ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

IPL

Instituto Politécnico de Lisboa

Escola Superior de Tecnologia da Saúde de Lisboa

Efficacy and safety of the carbamylated erythropoietin in an animal model of chronic TNBS-induced colitis

Dissertation specially performed to obtain the master's degree in Pharmacy, specialty of Pharmacology and Advanced Pharmacotherapy.

Student:

Carolina Rodrigues Alípio

Supervisors:

Professora Doutora Vanessa Mateus

Professora Dr.ª Inês Silva

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ABSTRACT

Introduction: Inflammatory bowel disease is an immune-mediated chronic inflammatory disorder of the gastrointestinal tract. It is characterized by abdominal pain, bloody diarrhea and influx of neutrophils and macrophages. The current pharmacological therapies present some concerns and new approaches are required. Erythropoietin is a hormone that promotes proliferation of erythrocytes. It also has non-erythropoietic effects, such as anti-inflammatory effect. Carbamylated erythropoietin is a modified molecule of erythropoietin with non-hematopoietic effects. **Objective**: To evaluate the potential anti-inflammatory effect of carbamylated erythropoietin in chronic animal models of rodents in IBD. **Materials and Methods:** Carbamylated erythropoietin was synthesized then, an experimental model of IBD was induced by the rectal administration of multiple doses of TNBS. The to evaluate the effect and safety of cEPO the clinical symptoms, biomarkers and the percentage of haematocrit were measured. **Results:** The carbamylation rate was approximately 99%. cEPO treatment presented anti-inflammatory effect confirmed by the decrease of concentrations on pro inflammatory cytokine (44.30 ± 4.0 pg/ml, *** $p<0.001$). The haematocrit was also performed and presented a similar result to the sham group. **Conclusion:** The cEPO 1000 UI/Kg molecule could have a better efficacy and safety in the treatment of chronic TNBS-induced colitis model.

KEY-WORDS: Inflammatory bowel disease, Carbamylated erythropoietin, Chronic TNBS-induced colitis, Inflammation.

RESUMO

Introdução: A doença inflamatória intestinal é uma doença inflamatória crónica imuno-mediada do trato gastrointestinal. Caracteriza-se por dor abdominal, diarreia com sangue e influxo de neutrófilos e macrófagos. As atuais terapêuticas farmacológicas apresentam algumas limitações pelo que, são necessárias novas abordagens. A eritropoietina é uma hormona que promove a proliferação dos eritrócitos. Tem também efeitos não eritropoéticos, tais como o efeito anti-inflamatório. A eritropoietina carbamilada é uma molécula modificada de eritropoietina com efeitos não hematopoiéticos. **Objetivo:** Avaliar o potencial efeito anti-inflamatório da eritropoietina carbamilada em modelos animais de roedores na doença inflamatória intestinal crónica. **Materiais e Métodos:** A eritropoietina carbamilada foi sintetizada, depois, foi feita uma administração retal com doses múltiplas de TNBS. Para avaliar o efeito e segurança da eritropoietina carbamilada foram avaliados os sintomas clínicos, biomarcadores e a percentagem de hematócrito. **Resultados:** A taxa de carbamilação foi de aproximadamente 99%. O tratamento com eritropoietina carbamilada apresentou efeito anti-inflamatório confirmado pela diminuição da concentração de citocinas pró-inflamatórias (44.30 ± 4.0 pg/ml, *** $p<0.001$). O hematócrito também foi avaliado e apresentou um resultado semelhante ao do grupo controlo. **Conclusão:** A eritropoietina carbamilada de 1000 UI/Kg poderá ter uma melhor eficácia e segurança no tratamento do modelo de colite crónica induzida por TNBS.

PALAVRAS-CHAVE: Doença inflamatória intestinal, Eritropoietina carbamilada, Colite crónica induzida por TNBS, Inflamação.

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ACRONYMS AND ABBREVIATIONS

ALP – Alkaline phosphatase

ALT - Alanine aminotransaminase

AZA - Azathioprine

CD - Chron's disease

cEPO – Carbamylated erythropoietin

CTE - Computed tomography enterography

EIMs – Extraintestinal manifestations

EPO – Erythropoietin

EPOR – Erythropoietin receptor

GIT - Gastrointestinal tract

HT - Haematocrit

IBD - Inflammatory bowel disease

IFN- γ - Interferon-gamma

IL – Interleukin

IP – Intraperitoneal

IR - Intrarectal

JAK 2 - Janus kinase-2

MT - mercaptopurine

MRE - Magnetic resonance enterography

MTX – Methotrexate

NF-κB - Nuclear factor kappa B

mL – Milliliter

µL – Microliter

MPO - Myeloperoxidase

NOD2 - Nucleotide-binding oligomerization domain 2

PGs - Prostaglandins

RBCs – Red blood cells

rHuEPO **–** Recombinant human EPO

STAT **-** Signal transducer and activator of transcription

TGF – Transforming growth factor

Th – T helper

TNBS - 2,4,6-Trinitrobenzene sulfonic acid

TNF-α - Tumor necrosis factor alpha

UC - Ulcerative colitis

INTRODUCTION

This dissertation is specially performed to obtain the master's degree in Pharmacy, specialty of Pharmacology and Advanced Pharmacotherapy. Its development was developed between the school year 2021-2022 at Escola Superior de Tecnologia da Saúde de Lisboa, under the guidance of Professors Vanessa Mateus and Inês Silva.

Inflammatory bowel disease (IBD) is a complex intestinal disorder characterized by chronic inflammation of the gastrointestinal tract (GIT) (1). The incidence and prevalence of IBD markedly increased over the second half of the 20th century (2,3). Worldwide incidence of IBD is rising in developed and developing countries (4,5). IBD is based on chronic inflammatory diseases of the gastrointestinal tract, which include Ulcerative Colitis (UC) and Chron's Disease (CD) (5). These chronic inflammatory conditions are disorders of unknown cause (5). IBD can be associated with significant morbidity, and before the diagnosis of the disease, patients may experience symptoms for years (5,6). Symptoms of IBD can highly variable by an immune dysregulation (4,5). Though the cause is unknown, both genetics and environment factors are involved into this immune dysregulation (4,5). The production and release of cytokines, proteolytic enzymes, and free radicals results in inflammation (7). Pharmaceuticals approaches commonly used in IBD management presents potentially side effects and frequently do not provide sufficient disease control (5). Also, these therapies aim to induce and maintain the patients in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism (5).

Animal model studies mimic the pathogenesis of IBD in humans and allow testing of new pharmacological approaches (8). A variety of animal models have been utilized to provide more knowledge and develop more therapeutic approaches (9). Three main methods are used to induce IBD disease: induction by dextran sulfate sodium, oxazolone and induction by 2,4,6-trinitrobenzenesulfonic acid (TNBS).

Thus, is still an incurable, life-long disease that warrants better understanding and more efficient therapy (9). There are some new drugs that can modulate some important cellular pathways in the establishment and development of inflammation such erythropoietin (10). Erythropoietin (EPO) is a hormone produced mainly by the kidney that regulates the production of red blood cells (11). It has been used in several studies involving the inflammatory process, but has shown severe adverse effects (10).

Carbamylated erythropoietin is a modified molecule of EPO that only binds to heterodimeric of EPO receptor (EPOR) maintaining its beneficial effects (12). This erythropoietin derivate allows the blocking of the erythropoietic effects while maintaining the protective effects so, the evaluation of its influence in inflammatory bowel disease is relevant (12). Hence, it could be a potential molecule to reverse IBD disease through its anti-inflammatory properties (12).

The aim of this dissertation is to study the effect of carbamylated erythropoietin in an experimental chronic model of IBD in rodents. Thus, we developed the work that is presented in this dissertation. The exhibition of the dissertation 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, the dissertation is organized as follows:
Chapter I: theorical framework: will describe IBD disease; its characteristics; pharmacological approaches; preclinical trials; animal models of disease and preclinical approaches under research.

Chapter II: aim: main aim e specifical aims

Chapter III: materials and methods: chemical and reagents; carbamylated erythropoietin synthesis; determination of carbamylation rate; animal and maintenance for the vivo experiments; induction of experimental colitis; experimental groups; monitoring of clinical signs; measurements of cytokines; biochemical markers; histopathological analysis; macroscopic assessment of colitis severity and statistical analysis.

Chapter IV: results and discussion: synthesis of carbamylated erythropoietin; evaluate of the safety and safety of the molecule, analysis of the results of carbamylated erythropoietin compared to the results obtained for erythropoietin.

Chapter V: conclusion

Chapter VII: references

CHAPTER 1 – THEORICAL FRAMEWORK

1 - CHARACTERIZATION OF INFLAMMATORY BOWEL DISEASE

IBD is a complex intestinal disorder characterized by chronic inflammation of the GIT (1). The inflammatory process is a body reaction to injury or irritation (1). IBD is characterized based on the location and phenotype of the disease. (6) There is more than one phenotype associated with IBD consequently, the two subtypes of IBD are defined as chronic inflammation of the intestine as well (1). Crohn's disease (CD) and ulcerative colitis (UC) are the most common types of IBD (1). Both conditions cause chronic inflammation that affects the gastrointestinal (GI) tract (1). Chronic inflammation leads to similar histopathological changes regardless of the stimulus that causes them (1). Due to the similarities between the UC and CD, inflammation may occur in an isolated form or spread to the entire gastrointestinal tract (1). The major difference between these two conditions is the area where the inflammation takes place within the digestive system as well as the degree toward which the deeper layers of the gut are involved in the inflammatory process (1). In a less complex way, in CD there are healthy parts of the intestine mixed in with inflamed areas while in UC there is continuous inflammation of the colon since beginning in the rectum with the eventuality of spreading to rest of the colon (1,8). Ulcerative colitis (UC) is more prevalent than Chron's disease (CD) (4,13). UC is characterized by mucosal ulceration and a spontaneous and recurrent inflammation damages confined to the large intestine and its terminal part, like the colon and the rectum, not affecting the deeper layers of intestinal tissue (4,13). CD can affect any part of GIT from mouth to anus, although 50% of the damages occur mostly in the terminal ileum (4). Also, in this condition is inherent the penetration through the intestinal wall by enveloping the connective tissue surrounding the intestine (1,4). This may lead to the narrowing of the intestine more commonly known as a stricture, abnormal connections of the bowel to other structures usually known as fistula, and abscess formation (4).

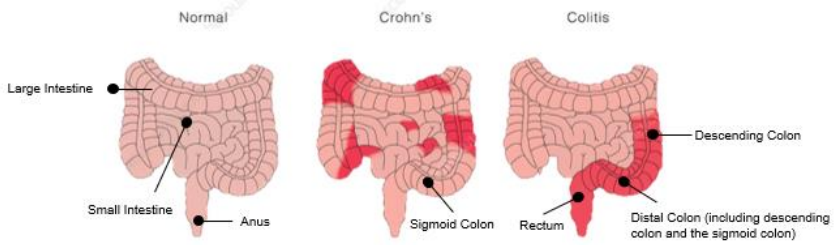

Figure 1 - Differences between the intestine without disease and with UC and CD (6).

1.1 - CLASSIFICATION

The cause of IBD is idiopathic which means it has an unknown origin (14). To standardize the classification of this disease, the Montreal classification was created for CD (Table 1) (6). The Montreal classification system describes the extent of colonic involvement and is most often used to objectively classify disease extent and severity and can improve guide clinical management (15). This classification includes factors such as the age at diagnosis, the disease location and behavior as the dominant phenotypic features, and last, the modifier factor named “P” (6).

Table 1 - Montreal Classification of Crohn Disease (6).

Age at diagnosis (years)	A1: ≤ 16
	A2: 17- 40
	A3: > 40
Location	L1: Ileal
	L2: Colonic
	L3: Ileocolonic
	L4: Isolated upper GIT disease *
Behavior	B1: Non-stricturing/nonpenetrating
	B2: Stricturing
	B3: Penetrating
	P: Perianal disease**

Legend: *Modifier factor which can be extended to localization when there is concomitant upper GIT disease; ** considered when the concomitant perianal disease is also present.

IBD can present at any age with the peak incidence being in adolescence and early adulthood but usually appears early in life before the age of 15 and often with severe disease (4). Approximately 25% of these patients are diagnosed before the age of 18 years (3). The disease often begins in adolescence and approximately 25% of patients with IBD are younger than 20 years (3). According to the Montreal classification, it is very common for the disease to appear in adolescence but also, it may emerge many years after birth (16). Yet, affect men and women equally (3,16). Given the fact that the prevalence of this disease is higher at younger ages and that it disrupts half the life of the patient, it will, most likely, become a major health problem in the near future, even in developing countries (3,16).

Perianal disease may be presented as an anal fissure, perianal fistula, or perirectal abscess (6). Both perianal fistula and abscess often require surgical procedure with drainage (6). For these reasons, many signs and symptoms are frequent and present in IBD.

1.2 - SYMPTOMS AND SIGNS

The diagnosis of IBD is a clinical challenge as the symptoms presented may be non-specific and are highly variable (4,6). Symptoms vary between individuals still, may be related to the phenotype and location of the disease (6). More than 90% of patients have symptoms before the age of 40 (16). However, symptoms are not confined to the GIT (17,18). Patients often report gastrointestinal symptoms such as abdominal pain, diarrhea, weight loss, urgency for bowel movements, nausea, vomiting, swelling, constipation, anemia, tiredness, fever, bowel obstruction, fissures, fistula, and rectal bleeding with the elimination of blood and/or mucus in the stools (6,17). Although, the sensation of urgent bowel movements is hampered due to rectal inflammation (19). Similar symptoms between CD and UC include bloody diarrhea, mucus in stool and weight loss (3).

Table 2 - Some types of symptoms related to IBD. (5)

Lower GI symptoms	Upper GI symptoms
Fecal urgency/ frequency	Weight loss
Nocturnal stooling	Poor growth
Diarrhea	Nausea
Rectal bleeding	Appetite changes
Tenesmus	Crampy abdominal pain

Inflammation of UC is confined to the colonic mucosa, making symptoms less heterogeneous than in CD (15). Children may not meet growth expectations since in severe Crohn's disease, fatigue, and nutritional deficiencies may occur (20). UC usually presents severe diarrhea, with or without hemorrhage (15). However, CD in the colon causes watery diarrhea and may be difficult to distinguish from UC (15).

The primary adverse effects occur in the gut, but are not restricted to the GIT and therefore IBD is a systemic disorder (18). However, extraintestinal manifestations (EIMs) can emerge and are increasingly recognized as problems associated with IBD (17). Altogether, 6-47% of IBD patients experience at least one EIM (17). These EIMs symptoms occur around 25-35% patients, and are more likely at a younger age (4,18). The prevalence of IBD-related EIMs is reported to occur in 31% (CD) and 43% (UC) of IBD patients, respectively (17). There may be manifestations in the mouth, eyes, liver, kidneys, skin, circulation, joints, and biliary tract (17). In some cases, EIMs warning signs may occur such as the development of fistulae between the bowel and any nearby organ (including the vagina, bladder, and other bowel areas), and for instance, an intra-abdominal abscess may occur (6,17). When the origin of an abscess is ascertained, in addition to the pain felt, the systemic symptoms mentioned before can the triggered (6).

Other EIMs associated with IBD may include joint pain in the spine, lower back, arthritis, aphthous ulcers, erythema, phlebitis uveitis, osteoporosis, sacroiliitis, kidney stones, vitamin B12 deficiency, blood clots, deep vein thrombosis, and skin lesions such as erythema nodosum and pyoderma gangrenosum (6,17). In severe cases of CD, bloody stool may be present but classically this is more commonly associated with UC (6). Patients with IBD have an increased risk of colon cancer thus, they require ongoing surveillance such as frequent colonoscopy and biopsies every 1 to 3 years (6,19). Nevertheless, it is unclear if EIMs are a direct result of inflammatory response in GIT or rather a consequence of genetic background that exhibits a dysfunctional immune response to environmental stimuli also, is remarkable that EIMs affect more women (50%) than men (34%) (18). Consequently, these EIMs manifestations considerably affect morbidity and mortality associated with IBD (18).

1.3 - EPIDEMIOLOGY

IBD represents a global health issue as incidence has increased in several countries (6). This disease increased considerably in the mid-twentieth century (21). An annual incidence of 3 to 20 cases per 100,000 is estimated (6). The highest prevalence of IBD was reported in Europe (ulcerative colitis 505 per 100,000 persons in the southeast of Norway; Crohn's disease 322 per 100,000 persons in Hesse, Germany) and North America (ulcerative colitis 286.3 per 100,000 persons in Olmsted County, USA; Crohn's disease 318.5 per 100,000 persons in Nova Scotia, Canada) (3,7,9). According to geographic location, the incidence of IBD changes 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 such Asia, and southern Europe (22). Approximately 1.6 million Americans are affected by IBD, with 785,000 patients with CD, and 910,000 with UC (15).

Therefore, it was assumed that the urban areas have a higher incidence of disease than rural population (15). The annual healthcare costs were estimated at around 4.5–5.6 billion euros, in Europe, and 6.3 billion dollars, in the United States of America (8).

(17). These epidemiological data can be considered to anticipate healthcare needs based on the disease characteristics of the population which could enable the supply of high-quality patient-centered care (3,22). However, there has been an increased prevalence and incidence of UC and CD cases, which was formerly considered to be unusual suggesting that environmental changes play a significant role in the development of IBD (22).

1.4 - ETIOLOGY

A number of environmental factors have been associated with the development of IBD (23). IBD has been considered one of the most prevalent gastrointestinal diseases with accelerating incidence (7). During the twenty-first century the incidence of IBD has been increasing in every industrialized country and have its own challenges and side effects (3,15).

The exact pathogenesis of IBD remains incompletely unknown therefore, it is known that the ability of the GIT to distinguish foreign from auto-antigen is impaired (6). Although some genetic, environmental and host-related factors have been shown to increase the risk of the disease and lead to the abnormal immune response characteristic of the disease (6). Also, some risk factors for the development of IBD appear to be related to alterations in the gut microbiome or disorders of the intestinal mucosa and genetics (6). The aggressive disease activity includes some risk factors such as the age of diagnosis less than 30 years, perianal and/or stricturing and/or penetrating disease, extensive anatomic involvement, prior intestinal resection, deep ulcers, and prior surgery (6). There are genetic, environmental, and host-related factors that contribute to the development of gut inflammation (3,7).

1.4.1 – GENECTIC RISK FACTORS

Family history is the most significant risk factor (6). Even though genetic risk factors are still being elucidated, more than 200 genes were associated with the development of IBD (3,6). Thirty genetic loci are shared between Crohn's disease and ulcerative colitis (7). Nucleotide-binding oligomerization domain 2 (NOD2) was the first gene identified on chromosome 16 whereby, heterozygous changes at this gene increase the risk by two to four times while homozygotic changes have a 20 to 40 times superior risk of developing IBD (6). Although family history represents an increased risk for predisposition to IBD, just 10% to 25% of patients have a first-degree parent with the disease. (6)

1.4.2 – ENVIRONMENTAL RISK FACTORS

The environmental risk increases the risk of developing IBD (3,6). IBD appears to be triggered by modifications in the intestinal microbiome or disturbance in the intestinal mucosa (6). IBD patients often have dysbiosis resulting in a reduction in the diversity of the gut microbiome (6). However, the specific mechanism by which alterations in the gut microbiome predispose to IBD is not yet fully understood (3,6).

Studies have already shown that the majority of patients with IBD lived in urban areas compared with those who live in rural areas (22,23). Air pollution, a consequence of the progressive contamination of the environment by countless compounds, is another factor associated with IBD (23). As particulate matter or other components can alter the host's mucosal defenses and trigger immune responses (23). Hypoxia associated with high altitude is also a factor under investigation as a potential new trigger of IBD flares (23). As another environmental risk factor, cigarette smoking has been identified as a double-up risk for developing IBD (6). Smoking can increase the amount of CD4+ T cells which are a type of white blood cell (3). They can release the inflammatory protein called interferon gamma, which is activated by smoking in the lungs (3). Smokers are twice as likely to be affected by IBD, compared to other people (3). Also, dietary factors have been shown to contribute to the development of IBD across populations (3,15). Diets high in saturated fat and processed meats have been implicated in the development of disease (15). Studies have provided evidence that intake of fruit and vegetable has been associated with decreased risk of IBD (7).Given the fact that food first enters the digestive system, it can be said that diet can affect the prevalence of IBD to some extent. (3) Some studies have suggested that diets rich in sugar, omega-6 fatty acids, polyunsaturated fatty acids, total fat, oil, and meat increase the risk of IBD while a diet rich in fiber and fruit decreases the risk of IBD (6). Despite this, further studies are needed to clarify the association between diet and the risk of developing IBD (6).

1.4.3 – MICROBIAL RISK FACTORS

Researchers have recently focused on the gut microbiome as a potential target and therapeutic vehicle in IBD (3,4). Furthermore, changes in the composition of gut microflora and/or deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system. (8) It is claimed that an inadequate inflammatory response occurs to intestinal microbes in a genetically susceptible host (22). Changes in the gut microbiome, and their metabolic products within the intestine contribute to the formation of the inflammatory process. Furthermore, these changes are potentially able to activate abnormal immune pathways in patients predisposed to IBD (22). An infectious agent in combination with the with the intestinal bacterial flora, sets up an inflammatory response in humans already predisposed to IBD (22). This gut microbiota is necessary for intestinal homeostasis, function, health, and disease. (7) Yet, GIT is colonized by a complex community of microorganisms, and some pathobionts of the GIT, composed mainly by *Escherichia coli* and *Clostridium dificille*, might present a potential risk of disrupting the integrity of tissues (22). A decrease in the diversity of the host microbiome

Firmicutes and an increase in *Proteobacteria* and *Bacteroidetes* may lead to a decrease in the production of short-chain fatty acids and can impair T-regulatory cell and epithelial cell function (15). Furthermore, *Proteobacteria* has been noted for affects the permeability of the gut and alters the overall composition of the microbiome (15).

1.4.4 – IMMUNOLOGICAL RISK FACTORS

The innate and adaptative immune dysregulation can trigger an inflammatory response (17). The immunological dysregulation in IBD is characterized by epithelial damage (7). A large number of cells infiltrating into the lamina propria including T cells, B cells, macrophages, dendritic cells, neutrophils, and a failure of immune regulation to control the inflammatory response (7). The intestinal immune system is responsible for the initial part of the inflammatory process (7). Once the immune system is activated, the host body can't turn off this pro-inflammatory path (7). This way, the protective function is compromised through the dysregulated immune activation and ongoing inflammation (7).

1.4.5 – THERAPEUTIC ASSOCIATED RISK FACTORS

Equally, for women, either hormone replacement therapy or oral contraceptives may increase the risk of IBD (6). As an example, medications that perturb the host microbiome (antibiotics, nonsteroidal anti-inflammatories, contraceptives, and statins) have all been shown to increase the risk of development of IBD. (15)

Briefly, all the risk factors and potentially harmful agents trigger an immune response that consequently leads to an inflammatory response that in turn, leads to tissue injury. So, the cause of IBD is multifactorial and includes genetic, microbial, and environmental factors that work simultaneously. (15)

1.5 - PATHOGENESIS

Considerable progress has been made in recent years to unravel the pathogenesis of this disease (7). IBD is a group of inflammatory conditions of the colon and small intestine caused by a dysregulated immune response (24). Recent studies suggesting that innate immune system are involved in inducing gut inflammation and the adaptive immune system has been considered to play the main role in the pathogenesis

of IBD (25). The mechanisms suspected to be involved in the pathogenesis of IBD are the loss of immune tolerance at the enteric level, microbiota disturbances, overstimulation of the immune system due to mucosal integrity damage, and genetic predisposition (24). Several pathways play important roles in maintaining intestinal homeostasis (7). Epithelial barrier function, innate mucosal defense, immune regulation, cell migration, autophagy, adaptive immunity, and metabolic pathways are associated with cellular homeostasis (7).

Various inflammatory mediators are triggered during inflammatory response (figure 2) (7). Through the inflammatory response, local production of various nonspecific inflammatory mediators, such as free radicals, leukotrienes, chemokines, and proinflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) are part of the cycle of inflammatory response into the intestinal tissue (7).

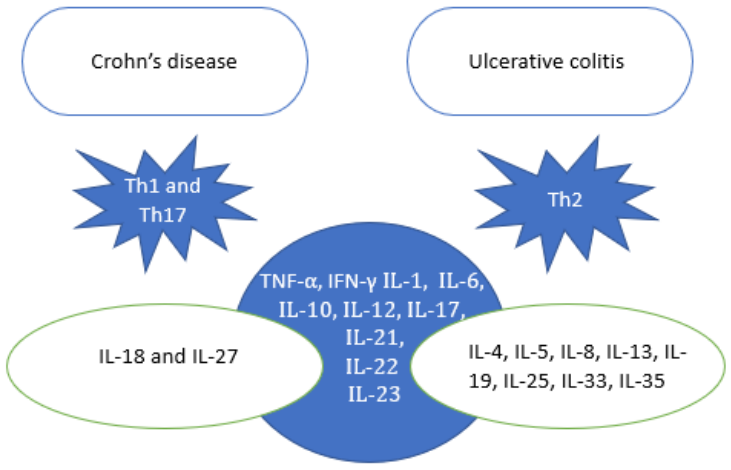

Figure 2 - inflammatory mediators triggered during inflammatory response.

Furthermore, CD and UC exhibit different inflammatory cellular responses (7). When a foreign agent is identified, neutrophils and macrophages triggering, activated T-cells, produce cytokines, proteolytic enzymes, and free radicals that result in inflammation process (7). Both CD and UC are amplified with T cell–mediated responses (15).

In CD, inflammation is triggered by an amplified T-helper (Th)1 and Th17 response (7). Also, triggers proinflammatory cytokines IL-17, interferon gamma (IFN-γ), and TNF-α, leading to a self-perpetuating cycle of inflammation (15). In UC, the response

is Th2 mediated, prominent to more efficient activation of B cells and natural killer T cells, mediated by IL-5 and IL-13. (15) Proinflammatory cytokines, secreted during intestinal inflammation such as TNF-α or IFN-γ, can increase the epithelial permeability by regulating tight junctions and promoting apoptosis (7). The high apoptotic rate of epithelial cells also leads to diminished epithelial barrier function observed in IBD (7). Specially, IFN-γ increases paracellular permeability and induces endocytosis of tight junction transmembrane proteins (7). IL-13, a key effector Th2 cytokine in ulcerative colitis, also shows the ability to impair epithelial barrier function by affecting epithelial apoptosis and tight junctions (7).

The activated T cells releases lymphocytes, TNF-α, which activate and recruit monocytes, macrophages, polymorphonuclear leukocytes, and mast cells (7). This way, these cells amplify the inflammatory process and the factor nuclear kappa B (NF-κB) pathway lead to secretion of some proinflammatory cytokines such as IL-12 (7). Once the inflammatory response is triggered, high levels of proinflammatory cytokines including TNF-α, IL-1β, IFN-γ, and cytokines of the IL- 23/Th17 pathway are produced (7). Innate immunity includes the barrier function of the intestinal mucosa, antibacterial proteins innate immune cells such neutrophils, macrophages, and natural killer T cells, and innate cytokines and molecules like IL-1, TNF-α (7). While, adaptive immunity is pathogen-specific as after exposure to a pathogen, including T and B cells (7).

Through damage to the intestinal mucous membrane, cells release inflammatory mediators (26). The intestinal barrier is also intimately involved with host innate immunity (15). This environment consists of intestinal epithelial cells such enterocytes, neuroendocrine cells, immune cells and goblet cells (15). Pro-inflammatory cytokines IL-1, IL-6, (TNF-α), chemokines (IL-8), and reactive oxygen species are released leading to an immune response, which can be dominated by T helper (Th) lymphocytes 1 or 2 (5). The release of pro-inflammatory cytokines influences GIT permeability, considering that interferon-γ (IFN-γ) and TNF-α increase permeability (26). This implies bidirectional consequences, on the one hand, increases the loss of interstitial fluid into the lumen, and conversely increases the access of microorganisms to the subepithelial cells, causing the antigenic stimulus, perpetuating the inflammatory cycle (26). For this reason, diagnosis is essential to anticipated the damages associated to IBD and for the control and maintenance of this disease (23).

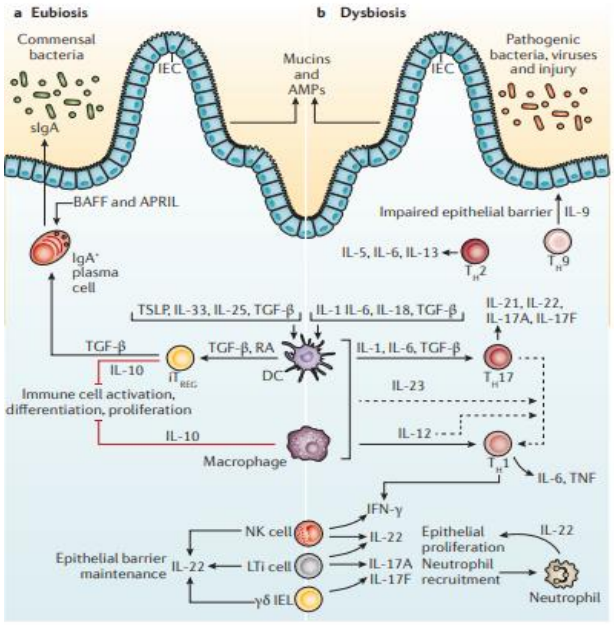

Figure 3 - Intestinal immune homeostasis and inflammation of IBD. Adapted from Immunopathogenesis of IBD: Current state of the art (26).

1.6 - DIAGNOSTIC OF INFLAMMATORY BOWEL DISEASE

When IBD is suspected, the establishment of the diagnosis may include routine laboratory tests, imaging studies, and endoscopic evaluation (27). Gastrointestinal tract (GIT) is usually the first source of initial symptoms leading to the diagnosis and evaluation of IBD (17). Physical exams and stools evaluation are considered through stool samples (4). Stool testing is useful in order to exclude other causes of gastrointestinal symptoms, distinguish inflammatory causes of diarrhea such as infections and celiac disease (6). Blood tests can measure the degree of inflammation present in the body (27). Along with the manifestation of symptoms, management decisions should be correlated with objective findings of disease activity such from biochemical markers, endoscopy, or radiologic findings (6). Diagnosis is commonly made on basis of symptoms, endoscopic and/or radiological findings (6). Ileocolonoscopy with biopsy is the gold standard for IBD diagnosis (27). Thus, that tissue biopsies look for the presence, type, and severity of inflammation, excluding other sources of inflammation (27). Endoscopic findings of colonic or ileal CD are usually characterized by raised lesions with different degrees of inflammation (including erythema, erosions, and ulcers) (6). A noninvasive safe fluoroscopic procedure is also

used to evaluate the small intestine. (4) Ultrasound procedure is noninvasive and radiation free hat provides better access for patients in some geographical areas such as detecting the presence of abscess or stenosis of bowel (4). Another procedure consist in capsule study that results in a pill that contains a tiny camera (4). Is helpful for patients with indeterminate colitis where they swallow or placed endoscopically in the stomach or small intestine (4). It is an advantage since It enables direct visualization of the areas that cannot be reached within the small intestine via endoscopy (4). Furthermore, the combination of upper endoscopy and colonoscopy with small bowel endoscopy with biopsies plays a fundamental role in diagnosing IBD (4). In addition, the endoscopic evaluation is useful to determine the severity of the disease as mild-to-moderate versus moderate-to-severe disease (27). Radiological findings using imaging can also be used to diagnose IBD through computed tomography enterography (CTE) and magnetic resonance enterography (MRE) (6). These diagnostic imaging methods allow visualization of the intestinal wall, mucosa, extraluminal complications, and extent of disease (6). The MRE method is more expensive compared with the CTE but above all, it avoids radiation exposure and does not use iodinated contrast (6).

In short, an effective diagnosis must be made since abdominal pain is usually colicky and often persists for many years before diagnosis (15). Thus, an early diagnosis of the disease allows the initiation of therapy for this condition (27).

1.7 - CURRENT PHARMACOLOGICAL THERAPIES IN INFLAMMATORY BOWEL DISEASE

There are a number of different drug classes for long-term management of moderate to severe IBD (28). Pharmacological treatment regimens for IBD depends on severity, location, extent of GIT and subtype of the disease (6). Although there is no known cure for this chronic disease, but there are a few effective treatment options (29). In general, currently used medical therapy in IBD consists of salicylates, corticosteroids, immunomodulators, and biological therapy (8). According to the phenotype presented and the disease severity, the pharmacologic therapy is determined (6). In order to treat IBD, it was necessary to determine the type of disease before initiating the treatment (3). The goal of conventional pharmacological therapy is to manage and control the active inflammation, avoiding acute episodes by the maintenance of patients in remission, not reversing or modifying the pathogenic mechanism (6). Also, current goals imply the improvement of clinical symptoms, control the inflammatory response, preventing others complications and, improve quality of life (5,25). Still, a therapeutic option is a surgery required after diagnosis, prolonging hospital stay, and costs for health services (6,29).

And even then, patients still require ongoing therapy even after surgery for disease recurrence (6,29). It is estimated that around 50% of patients will develop a clinical recurrence within 5 years after the surgery procedure (6,29). And yet, around 40% need a second surgery within 10 years (6). Furthermore, one of the objectives of keeping patients in remission is the hope of preventing complications and surgery (6). However, surgery is not curative for IBD, which implies that patients need surgery, when complications occur, over their lifetime (6). Therefore, ideally the drug therapy would be used to prevent this clinical recurrence following surgery (6). Moreover, the treatment for EIMs has expanded with new classes of pharmaceutical drugs that effectively treat IBD (17). Increased use of IBD drugs alongside those for the control of IMTs increases the likelihood of adverse reaction, incompatibilities and drug-drug interactions (29). Each therapeutic agent has its own mechanism of action, indications and side effects (25).

Until recently, therapeutic options were limited to methotrexate (MTX), thiopurines, natalizumab, and anti-tumor necrosis factor (anti-TNF) agents (6,30). Subsequently, other molecules with novel mechanisms of action were discovered, including a gut-selective anti-integrin (a4b7) inhibitor and a monoclonal antibody to current and emerging therapeutic targets for IBD (6,29).

As current treatment drugs are associated with serious side effects (29). Also, the routine and timing of these treatments strongly affect patient's quality of life (29). Furthermore, these adverse side effects present serious risks to patients by affecting the host immune system and thus increasing susceptibility to infections such as sepsis (29). However, many guidelines help to maintain a line of treatment for IBD, considering all available therapies (31–36). According to a recent guideline, the disease is classified from mild to severe disease (32). Therapy is stipulated following cases of response to therapy and in case of remission or no remission (32).

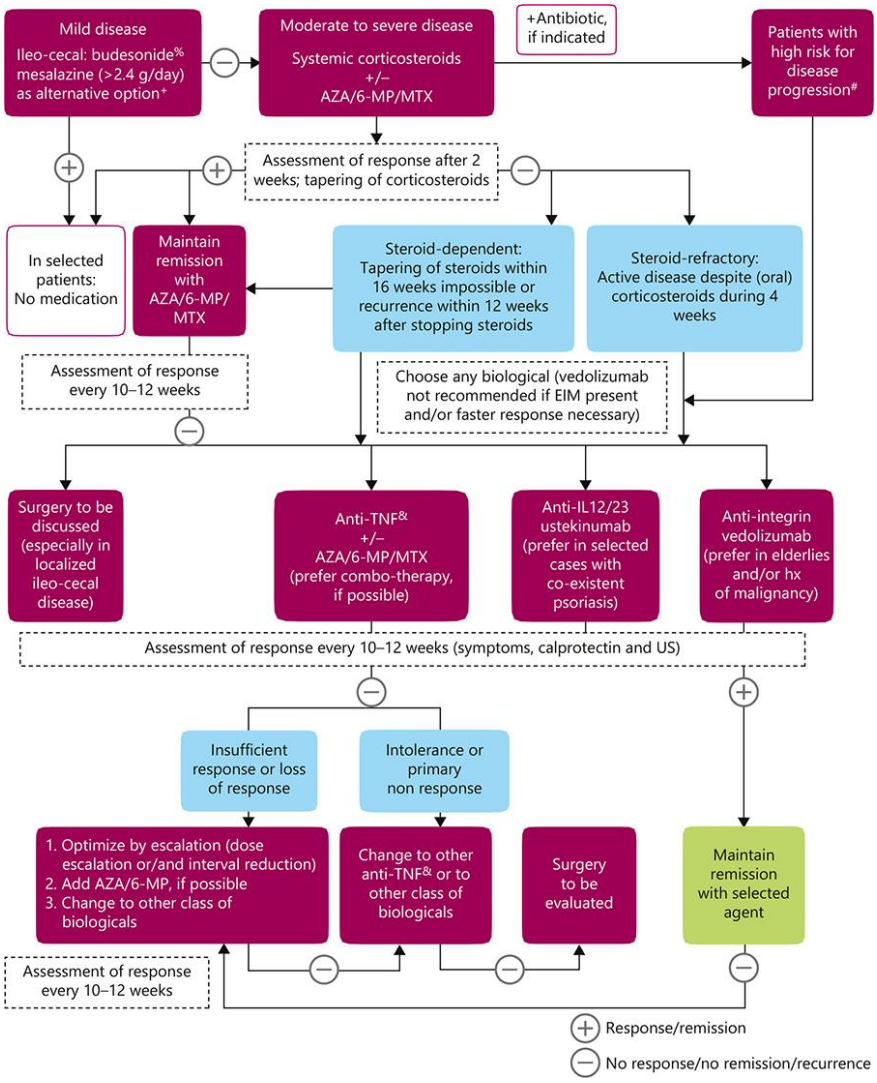

Figure 4 - Guideline considering the steps to be taken according to response, no remission or recurrence (32).

1.7.1 - AMINOSALICYLATES

Aminosalicylates are a class of drugs used to reduce inflammation in the intestinal lining (4). Aminosalicylates can be targeted for locations across the GIT (4,37). They also inhibit the production of oxygen radicals and are free radical scavengers (4). Besides, these are mainly used in mild ulcerative colitis or as aid therapy (4). Aminosalicylates are

used as topical therapy in treatment of pediatric IBD patients (4). Mild to moderate IBD has a good response to 5-aminosalicylate-containing agents (29). In the class of aminosalicylates, the 5-aminosalicylic acid (5-ASA) derivatives (mesalamine, mesalazine and sulfasalazine) offer anti-inflammatory actions for connective tissue (4). Especially in women, the sustained use of nonsteroidal anti-inflammatory drugs may raise their risk of having IBD (6,37). Nevertheless, studies have demonstrated ineffective in maintaining disease remission but its prescription can be related to its safety profile (6).

Adverse events may include diarrhea, headache, interstitial nephritis, anorexia, and in the case of may still occur hemolytic anemia, leukopenia, and hepatitis (6). If diarrhea develops treatment should be stopped and no other aminosalicylates agent should be used since may be due to a class effect (6,37).

1.7.2 - CORTICOSTEROIDS

This class of drugs enable blocking the substances that trigger allergic and inflammatory responses (25). These classes of drugs are not effective maintenance agents so, they are used to induce remission (6). Steroids have a fast onset of action so they are usually effective at inducing remission in CD (6). Corticosteroids are also ineffective in perianal CD treatment (6). Prednisone, budesonide, and methylprednisolone are examples of corticosteroids used in IBD (29).

Nevertheless, corticosteroids can cause serious side effects (4). Furthermore, their use should be diminished to minimize known side effects (4). For this reason, these class of therapeutics are no longer first-line therapy for all newly-diagnosed patients with IBD (4). They are mainly reserved for inducing remission during periods of active disease (4). Systemic corticosteroids increase the risk of adverse effects which could be identified such as glaucoma, adrenal insufficiency, delayed wound healing, osteopenia/porosis and avascular necrosis, serious infections and mortality in patients with moderate to severe CD (6). The goal is to avoid long-term steroid use, maintaining patients in remission with more effective anti-inflammatory therapies (4).

1.7.3 - IMMUNOSUPPRESSANTS

Immunosuppressants, as the name suggests, suppress the immune system, and reduce levels of inflammation (4,6). This class is often used in patients who relapse when corticosteroid therapy is withdrawn (6). It is understood that these drugs change the immune response by suppression of T-cell function and inhibition of natural killer cell activity (4). Immunomodulators (mercaptopurine, azathioprine, and methotrexate) are

used as maintenance treatment in IBD (4). Methotrexate (MTX) can also be used in patients who cannot tolerate azathioprine and mercaptopurine (3). Thiopurines such as Azathioprine (AZA) and Mercaptopurine (MP) and MTX are typically employed to maintain disease remission due to the slow onset of action (6 to 12 weeks) (6). Yet, recent studies question the overall efficacy of azathioprine and mercaptopurine in a monotherapy scheme and their use in early IBD diagnosis (3,6). Some studies suggest that azathioprine was shown to be less effective than infliximab monotherapy or the combination of azathioprine and infliximab at maintaining a steroid-free remission (6). MTX can be used both for induction of remission and for maintenance patients on remission (6). Only the subcutaneous injection was found to be effective for the induction of remission (6). MTX appears to be slightly effective for the maintenance of remission compared with AZA and MP (6). The onset of action is within 8 to 16 weeks, incentivizing non-use for treatment purposes (6). Immunomodulatory therapy has been well tolerated and is recognized as an alternative more effective than steroids as a maintenance therapy (36). Yet, corticosteroids can be used as an adjuvant therapeutic class since the immunomodulators take several weeks for their full effects to be realized (4). Newly, immunosuppressants have been used in combination with anti-TNF drugs to avoid their immunogenicity and to increase anti-TNF drug concentrations (6).

The thiopurines are limited by their side effect profile, which makes around 15% to 20% of patients discontinue the therapeutic protocol (6). Nonspecific symptoms of MTX such as fever, rash, leukopenia, nausea, vomiting, fatigue, and headaches may occur (6). Also, bone marrow suppression, pancreatitis, pulmonary toxicity, cervical dysplasia, and non-Hodgkin lymphoma are identified as potential adverse events (6). Similar to thiopurines, both nonmelanoma skin cancer and lymphoma are at increased risk when MTX is used (6).

1.7.4 - BIOLOGICAL THERAPIES

Biologics as infliximab were approved by the FDA since 2006 for CD and in 2011 for UC (4). This therapeutic class is indicated for higher risk patients with penetrating or stricturing disease, perianal disease, growth/height failure, extensive small bowel involvement, or multiple extra-intestinal manifestations (4). Among the biological therapies we have the group of anti TNF agents and the group of monoclonal antibodies agents (29).

1.7.4.1 - ANTI-TNF AGENTS

Anti-TNF agents are biological drugs that target a protein in the body called TNF, preventing inflammation (29). Anti-TNFs are commonly a good choice when there is a balance between uncontrolled inflammation and the risk of immune suppression (4). As anti-TNF drugs stand out the infliximab, certolizumab, and adalimumab that targets the TNF-α (6). Although they are monoclonal antibodies, are included in the group of anti-TNF agents for its specific indication (6). Anti-TNF therapy appears to be an effective therapy for moderate to severe IBD (6). These therapeutics agents can be used as a single therapy or in combination with an immunomodulator to maintain disease remission (6).

Some data suggest that infliximab presents fewer adverse effects, compared with other anti-TNF agents (6). The response to anti-TNF agents may be seen within the first week of treatment although the therapeutic effect may take up to 6 weeks (6). Drug-induced lupus erythematosus, demyelinating disease, infection, and hepatosplenic T-cell lymphoma may result as adverse events when these drugs are used (6). However, skin lesions are most prevalent as side effects (18).

1.7.4.2 - MONOCLONAL ANTIBODIES

A newer therapeutic class is named anti-integrin therapy (38). This therapy blocks the action of integrin on the surface of circulating immune cells and endothelial cell adhesion molecules, inhibiting the interactions between leukocytes and intestinal blood vessels (25). The most recently approved drugs are monoclonal antibodies as a directed target against certain integrins such as α4 or α4b7 and also, IL-12/IL-23 interleukins (29). Natalizumab was the first adhesion molecule as an anti-integrin approved for IBD since blocks the α4 integrin (6). As it is not gut specific is associated with a fatal brain infection named progressive multifocal leukoencephalopathy (6). Vedolizumab is a gut selective adhesion molecule inhibitor, an anti-integrin, and therefore is has not been associated with that fatal brain infection (6). It is a humanized monoclonal IgG antibody that targets the α4b7 adhesion molecule, inhibiting leukocyte migration (6). Is mostly used to maintain remission in moderate to severe IBD considering a limited efficacy in inducing remission (6). Ustekinumab agent was recently approved as an humanized monoclonal anti- body of IL-12/IL-23 inhibitor has been revealed to be as effective as anti-TNF therapy that maintaining remission in moderate to severe IBD (6). The response to this drug is usually seen within 6 weeks (6).

Natalizumab and vedolizumab can generate adverse events such as opportunistic infections, increased risk of melanoma, infusions reactions, and nasopharyngeal polyps, and liver toxicity (6). Ustekinumab may origin leukoencephalopathy, infections, and skin cancer (6).

1.8 - SUPPORT THERAPIES

In the development of IBD were implicit gastrointestinal infections, nonsteroidal anti-inflammatory drugs, and antibiotics (6). Antibiotics such as ciprofloxacin and metronidazole are still used in IBD although, the evidence supporting their use is also limited (6). Cyclosporine is the most used drug in the treatment of UC (3). It should be pointed out that this drug can be toxic for patients with CD due to the need for higher doses (3). Metronidazole is one of the antibiotic drugs used in IBD, usually after surgical treatment or in cases where the side effects of inflammation appear in the body (3). Its use implies the treatment of perianal or suppurative complications such as abscesses or fistulas (6). As well as, an early antibiotic exposure has been associated with an increased risk of development of IBD (6). Environmental factors, including the use of antibiotics during childhood, is associated with higher rates of IBD (4). In addition, the long-term use of these agents presents limitations such as antibiotic resistance and side effect profile (6).

In general, most drugs that are initiated for induction of remission are continued as maintenance therapy, if they are effective (28). Chronic use of these therapies for inflammatory diseases leads to some serious adverse effects like gastrointestinal, renal, respiratory, cardiovascular damages, and reduction in host defense against infections (39). Besides all these treatments, they are still not enough to alleviate the symptoms of the disease. Due to the wide use of biological products, anti-TNF agents, in particular, treatment-induced inflammatory manifestations have been noted (18). For the fibrostenotic changes that cause strictures, does not exist medical therapy to reverse this condition (6). Dual therapies are also used when the disease severity or other factors indicate or limit the use of other medications and decreased risk of hospitalization and surgery (4). Furthermore, the current therapies require expensive costs relating to medications, often hospitalization and/or surgery resulting in large health burdens (40).
For all the above considerations, there is still no cure for this disease (6). Nevertheless, the main objective of the diagnosis and treatment of the disease is to reduce the symptoms and improve the patients health, to completely eliminate the symptoms of the disease or keep the disease at a fixed stage (3). Therefore, it is necessary to continue researching new therapeutic approaches that may circumvent this disease and prevent

complications and surgery, improving the patient's quality of life (6). Furthermore, continued research for new medicines is focused on developing safer medications (3).

2 - PRECLINICAL TRIALS IN ANIMAL MODELS

To find new approaches, preclinical studies in vitro and in vivo are essential for testing and to provide reasonable evidence prior to early feasible testing in humans, namely clinical trials (41). In vitro studies use isolated organs or tissues and in vivo studies are performed using whole animals. Yet, these studies give a better understanding of human diseases when they are not fully known to provide new discoveries and more effective ways for prevention and cure diseases as well, to develop new techniques for treating and to improve surgical methods (42,43). To improve drug development outcomes, preclinical studies intend to evaluate pharmacodynamic and pharmacokinetic studies such safety, efficacy, predict toxicity, overall benefit-risk relationship which could decide if progress to clinical trials to assess novel therapeutic agents and treatments (41,44,45). Through providing detailed information on dosing and toxicity levels predict treatment outcomes in patients and risk assessment, supporting human drug approvals (41). For this reason, responsibility for these trials is shared by researchers, the pharmaceutical industry, regulatory authorities, and ethics committees (41,46).

2.1 - ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

To find new approaches, preclinical studies in vitro and in vivo are essential for testing and to provide reasonable evidence prior to early feasible testing in humans, namely clinical trials (47). Inflammation is the local defensive response of living tissues to injury or due to any other chemical agents (48). There are two types of inflammation, acute and chronic (48). Acute inflammation may be an initial response of the body to harmful stimuli (48). Chronic inflammation starts in 2–4 days after the onset of the acute response and can last for weeks to months or years (48).

Animal models have provided valuable insights to the molecular level and have allowed researchers and allowed researchers to study its role in this inflammatory disease (9). In addition, animal models of inflammation are used to study evaluate potential anti-inflammatory drugs for clinical use (48). These experimental models of IBD have provided important contributions not only for understanding the basic mechanism of IBD but also for developing important therapeutic interventions against IBD (21). To

investigate the pathogenesis and etiology of human IBD, several animal models of IBD have been developed (24). Providing indispensable insights into the histopathological and morphological changes as well as factors associated with the pathogenesis of IBD and evaluation of therapeutic options in the last few decades (24). The current information and knowledge about this disease have been obtained mainly through animal experimental models (24). These data often allow the extrapolation of pharmacological approaches to humans (8). Thus, recent research converging on developing new strategies for the treatment of IBD (8). Collecting samples from animals helps in the study of diseases but also, mimics some procedures done in humans such as blood sampling and stool consistency (8).

There's a vast array of different model types and subtypes available (8). The various models of IBD inflammation can be divided on the basis of how the disease is induced (8). They are divided into three types: those that express intestinal inflammation spontaneously (spontaneous models), ones in which intestinal inflammation can be induced by immunological agents (immunological models) or specific chemicals (chemically induced models) those that are genetically engineered by gene knockout, knockin, or transgenic methods (genetically engineered models), and the last includes adaptative transfer models (adoptive transfer models) (8,49).

Chemically induced colitis models are rapid to develop (8). The disruption of the intestinal barrier has been shown to lead to IBD in animal models (15). Animal models of IBD produce a large amount of proinflammatory mediators, such as TNF-α, IL-6, and nitric oxide (7). In IBD chronic model, to characterize the preclinical model the parameters more treated include body weight, stool consistency and morbidity, inflammatory biomarkers like IFN-γ, myeloperoxidase (MPO), TNF-α, IL-6, and IL-10 (8). But also, the presence of ulcers, thickness or hyperemia in the colon, and histological evaluation of the inflammation. (8) The different animal models are used for evaluation of anti-inflammatory activity for pre-clinical study (48). These models are one of the most commonly used to study IBD, because they are toxic to colonic cells that generate intense inflammatory response and recruitment of inflammatory cells. (8) The most widely used IBD preclinical mouse models are those with chemically induced disease e.g., by dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid (TNBS) and oxazolone (8). However, dextran sulfate sodium (DSS)-induced colitis and TNBS-induced colitis models are the most widely used to induce IBD (8,49). Following are the three most commonly utilized animal models that have shown a significant consistency that reflects to their extensive use during the last decades with shares significant properties with human IBD (9).

2.1.1 - INDUCTION BY DEXTRAN SULFATE SODIUM

Numerous animal experiments over the past 25 years have used the Dextran Sodium Sulfate (DSS) colitis model as a chemical induction of a model of intestinal inflammation that morphologically and symptomatically resembles the epithelial damage seen in human ulcerative colitis (21,24). The DSS model, initially reported by Okayasu et al, and has been used to investigate the role of leukocytes in the development of colitis in animal models (24). DSS induce epithelial damage and is a reproducible model that morphologically and symptomatically resembles UC in humans (8,50).

DSS is a water soluble, negatively charged sulfated polysaccharide with a highly variable molecular weight ranging from 5 to 1400 kDa which damages epithelial cells when administered to mice (24). Murine colitis results from administration of 40-50 kDa DSS added to drinking water (24). In the DSS model, the sulfated polysaccharide does not directly induce intestinal inflammation, but rather acts as a direct chemical toxin to colonic epithelium resulting in epithelial cell injury (24). The proposed and most accepted mechanism by which DSS induces intestinal inflammation results in the disruption of the monolayer lining of the intestinal epithelium, leading to the entry of luminal bacteria and associated antigens into the mucosa and allowing the dissemination of pro-inflammatory intestinal contents into the underlying tissues (24). The Innate immune cells then release cytokines causing inflammation in the colon, characterized by ulcers and granulocyte infiltration (24). Common uses for the DSS-induced colitis model include studying how the innate immune system is involved in intestinal inflammation, and also looking at factors that maintain or reestablish epithelium integrity during/after injury (24). The DSS-induced colitis model promotes a Th2 response, look like UC in humans (8). This pattern of T-cell differentiation is associated with distinct functional activities (8). Th2 T cells are potent inducers of antibody-mediated immunologic reactions while, Th1 cells are the key players in delayed-type hypersensitivity reactions (8). Additionally, Antoniou et al. (2016) describe that TNBS-induced colitis includes the development of a transmural inflammation that closely resembles histopathological lesions that develop in human CD (8,9). Models of acute, chronic and relapsed gut inflammation can be achieved by modifying the concentration of DSS and the frequency of administration (24). For DSS-induced colitis in mice the protocol generally is to add DSS to drinking water in a dose range of 2%–10%, by repeated exposure administering in three to five cycles, punctuated with recovery periods (8,51). The severity of DSS-induced colitis model depends the dose, duration of administration, molecular weight and animal strain (8,24,51). C3H/HeJ, C57BL/6 and Balb/c mice strains are more susceptible (8,24).

Additionally, males are more susceptible to DSS-induced colitis model (8). DSS colitis model in IBD research has advantages over other various chemically induced experimental models due to its speed, simplicity, reproducibility and controllability (24).

Nevertheless, this model has the disadvantage of being very expensive, what limits the choice of this method (8).

2.1.2 - INDUCTION BY OXAZOLONE

Oxazolone is an agent widely used to induce colitis in mice in order to evaluate the pathological processes involved in the UC (21,52). Oxazolone-induced model it is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity (48). The oxazolone-repeated challenge increased the level of Th2 cytokines (21,48). It has been shown that colitis induced by oxazolone resembles human UC on the basis of manifestations of inflammation of the mucous membranes, epithelial microulcerations, and histopathological changes in the distal colon (21,53). Different strains of mice were used to observe immunological responses, including C57BL/ 6J, BALB/CJ, and SJL/J (21). Mice of the C57 strain resist the induction of colitis with oxazolone (21). The mouse strain SJL/J is less favored for the induction of colitis because this strain is characterized by the possibility of developing a number of autoimmune diseases initiated by the Th1 phenotype (21). This strain also has a high mortality rate (21). The cellular and immune responses, as well as the secretion profile of oxazolone-induced colitis cytokines, differ from those of TNBS colitis (21). For this reason, the insights into the underlying immune response and pathological features are surprisingly still very limited (52).

2.1.3 - INDUCTION BY TRINITROBENZENE SULFONIC ACID

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) mimics several features of Crohn's disease wherefore, is one of the main models in the experimental studies of IBD (8,9). TNBS induced colitis introduced in 1989 by Morris et al. has a pivotal role especially in preclinical testing of his various chemical compounds in terms of their anti-inflammatory and/or antioxidant effects (9,54). TNBS model allows a rapid, reliable, robust and reproducible disease progression (8,55). Also, is an efficient method, since it promotes a transmural colitis, Th1-mediated immune response, with severe diarrhea, weight loss, and rectal prolapse, an illness that mimics some characteristics of CD in humans (8). Also, the scientific evidence suggests that TNBS-induced colitis promotes a Th1 response, resembling CD in humans (8).

According to preclinical studies, multiple intrarectal (IR) administrations of TNBS are given at an average dose of 1.2 mg using a volume of less than 150 μL with a 50% ethanol vehicle (8). The corresponding dose is ethanol and TNBS at a dose of 100 mg/kg are administered IR (9). However, specifically, a range of 50 and 150 mg/kg has been utilized by various researchers (9). A polyurethane catheter is attached for induction approximately 4 and 8 cm proximal to the anal verge (9). After that, the rodents are maintained for some minutes at a head down position, Trendelenburg position, in order to avoid expulsion of the fluid and ensure even distribution of the chemical (9). Dosing ethanol is used as a means to effectively disrupt the intestinal barrier and allow interaction of TNBS with colon tissue proteins (9). These administrations produce a reaction with certain groups of amino acids in the intestinal mucosa and colonic bacterial proteins, making them immunogenic through a process called haptenation (8,56). TNBS haptenates autologous colonic proteins with a trinitrophenyl (TNP) moiety and induces an IL-12 mediated Th1 T cell transmural colitis (Figure 5), which resembles human IBD, both on a histologic and immunologic level (8). The phenotype of Th1 inflammation includes a dense colonic tissue infiltration by CD4 T cells and the secretion of various potent pro-inflammatory cytokines (9,57).

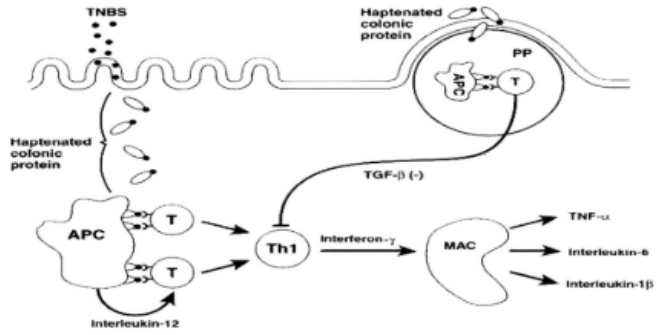

Figure 5 - Mechanism of action of 2,4,6-trinitrobenzenesulfonic acid (TNBS). Inducing experimental colitis [40].

Balb/c and C57BL/6 with 5–6 weeks are the strains mostly used (8). The induction of the disease occurs quickly and appears 4 to 7 days after IR administration of the TNBS, promoting acute or chronic colitis depending on the dose and frequency of administration (8,50). After the colitis induction the animals develop several manifestation including inconsistent stool forma- tion and occult or even bloody diarrhea (9). Intracolonic administration of TNBS/ethanol induces a severe illness characterized by bloody diarrhea and a dramatic loss of body weight during the first week (9). Also, mice treated with TNBS in 50% ethanol developed severe bloody diarrhea and rectal prolapse

accompanied by an extensive wasting disease (9). The histopathologic analysis during the first days after induction of colitis showed infiltration of neutrophils and macrophages into the colonic mucosa and submucosa layers (56). By day 3, transmural inflammation, characterized by neutrophilic infiltration, was associated with a thickening of the colon wall, ulcerations, loss of goblet cells, and fibrosis found through the colon (9). By day 7, massive infiltration of lymphocytes characterized the colon sections studied, which is a major sign of the chronic inflammation in the late stages of this colitis model (9).

The development of preclinical studies allows evaluating other therapeutic alternatives essential to improve the pharmacological approaches in the treatment of IBD (8). This way, animal models of IBD play a pivotal role in the development of new therapeutic approaches to the treatment of IBD (8). The furthermost used parameters to characterize this preclinical model include: clinical signs and symptoms (body weight, stool consistency, and morbidity), concentration of inflammatory biomarkers (IFN-γ, MPO, TNF-α, IL-6, and IL-10), macroscopic evaluation of the colon (ulcers, thickness, and hyperemia) and histological evaluation of the colon. (8) However, the variability in the results in preclinical studies regarding to the type of induction method, administered doses and treatment period, makes it difficult to choose the method used (8). Nevertheless, these colitis models are appropriated to developing and testing novel therapeutic strategies for the treatment of IBD (8). Also, these potential therapeutic approaches could ameliorate the inflammation and minimize the morbidity and mortality associated with IBD (8).

3 - PHARMACOLOGICAL APPROACHESUNDER PRECLINICAL RESEARCH

3.1 – ERYTHROPOIETIN

Erythropoietin (EPO) started to be studied in the previous models mentioned. It has been studied in the acute model of IBD with satisfactory results (58). Our team has already studied the chronic model of the disease with results that have not been published so far.

The major challenge has been the drug delivery process to the appropriate sites along the gastrointestinal tract (8). The development of and improvement in drug delivery, increased efficacy and decreased side effects have therefore been sought (8). EPO is the main glycoprotein hormone involved in regulating red blood cell production (59). In 1985 rHuEPO (recombinant human erythropoietin) was first successfully expressed by recombinant DNA technology (60). Epoetin-α was the first therapeutic to be licensed in the European Union in 1988 (60). After that, numerous rHuEPO agents have emerged in global markets (60). Each agent presents a characteristic glycoform profile considering the distribution of sialic acids over their structure (60). The way every molecule is glycosylated, play a vital role in serum half-life, in-vivo activity, stability, solubility and immunogenicity (60). In addition, it is recognized that the N-glycans have an impact in the efficacy of rHuEPO where the sialic acid residues on the N-glycans presents a critical role to maintain its in-vivo activity (60).

The story of erythropoietin (EPO) began in the late nineteenth century in France when Francois-Gilbert Viault, a histologist, observed an increase in the number of his own RBC following 3 weeks at altitude (61). Erythropoietin is an approved drug with marketing authorization for treatment of patients with anemia of chronic renal, AIDS patients and cancer patients undergoing chemotherapy but according to recent studies EPO has other potential effects (62–64). EPO is a glycoprotein hormone mainly produced by the fetal liver and adult kidney by hypoxia stimulus (39). EPO is an a hematopoietic growth factor and an endogenous glycoprotein hormone that regulates the production, survival and differentiation of red blood cells to mature erythrocytes in the human and murine bone marrow through binding of EPO to EPO receptor (EPOR) that determine the EPO response (65–69).

Animal models provide evidence for EPO activity in non-hematopoietic tissue mediated by EPOR expression (10,39). EPOR is expressed in many cells both

hematopoietic and non-hematopoietic such endothelial and cardiac cells, macrophages, adipocytes and neurons (70). Endogenous EPO production also occurs in the central nervous and cardiovascular systems, demonstrating tissue-protective effects on these organs (71). The increased blood concentration of RBCs improves the ability to transport oxygen to the tissues (71). Its endogenous production consists of the state of hypoxia and/or anemia through interaction between the hypoxia-inducible factor (HIF)-system and the GATA-system through the erythropoiesis stimulation in the bone marrow (39). Besides this, the EPO mechanism of action is triggered when EPOR is activated by EPO trough the cleavage of GATA1 the essential transcription factor in erythroid development, inducing activation of Janus kinase-2 (JAK)-2 and consequently, a signal transducer and activator of transcription-3/5 (STAT)-3/5, achieving its effects by binding to homodimeric EPOR leading to erythropoietic effects or binding to heterodimeric complex of EPOR in combination with β common receptor, also named CD131, triggering non erythropoietin effects (39–44). Nevertheless, it is also suggested that EPO has anti-inflammatory properties trough the interaction with the heterodimeric receptor EPOR/ CD131 complex via JAK2/STAT3/5 signaling and NF-κB pathway (58,71).

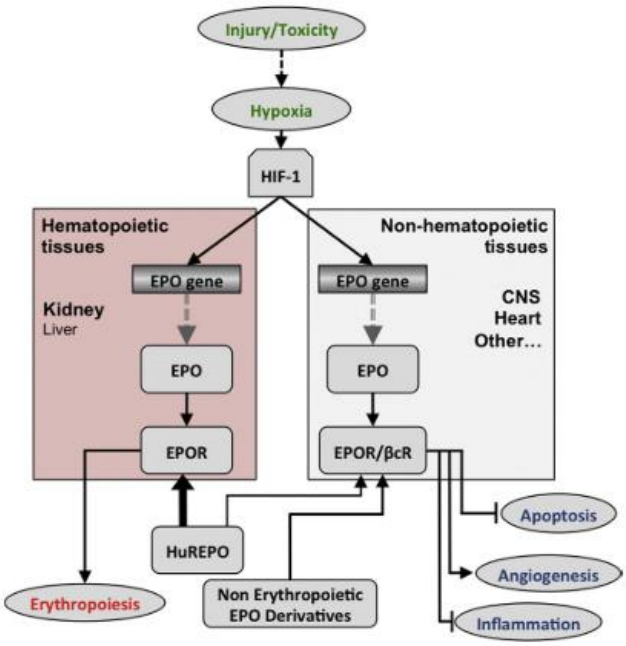

Figure 6 - EPO is a haematopoietic growth factor whose production is regulated by hypoxia. Thus, there is an increase in its expression and EPOR, in various tissues. This increase leads to erythropoiesis in haematopoietic tissues or the induction of angiogenesis and inhibition of apoptosis and inflammation in non haematopoietic tissues (42).

As cytokines receptors, the EPO binds to the receiver to the erythropoietin receptor (EPOR) and cytokine receptor family named common β-chain (CD131) (59). The erythroid cells bind to the EPOR homoreceptors, while EPOR/βc heteroreceptors are expressed in other organs (59).

3.1.1 - CHEMICAL COMPOSITION

EPO is available as a therapeutic agent produced by recombinant DNA technology in mammalian cell culture (59). Recombinant DNA technology allowed a large-scale production of exogenous EPO through the human EPO gene cloned, producing the same biological activity of endogenous EPO, acting on the same EPOR (45,46). To obtain large quantities of this hormone, the human EPO gene has been identified, cloned, and successfully expressed in mammalian cells, such as the young hamster kidney (BHK) and young hamster kidney (BHK) and Chinese hamster ovary (CHO) cell lines (59). EPO is a hormone that acts in the bone marrow as a primary regulator of primary regulator of erythropoiesis in mammals (59). Although, purification of the human endogenous EPO on an industrial scale for therapeutic use is not feasible and presents limitations particularly, in purification and isolation since this hormone is present in the human body in a very low concentration so, exogenous erythropoietin is used (79–82). Since erythrocytes lack a nucleus, endoplasmic reticulum, mitochondria and ribosomes, these cells are not able to divide, and erythropoiesis is the only way to form new erythrocytes (59). As EPO is produced mainly in the kidneys, malfunctioning kidneys cause insufficient production of the production of the hormone and, consequently, the body is not able to transport oxygen to the tissues (61).

As mentioned above, EPO is a 30,4 kDa molecule mainly produced by the fetal liver and adult kidney by hypoxia stimulus however, can be segregated by other organs such as liver, spleen, lung and brain (38–40). rHuEPO is a glycoprotein that contains 166 amino acids arranged in a single chain (60). It also features, 3 N-glycans, and an O-glycan (85). The molecular mass of rHuEPO varies in the range 30-34 kDa which approximately 40% can be attributed to the carbohydrate (60,61). The presence of sialic acid at the ends of glycidic chains are necessary for the hormone to reach its target sites (59). When the terminal sialic acid residues present in the glycidic chains of rHuEPO are removed, the half-life of the glycoprotein in plasma is affected, thereby preventing rHuEPO from playing its role in maintaining erythrocyte levels (61). In endogenous EPO molecule most sialic acid residues are attached to the three N-linked glycosylation chains which serves a diversity of functions, including the protection of EPO from proteases and the modulation of its receptor binding affinity (86,87). For this reason, glycosylation is

important for the biologic activity of EPO analogues and a removal or modification of the glycan chains results in altered in vivo and in vitro activity (82,88).

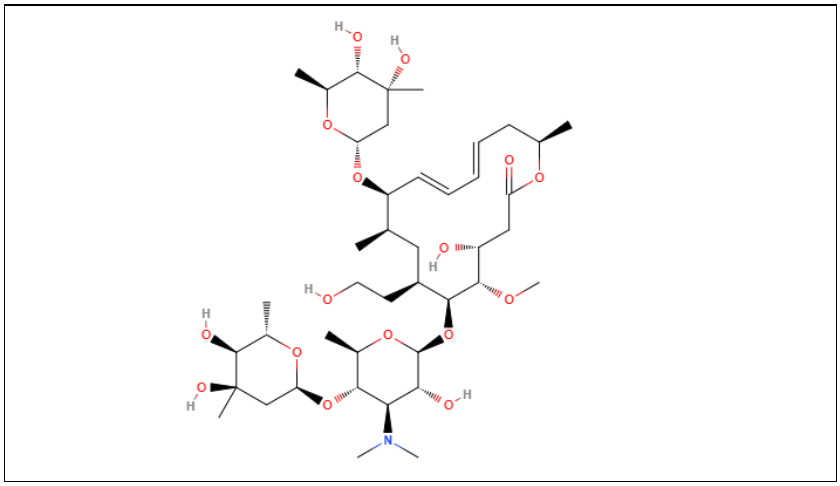

Figure 7 – Oganic structural formula of an rHuEPO molecule.

3.1.2 - EXPERIMENTAL STUDIES IN INFLAMMATORY BOWEL DISEASE WITH EPO

As referred, EPO is commonly used for the treatment of anemia in humans since stimulates erythropoiesis but it also reveals non-erythropoietic protective functions such as the inhibition of apoptosis, autophagy, induction of angiogenesis, neuroprotection and tissue regeneration (34,35). Moreover, EPO also has non-haematopoietic properties, through EPO receptors interaction that are expressed on various non- erythroid tissues, such as the brain, retina, heart, skeletal muscle, kidney and endothelial cells (58).

EPO has been widely used in studies such as abdominal aortic aneurysms, acute renal ischemia, neuroprotection, brain injury, ischemic encephalopathy, cerebral hypoxia-ischemia, infections, lupus, Alzheimer's disease and rheumatoid arthritis (90–97). Multiple experimental studies have identified rHuEPO as a potential neurotherapeutic after inflammation and HI-induced perinatal brain damage, with inconsistent outcomes reported in clinical studies (92). HIF-1α may induce EPO receptor overexpression, which provides the therapeutic opportunity to administer pharmacological doses of EPO to rescue and/or repair affected brain tissue (92). Besides this, it has been demonstrated that EPO activity includes neuroprotection in brain

ischemia and trauma, endothelial nitric oxide production and cardioprotection, skeletal muscle wound healing, and context dependent bone remodeling affecting bone repair or bone loss (10). Also, EPO has been studied in the acute colitis model (58). Mice with TNBS-induced colitis were treated with a daily dose of erythropoietin at 500 IU/kg bw/day and 1000 IU/Kg bw/day IP during 4 days (58). Erythropoietin is a potent stimulator of erythroid progenitor cells, which is able to inhibit NF-kB activation, due to its pleiotropic properties, thus promoting an anti-inflammatory effect (58). The anti-inflammatory properties of erythropoietin in the TNBS-induced colitis were confirmed by suppression of pro-inflammatory mediators, such as TNF-a, IL-1b and MPO, as well as a significant increase in the anti-inflammatory cytokine, IL-10, was promoted (58). Through these multiple mechanisms of action, EPO presents an important role in the reduction in inflammation, apoptosis, and oxidative stress, due to hypoxia, toxicity, or injury (58,90,96).

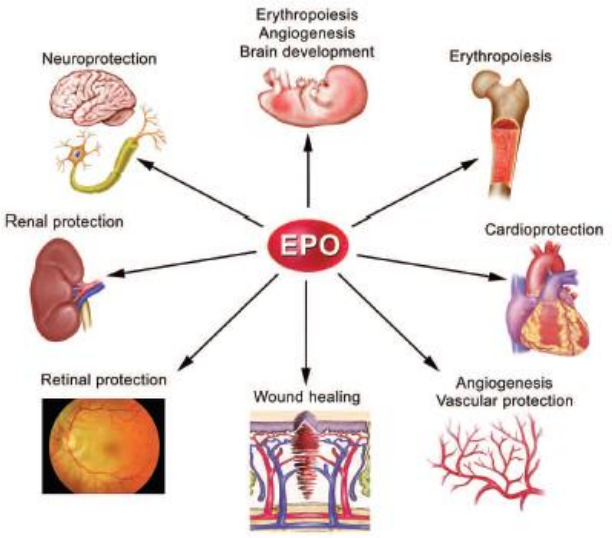

Figure 8 - Erythropoietic and non-erythropoietic effects of erythropoietin [40].

Despite this, EPO presents high side effects due to erythropoiesis stimulation and leads to the risk of severe cardiovascular events and therefore, increases incidence of thromboembolism, hypertension, myocardial infarctions, strokes and heart failure and tumor progression (67,77,83,98). These conditions cause renal cells to increase production and secretion of erythropoietin into the bloodstream, leading to stimulation of erythropoiesis in the bone marrow and ultimately to an increase in the number of red blood cells (RBCs) in the blood (61). Thus, EPO presents high side effects due to erythropoiesis stimulation and leads to the risk of severe cardiovascular events (39).

Therefore, despite being effective in the acute model of IBD it is necessary to synthesize new molecules

3.1.3 - CARBAMYLATED ERYTHROPOIETIN

The novel the biotechnology-derived drug products development appears as an added value (99). The first biological biotechnology-derived pharmaceutical drug product approved in the United States was Humulin, in 1982 (99). Pharmaceutical companies have been investing and resorting to molecular and cellular biological techniques due to the potential clinical benefits of biotechnology drug products (99). Biotechnology-derived products with biological origin are those that are “well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology” (99). These products include monoclonal antibodies, vaccines, blood plasma factors, growth hormones, and insulins (99). Beyond these, an example is the rHuEPO which is produced through recombinant DNA technology (99). Nevertheless, the production of several molecules could fail during the development process (99). To minimize these technological limitations, a focus on efficient methodologies is needed (99). All the above mentioned information is critical in determining the potential methods for reasonable drug delivery (99). The applied methodologies should be relevant, validatable, and transferable to be reproducible (99). Thus, as inflammatory bowel disease is a chronic disease with reduced quality of life, and the current pharmacotherapy only induces or maintains the patient in remission, there is a crucial need of new pharmacological approaches (58).

To minimize EPO potential risk and side effects a possible approach is the use of carbamylated erythropoietin (cEPO) molecule (100). EPO presents high side effects due to erythropoiesis stimulation and leads to the risk of severe cardiovascular events (39,98). In this sense, it is important to modify the EPO molecule, maintaining similar pharmacokinetics characteristics to EPO, although doesn’t binding to the conventional homodimeric EPOR, lacking erythropoietic side effects (101). cEPO doesn't bind to homodimeric EPOR instead, its effects are mediated by binding to a heterodimeric EPO common-β receptor (CD131), also shared by GM-CSF, IL-3, and IL-5 and yet, cEPO doesn’t activate JAK-2 (74). This is possible because this molecule has a low affinity for EPOR, and it is suggested that its effects are mediated by a heterodimer receptor formed by EPO-R and the common beta (EPOR-βcR) also known as CD131 heteroreceptor (102).

Thus it is possible to maintain efficacy and improve the safety of EPO (39).

cEPO is a non-erythropoietic erythropoietin carbamoyl derivative from modification of the EPO molecule which also doesn't bind to the homodimeric EPO receptor (101). cEPO only binds to heteromeric EPOR-CD131 complex as well, lacking erythropoietic side effects (12,71,103). cEPO has the same half-life as rHuEPO and presents the same protective effects of endogenous EPO (71).

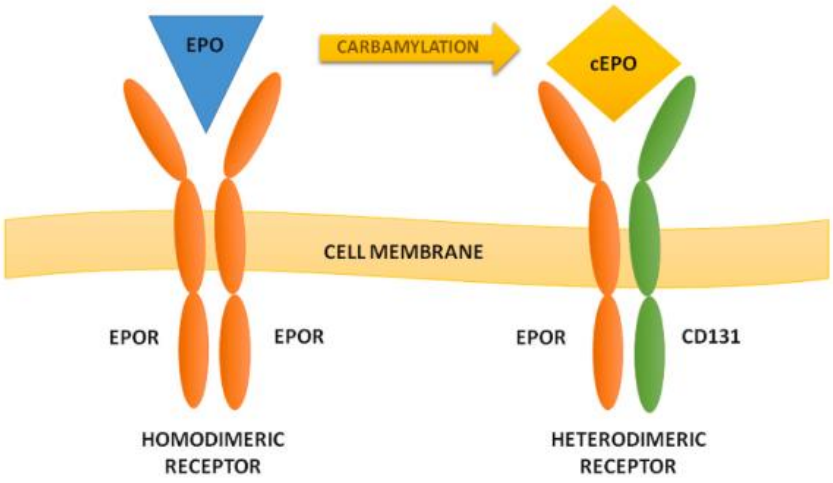

Figure 9 - Mechanism of action of the cEPO after carbamylation process (40).

It has gained focus in recent years as it provides protective and anti-inflammatory effects in many diseases (101). cEPO has been studied in several animal models having demonstrated several satisfactory properties (104,105). It has shown neuroprotective activity in models of cerebral ischemia, diabetic neuropathy, encephalomyelitis autoimmune and medullary hemisection (106–108). cEPO also demonstrated some potential in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases and protective effects in spinal cord injury, myocardial damages, cerebral ischemia, diabetic neuropathy, renal injury and (109–113). Another study showed that cEPO affects degenerations and differentiation of cholinergic neurons which improves memory and cognition. (114,115). A brain death study, proved that cEPO exhibited inflammatory responses which preserved vascular integrity, when compared to the EPO which was less effective (8). There is scientific evidence that EPO inhibits the NF-κB pathway, decreasing the production of NF-κB that stimulated autoimmune mediators, limiting tissue damage and ameliorating disease severity (100,102). Some studies in mice and rats shows that after being given high doses for long periods of cEPO, it has not triggered an erythropoietic response (109,116). Nevertheless, it is also suggested that EPO has

anti-inflammatory properties trough the interaction with the heterodimeric receptor EPOR/ CD131 complex via JAK2/STAT3/5 signaling and NF-κB pathway (58,71).

Activation of heteroreceptor, composed of EPOR and CD131 promotes protective replies mediated by an anti-inflammatory and anticoagulant response, similar to those of EPO (112,117). cEPO results from carbamylation of EPO lysine residues, which one carbamyl group is irreversibly coupled to each of the residues, achievable through the N-terminal alanine in the polypeptide chain of EPO, with eight lysine residues, providing nine amino groups for carbamylation, resulting in functional alteration by incubation with potassium cyanate (118). Both EPO and cEPO decreased production of TNF-α, IL-1β and lymphocytes, previously seen as inflammation mediators in IBD and increased IL-10, which proves that the cEPO can antagonize inflammation (119). Leist et al. (2004) reported that even without erythropoietic properties the cEPO was able to maintain neuroprotection, renoprotection and the tissue-protective effect, applied to in vitro and vivo models (120–122).

Therefore, cEPO presents helpful effects on cell proliferation, inflammatory process, angiogenesis, antiapoptotic outcome (inhibits caspase-3) and neurogenesis, as previously seen by EPO however, without the side effects related to erythropoiesis. (94–97)

cEPO can be a possible new strategy of IBD, and some studies have been developed to evaluate its efficacy and security (37). For this, is a possible alternative because mimics the three dimensional structure of EPO and inhibits erythropoiesis as well, diminishing the EPO-related side effects and promotes non-erythropoietic functions like tissue protection, presenting a therapeutic advantage (118). As a modified molecule, is presented at with the same 165 amino acid chain as the other derivatives, changing only identical to endogenous EPO but differences in carbohydrate structure (118). For this reason, glycosylation is important for the biologic activity of EPO analogues and a removal or modification of the glycan chains results in altered in vivo and in vitro activity (82,88). In endogenous EPO molecule most sialic acid residues are attached to the three N-linked glycosylation chains which serves a diversity of functions, including the protection of EPO from proteases and the modulation of its receptor binding affinity (87,127). Regarding the chemical composition, the nucleophilic nitrogen group is already present so that it later forms the carbamoyl group suppressing the adverse reactions already presented and binding to the heterodimeric receptor (118).

3.1.4 - CARBAMYLATED ERYTROPOIETIN SYNTESIS

The knowledge of the specific molecular pathophysiology of different diseases allows the design of molecules through the modification of existing pharmaceutical agents (128). The production of biopharmaceuticals makes it possible to reproduce proteins that are identical or very similar to natural proteins and also to develop completely new ones and/or greater biological activity, longer half-life or fewer side effects (128). Drug repurposing is a valuable strategy as the method consists of testing already approved molecules (128).

The carbamoylated protein procedure has been used in the study of chronic diseases such as chronic renal failure and atherosclerosis (129). Theoretically, all proteins may be carbamylated (129). Nevertheless, the carbamoylation potential of each protein depends on a variety of parameters such as the number and accessibility of amino groups and the lifespan of the protein (129). The preclinical development of a molecule involves complex procedures (130). rHuEPO it is a glycoprotein with 166 amino acids, three N-glycosylation sites and one O-glycosylation site, with a molecular weight of 34 kDa (71). Its tertiary structure is globular, characterized by four α-helices and two anti-parallel β-sheets (71).

Several studies clearly shown that carbamoylated proteins alters protein properties and configuration, changing molecular and cellular properties for the desired target (129). Carbamoylation reaction decreases the biological activity of EPO, which may contribute to the suboptimal erythropoietic responses to EPO therapy (129). EPO carbamoylation leads to a loss of its erythropoietic effects, maintaining the protective properties (129).

Carbamoylation is a modification of proteins between potassium cyanate or potassium isocyanate to a specific free functional groups, through the nonenzymatic reaction (129). Protein structural and functional properties are changed by this reaction (129). This reaction results in the addition of carbamoyl moiety (-CONH2) on the rHuEPO protein (126). Many in vitro studies used incubation of purified proteins with high concentrations of potassium cyanate (KCNO) to achieve the carbamoylation (129).

CHAPTER 2 - AIM

The main objective of this dissertation is to study the effect of carbamylated erythropoietin in an experimental chronic model of IBD in rodents. To this end, it will synthesize this molecule to discover the efficacy and safety of carbamylated erythropoietin which could be a more effective and targeted novel approach considering the known therapies used. Therefore, the specific objectives are:

Main aim:

- Evaluate the efficacy and safety of carbamylated erythropoietin in IBD

Specific aims:

- Synthesize the carbamylated erythropoietin molecule from an erythropoietin molecule
- Evaluate the effect of cEPO on the efficacy of treatment in the chronic model of TNBS in rodents
- Evaluate the effect of cEPO on the safety of treatment in the chronic model of TNBS in rodents
- Compare the efficacy and safety of carbamylated erythropoietin with erythropoietin in TNBS-induced chronic colitis model

CHAPTER 3 – MATERIALS AND METHODS

1 - CHEMICALS AND REAGENTS

TNBS 5% and sodium hydroxide (NaOH) were purchased from Sigma Chemical Co. Erythropoietin (Eprex ® 2000 IU/0,5ml) was purchased from Janssen-Cilag Farmacêutica. Ketamine (Imalgene ® 1000) was purchased from Bio2. Xilazine (Rompun ® 2%) was purchased from Bio2.

ADVIA® kit was purchased from Siemens Healthcare Diagnostics.

Sodium borate ($Na_2[B_4O_5(OH)_4]\cdot 8H_2O$), Boric acid ($H_3BO_3$), Hydrochloric acid (HCl) Potassium cyanate (KOCN), Phosphate-buffered saline (PBS), Sodium chloride (NaCl) and Sodium bicarbonate ($NaHCO_3$).

2 - CARBAMYLATED ERYTHROPOIETIN SYNTHESIS

To begin synthesis of cEPO 100 mL of borate buffer at pH 8.9 was added to 1 mL of EPREX.The pH was adjusted with HCl. 2M of KOCN was mixed with the previous solution. Then, the solution was left at 37ºC for 20 hours. After that, the solution was filtered in amicon tubes (4 mL) for 20 minutes. 2mL of PBS were added and filtered in amicon tubes for 15 minutes. 2mL of NaCl at 0,9% were added to the solution and then filtered in amicon tubes. This process was repeated once again. At the end, 2 mL of NaCl was added to the solution to obtain a concentration of 2000 IU/0.5 mL.

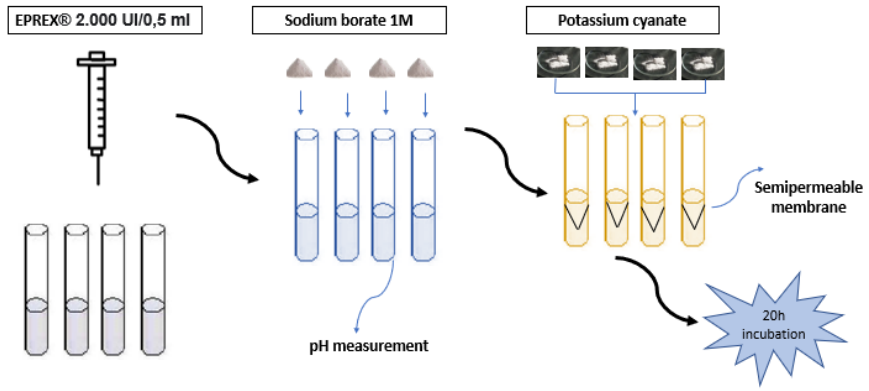

Figure 10 - cEPO molecule synthesis

The mechanism and formulation of cEPO involved several methods. Firstly, sodium borate was added and incubated to the (Eprex ® 2000 IU/0,5ml) molecule as a buffer solution with a pH of 8,9. (131) Then, potassium cyanate was then added to the solution and this one stayed 20h at 37ºC. The solution was filtered using amicron tubes. PBS was added and filtered again. Once more, NaCl was added to the solution, and it was filtered again. Thus, a solution of cEPO at 2000 IU/0.5 mL was obtained. Carbamoylation results from the covalent binding of potassium cyanate to proteins (129).

3 - DETERMINATION OF CARBAMYLATION RATE

A 500 IU/mL cEPO solution was prepared and 1mL of $NaHCO_3$ 4% solution was added with 8,4 of pH. Afterwards, 50µL of TNBS 0.1% were added. The solution was left for 1h at 37ºC. The absorbance of this solution was measured at 335nm using the Pierce method. The same procedure was performed for a sample of EPO 500 IU/mL. As a reference, a solution of 1mL of NaCl + 1 mL $NaHCO_3$ + 50µL of TNBS 0.1%. The aborvance of one cEPO and one EPO solution was read. Along with these solutions, 50µl of 1% TNBS, 1mL of NaCl and 1mL of 4% $NaCO_3$ were added. The $NaCO_3$ is added to the solution of cEPO and EPO as a buffer solution at a pH of 8.4, to deprotonate the lysine residues. After the buffer solution, TNBS was added at 37°C for one hour and the absorbance was measured.

4 - ANIMALS

Fifty-six (56) female CD-1 mice 6 weeks of age and 30-40 g were obtained from Instituto de Higiene e Medicina Tropical (IHMT). 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. Animals were housed in standard polypropylene cages with *ad libitum* access to food and water, under uniform and controlled temperature, humidity, and lighting conditions, in the Bioterium of the Faculty of Pharmacy, University of Lisbon. The experiment was approved by the Ethics Committee for Animal Experimentation of the Faculty of Pharmacy of the University of Lisbon (ORBEA) with code nrº 3/2020; approved by the Direcção Geral de Alimentação e Veterinária (DGAV) on November 6th of 2020; and, finally, approved by Ethics Committee of ESTeSL (code CE-ESTESL-IDI&CA_Nº. 11-2020).

5 - INDUCTION OF EXPERIMENTAL COLITIS

TNBS was instilled intracolonic during 5 weeks according to the validated chronic model described by Inês Silva et al. (43) In short, 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 cannula delicately inserted until 4 cm into the colon. Mice were kept for 1 min in a Trendelenburg position to avoid reflux. (58)
In the week 6 of the experiment, mice were anesthetized, and blood samples were collected by cardiac puncture. Following, the mice were euthanized by cervical dislocation and necropsied. The abdomen was opened by a midline incision. Colon was removed and is placed in formaldehyde.

6 - EXPERIMENTAL GROUPS

Groups were categorized based on the main objectives of this study, namely the development of the TNBS-induced chronic colitis model and the evaluation of the influence of cEPO in the IBD (figure 11 e 12). This way, the experimental groups were:

A: DEVELOPMENT OF ANIMAL MODEL OF TNBS-INDUCED COLITIS

The TNBS groups (n= 5) received 100µl (IR) of 1% TNBS in 50% ethanol and its necropsy was made on in the 6th week, 36 day respectively. The sham group (n = 5) received 100µl IR of saline solution (NaCl 0.9%). The ethanol group (n = 4) received 100µl intrarectal of 50% ethanol (TNBS vehicle). Thus, the TNBS, ethanol and sham groups were then used as a reference group to compare and evaluate the influence of cEPO, in the treatment of IBD.

B: EVALUATION OF THE INFLUENCE OF cEPO IN THE IBD

The TNBS+cEPO 500 UI/Kg group (n=15), TNBS+cEPO 1000 UI/Kg group (n=15), and TNBS+EPO 1000 UI/Kg (n=6) were a TNBS-induced colitis models treated through IP administration daily during the last 2 weeks of the experiment. cEPO 1000 UI/Kg group (n= 4) and EPO 1000 UI/Kg (n=2) as a control group have been used to receive IP treatment within the last two weeks.

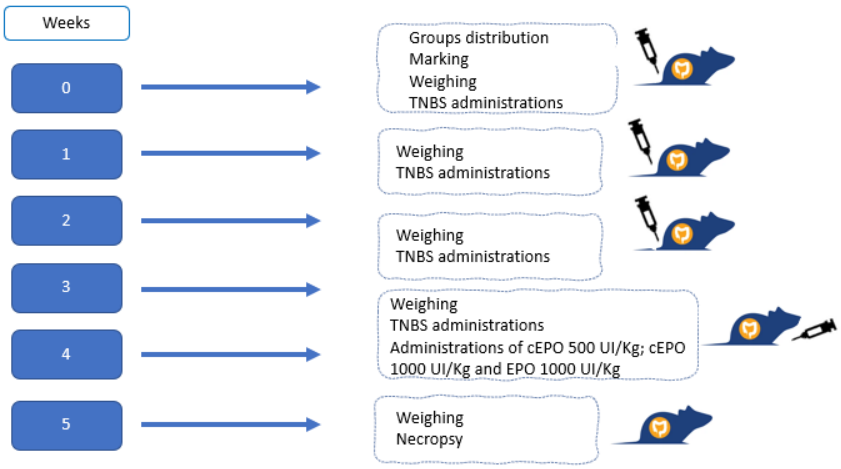

Figure 11 - Procedures made during experiment during each week.

The experiment ran for 6 weeks. For 5 weeks TNBS was administered in the respective groups and control. The administration of cEPO 500 IU/Kg and cEPO 1000 IU/Kg occurred in the fourth and fifth weeks with the experimental treatment group of cEPO 1000 IU/kg only being used from the fourth week onwards.

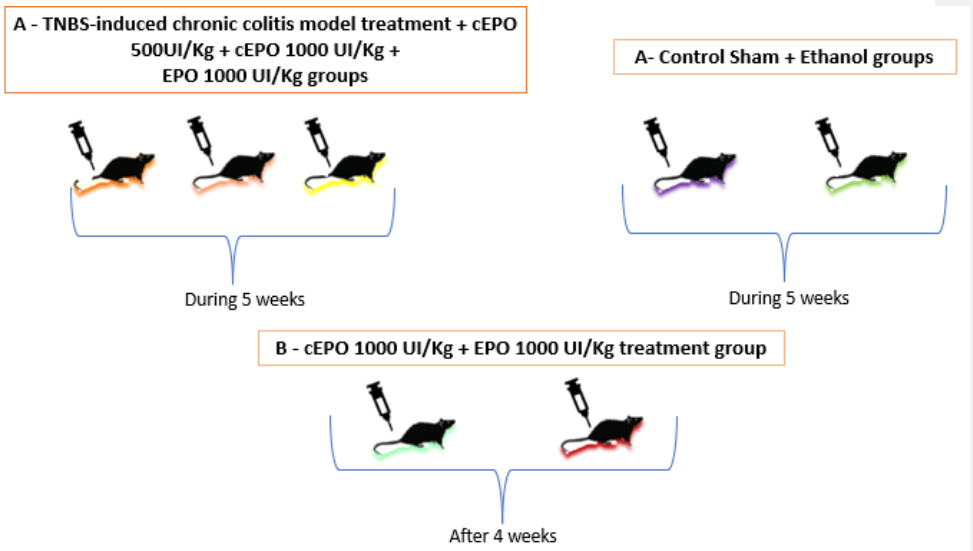

Figure 12 - Scheme of second phase of the study design with the involved experimental groups.

7 - MONITORING OF CLINICAL SIGNS

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

8 - 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);
- The TNF-α and IL-10 (pro-inflammatory and anti-inflammatory cytokines) were measured and expressed as pg/ml. The total cytokine samples were collected from the serum;
- 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;
- 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;
- 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;
- 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;

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

9 - HISTOPATHOLOGICAL ANALYSIS

Histopathology was carried out by an independent histopathologist, from the Gulbenkian Institute of Science, blinded to the groups. Colon samples were fixed in 10% phosphate-buffered formalin, processed routinely for paraffin embedding, sectioned at 5 µm, and stained with hematoxylin and eosin.

10 - STATISTICAL ANALYSIS

All results were expressed as mean ± SD 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 4 – RESULTS AND DISCUSSION

1 - SYNTHESIS OF CARBAMYLATED ERYTHROPOIETIN

Carbamylation from the erythropoietin molecule was successfully achieved (figure 13). Carbamoylation results from the covalent binding of potassium cyanate to proteins which generates a nucleophilic nitrogen nucleus that will react with cyanate (129).

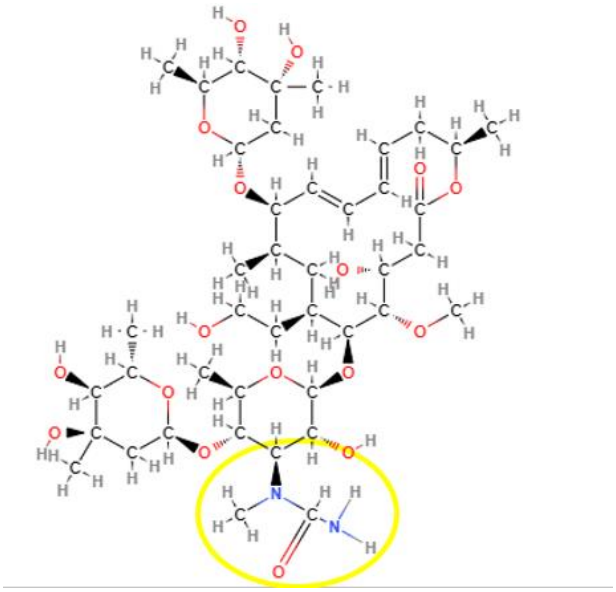

Figure 13 - Chemical composition of cEPO molecule.

Each organic substance containing carbon in its composition, affects all living beings (130). Organic chemistry is the field of chemistry that studies the structures, properties, reactivity, and production of organic substances (130). Nevertheless, this field depends on laboratory and experimental work. Yet, from the 19th century onwards the synthesis of organic molecules (130).

Carbamoylation results between the reaction of potassium cyanate and rHuEPO molecule (Figure 14) (129).

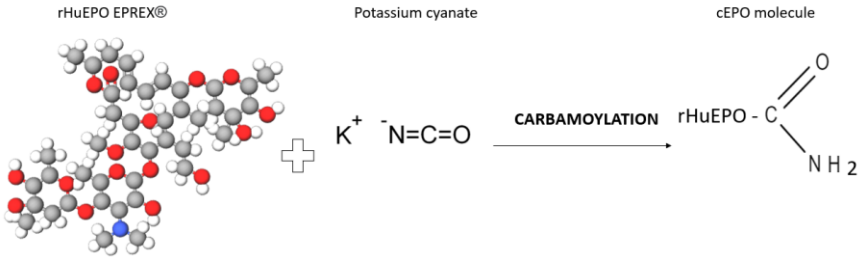

Figure 14 - Carbamylation conversion process of the EPO molecule to cEPO

The first molecule represents the lysine residues of EPO at 30.4 kDa without sodium. In the second step, the nitrogen molecule becomes nucleophilic and so will attack the cyanate after putting the sodium borate (71). After the addition of potassium cyanate, the carbamoyl group was formed (71). Post-translational modification-derived products arise from a stable covalently bound adducts on proteins through post-translational modifications, between two compounds (129). These underlying modifications of covalent bonding stabilize secondary and tertiary structures of protein (129)

Then, a carbamoyl group was obtained through the reaction of potassium cyanate (figure 15).

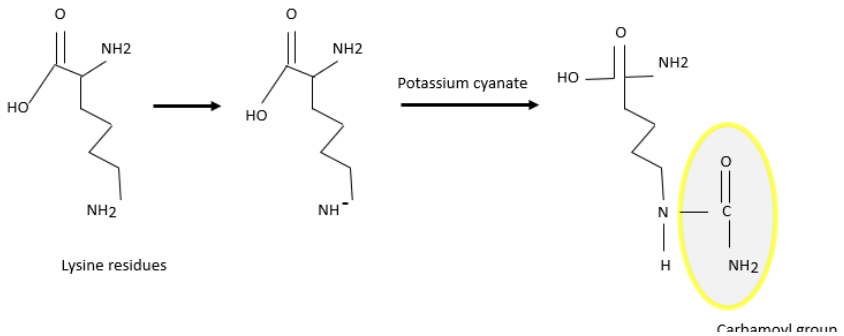

Figure 15 – Organic synthesis procedure of the cEPO molecule.

1.2 - MOLECULE CARBAMOYLATION RATE

Through laboratory techniques, it has been possible to analyze, characterize, isolate, and dose constituents in formulations. Since the rate of carbomylation is the interest of the study, it should be isolated by assessing its purity. This way, the carbamoylation rate of the molecule proteins prepared in vitro should be checked before transitioning to in vivo studies to ensure consistency of results (129).

The Pierse Protein Assay is a free method for the colorimetric detection and quantification of total proteins (132). This method combines the reduction of cations by proteins in an alkaline medium (sodium borate). The rate of carbamoylation depends on the number of lysine residues that have been attached to the molecule (129). The carbamoylation protein rate mainly results from the covalent binding of isocyanic acid to the N-terminal free lysine residues (129). Tryptic hydrolysis of rHuEPO produces nineteen peptides and two amino acids as this glycoprotein has twelve arginine and eight lysine residues arginine and eight lysine residues.

To measure the rates of carbamoylation, different methods could be applied such as liquid chromatography, immunoassays, or mass spectrometry (129)**.**

The purification rate is measured according to the lysine residues that are free through the reaction with TNBS (133). This is a rapid and sensitive assay reagent for the determination of free amino groups. (133) Thus, the non-free lysine residues are attached to the TNBS molecule (133).

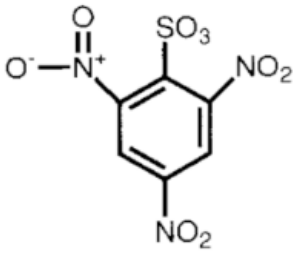

Figure 16 - TNBS molecule with attached lysine residues.

As rHuEPO is an already approved molecule for the clinical condition for which it has been approved, already proved its efficacy and safety. So, all the information inherent to the original molecule are known. Given the utility of the original rHuEPO molecule, cEPO was synthesized to suppress the adverse effects associated with the original rHuEPO molecule. Yet, the cEPO as a new molecule requires preconditions to ensure that the

dose needed is non-toxic to prove that it could bring benefit. Thus, the molecule potentially used for IBD therapy was used in a preclinical study on mice to assess the efficacy and toxicity. Pierce's method is a time-consuming method to perform so the twenty hours were used. As benchmark indicators were used NaCl and $NaHCO_3$ and an absorbance of 335 nm was used. This makes sense because they are formulations that are part of the vehicle of these molecules. TNBS 1% evaluates the free lysine residues, so it is essential to have it during the reaction procedure. The absorbance values are meaningful as the values should be between 0 and 1. The absorbance of cEPO resulted in a value of 0.009nm and the absorbance of EPO resulted in a value of 0.788. Fortunately, the absorbance value of cEPO was much lower which indicates that the lysine residues reacting with TNBS were few.

The carbamoylation-induced process and reaction protein has been used in the study of chronic diseases. The determination of the carbamylation rate resulted in a percentage of 98,9%. This indicates that the carbamylation process was a success, with a satisfactory and substantial percentage.

1 - EVALUATION OF THE EFFICACY AND SAFETY OF THE MOLECULE

1.1 - MONITORING OF CLINICAL SIGNS

The mice were observed daily during the four weeks of disease induction. Parameters such as morbidity, stool consistency, anus appearance, and weight have been accessed. No morbidity or edema of the anus was observed during the experiment as well as, the stool consistency remained unchanged. In the control groups, namely sham and ethanol groups, no changes were identified either during all experimental study. 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.

A variation in weight was notable. Regarding body weight, control groups as sham 1.2 ± 4.8%, ethanol -0.87 ± 6% and cEPO 1000 UI/Kg -0.02 ± 4.3% demonstrated a very similar curve in the register of body weight, throughout the experiment. From the second administration of TNBS onwards, the mice lost weight, as expected. With the administration of cEPO all animals on this formulation gained weight. As shown, the TNBS group -2.6 ± 4.9% decreased weight, being lower than the control and the treated groups at the end. The Sham, Ethanol and cEPO 1000 IU/kg control groups maintained

the higher weight as expected. The groups treated with cEPO all increased weight. Notably, after induction of TNBS and administration of cEPO 1000 IU/kg -1.5 ± 4.8%, the weight of the mice was seen to increase relative to those treated with cEPO 500 IU/kg -2.6 ± 5.5%. For this, cEPO treatment attenuated the loss of body weight and the effects were statistically significant with the higher dose.

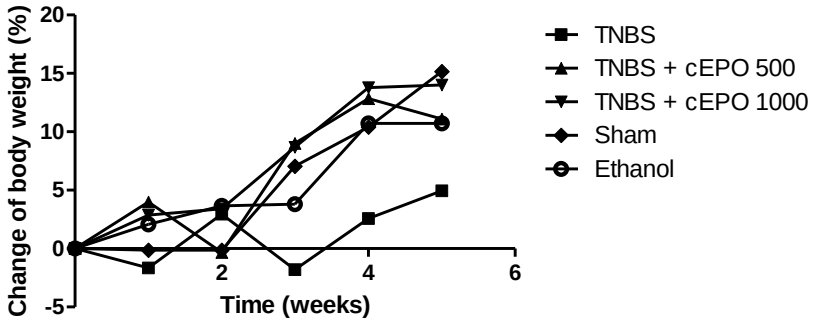

Figure 17 Change of body weight during cEPO treatment in the IBD.

The colon length was determined at the end of the treatment period, using a measuring scale (figure 18 e 19). Moreover, the TNBS-induced colitis had an influence on colon length comparing with control groups (cEPO 1000 UI/Kg 10.0 ± 0.4 cm; sham 12.0 ± 0.4 cm and ethanol 11.4 ± 0.4 cm). The mice treated with cEPO 1000 IU/Kg presented a colon length superior to that of the ill mice, showing a positive result after administration of the molecule. More specifically, the TNBS group presented around 9.7 ± 0.2 cm of colon and the treated groups revealed a significantly increase of colon length. Also, the TNBS + cEPO 1000 UI/Kg treated mice 11.3 ± 0.4 cm demonstrated a larger colon size than the mice treated with TNBS + cEPO 500 UI/Kg 10.1 ± 0.4 cm.

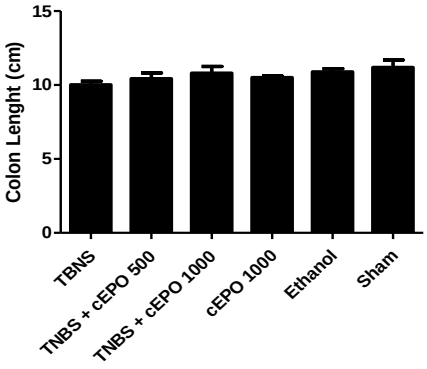

Figure 18 - Effect of TNBS-induced colitis on colon length in the IBD.

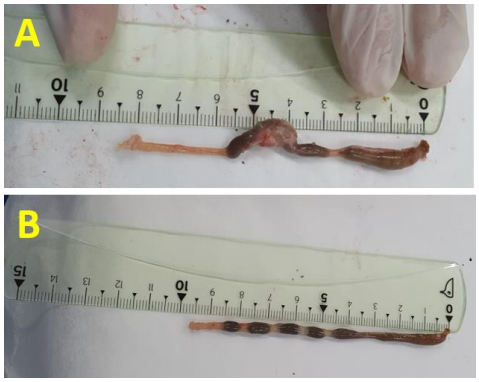

Figure 19 - Appearance and length of colon in the TNBS (A) and TNBS + cEPO 1000 UI/Kg groups (B).

After cEPO treatment, there was a tendency to increase the colon length in EPO-treated mice, but without statistically significant differences in the TNBS group.

1.2 – MEASUREMENT OF CYTOKINES

The TNBS-induced colitis showed a significant production of pro-inflammatory cytokine, as TNF-α, after TNBS administration (figure 20).

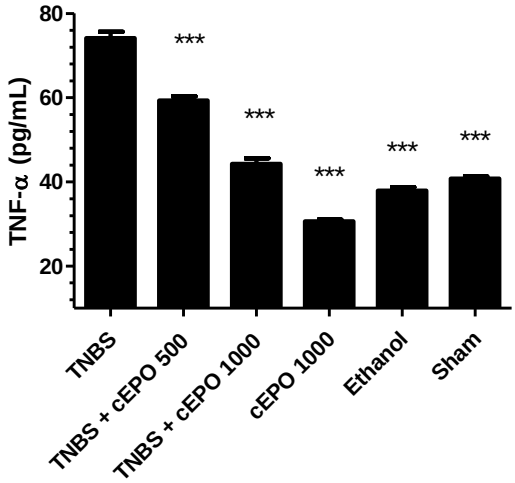

Figure 20 – Effect of TNBS-induced colitis on TNF-α concentration in the IBD.

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

Through the results it is possible to observe that the treatment with TNBS + cEPO 1000 IU/Kg demonstrated greater efficacy in the inflammatory response with 44.30 ± 4.0 pg/ml, compared with cEPO 500 UI/Kg treated group that presented 59.33 ± 2.96 pg/ml.
The TNBS group as a positive control showed a marked inflammatory response with 74.13 ± 4.6 pg/ml. The Sham and Ethanol control groups showed similar baseline values (40.75 ± 1.3 pg/ml and 37.90 ± 1.98 pg/ml), respectively. Control cEPO 1000 UI/Kg (30.60 ± 0.98 pg/ml) showed a better result than the Sham group. Also, the groups TNBS + cEPO 500 UI/Kg, TNBS + cEPO 1000 UI/Kg, cEPO 1000 UI/Kg, Ethanol and Sham demonstrated a statistically significant result considering the TNBS comparator group, with $p<0.001$.

As an inflammatory cytokine (TNF-α) it is expected that this cytokine is in higher concentration in the untreated groups such Ethanol and Sham (134). Also, in a study of IBD with EPO treatment the results of TNF-α concentration were higher in the TNBS group compared to the EPO treated groups, proving its pro-inflammatory effect (58).The fact that lower concentrations were found in the treated groups, especially in the group treated with cEPO 1000 IU/Kg demonstrates a higher efficacy compared with other groups in reducing the inflammatory response. This is an added value as it proves the anti-inflammatory effect with the cEPO molecule 1000 IU/kg treatment.

Regarding IL-10, The chronic TNBS-induced colitis showed a significant production of anti-inflammatory cytokine, after TNBS administration (figure 21).

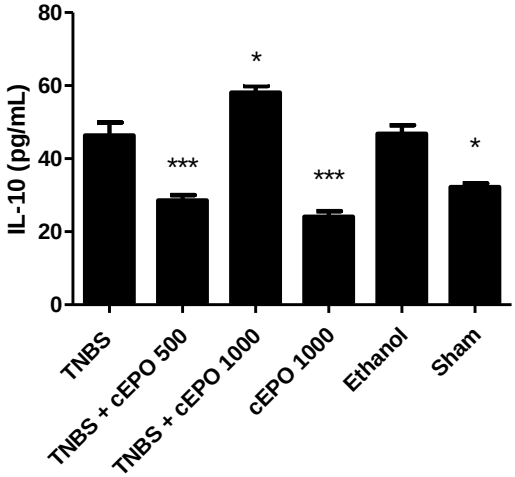

Figure 21 – Effect of TNBS-induced colitis on IL-10 concentration in the IBD.

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

As expected, treatment with cEPO 1000 UI/Kg (58.1 ± 3.56 pg/ml) showed an increased concentration of IL-10 level. The TNBS group presented the highest concentrations of IL-10 with 46.4 ± 7 pg/ml ($p<0.05$), compared with sham group 38.30 ± 1.4 pg/ml). Comparing the treatment of cEPO, the cEPO 500 UI/Kg (28.5 ± 3.0 pg/ml) showed a lower effect comparing to cEPO 1000 UI/Kg (58.10 ± 3.6 pg/ml) As control groups, cEPO 1000 UI/kg (24.10 ± 2.2 pg/ml) showed a lower concentration of IL-10 rather than Sham with 32.2 ± 1.4 pg/ml and cEPO 1000 UI/Kg with 31.95 ± 0.7 pg/ml ($p<0.05$, compared with sham group).

The higher the dose of cEPO, the greater the anti-inflammatory activity showing a dose response effect. Still, as expected, it is possible to verify a greater efficacy in the cEPO molecule 1000 IU/Kg compared with the other treatments. The concentration of IL-10 in the TNBS group is due to the factor of the body starting to produce anti-inflammatory cytokines to target the induced disease (134).
Therefore, in a other acute colitis model of IBD the higher dose of EPO with 1000 UI/Kg showed a significant increase in IL-10, compared to EPO treatment with 500 IU/Kg (58). As estimated, these cytokines were produced during the characteristic innate immune response of the IBD (58).

1.3 – SERUM ALKALINE PHOSPHATASE

Serum alkaline phosphatase (ALP) in the blood was identified in all experimental groups as a marker of intestinal homeostasis (figure 22).

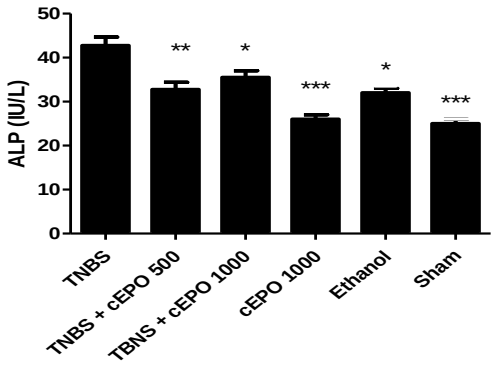

Figure 22 - Effect of TNBS-induced colitis on serum total ALP concentration in the IBD.

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

All groups demonstrated statistically significant values compared to TNBS group. TNBS + cEPO 500 UI/Kg with 32.7 ± 3.3 IU/L treated group showed a lower value when compared to TNBS + cEPO 1000 UI/Kg with 35.5 ± 3.0 IU/L. In general, the ALP levels in TNBS groups were higher than those observed in control groups.

As indicator of intestinal homeostasis this demonstrates a beneficial effect compared to the TNBS group. Since the lower dose of the cEPO treated group shows lower ALP values, presented a beneficial effect rather than TBNS + cEPO 1000 UI/Kg treated group.

ALP is an enzyme distributed among different tissues throughout the body (135). Intestinal ALP has been studied in inflammatory disorders such IBD (58,135). This is an endogenous protein expressed by the intestinal epithelium that is believed to play a vital role in maintaining gut homeostasis (135). Data from several animal and human research trials have demonstrated exogenous IAP may have an effect in mitigating intestinal and systemic inflammation (135). A higher ALP function and expression is associated with increased intestinal inflammation and/or dysbiosis and bacterial translocation (135). So, according to the results an increase concentration of ALP may indicate an inflammatory response that were reduced with cEPO treatment. Furthermore, ALP concentration also decreased on blood in the EPO-treated mice at both EPO doses.

1.4 – ALANINE AMINOTRANSAMINASE

The hepatic function was evaluated according to serum alanine aminotransaminase (ALT) concentration from mice with TNBS induced colitis and mice treated with cEPO.

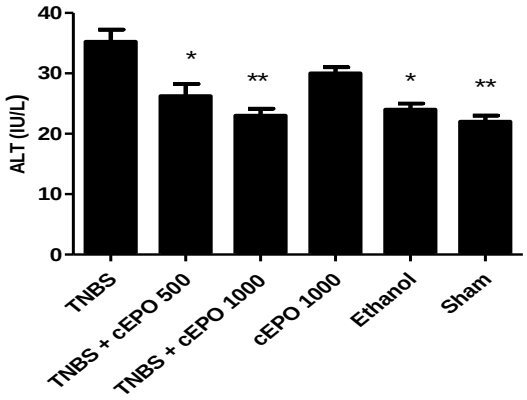

*Figure 23- Effect of EPO treatment on serum ALT concentration in the IBD.Legend: One-way ANOVA and Tukey's post hoc test; *p<0.05; **p<0.001 compared with TNBS group.*

The ALT concentration in blood was significantly higher in the TNBS group compared with the sham group (35.3 ± 4.0 vs 24.0 ± 1.41 IU/L, p<0.001). The cEPO 500 UI/Kg and 1000 UI/Kg treatments at both doses promoted a decreased of ALT levels (26.3 ± 4.0 and 23.0 ± 2.3 IU/L, respectively). Except for the 1000 IU/kg CEPO group, all the other groups demonstrated statistical significance.

Liver test abnormalities are frequently observed in patients with IBD such as an increased bowel permeability (136). Through biochemical analysis, recent studies revealed significant increase in serum activities of ALT when IBD disease is present (137,138). Also, a study in the acute model of the disease with EPO treatment was demonstrated an increase in ALT in mice with TNBS-induced colitis (58).

As a marker of hepatic function, the fact that lower values were obtained in the TNBS + cEPO 1000 IU/Kg group is a favorable indicator to exclude hepatic damages (136). The treatment groups being close to the ALT levels of the sham group may be a good indicator for liver function (136).

1.5 – CREATININE

Creatinine is a marker of renal function as such, mice treated with cEPO were used to determine if there were alterations in the values for the renal damage markers (Figure 24).

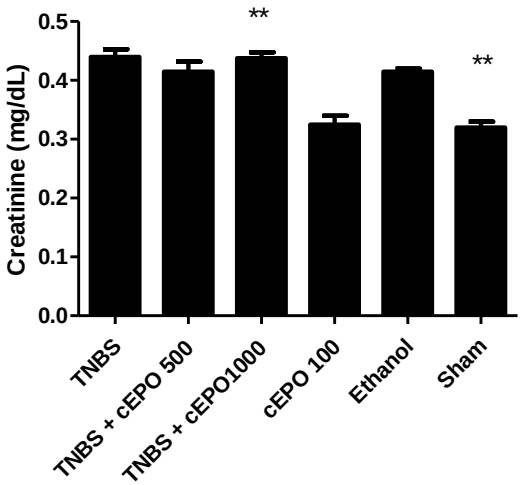

Figure 24 - Effect of TNBS-induced colitis on serum creatinine concentration in the IBD.

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

The TNBS group exhibited a significant increase in creatinine compared with sham group (0.44 ± 0.02 vs 0.32 ± 0.014 mg/dl, p<0.01). Sham group and TNBS + cEPO 1000 UI/Kg group demonstrated statistical significance p<00.1 considering the TBNS group as a comparator. The cEPO 1000 IU/Kg with 0.13 ± 0.5 mg/dl and Sham with 0.19 ± 0.44 mg/dl control groups demonstrated inferior effects to the other groups. TNBS + cEPO 1000 UI/Kg group exhibited a statistical significance p<0.001 with 0.43 ± 0.02 Compared with TNBS + cEPO 500 UI/Kg that has not demonstrated a statistical significance with 0.41 ± 0.03.

As the bibliography indicates, the increase of creatinine is an indicator of an extra-intestinal manifestation (58). Looking through the results, a higher dose of cEPO treatment may further stimulate renal function (58). In groups with high creatinine values, renal function may be damaged and compromised.

1.6 – UREA

It was analyzed serum from mice with TNBS induced colitis and mice treated with cEPO to determine if there were alterations in the values for the renal damage markers, like urea (figure 25)**.**

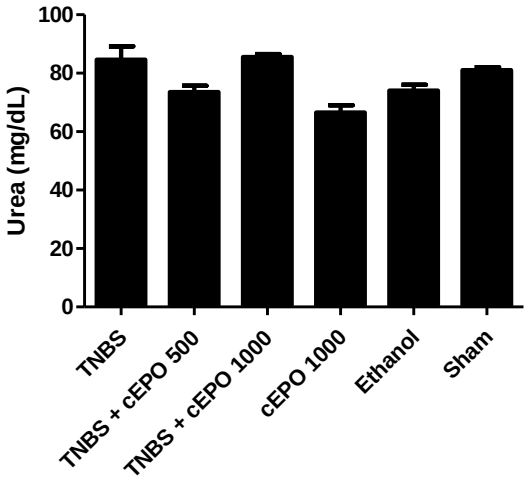

Figure 25 - Effect of TNBS-induced colitis on serum urea concentration in the IBD.

The TNBS group exhibited a significant increase in urea compared with sham group (84.7 ± 11.0 vs 81.0 ± 1.4 mg/dl. The mice treated with cEPO presented a significant decrease of urea. The cEPO treatment group with cEPO 1000 UI/Kg (86.0 ± 2.0 mg/dl) showed a higher urea value than treatment with cEPO of 500 IU/Kg with 74.4 ± 4.4 mg/dl. No statistical significance was observed between all groups.

According to the results the value presented for TNBS + cEPO 500 UI/Kg are in line with what is anticipated because urea is a marker of renal function so, as it is a lower dose the renal wear is also lower promoting thus a dose-dependent effect but without statistical significance between cEPO doses.

1.7 - MORTALITY RATE

At the end of the study, it was evaluated the mortality rate in the experimental groups as a sign of toxicity (figure 26).

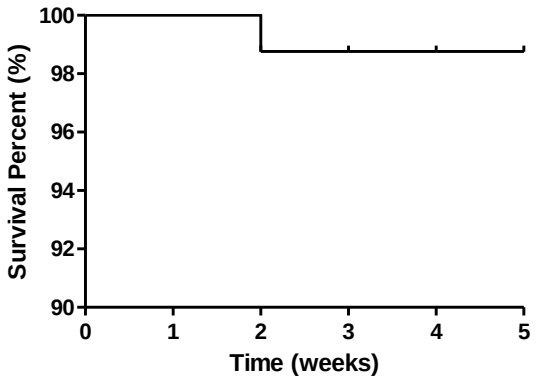

Figure 26 – Survival rate considering weeks of experiment.

Then, the survival rate remained stable after the second week. Since the administration of the cEPO molecule, the mortality rate has been null.

In previous results, the mortality rates up to the second week of experience are documented, which is expected since as it proceeds to induce the disease (21,58).

1.8 – RED BLOOD CELLS

For measurement of the adverse effects these results are intended to demonstrate the absence of haematopoietic effect (figure 27).

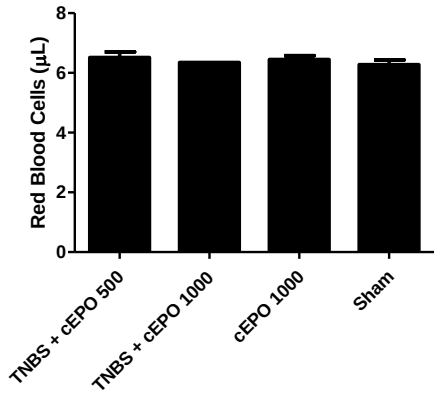

Figure 27 - Red blood cell count in each group.

In the groups treated with cEPO, the group with the highest dose showed the best results. cEPO 500 UI/Kg with 6.5 ± 0.4 µL and cEPO 1000 UI/Kg with 6.3 ± 0.4 µL groups that present a similar red blood cells (RBCs) counting, comparing with sham group with 5.6 ± 0.4 µL.

Erythropoietin has a haematopoietic effect, so by carbamylating the EPO molecule it was possible to inhibit the haematopoietic effect of erythropoietin (139). According to the bibliography, an increase in RBCs is expected when the haematopoietic process is stimulated (139). As observed, there was a reduced RBCs in the cEPO treated group compared to the EPO treated group. These results bring new information insofar as there is no stimulation of the erythropoietic effect caused by EPO whose adverse effects were diverse. It could, therefore, be a therapy used to treat IBD without the expected adverse effects of EPO.

1.9 - HAEMATOCRIT

The haematocrit (HT) level was measured at the end of the experimental period to evaluate the risk of related side effects (Figure 28).

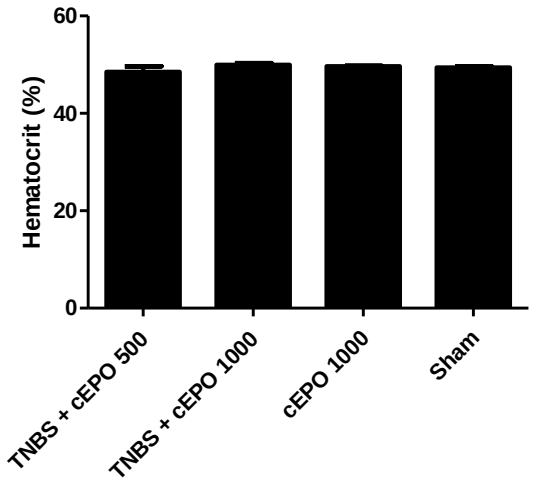

Figure 28 - Percentage of haematocrit in each group.

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

All evaluated experimental groups, like TNBS + cEPO500 (48.5± 2.2 %), TNBS+cEPO1000 (49.9 ± 0.7 %) groups, presented similar values of hematocrit compared with sham group (49.6 ± 0.3 %), without statistically significant differences.

HT indicates the percentage of red cells, also known as RBCs or erythrocytes, in the total blood volume (140). Moreover, it is known that EPO stimulates erythropoiesis, which leads to increased production of RBC and, subsequently, an increase in HT level (58). These data suggest that cEPO does not increase the risk of adverse events related to EPO molecule (58). Although there are differences in the results, these are not statistically significant. The results indicate that carbamylation had positive safety effects, as the hematocrit percentage was not elevated compared to the sham group. This suggest that treatment with cEPO is safer and carries fewer risks and adverse reactions as severe cardiovascular events.

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1 - ANALYSIS OF THE RESULTS OF CARBAMYLATED ERYTHROPOIETIN COMPARED TO THE RESULTS OBTAINED FOR ERYTHROPOIETIN

Our research group has developed previous preclinical studies in a chronic model of TNBS-induced colitis, with Erythropoietin with 500 UI/Kg and 1000 UI/Kg, which presented beneficial effects in the progression and treatment of IBD (58,141,142). Although the results are not published, a comparison will be made considering the results obtained so far. Here, the results of the cEPO treatment of 1000 IU/Kg will be compared with the EPO treatment of 1000 IU/Kg. As mentioned, the objective will be to compare the efficacy and safety between the two molecules in the chronic IBD model.

1.1 - MEASUREMENT OF CYTOKINES

The comparison between the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10 will be made considering the two treated groups.

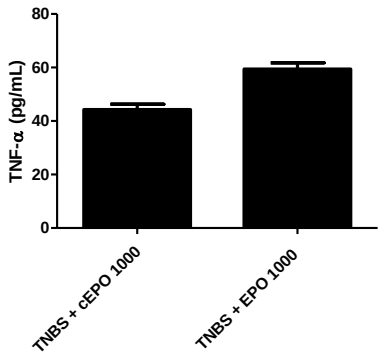

Figure 29 - Treatment groups with TNBS + cEPO 1000 UI/Kg vs TNBS + EPO 1000 UI/Kg.

Through the results it is possible to observe that the group treated with cEPO 1000 IU/Kg with 44.3 ± 4.0 pg/ml presented lower levels of this pro-inflammatory cytokine compared to the group treated with EPO 1000 IU/kg with 59.4 ± 4.0 pg/ml. The cEPO of 1000 IU/Kg showed a lower concentration of TNF-α cytokine. This means that treatment with cEPO 1000 UI/Kg presented a higher efficacy than treatment with EPO 1000/Kg.

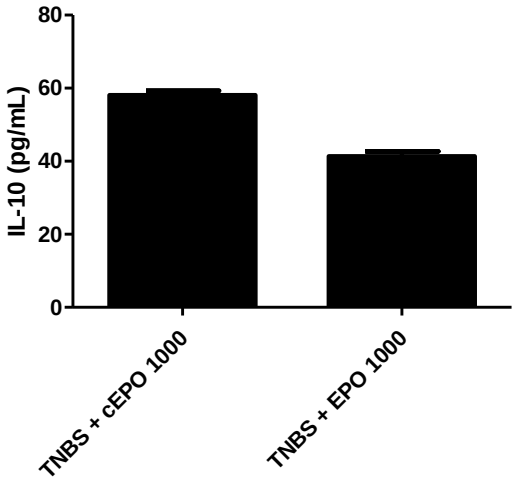

Figure 30 - Treatment groups with IL-10 cEPO 1000 UI/Kg vs treated groups with TNBS + EPO 1000 UI/Kg.

TNBS + cEPO 1000 UI/Kg treated group demonstrated a higher concentration of IL-10 cytokine. Comparing the groups, the group of TNBS + cEPO 1000 UI/Kg with 58.1 ± 3.3 pg/ml presented a higher value of these anti-inflammatory cytokine rather than the group of EPO 1000 UI/Kg with 41.3 ± 2.3 pg/ml.

This means that treatment with cEPO 1000 IU/kg demonstrated a superior anti-inflammatory property compared to treatment with EPO 1000 IU/kg. Once again, the efficacy of cEPO 1000 UI/Kg treatment was verified.

1.2 – HAEMATOCRIT AND RED BLOOD CELLS

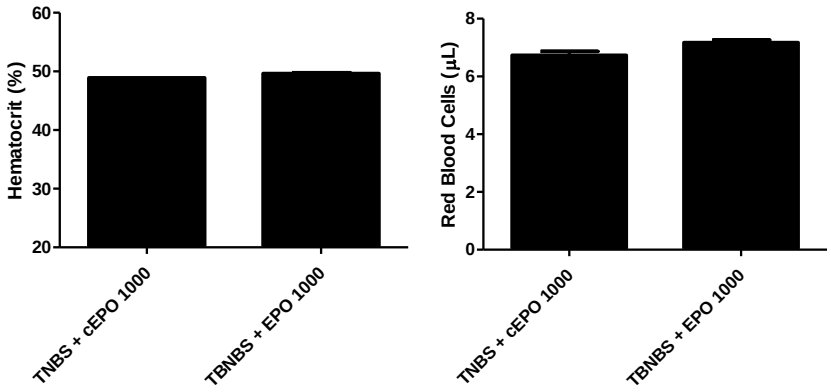

Figure 31 - Treatment groups with hematocrit and RBCs cEPO 1000 UI/Kg vs treated groups with TNBS + EPO 1000 UI/Kg.

Comparing TNBS + EPO 1000 UI/Kg with the TNBS + cEPO 1000 UI/Kg, the first demonstrated an increased in HT level with 49.7 ± 0.3 % and the second showed 48.0 ± 0.01 %. Concerning the RBCs, TNBS + cEPO 1000 UI/Kg with 6.3 ± 0.3 µL also showed a minor concentration of these cells, compared to the EPO 1000 UI/Kg treatment with 6.8 ± 0.4 µL.

In terms of the percentage of haematocrit and RBCs, it is possible to verify that treatment with cEPO 1000 IU/Kg showed better results, with greater safety in terms of potential adverse reactions, as cardiovascular events. These results demonstrate that carbamylation of the EPO molecule was able to reduce the hematopoietic effects associated with the EPO molecule with a superior efficacy and security.

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CHAPTER 5 –CONCLUSIONS

IBD is linked significant psychological comorbidities, with associated impacts on patient quality of life, disease course, and health care costs. Although the cause of IBD remains unknown, considerable progress has been made in recent years. Several environmental factors have been associated with the development of IBD. In summary risk factors play important roles in the pathophysiology of IBD through, affecting host immune system.

Pharmaceutical and medicinal chemistry has been modulating and changing the lifestyle of human beings until today, through the formulation of organic molecules.

Chronic TNBS-induced colitis is often used. The main advantages of chemically induced IBD models are that they are relatively cheap, quick, and easy to develop. Animal models of IBD mimic the pathogenesis of IBD in humans and allow testing new pharmacological approaches. Chronic colitis model is appropriated to developing and testing novel therapeutic strategies for the treatment of IBD. The advantage of chronic models compared to acute models is that the latter could provide only limited information about the pathogenesis of human IBDs, as the chemical injury to the epithelial barrier leading to self-limiting inflammation rather than to chronic disease.

All the proposed objectives were achieved. The carbamylation process was a success, with a satisfactory and substantial percentage of approximately 99%. In addition, clinical conditions in the renal and hepatic functions were attenuated when mice were treated with cEPO. The cEPO molecule 1000 IU/kg showed the best results in terms of body weight, colon length, pro-inflammatory and anti-inflammatory cytokines, and haematocrit and RBCs levels.

This way, it was demonstrated that treatment with cEPO 1000 UI/Kg molecule could have a better safety and safety in chronic TBNS-induced colitis model.

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