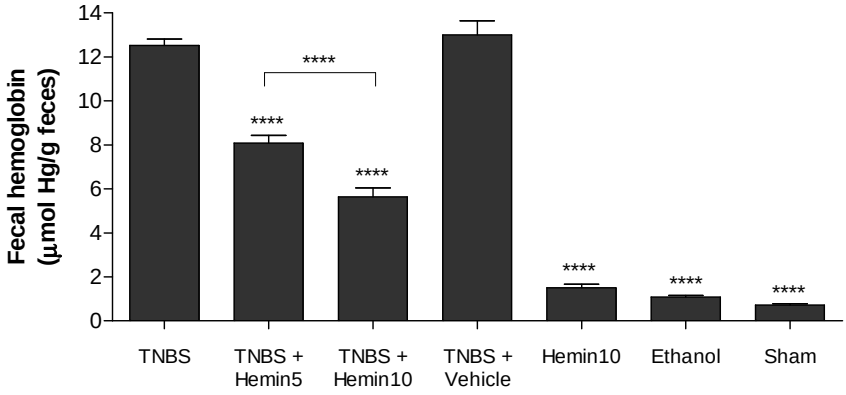

FIGURE 71. Effect of hemin treatment on fecal hemoglobin in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group or between groups.

The ALP plays an essential role on intestinal homeostasis and its concentration was measured in all experimental groups (FIGURE 72). The ALP concentration on the TNBS group was considerably higher compared with the sham group (72.7 ± 2.3 vs 19.2 ± 1.8 IU/L, p<0.0001). The TNBS+Vehicle group had 69.7 ± 0.4 IU/L of ALP, quite similar with the TNBS group. The hemin treatment was able to decrease the ALP concentration on blood at both hemin doses, achieving a dose dependent effect with statistical significance (p<0.0001, compared with TNBS group). The TNBS+Hemin5 group presented 49.7 ± 0.7 IU/L, whereas the TNBS+Hemin10 group presented 36.8 ± 1 IU/L of ALP (p<0.0001). The ALP in the hemin10 and ethanol groups are increased comparing with the sham group, with 41.1 ± 0.6 IU/L and 38.5 ± 0.6 IU/L, respectively.

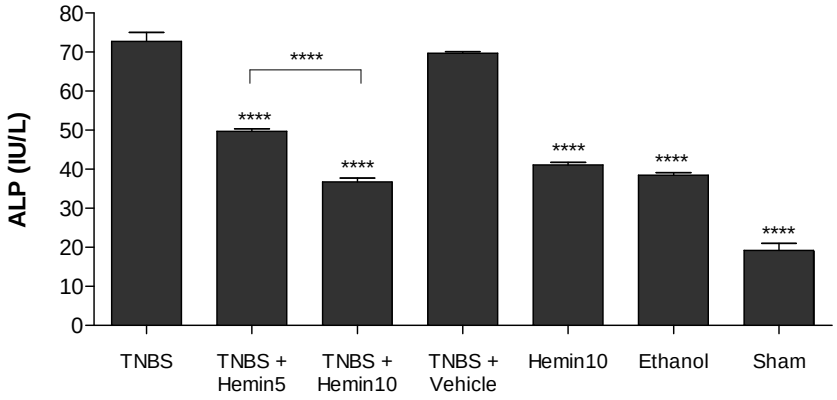

FIGURE 72. Effect of hemin treatment on serum total ALP concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with TNBS group or between groups.

The influence of hemin treatment on the renal function was evaluated by measurement of renal markers concentration, like urea and creatinine (FIGURE 73 AND 74). The TNBS group exhibited a significant increase in urea compared with sham group (62.4 ± 1.5 vs 49.3 ± 0.9 mg/dl, p<0.0001), as well as the creatinine compared with sham group (0.27 ± 0.01 vs 0.20 ± 0.02 mg/dl, p<0.0001). The TNBS+Vehicle group had both renal markers, like urea (68.8 ± 0.7 mg/dl) and creatinine (0.28 ± 0.01 mg/dl), higher than TNBS group. The mice treated with hemin presented a significant decrease of urea and creatinine levels to values quite similar with sham group, promoting thus a dose dependent effect with statistical significance between hemin doses in the urea levels (p<0.0001). The renal biochemistries of TNBS+Hemin5 group revealed 53.8 ± 1.1 mg/dl of urea and 0.21 ± 0.01 mg/dl of creatinine, while the TNBS+Hemin10 group revealed 41.4 ± 0.9 mg/dl of urea and 0.20 ± 0.01 mg/dl of creatinine. In the control groups, the urea concentration in the hemin10 and ethanol groups were lower than the sham group, with 40.6 ± 1 mg/dl and 41.4 ± 0.5 mg/dl, respectively (p<0.05). There are no statistically significant differences in creatinine levels between the control groups, like hemin10, ethanol and sham.

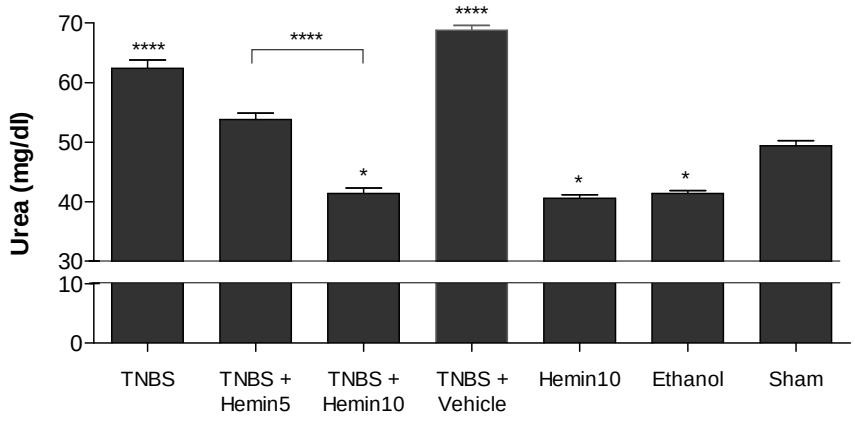

FIGURE 73. Effect of hemin treatment on serum urea concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; **** p<0.0001 compared with sham group or between groups.

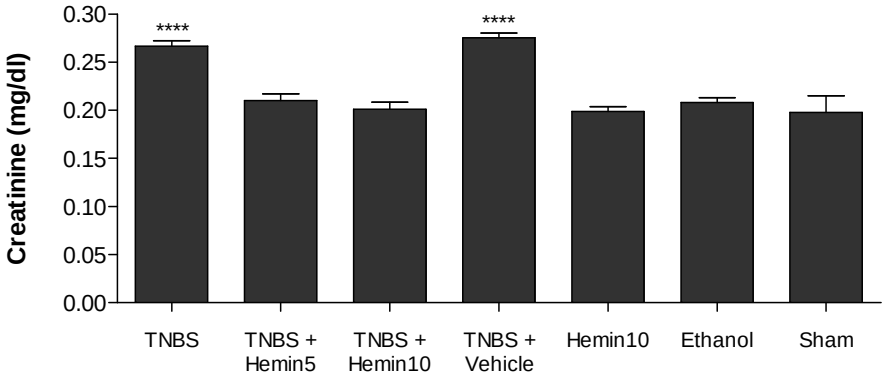

FIGURE 74. Effect of hemin treatment on serum creatinine concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; **** p<0.0001 compared with sham group.

The ALT concentration was measured in mice with colitis induced-TNBS and mice treated with hemin to evaluate the hepatic function (FIGURE 75). The ALT concentration in blood was significantly higher in the TNBS group compared with the sham group (38.7 ± 1.2 vs 17.5 ± 0.8 IU/L, p<0.0001). The TNBS+Vehicle group presented a slight decrease of ALT concentration (32.4 ± 0.6 IU/L) compared with the TNBS group. On the other hand, the hemin treatment at both doses promoted a considerable decrease of ALT levels, namely to 30.1 ± 0.8 IU/L in the TNBS+Hemin5 group and 25.5 ± 1 IU/L in the TNBS+Hemin10 group, comparing with the TNBS group (p<0.0001). However, these results are still higher than the sham group (p<0.001). The ALT levels on hemin10 (30.4 ± 0.6 IU/L) and ethanol (32.3 ± 1.2 IU/L) groups were higher than the sham group (p<0.0001).

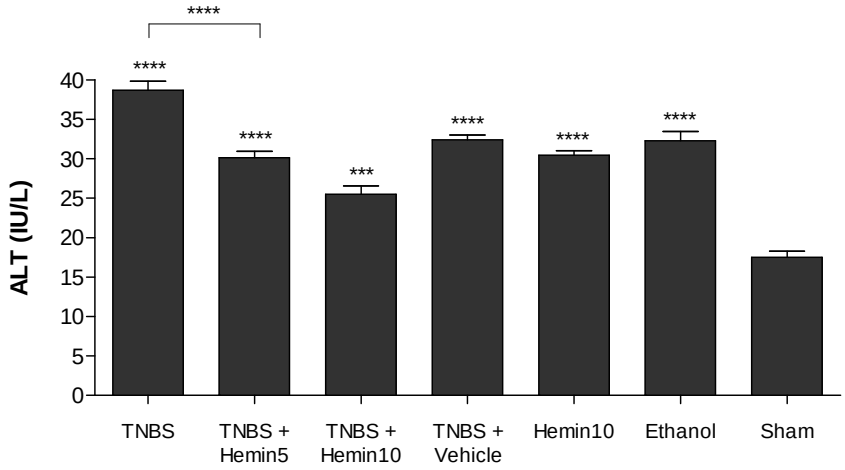

FIGURE 75. Effect of hemin treatment on serum ALT concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001; **** p<0.0001 compared with sham group.

MYELOPEROXIDASE ACTIVITY

The MPO activity was registered in all experimental groups (FIGURE 76). The TNBS group presented an increase of MPO concentration comparing with the sham group (42 ± 2.8 vs 2.3 ± 0.4 ng/ml, p<0.001). The TNBS+Vehicle group also had an elevated level of MPO with 39.4 ± 2.3 ng/ml. Furthermore, the mice treated with hemin presented a decrease of MPO concentration with both hemin doses. In the TNBS+Hemin5 group was registered 36 ± 3.2 ng/ml of MPO, whereas in the TNBS+Hemin10 group was registered a lower MPO activity with 16.8 ± 3.8 ng/ml (p<0.001). A dose dependent effect was identified in the decrease of MPO after hemin treatment, however a statistical significance difference was only identified in the highest dose (p<0.001, compared with TNBS group). The hemin10 and ethanol groups presented a significantly decrease of MPO activity to around 1.8 ± 0.1 ng/ml and 5.8 ± 0.9 ng/ml of MPO, respectively.

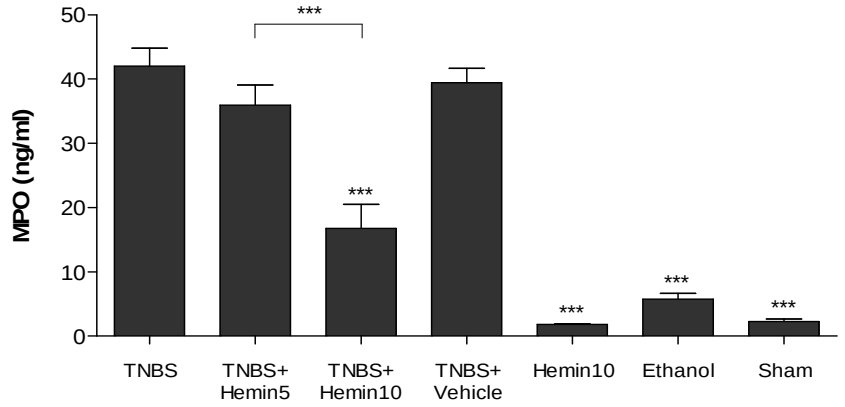

FIGURE 76. Effect of hemin treatment on MPO activity in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with TNBS group or between groups.

MEASUREMENT OF CYTOKINES

The pro-inflammatory cytokines were increased in the TNBS-induced colitis (FIGURE 77 AND 78). So, the TNBS group revealed a significant increase of TNF-α compared with sham group (253.2 ± 31.4 vs 11.2 ± 0.2 pg/ml, p<0.001), as well as IL-1β compared with sham group (263.9 ± 25 vs 12.7 ± 0.1 pg/ml, p<0.001). The TNBS+Vehicle group also revealed an increased concentration of TNF-α (258.5 ± 14.3 pg/ml) and IL-1β (264.8 ± 15.6 pg/ml). After hemin treatment, the mice exhibited a decrease of these pro-inflammatory cytokines with a dose-dependent effect (p<0.001, for the highest dose compared with TNBS group). In detail, the TNBS+Hemin5 group presented 215.6 ± 17.7 pg/ml of TNF-α and 209 ± 29.1 pg/ml of IL-1β, whereas the TNBS+Hemin10 group had a decrease of these cytokines with 85.4 ± 14.9 pg/ml of TNF-α and 118.7 ± 23.5 pg/ml of IL-1β, with statistically significant differences. The remaining control groups presented quite similar concentrations of these cytokines with sham group. The hemin10 group had 13.7 ± 1 pg/ml of TNF-α and 15 ± 0.8 pg/ml of IL-1β and the ethanol group had 14.9 ± 2 pg/ml of TNF-α and 16.2 ± 1.6 pg/ml of IL-1β.

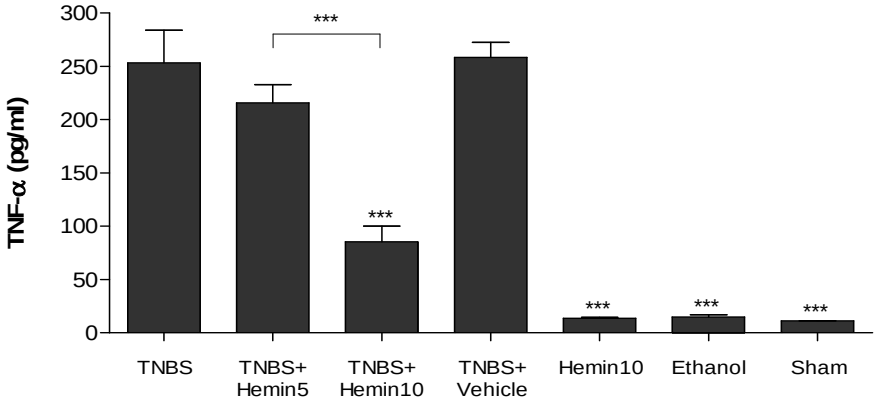

FIGURE 77. Effect of hemin treatment on TNF-α concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with TNBS group or between groups.

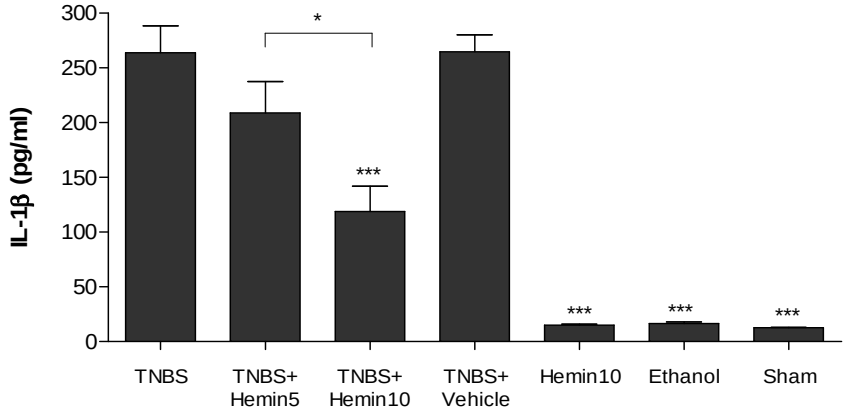

FIGURE 78. Effect of hemin treatment on IL-1β concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.05; *** p<0.001 compared with TNBS group or between groups.

The assessment of IL-10 concentration is crucial to confirm the obtained results with TNF-α and IL-1β measurements (FIGURE 79). Concretely, in the TNBS group was registered a low IL-10 concentration of around 31.4 ± 3.3 pg/ml, as well as the TNBS+Vehicle group with 31.5 ± 0.9 pg/ml. The mice treated with hemin had an increase of IL-10 concentration, with a dose-dependent effect ($p<0.001$, for the highest dose compared with TNBS group). The TNBS+Hemin5 and TNBS+Hemin10 groups had 33.7 ± 3.3 pg/ml and 58.4 ± 5.3 pg/ml, respectively ($p<0.001$). The control groups, as hemin10, ethanol and sham groups, had similar results with 23.6 ± 2.5 pg/ml, 17.3 ± 1.2 pg/ml and 15.7 ± 0.1 pg/ml of IL-10, respectively.

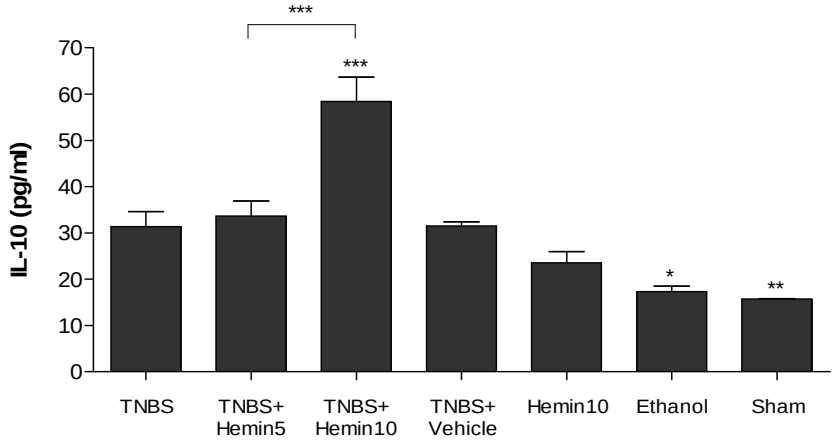

FIGURE 79. Effect of hemin treatment on IL-10 concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared with TNBS group or between groups.

ASSESSMENT OF COLITIS SEVERITY

The macroscopic inspection of cecum, colon and rectum in the TNBS group showed presence of mucosal congestion, hemorrhagic ulcerations and necrosis. After hemin treatment, the macroscopic alterations were the same than were observed in the TNBS group. No macroscopic alterations were observed in the control groups.

The histopathological score was calculated based on the severity and extension of the lesions (TABLE 16). The analysis of partial scores allows evaluating the beneficial effect of hemin treatment and which parameters are affected. Daily treatment with hemin (5 and 10 mg/kg) had an influence in the extension of the lesions, promoting a decrease of the percentage of intestine affected by the most severe lesion. However, the final histopathological score doesn´t allow to identify this influence. Furthermore, the hemin had no effect in the severity of colitis, since this drug was not able to promote a considerable and consistent decrease of severity parameters under evaluation. The partials scores in the TNBS+Vehicle group were quite similar with the TNBS group in

all parameters. The control groups revealed partial scores too low in all evaluated parameters.

TABLE 16. Average (± SEM) of partials scores of histopathological score of TNBS-induced colitis under hemin treatment.

	TNBS	TNBS + HEMIN5	TNBS + HEMIN10	TNBS + VEHICLE	HEMIN10	ETHANOL	SHAM
MUCOSAL LOSS	3.3 ± 0.2	2.9 ± 0.5	3,5 ± 0,3	3.8 ± 0.3	0 ± 0	0.2 ± 0.1	0 ± 0
EPITHELIAL LESIONS	3.4 ± 0.2	4 ± 0	3,9 ± 0,1	4 ± 0	0 ± 0	0.9 ± 0.5	0 ± 0
INFLAMMATION	3.8 ± 0.1	3.5 ± 0	4 ± 0	3.8 ± 0.3	0 ± 0	1.1 ± 0.2	0.1 ± 0.1
EXTENT 1*	3.7 ± 0.1	4 ± 0	4 ± 0	3.8 ± 0.3	0 ± 0	1.3 ± 0.3	0.1 ± 0.1
EXTENT 2**	3 ± 0.2	2 ± 0.3	2,4 ± 0,2	2.7 ± 0.3	0 ± 0	1 ± 0.2	0.1 ± 0.1

Legend: * Extent 1 - Percentage of intestine affected in any manner;
** Extent 2 - Percentage of intestine affected by the most severe lesion.

The histopathological score was evaluated in all experimental groups (FIGURE 80). The TNBS group presented a final score of around 17 ± 0.6, substantially higher than the sham group ($p<0.0001$). Even after the treatment with hemin, the histopathological score remained unchanged at both hemin doses, presenting 16.5 ± 1 and 17.6 ± 0.5 of histopathological score with 5 and 10 mg/kg of hemin, respectively. The results from TNBS+Vehicle (17.8 ± 1) are quite similar with the TNBS and TNBS+Hemin groups. The control groups revealed a histopathological score very low, namely 0 ± 0 for the hemin10 group and 0.2 ± 0.2 for the sham group, except the ethanol group with 4.5 ± 1.2 of final score.

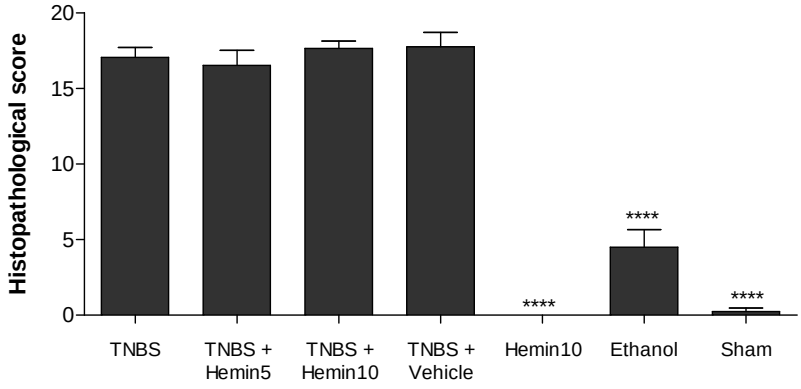

FIGURE 80. Effect of hemin treatment on histopathological score.

Legend: One-way ANOVA and Tukey's post hoc test; **** $p<0.0001$ compared with TNBS group.

Representative images of the histopatological score were analyzed for all experimental groups (FIGURE 81). Briefly, the TNBS group displays diffuse transmural necrosis with

severe hemorrhaging, involving the mucosa, submucosa, muscle layer and serosa, and often associated with peritonitis. Under hemin treatment, similar lesions are seen, without any improvement in its severity. However, it is perceptible a slight influence in the extension of the lesions, but without expression on the histopathological score. The increase of hemin dose has no effect in the histophatological images and, consequently, in the histopathological score. The histopathological images of TNBS+Vehicle group show the same lesions that TNBS group. The ethanol group showed only mild epithelial erosion, and no lesions were observed in the hemin10 and sham groups.

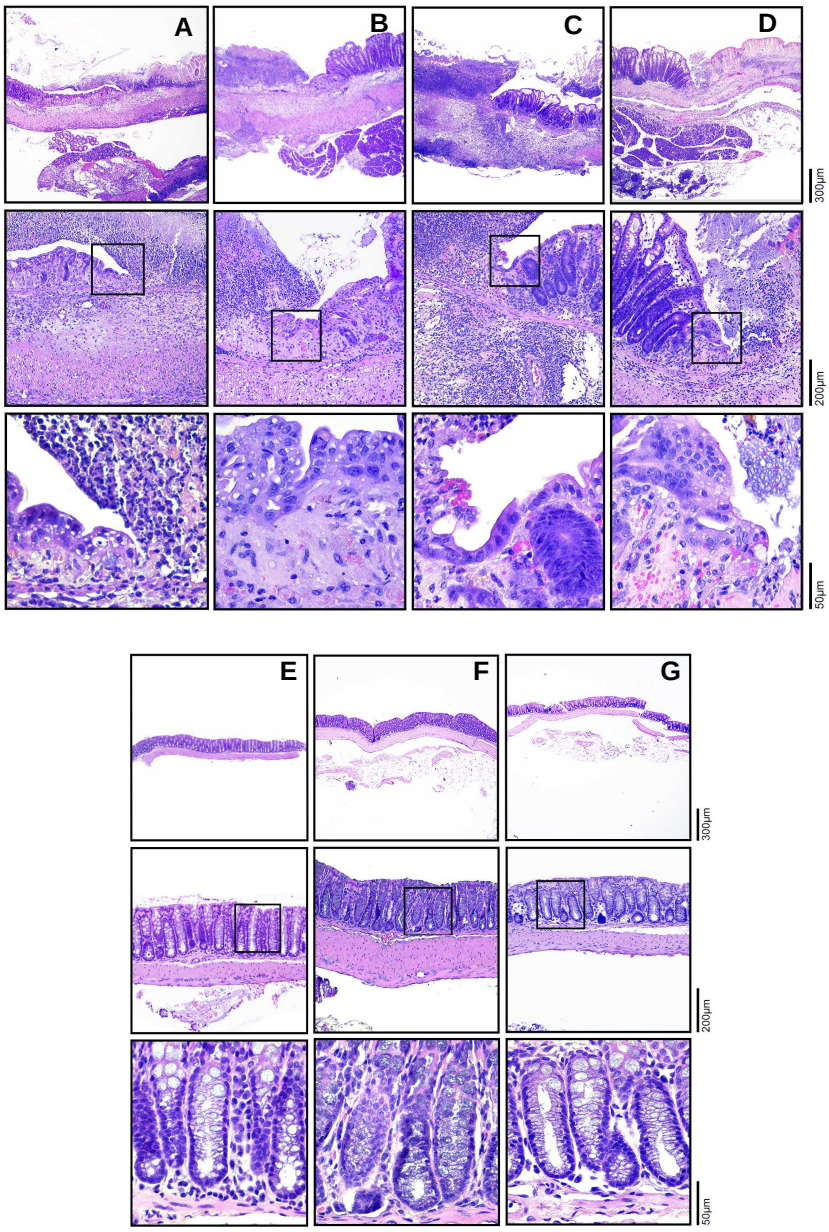

FIGURE 81. Effect of hemin treatment on histopathologic changes in the IBD.

Legend: Each column corresponding with a different experimental group, namely (A) TNBS group, (B) TNBS+Hemin5 group, (C) TNBS+Hemin10 group, (D) TNBS+Vehicle group, (E) Hemin10 group (F) Ethanol group and (G) Sham group.

MORTALITY RATE

The mortality rate was evaluated in the experimental groups as a sign of toxicity (TABLE 17). At the end of the study, the TNBS group presented 31.4% of mortality rate. After these mice were treated with a daily dose of hemin, the mortality rate fell completely for 0%. In this case, the administration of hemin produced a statistically significant decrease of mortality rate. Curiously, TNBS+Vehicle group presented 5% of mortality rate. In the sham group, all mice survived during the study.

TABLE 17. Effect of hemin treatment on mortality rate in the IBD.

		TOTAL	DEATHS	SURVIVORS	P-VALUE
		n (%)	n (%)	n (%)	
EXPERIMENTAL GROUPS	**TNBS**	35 (100%)	11 (31.4%)	24 (68.6%)	<0.001
	TNBS + Hemin5	35 (100%)	0 (0%)	35 (100%)	
	TNBS + Hemin10	35 (100%)	0 (0%)	35 (100%)	
	TNBS + Vehicle	20 (100%)	1 (5%)	19 (95%)	
	Sham	20 (0%)	0 (0%)	20 (100%)	

Legend: Chi-square test; *** p<0.001 compared between groups.

2. DISCUSSION OF RESULTS

In this study, we also assessed the influence of hemin in the TNBS-induced colitis model. Hemin is well known as an inducer of HO-1 [Guan et al., 2009; Hualin et al., 2012]. HO-1 is a rate-limiting enzyme for heme metabolism and is capable of producing anti-oxidant and anti-inflammatory products, such as biliverdin/bilirubin and CO [Willis, Moore & Willoughby, 2000; Maines, 2005; Ryter et al., 2006]. Biliverdin/bilirubin can scavenge peroxyl radicals in vitro as effectively as α-tocopherol, which is regarded as the most potent antioxidant against lipid peroxidation [Stocker et al., 1987], whereas CO can inhibit the production of pro-inflammatory cytokines in macrophages, such as TNF-α, IL-1β, and macrophage inflammatory protein-1, through modulation of MAPK activation [Otterbein et al., 2000]. Nowadays, hemin is already used in the management of porphyria attacks, particularly in acute intermittent porphyyria [Bissell & Wang, 2015]. However, many studies have reported the protective effect of hemin via HO-1 induction in various animal models, as hippocampal injury, renal fibrosis, cardiac ischemia/reperfusion, lung injury [Hangaishi et al., 2000; Guan et al., 2009; Correa-

Costa et al., 2010; Hualin et al., 2012]. HO-1 expression can confer cytoprotective, antiapoptotic and anti-inflammatory properties, suggesting thus that HO-1 can be a possible therapeutic target in several kinds of gastrointestinal diseases [Naito et al., 2011]. Some studies have been developed in DSS and TNBS colitis models to evaluate the effect of HO inducers on the management of IBD [Naito et al., 2011]. However, there is only one study testing the influence of hemin in the TNBS-induced colitis model [Wang et al., 2001]. Thus, the biological significance of HO-1 up-regulation by hemin in this colitis model remains to be not fully elucidated as well as the mechanisms underlying HO-1 activity [Naito et al., 2011].

Mice received daily IP injection of hemin, promoting an improvement of some clinical symptoms/signs such as a moderate edema of the anus and mild morbidity comparing with non-treated mice. Their weight change was also monitored and expressed as percentage of original body weight. Thus, we observed that hemin treatment significantly attenuated the weight loss caused by TNBS-induced colitis, suggesting that enhancement of HO-1 functional activity is implicated in controlling intestinal inflammation. However, no clear dose-response relationship was observed, since this beneficial effect was only observed in the lower hemin dose. The increase of hemin dose did not promote a better beneficial effect in the changes of body weight. We have not a clear explanation for this finding yet. Nevertheless, the findings from other studies do not also allow to compare with our results, since do not evaluate the influence of hemin treatment in the changes of body weight.

In this study, the length of colon was also assessed as a marker of tissue integrity. Since the variations in the length of any part of the colon may lead to variety of acute and chronic pathological conditions [Nayak et al., 2012], it is truly relevant measuring this parameter. Nevertheless, colon length was never evaluated under these conditions. Thus, we registered that shortening of the colon due to TNBS-induced colitis was influenced by hemin treatment at both administered doses. However, this evident tendency did not represent a statistically significant difference.

The intensity of hemorrhagic focus was evaluated by the fecal hemoglobin concentration. We observed that hemin treatment produced a considerable influence in the intensity of hemorrhagic focus, since the fecal hemoglobin concentration significantly decreased in a dose dependent manner after a daily dose of hemin. This finding suggests that hemin-treated mice presented an amelioration of the active inflammatory disease identified in mice with TNBS-induced colitis [Hirata et al., 2007;

Mooiweer et al., 2014]. The influence of hemin treatment in fecal hemoglobin was measured for the first time in this study.

Regarding ALP concentration, the data were consistent with the other results of the evaluated parameters. In this study, we observed that hemin treatment was able to decrease the elevated level of ALP on blood in a dose dependent manner, demonstrating an anti-inflammatory potential by HO-1 induction. This data is consistent with other previous findings, where anti-inflammatory drugs are able to decrease ALP level [Kumar et al., 2014]. In both hemin doses, the mice presented serum ALP values similar with control groups, like hemin10 and ethanol groups.

MPO activity was used as an index of quantitative inflammation and neutrophil infiltration in tissues [Rachmilewitz, Simon & Schwarts, 1989]. Hemin administration was able to attenuate neutrophil infiltration and inflammation with both hemin doses. Indeed, after hemin treatment, the decrease of MPO was registered in a dose dependent manner. However, a statistical significance difference was only identified in the highest hemin dose, where MPO decreased around 60% comparing with non-treated mice. These results suggest that generation of endogenous HO-1 may possibly protect colonic tissue against inflammation. Our findings are also consistent with other studies, where hemin or other HO-1 modulators are used in the experimental colitis model [Wang et al., 2001; Jun et al., 2006; Varga et al., 2007].

TNF-α and IL-1β are pro-inflammatory cytokines that can become dysregulated under pathological condition of inflammation [Műzes et al., 2012]. Actually, mice with TNBS-induced colitis exhibited a significant increase of TNF-α and IL-1β levels, at the end of experimental study. Nevertheless, hemin treatment significantly decreased the level of these cytokines in a dose-dependent manner. Furthermore, IL-10 was also evaluated in this study as an anti-inflammatory cytokine. Hemin treated-mice also revealed a significant increase in the concentration of this cytokine in a dose dependent manner as expected, confirming the obtained results with TNF-α and IL-1β measurements. These findings allow concluding that HO-1 induction by hemin treatment can produce anti-inflammatory effect, suppressing the production of these pro-inflammatory cytokines, too. Moreover, HO-1 induction may still confer a protective effect, since is able to increase anti-inflammatory cytokines [Zhang et al., 2014].

To assess whether hemin affected TNBS-induced colon damage, there was analyzed the colon morphology and quantified its colonic damage. In our study, we observed that histological score of hemin treated mice was similar with those of non-treated mice,

suggesting that hemin treatment possibly has no effect on the severity and/or extension of the intestinal lesion inducted by disease. However, the histological features of the lesions suggest that hemin was able to decrease the extension of the lesions, suggesting a beneficial effect in the inflammation of tissue due to HO-1 induction [Zhang et al., 2014].

Since IBD can promote extra-intestinal manifestations, the periodic evaluation of renal and hepatic functions should be emphasized [Larsen et al., 2010; Oikonomou et al., 2011; Rojas-Feria et al., 2013]. Single daily dose of hemin significantly recovered the renal and hepatic functions to normal levels, similarly to control group, suggesting a beneficial effect in the extra-intestinal manifestations due to metabolic and physiologic changes induced by the IBD. We also can conclude that hemin does not promote renal and/or hepatic changes as adverse drug reaction, in this experimental colitis model

Mortality rate was also evaluated during the experimental study. We observed that hemin treatment was able to reduce the mortality rate. Actually, mice with TNBS-induced colitis presented around 31.4% of deaths, whereas hemin treated mice survived, presenting 0% of deaths. This result suggests that hemin treatment considerably reduces the toxicity associated with the illness, producing a statistically significant decrease of mortality rate.

Until nowadays, there is only one published paper relating the hemin treatment with TNBS-induced colitis and, therefore, several parameters had never been evaluated under these conditions, such as weight loss, colon length, fecal hemoglobin, TNF-α, IL-1β, IL-10, histological features and extra-intestinal symptoms. Thus, this study was innovative, since some new parameters were measured for the first time. Briefly, hemin treatment had a positive influence in the development of experimental colitis, but not in all evaluated parameters. This drug promoted a reduction of fecal hemoglobin, ALP, MPO and pro-inflammatory cytokines (TNF-α and IL-1β). Hemin was also able to increase the anti-inflammatory cytokine (IL-10), as well as regulated of renal and hepatic functions. In sum, hemin treatment decrease the severity of the disease, since is able to improve several inflammation markers, suggesting an anti-inflammatory effect of hemin by HO-1 induction. Hemin also decreases the extension of the intestinal lesions, corroborated by histological images. These findings suggest that hemin significantly inhibits acute inflammatory response in the experimental colitis.

Regarding future prospects, we believe the findings of our study should be warranted by large number of experimental and prospective clinical studies involving these drugs.

Future studies are needed to clarify the exact mechanisms beside the anti-inflammatory property of these drugs, as well as the full pharmacological profile and selectivity of these agents awaits further evaluation [Cuzzocrea et al., 2004; Dugo et al., 2005; Whittle et al., 2006].

CHAPTER 9 – DISCUSSION AND CONCLUSION

IBD is characterized by the presence of one or more genetically determined defects, resulting in a mucosal immune system that overreacts to normal constituents of the mucosal microflora. Therefore, IBD is produced through a final common immunopathologic pathway comprised of a Th_1 T cell response mediated inflammation (CD) or a Th_2 T cell response mediated inflammation (UC). This implies that, regardless of the nature of the fundamental defects present, one could potentially treat IBD with therapy that addresses an essential element of the final common pathway [Strober et al., 2007].

Within this context, existing conventional treatments such as corticosteroids, mesalamine, and immunosuppressants aim broadly to block downstream inflammatory events such as the secretion of cytokines and the elaboration of immunocytes and neutrophils, regardless of the nature of the underlying T cell response that generated these events. These agents have sustained treatment of IBD for many years despite shortcomings and toxicities [Hanauer et al., 2002; Adelman, Sandrock & Panzara, 2005; Feagan et al., 2005; Sandborn et al., 2005; Rutgeerts et al., 2005; Blonski & Lichtenstein, 2006]. For many years there have been numerous efforts to find a new effective method that would allow controlling specifically unwanted immune responses that occur during autoimmune reaction [Szczepanik et al., 2012]. Emerging treatments are evaluating the hierarchy of the inflammatory cytokine effect, by targeting IL-12/IL-23, IFN-γ, or IL-6 or by restoring IL-10 levels [Ito et al., 2004; Mannon et al., 2004; Hommes et al., 2006; Braat et al., 2006]. These treatments has been able to induce the reduction the inflammatory mediators that cause tissue damage, maintenance of the active inflammation, and exacerbation of dysfunction of the epithelial barrier, ultimately leading to control of the second secondary effects [Strober et al., 2007]. The future treatment options for IBD will not only be extended by simultaneously targeting several pathogenetic players through combinations of existing strategies, but also by the introduction of drugs with completely new targets [Zundler & Neurath, 2015]. Thus, the assessment of the influence of a set of new drugs in IBD, like EPO, TDZD-8 and hemin, through of a TNBS-induced colitis model in mice, can facilitate a more effective and selective treatment than the currently known.

A steadily increasing number of experimental animal models have been used for preclinical studies, presenting some clinical manifestations similar to those observed in

human IBD. These animal models have recently been developed and have contributed greatly to important advances in our current understanding of the immunological, pathological, and physiolgical features of chronic intestinal inflammation [Hibi et al., 2002; Murphy, 2006]. TNBS-induced colitis model is one of the animal models of experimental colitis that has been studied and has so far produced much information [Borm & Bouma, 2004]. These animal model is an efficient method, since can mimic the pattern of inflammation with human IBD, producing a rapid, reliable and reproducible disease [Hibi et al., 2002; Linden et al., 2003; Randhawa et al., 2014]. However, since the protocols of the TNBS-induced colitis model are not standardized, the degree of disease and time required to produce the injury may vary between laboratories [Murphy, 2006; Wirtz et al., 2007; Qin et al., 2011].

In our study, the development of a TNBS-induced colitis model was essencial, as well as the standardization and validation of the induction method. Several conditions were tested to achieve a standarded induction method, such as the dose of TNBS, the depth of TNBS administration, the time point for model evaluation, and the concentration of ethanol (as TNBS vehicle). Although IBD is a chronic inflammatory disease, we would like to implement an acute intestinal inflammation model, where proof of concept is more easily tested. During the induction method were evaluated and monitored several parameters, such as clinical symptoms/signs, biochemical markers, histopathological analysis and concentration of pro- and anti-inflammatory cytokines. All parameters under evaluation corroborated that the damage became maximal at day 4 after the induction. So, TNBS-induced colitis was developed in 4 days, providing an acute intestinal inflammation model. Beyond the validation of an animal model of TNBS-induced colitis in mice, the additional findings of this study was evaluating if all tested drugs significantly inhibit acute inflammatory response in this experimental colitis model.

EPO is one of these drugs that we tested in our TNBS-induced colitis model. EPO is currently being used in the therapy of patients with chronic renal failure suffering from anemia, with an initial dose of 50 to 100 IU/kg IV or SC 3 times weekly [Cody et al., 2001; Nairz et al., 2011; Willis et al., 2012; Jelkmann, 2012]. However, EPO also has extrahematopoietic properties, which may be of therapeutic relevance in inflammatory disease [Brines & Cerami, 2005; Jelkmann, 2007; Nairz et al., 2011]. In fact, EPO decreases the production of NF-κB inducible immune mediators, thus limiting tissue damage and ameliorating disease severity [Nairz et al., 2011].

After 4 days of daily EPO treatment, our findings clearly demonstrate that EPO exerts potent anti-inflammatory effects on this specific experimental colitis model. EPO treatment presented a beneficial effect in the development of experimental colitis in all evaluated parameters, thus reducing its severity and extension. More precisely, EPO reduced the percentage of weight loss, fecal hemoglobin, ALP, MPO, pro-inflammatory cytokines (TNF-α and IL-1β) and histopathological score. On the other hand, EPO also increased anti-inflammatory cytokine (IL-10), as well as regulated the renal and hepatic functions. Furthermore, in our study, we used two different doses of EPO, namely 500 IU/Kg and 1000 IU/Kg, in the TNBS-induced colitis model, which are considerably lower than usually used in these experimental studies. Since it is known that EPO treatment can affect the hematocrit level and promote cardiovascular adverse effects, it is important to use the lowest possible doses. Moreover, the administrated doses are relevant for the clinical practice in a context of translational pharmacology. Nevertheless, we evaluated the hematocrit level after EPO treatment and the results were normal. This data indicate that EPO significantly inhibits the acute inflammatory response in the experimental colitis, without adverse events related to blood viscosity.

TDZD-8 is another new drug that we tested in the TNBS-induced colitis model. TDZD-8 is the first non-ATP competitive GSK-3β inhibitors with highly effectivity and selectivity [Martinez et al., 2002a; Dugo et al., 2007]. The capacity of TDZD to suppress the expression of inflammatory cytokines and present tissue protective action by GSK-3β inhibiton suggests that this agent may be effective in the treatment of several inflammatory diseases [Luna-Medina et al., 2005]. In IBD, TDZD-8 was only tested once in 2006, where promoted a reduction of the colonic inflammation, tissue injury and a reduced decline in body weight [Whittle et al., 2006]. However, many GSK-3β inhibitors have already been tested in experimental colitis models until nowadays.

In our study, mice were treated with a daily TDZD-8 dose during 4 days. TDZD-8 treatment was able to modulate the development of experimental colitis. Particularly, TDZD-8 promoted a reduction of fecal hemoglobin, ALP, MPO and pro-inflammatory cytokines (TNF-α and IL-1β). Furthermore, it was also able to increase the expression of anti-inflammatory cytokines (IL-10), as well as regulated the renal and hepatic functions. According to histopathological analysis, TDZD-8 treatment only produced a slight decrease in the extension of the disease. However, the obtained results with the cytokines concentration also suggest and confirm its beneficial effect in the reduction of the severity of the disease. These data indicate that TDZD-8 also significantly inhibits the acute inflammatory response in the experimental colitis.

Hemin was also tested in the TNBS-induced colitis model. Hemin is well known as a HO-1 inducer and, currently, it is commercialized for the treatment of acute porphyria [Naito et al., 2011; Bissell & Wang, 2015]. Additionaly, HO-1 expression can also confer cytoprotective, antiapoptotic and anti-inflammatory properties through its production of biliverdin/bilirubin and CO, suggesting thus that HO-1 can be a possible therapeutic target in several kinds of gastrointestinal diseases [Naito et al., 2011]. In IBD, HO-1 plays a protective role in the colonic damage induced by TNBS enema [Wang et al., 2001].

Mice were treated with a daily dose of hemin. At the end of experimental period, hemin treatment presented a beneficial influence in the development of experimental colitis, decreasing its severity and extension. Hemin promoted a reduction of fecal hemoglobin, ALP, MPO and pro-inflammatory cytokines (TNF-α and IL-1β). It was also able to increase the anti-inflammatory cytokine (IL-10), as well as regulated of renal and hepatic functions. Furthermore, hemin treatment produced a statistically significant decrease of mortality rate to 0% of deaths, promoting similar results to those obtained with healthy mice. These findings suggest that hemin also significantly inhibits acute inflammatory response in the experimental colitis, totally reducing the mortality associated with the disease.

That way, our work allows contributing for the scientific knowledge about the impact of these drugs in the management of IBD, helping the scientific community to decide about its relevance to treat this disease. Therefore, this work is also innovative, presenting an additional contribution for the scientific knowledge, since:

- Mice with TNBS-induced colitis (and without any other comorbidity) were never subjected to an EPO treatment and we assess that in this study;
- We also measured, for the first time, the influence of EPO in the hematocrit level to evaluate the risk of cardiovascular events, as well as its beneficial effect in the IL-10 expression;
- In the hemin treatment, we implemented a daily administration of hemin, monitoring TNF-α, IL-1β and IL-10, which had never been done;
- Bloody diarrhea is the hallmark of IBD, the determination of fecal hemoglobin can be useful in the detection and quantification of lesions accompanied by bleeding [Hirata et al., 2007; Kornbluth & Sachar, 2010; Jagtap et al., 2011]. However, this biological marker had never been measured in this animal model after the propused treatments. In this study, we measured the influence of these new drugs in the reduction of fecal hemoglobin;

- Finally, many parameters never evaluated before were were monitored in this study. For example, the well-documented extra-intestinal manifestations and complications of IBD, as well as the possible renal and hepatic side effects of pharmacotherapy, emphasize the need for periodic evaluation of renal and hepatic functions [Larsen et al., 2010; Oikonomou et al., 2011; Rojas-Feria et al., 2013]. The assessment of the influence of these new drugs in the extra-intestinal manifestations is truly relevant, since had never been investigated. For the first time, we monitored the effect of these new pharmacological approaches in the renal and hepatic functions.

These findings may provide insight into these potential therapeutic approaches to ameriolate the inflammation and to minimize the morbidity and mortality associated with IBD. However, as George Bernard Shaw once said, "*Science never solves a problem without creating ten more*". So, although the experimental data reported are impressive, comparison of these results with others publications suggests a slight variability in the results. This is quite expected in preclinical studies, due to several conditions, such as the type of induction method, administrated doses, treatment period, which difficult the translation of the data for the clinical practice. Careful attention may be required to translate animal studies to clinical settings by ensuring that both safety and efficacy can be modeled [Mizoguchi & Mizoguchi, 2010]. Thus, because there are much more to research and the science is a machine that never stops, the need to further future research projects to confirm the relevance of these drugs for IBD is a reality and, consequently, their application to the clinical situation. In any case, robust clinical trials proving such potential benefits will be required before the use of these drugs for the management of IBD can be recommended. Inclusively, some other interesting and promising examples of conceptually new strategies in IBD treatment include the fortification of impaired epithelial barrier, stimulation of innate immune processes via TLR agonists or the modulation of the intestinal flora in IBD [Neurath, 2014b]. It will be interesting to investigate if these new drugs can eventually produce effect in one of these mechanisms. Furthermore, future studies are also needed to clarify the relevance of some changes in the drug delivery system could promote in the therapeutic effect, since topical application by rectal administration is eventually a good strategy to achieve a more effective and selective treatment with less adverse reactions.

In conclusion, this study allowed exploring the effect of these new drugs in the development of IBD, as well as their influence on response mechanisms to intestinal injury. Moreover, it represents a truly innovative contribution to the pharmacological

treatment of IBD, identifying pro- and anti-inflammatory associated responses that can modulate the establishment and development of the disease, as well as new therapeutic targets that allow decrease or attenuate the IBD and contribute to the enrichment of the therapeutic opportunities of this disease.

CHAPTER 10 - REFERENCES

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ANNEXES

ANNEXE 1 - CROHN´S DISEASE ACTIVITY INDEX

In 1972, the CDAI (TABLE 18) was first used in clinical trias and was the instrument of evaluation in two large trials that resulted from the National Cooperative Crohn´s Disease Study [Summers et al., 1979; Singleton et al., 1979]. Today, the CDAI remains widely used in clinical research. A random overview of important published clinical trials of the past four years [Greenberg et al., 1994; Rutgeerts et al., 1994; Feagan et al., 1994; Feagan et al., 1995; Targan et al., 1997; Stack et al., 1997] reveals that more than 25 years after the CDAI was developed, it remains a popular instrument of evaluation of CD.

The CDAI is a disease-specific index, ie its use is restricted to CD and it is not applicable to IBD in general. The CDA attempts to evaluate the activity of CD in several domains, each of which evaluates a specific aspect of CD. The CDAI sums up the weighted value of each item of the domain and quantifies the global disease severity in a final numerical score [Best, Becktel, Singleton & Kern, 1976].

A value less than 150 was defined as quiescent CD (ie, nonactive disease, clinical remission), whereas values greater that 450 were associated with extremely severe disease [Best et al., 1976].

TABLE 18. Crohn´s Disease Activity Index and their weights.

Item (daily sum per week)	Weight
Number of liquid or very soft stools	2
Abdominal pain score in one week (rating: 0-3)	5
General well-being (rating: 1-4)	7
Sum of findings per week:	20
Arthritis/arthralgia Mucocutaneous lesions (eg, erythema nodosum aphthous ulcers) Iritis/uveitis Anal disease (fissure, fistula, etc) External fistula (enterocutaneous/vesicle/vaginal, etc) Fever >37.8°C	
Antidiarrheal use (eg, diphenoxylate hydrochloride)	30
Abdominal mass (none = 0, equivocal = 2, present = 5)	10
47 minus hematocrit (males) or 42 minus hematocrit (females)	6
100 x (1 – [body weight divided by standard weight])	1