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Efficacy and safety of erythropoietin in a chronic model of Inflammatory Bowel Disease

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ABSTRACT

Background: Inflammatory Bowel Disease (IBD) is recognized as a group of chronic inflammatory disorders, localized in the gastrointestinal tract, which does not have a cure known. Indeed, the pharmacological approaches, commonly used, demonstrate significant toxicity, which highlights the need of investigating new possible treatments. Erythropoietin (EPO) is clinically used in anemic patients, with chronic renal insufficiency, due to its erythropoietic effect. However, it has also been described other non-erythropoietic effects, such as an anti-inflammatory role. There is already preclinical evidence about its anti-inflammatory effect in the IBD context, namely in an acute model of colitis in mice. Therefore, it is relevant to ascertain its anti-inflammatory effect in a chronic model, but mainly its hematopoietic side effect, during chronic treatment. Aim: This experiment aims to evaluate the efficacy and safety of EPO treatment in a chronic 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis model in rodents. Methods: The induction of chronic colitis consistedofn five weekly intrarectal administrations of 1% TNBS, and then mice were treated daily with 500 IU/Kg or 1000 IU/Kg of EPO, through intraperitoneal injections, for 14 days. Results: EPO demonstrated a significant anti-inflammatory effect, translated by a significant reduction of the concentration oftumorr necrosis factor-α, fecal calprotectin, and fecal hemoglobin. Moreover, it has also been demonstrated to be safe, considering the cardiovascular system, in terms of extraintestinal manifestations, namely at renal and hepatic functions. Conclusions: EPO demonstrated to be a promising pharmacological approach to be considered in the management of IBD, being an interesting target for drug repositioning.

1. Introduction

Inflammatory Bowel Disease (IBD) is recognized as a chronic and relapsing inflammatory response, localized in the gastrointestinal tract, which can be represented by two phenotypes, namely Chron's disease and ulcerative colitis [1–4]. Indeed, both phenotypes are considered to be debilitating conditions but not fatal [5]. Currently, it is estimated that nearly 3,9 million females and 3 million males are living with IBD, and there is an upward trend of new cases, especially in newly industrialized nations, such as those inserted in Asia, like for example China, and India [1,5,6]. Clinical presentation of IBD presents a wide range of symptoms and signs that can be specific to the gastrointestinal tract, and others that are non-specific, which can be related to extraintestinal manifestations. As gastrointestinal signs and symptoms, it can be emphasized diarrhea, abdominal pain/cramping, blood in feces, and rectal urgency. As extraintestinal manifestations, it can be identified fever, anemia, arthritis, ankylosing spondylitis, uveitis, iritis, pyoderma gangrenosum, sclerosing cholangitis, and erythema nodosum [1,7–9].

Nowadays, there is no cure for IBD and pharmacological approaches aim to induce and maintain the patient in remission, along as ameliorate the disease's secondary effects [1,7,10,11]. Furthermore, pharmacological treatment in the IBD context includes anti-inflammatory drugs, such as aminosalicylates, immunomodulators, corticosteroids, biological agents, antibiotics, and agents that inhibit leukocyte adhesion and migration [1,7,12,13]. These therapies have shown to be effective but are inappropriate for long-term use due to their side effects and complications, like for example, an increased rate of infectious diseases and/or malignancies [7,14]. In this sense, it is essential to investigate

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new possible pharmacological tools for the future management of IBD, which includes not only an acceptable efficacy profile but also a safer approach in a longer spectrum of time of application.

Erythropoietin (EPO) is commonly used in anemic patients, with chronic renal failure, due to its capability of regulating the production, differentiation, and survival of red blood cells [7,15–17]. Additionally, EPO has also demonstrated non-erythropoietic effects, such as the inhibition of apoptosis, autophagy, induction of angiogenesis, neuroprotection, and tissue regeneration [18–20]. Moreover, it has been also identified a potential anti-inflammatory effect, which can be explained by the interaction between EPO and a heterodimeric receptor, constituted by its endogenous receptor and a β common receptor (EPOR/CD131) [18,19,21]. Upon this interaction, EPO demonstrates the capability of activating Janus Kinase(JAK)− 2/Signal Transducer and Activator of Transcription-5 pathway, along as the inhibition of the expression of Interleukin(IL)− 6, − 1β, and Tumor Necrosis Factor (TNF)-α, via STAT3/5 signaling [22,23]. Additionally, EPO also decreases the expression of Nuclear Factor(NF)-kB, which represents a family of inducible transcription factors related to the inflammatory response, cell proliferation, apoptosis, and differentiation [7,24]. In this sense, EPO arises as an interesting pharmacological approach to be evaluated in chronic inflammatory disorders.

Currently, there is already preclinical evidence demonstrating that EPO can be relevant in the clinical outcomes from the treatment of acute crisis of IBD, through the significant attenuation of the inflammatory response and extraintestinal manifestations [7]. After the evaluation of EPO treatment in an acute colitis model, it arises the interest of evaluating its effect, in a longer spectrum of time, considering its potential long-term application. Therefore, the main objective of this experiment is to evaluate the efficacy and safety of EPO treatment, in a chronic 2,4,6-Trinitrobenzenesulfonic acid(TNBS)-induced colitis model. According to the vast clinical information available concerning the safety profile of EPO, an opportunity might arise for a possible future process of drug repositioning, where the results of this experiment can be fundamental for further consideration.

2. Material and methods

2.1. Chemicals and reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS 5%) was acquired from Sigma Aldrich Chemical®. Xylazine (Rompun® 2%) and Ketamine were purchased from Bayer® and Merial®, respectively. Erythropoietin (Eprex® 10,000 IU/ml) was purchased from Janssen-Cilag Farmacêutica, Lda. Elisa assay kits, for TNF-α and fecal calprotectin measurements, were obtained from Hycult Biotechnology®. ADVIA® kit was purchased from Siemens Healthcare Diagnostics®.

3. Animals

Female CD-1 mice (6–8 weeks old, 24 ± 4 g) were used throughout the experiment. The animals had one week for acclimatization and were housed in standard polypropylene cages, with access to food and water ad libitum. The temperature, humidity, and lighting were controlled through the study. Indeed, mice were kept at 18–23 ºC and 40–60 % humidity, in a controlled 12-hour day/night cycle. Animal care was in accordance with the internationally accepted principles for laboratory use and care found, present in Directive 2010/63/EU. Indeed, the experiment was approved by the Ethics Committee for Animal Experimentation of the Faculty of Pharmacy of the University of Lisbon (ORBEA), with code number 3/2020. Moreover, it was also approved by the *DireçãoGeral de Alimentação e Veterinária* (DGAV), on November 6th of 2020.

4. Induction of experimental colitis

The induction of chronic colitis was developed through weekly intrarectal administrations of TNBS. The animals were left unfed 24 h before each administration, in order to facilitate the intrarectal administrations due to the absence of feces. Prior to each intrarectal administration, mice were anesthetized, through an intraperitoneal injection of 40 μL of a solution containing ketamine 100 mg/kg + xylazine 10 mg/kg. Afterwards, 100 μL of TNBS 1 % was administrated intrarectally, with the help of a cannula, carefully inserted until 4 cm in the colon. Then, in order to avoid rectal reflux, the animals were kept in Trendelenburg position for 1 min. Mice were then subjected to four more intrarectal administrations of 100 μL of TNBS 1 %, one per week, five weeks in the total, namely at days 1, 8, 15, 22, and 29 of the experiment.

At day 36, mice were anesthetized, and cardiac puncture was performed for blood collection. Afterwards, the animals were sacrificed through cervical dislocation. The necropsy was initiated with a midline incision into the abdomen and the colon was carefully removed and freed from surrounding tissues.

5. Experimental design

The animals were randomly allocated to six distinct groups: TNBS group (n = 10) – disease control group, where the animals were subjected to the induction of chronic colitis; TNBS + EPO500 group (n = 20) – experimental group, where the animals were subjected to the induction of chronic colitis and treated daily with EPO 500 IU/Kg (diluted in NaCl) for 14 days, through intraperitoneal injections, between days 22–35 of the experiment; TNBS + EPO1000 group (n = 20) – experimental group, where the animals were subjected to the induction of chronic colitis and treated daily with EPO 1000 IU/Kg (diluted in NaCl) for 14 days, through intraperitoneal injections, between days 22–35 of the experiment; EPO1000 group (n = 10) – drug control group, where the animals were subjected to a daily treatment with EPO 1000 IU/Kg (diluted in NaCl), through intraperitoneal injections, between days 22–35 of the experiment; Ethanol group (n = 6) – vehicle control group, where the animals were subjected to five weekly intrarectal administrations of 50 % ethanol, at days 1, 8, 15, 22, and 29 of the experiment; Sham group (n = 6) – control group, where the animals were subjected to five weekly intrarectal administrations of 0.9 % NaCl, at days 1, 8, 15, 22, and 29 of the experiment.

6. Monitoring of clinical signs

Mice were carefully observed daily in order to evaluate clinical signs, such as body weight, morbidity, stool consistency, and annus appearance. These observations were performed and registered throughout the whole experiment.

7. Quantification of biochemical markers

All the blood samples collected through cardiac puncture were separated by centrifugation (3600 rpm for 15 min) and then serum was analyzed by an automated clinical chemistry analyzer (ADVIA®1200) to evaluate the concentration of Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP), urea, creatinine, and hematocrit. In addition, to evaluate the inflammatory response, it was also evaluated the serum concentration of TNF-α and IL-10. Moreover, fecal hemoglobin was quantified using a quantitative method by immunoturbidimetry. Finally, the measurement of calprotectin in stool was developed through enzyme-linked immunosorbent assay (ELISA).

8. Macroscopical and microscopical evaluation

In order to evaluate colitis severity, macroscopical and microscopical evaluation of colon tissue were performed. For the macroscopical

evaluation, the analysis took in account the length of the colon tissue, the presence of hyperemia, adhesions, and/or ulcers, according to Morris et al., 1989.[25] For the microscopical evaluation, a portion of four centimeter of distal colon tissue was fixed in 10 % phosphate-buffered formalin, sectioned at 5 μm and then stained with hematoxylin and eosin. To increase the possibility of detecting fibrosis, Masson's trichrome staining was used. The intestinal samples were evaluated, by an independent histopathologist, considering several parameters, such as tissue loss, epithelial lesions, inflammation grade, fibrosis, and the total extent of the disease.[26,27] In addition to the qualitative microscopic evaluation, a quantitative analysis was also carried out between groups, attributing a punctuation between 0 and 4 in each factor, previously referred, which its sum resulted in a final histopathological score (maximum of 20).

9. Statistical analysis

All the results were expressed as mean ± standard deviation of N observations, where N represents the number of animals analyzed, in each group. Data analysis was performed using GraphPad 5.0® (GraphPad, San Diego, CA, USA) software. The results were analyzed by one-way ANOVA to determine statistically significant difference between the groups, followed by Tukey's post hoc test for multiple comparisons. It was considered a p-value < 0.05 to determine statistically significant differences.

10. Results

10.1. Efficacy of erythropoietin in a chronic model of colitis

10.1.1. Monitoring of clinical signs

Throughout the whole experiment, the mice were observed daily for body weight, stool consistency and morbidity. The animals of TNBS group presented changes of intestinal motility, characterized by the presence of diarrhea and/or soft stool allied with moderate edema of the anus. Between the EPO-treated groups with the disease-induced, the mice also presented alterations in intestinal motility, but with less severity. The control groups, namely EPO1000, ethanol and sham groups, did not present any changes.

The body weight was measured throughout the whole experiment (Fig. 1), and it was noticed a gradual increase between the groups, although with some variances, until the end of the experiment. The animals of the TNBS group demonstrated a smaller increase of the body weight, namely 4.95 ± 2.61 % of its initial weight. In addition, between TNBS+EPO500 and TNBS+EPO1000 groups, they presented a variation of 12.85 ± 5.69 %, and 14.00 ± 5.95 % in terms of body weight, respectively. In the EPO1000, ethanol and sham control groups, it was observed a mean variation on their bodyweights of 8.38 ± 5.33 %, 5.16 ± 4.51 %, and 5.39 ± 6.53 %, respectively. There were no significant differences between all groups. Therefore, it is not possible to determine a significant effect of EPO in the variation of the bodyweight between the treated and non-treated groups.

10.2. Analysis of the Inflammatory Response

The measurement of TNF-α, which is a pro-inflammatory cytokine, provides the analysis of the inflammatory response. This cytokine was measured and compared between all experimental groups (Fig. 2). As expected, the TNBS group presented the highest concentration of this pro-inflammatory cytokine (76.96 ± 8.21 pg/ml). EPO treatment showed a beneficial effect on this parameter, where TNBS+EPO 500 and TNBS+EPO 1000 groups showed a significant decrease on TNF-α levels, in comparison to TNBS group (48.18 ± 8.74 pg/ml vs. 50.90 ± 8.95 pg/ml vs. 76.96 ± 8.21 pg/ml), but without statistically significance. Additionally, it is not possible to confirm a dose-dependent effect by EPO, since there were not statistically significant differences between the treatment at both dosages. Finally, the EPO1000 (30.15 ± 2.05 pg/ml), ethanol (41.50 ± 3.96 pg/ml), and sham (40.18 ± 5.22 pg/ml) control groups presented a significant reduction in terms of the concentration of this cytokine, comparing to the TNBS group ($p < 0.001$).

An anti-inflammatory cytokine, IL-10, was measured and compared between all the experimental groups (Fig. 3). The TNBS group presented the highest concentration of this cytokine (70.31 ± 6.88 pg/ml). As expected, it was noticed a significant decrease on the concentration of

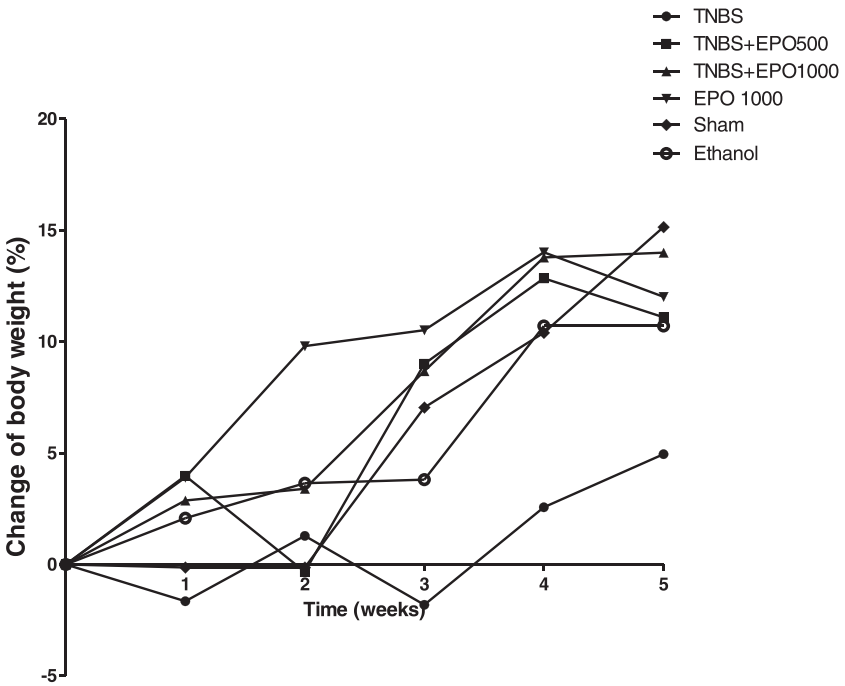

Fig. 1. – Change of bodyweight between the groups throughout the experiment. Experimental data: TNBS group (n = 10), + 4.95 ± 2.61 %; TNBS + EPO500 group (n = 20), + 12.85 ± 5.69 %; TNBS + EPO1000 group (n = 20), + 14.00 ± 5.95 %; EPO1000 group (n = 10), + 8.38 ± 5.33 %; Ethanol (n = 6), + 5.16 ± 4.51 %; Sham group (n = 6), + 5.39 ± 6.53 %.

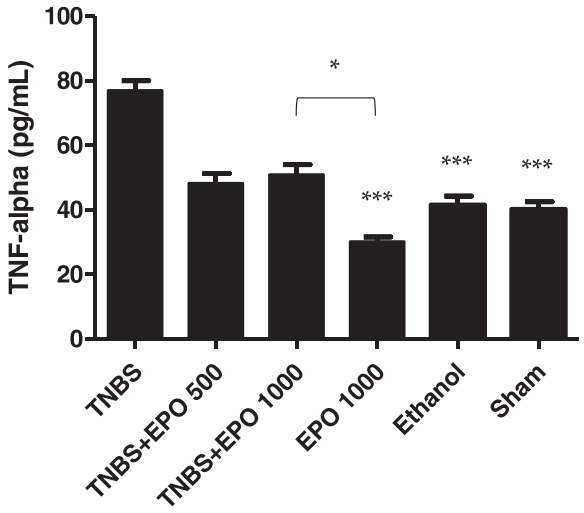

Fig. 2. – TNF-α concentration between the groups. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$; * **$p < 0.001$; compared with TNBS group and between groups. Experimental data: TNBS group (n = 7), 76.96 ± 8.21 pg/ml; TNBS + EPO500 group (n = 8), 48.18 ± 8.74 pg/ml; TNBS + EPO1000 group (n = 8), 50.90 ± 8.95 pg/ml; EPO1000 group (n = 2), 30.15 ± 2.05 pg/ml; Ethanol (n = 2), 41.50 ± 3.96 pg/ml; Sham group (n = 5), 40.18 ± 5.22 pg/ml.

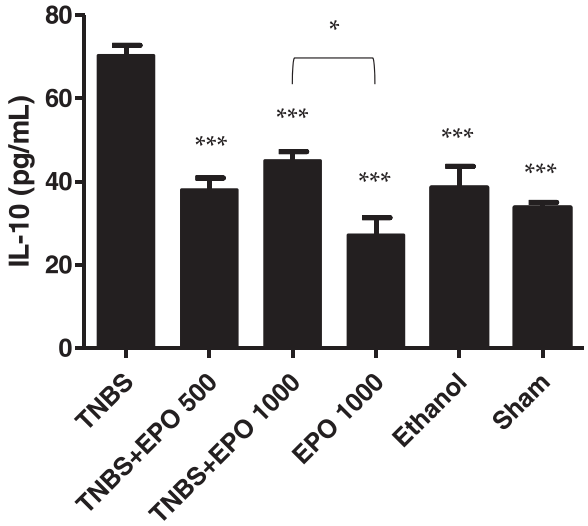

Fig. 3. - IL-10 concentration between the groups. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$; * **$p < 0.001$; compared with TNBS group and between the other groups. Experimental data: TNBS group (n = 8), 70.31 ± 6.88 pg/ml; TNBS + EPO500 group (n = 8), 38.06 ± 8.00 pg/ml; TNBS + EPO1000 group (n = 8), 45.06 ± 6.13 pg/ml; EPO1000 group (n = 2), 27.15 ± 6.01 pg/ml; Ethanol (n = 2), 38.60 ± 7.21 pg/ml; Sham group (n = 5), 33.80 ± 2.70 pg/ml.

IL-10 in treated groups, TNBS+EPO500 (38.06 ± 8.00 pg/ml) and TNBS+EPO1000 groups (45.06 ± 6.13 pg/ml), in comparison to the disease control group ($p < 0.001$). In this sense, EPO demonstrates a significant effect on decreasing the concentration of IL-10 when administrated in both doses. The EPO1000 (27.15 ± 6.01 pg/ml), ethanol (38.60 ± 7.21 pg/ml) and sham (33.80 ± 2.70 pg/ml) control groups also showed a significant decrease on the levels of IL-10, in comparison to the TNBS group ($p < 0.001$). Finally, it was additionally observed a significant reduction on the concentration of this cytokine between the TNBS+EPO1000 group and the EPO1000 group ($p < 0.05$).

10.3. Fecal hemoglobin

Fecal hemoglobin is a marker that allows the evaluation of the intensity of hemorrhagic focus, making it possible to analyze and compare the severity of the lesions among the experimental groups (Fig. 4). Throughout the analysis, it was possible to observe that the TNBS group presented a significantly higher concentration of fecal hemoglobin in comparison with other groups namely, treated groups TNBS+EPO 500 (6.93 ± 2.1 μmol/g feces vs. 2.18 ± 0.30 μmol/g feces, $p < 0.001$) and TNBS+EPO1000 (6.93 ± 2.1 μmol/g feces vs 2.53 ± 0.35 μmol/g feces, $p < 0.001$). In addition, TNBS group also shown a significant increase in the concentration of this biomarker comparing to EPO 1000 (6.93 ± 2.1 μmol/g feces vs 1.05 ± 0.21 μmol/g feces, $p < 0.001$), ethanol (6.93 ± 2.1 μmol/g feces vs. 2.05 ± 0.50 μmol/g feces $p < 0.001$) and sham (6.93 ± 2.1 μmol/g feces vs. 1.70 ± 0.26 μmol/g feces, $p < 0.001$) control groups. In this sense, the treatment with EPO demonstrated a beneficial effect in terms of the hemorrhagic focus in both doses. However, it was not possible to determine a dose-dependent effect, since there are not statistically significant differences between both EPO treated groups.

10.4. Fecal Calprotectin

Fecal calprotectin is an important marker related to mucosal inflammation, and its concentration is directly associated to the leukocyte accumulation in stool.[28] In this sense, in order to evaluate the influence of EPO in this marker, it was quantified and compared between all the groups (Fig. 5). As expected, the TNBS group revealed the highest concentration of fecal calprotectin, namely 110.6 ± 15.14 ng/mg. The values obtained in TNBS + EPO500 (10.50 ± 1.93 ng/mg) and TNBS + EPO1000 (10.75 ± 2.12 ng/mg) groups revealed that the treatment with EPO had the capability to significantly reduce the concentration of this marker, in comparison to the TNBS group ($p < 0.001$). Indeed, it is possible to observe that the EPO-treated groups had similar results to the EPO1000 (5.33 ± 0.58 ng/mg), ethanol (9.00 ± 0.00 ng/mg) and sham (6.5 ± 0.71 ng/mg) control groups. However, it is not possible to determine a dose-dependent effect by EPO since there are not significant differences between TNBS + EPO500 and TNBS + EPO1000 groups.

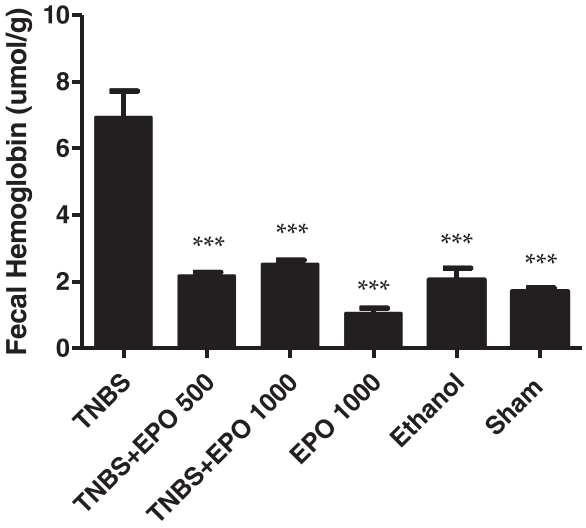

Fig. 4. - Quantification of fecal hemoglobin between groups. Legend: One-way ANOVA and Tuckey's post hoc test. * **$p < 0.001$ compared with TNBS group. Experimental data: TNBS group (n = 7), 6.93 ± 2.1 μmol/g feces; TNBS + EPO500 group (n = 8), 2.18 ± 0.30 μmol/g feces; TNBS + EPO1000 group (n = 8), 2.53 ± 0.35 μmol/g feces; EPO1000 group (n = 2), 1.05 ± 0.21 μmol/g feces; Ethanol (n = 2), 2.05 ± 0.50 μmol/g feces; Sham group (n = 5), 1.70 ± 0.26 μmol/g feces.

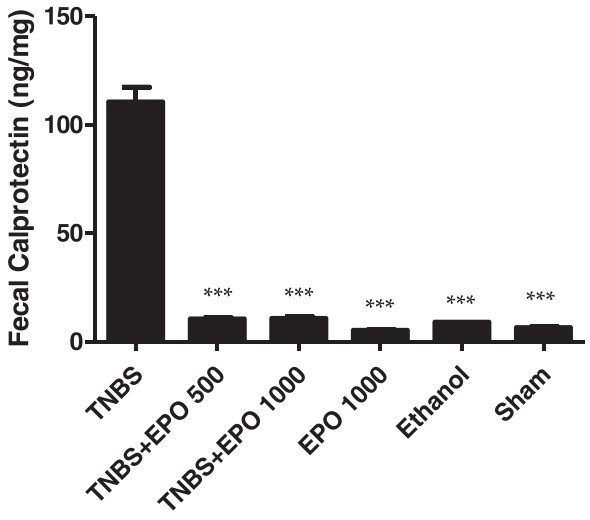

Fig. 5. – Determination of fecal calprotectin between groups. Legend: One-way ANOVA and Tuckey's post hoc test. * **$p < 0.001$ compared with TNBS group. Experimental data: TNBS group (n = 5), 110.6 ± 15.14 ng/mg; TNBS + EPO500 group (n = 8), 10.50 ± 1.93 ng/mg; TNBS + EPO1000 group (n = 8), 10.75 ± 2.12 ng/mg; EPO1000 group (n = 3), 5.33 ± 0.58 ng/mg; Ethanol (n = 3), 9.00 ± 0.00 ng/mg; Sham group (n = 2), 6.5 ± 0.71 ng/mg.

10.5. Alkaline Phosphatase

ALP has a protective effect on the intestinal system, being responsible for the mucosal defense [7] This marker was measured in all experimental groups and compared between each other (Fig. 6). Indeed, the highest concentration of ALP was noticed on the TNBS group (42.88 ± 5.7 U/L), and the other groups presented similar results, without statistically significant differences between them. Interestingly, the TNBS+EPO500 (31.00 ± 3.42 U/L) and TNBS+EPO1000 (32.38 ± 4.1 U/L) groups demonstrated statistically significant differences in comparison to the TNBS group ($p < 0.001$), showing a beneficial effect of EPO in terms of the expression of intestinal ALP. However, it was not possible to determine a dose-dependent effect, since there were not statistically significant differences between the two groups treated with EPO at different doses. Additionally, it is possible to observe that the TNBS+EPO500 and TNBS+EPO1000 groups presented similar results with the EPO1000 (26.00 ± 1.41 U/L), ethanol (30.50 ± 3.54 U/L), and sham (29.60 ± 3.44 U/L) control groups. In this sense, it was possible to observe a positive influence of administrating EPO in the concentration of ALP.

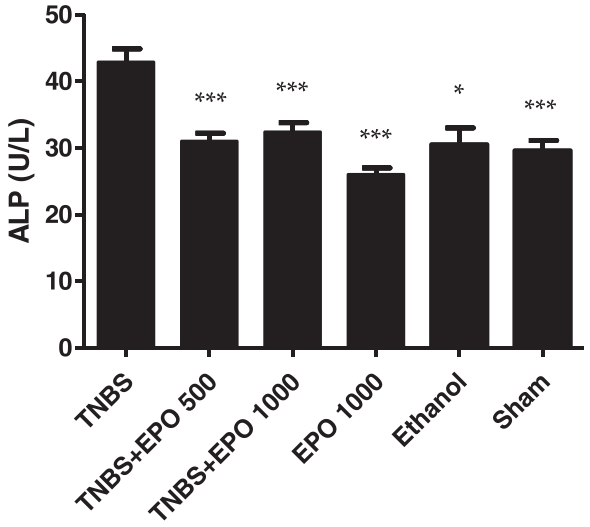

Fig. 6. – Alkaline phosphatase concentration between the groups. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$; * **$p < 0.001$ compared with TNBS group. Experimental data: TNBS group (n = 8), 42.88 ± 5.7 U/L; TNBS + EPO500 group (n = 8), 31.00 ± 3.42 U/L; TNBS + EPO1000 group (n = 8), 32.38 ± 4.1 U/L; EPO1000 group (n = 2), 26.00 ± 1.41 U/L; Ethanol (n = 2), 30.50 ± 3.54 U/L; Sham group (n = 5), 29.60 ± 3.44 U/L.

10.6. Macroscopic assessment of colitis

The macroscopical evaluation of the colons revealed that the maximal damage was observed in TNBS group, corresponding to localized hyperemia but without ulcers. In addition, the TNBS + EPO500 and TNBS + EPO1000 groups presented also localized hyperemia, however with less severity than TNBS group. The EPO1000, sham, and ethanol control groups did not present any damage on the colon tissue.

The colon length represents a marker of tissue integrity and was measured and compared between all the experimental groups (Fig. 7). There were differences between some of the groups, but they were not statistically significant. Contrariwise to what was expected, the TNBS and EPO-treated groups did not have any significant differences between them in terms of colon length, presenting each one a mean of 13.40 ± 7.98 cm (TNBS group), 9.88 ± 0.93 cm (TNBS+EPO 500 group) and 10.30 ± 0.94 cm (TNBS + EPO 1000 group) at the end of the experiment. The EPO1000, ethanol, and sham control groups revealed differences between them, with 10.3 ± 1.57 cm, 8.71 ± 0.84 cm, and 8.75 ± 0.60 cm, respectively. However, these differences were not statistically significant.

10.7. Microscopic assessment of colitis severity

The differences according to the histopathological evaluation, between the groups, can be represented by the illustrative images present on Fig. 8. The histopathological analysis allows the evaluation of colonic injury, based on inflammatory cell infiltration and tissue damage. The colons of TNBS group presented a significant infiltration of inflammatory cells, mostly in the mucosa and submucosa layers, foci of ulceration with necrosis, and tissue disruption. Indeed, it was observed a reduction in the level of inflammatory cell infiltration and area of epithelial

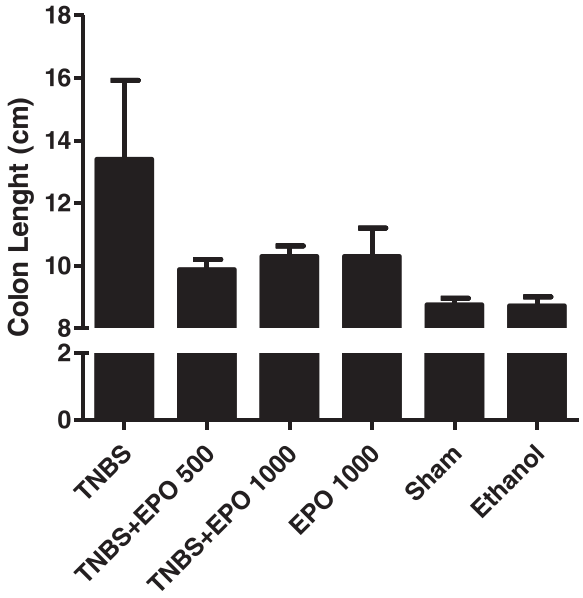

Fig. 7. - Colon length measurement between groups. Legend: One-way ANOVA and Tuckey's post hoc test. Experimental data: TNBS group (n = 10), 13.40 ± 7.98 cm; TNBS + EPO500 group (n = 8), 9.88 ± 0.93 cm; TNBS + EPO1000 group (n = 8), 10.30 ± 0.94 cm; EPO1000 group (n = 3), 10.3 ± 1.57 cm; Ethanol (n = 6), 8.71 ± 0.84 cm; Sham group (n = 6), 8.75 ± 0.60 cm.

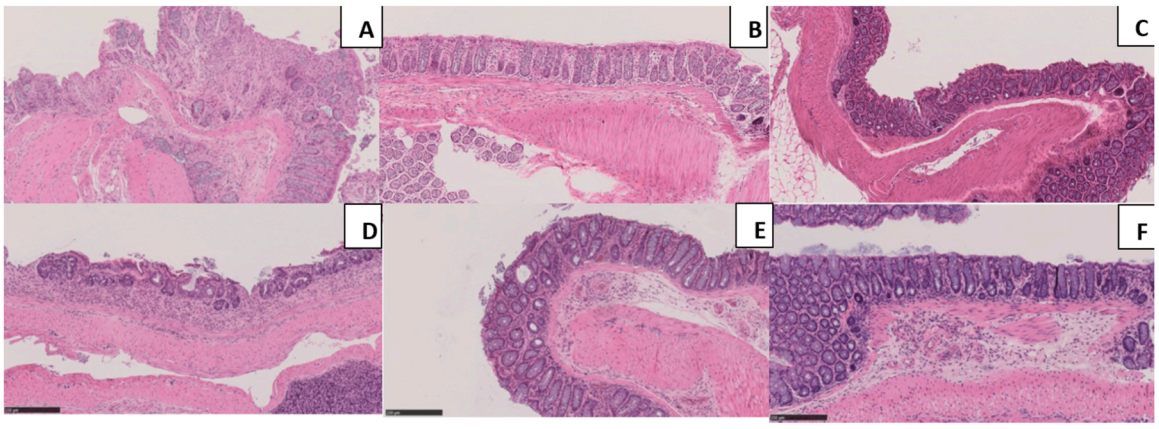

Fig. 8. – Histopathological analyses of Masson's trichrome staining 10x. Legend: A, TNBS group; B, ethanol group; C, sham group; D, TNBS + EPO500 group; E, TNBS + EPO1000 group; F, EPO1000 group.

ulceration in the EPO-treated groups. Additionally, it was observed that the treatment with EPO, at its highest dosage, showed a significant anti-inflammatory effect, namely at the TNBS + EPO1000 group, having the least rate of cell infiltration.

Concerning the microscopical assessment of colitis severity, it was generated a final histopathological score in order to generate a more objective evaluation, which promoted a more objective comparation between the groups (Fig. 9). As expected, the animals present on TNBS group revealed the highest histopathological score, namely 9.25 ± 2.44. Indeed, according to the data obtained in EPO1000 (2.25 ± 2.49), ethanol (0.5 ± 1.23) and sham (0.4 ± 0.89) groups, it was identified a significant reduction in comparison to the TNBS group ($p < 0.001$). The EPO-treated groups, namely TNBS + EPO500 (7.00 ± 2.83) and TNBS + EPO1000 (5.00 ± 4.42), shown a reduction of the histopathological score comparing to TNBS group, however it was not statistically significant. Finally, TNBS + EPO1000 group shown a statistically significant higher histopathological score in comparison to the EPO1000, ethanol, and sham control groups ($p < 0.001$).

11. Safety of erythropoietin in a chronic colitis model

11.1. Cardiovascular function

According to the hematopoietic activity demonstrated by EPO, it is essential to evaluate the hematocrit , and it was analyzed and compared between the TNBS + EPO1000, EPO1000, and sham groups (Fig. 10). Indeed, the highest value obtained was in the TNBS + EPO1000 group, namely 51.95 ± 0.52 %. Moreover, it was noticed a significant reduction of this marker in both EPO1000 (49.45 ± 0.40 %) and sham (49.40 ± 0.58 %) control groups ($p < 0.001$).

11.2. Renal and Hepatic Functions

In order to evaluate the renal function of the animals between all groups, it was measured and compared the levels of urea and creatinine, which are recognized as renal damage markers (Fig. 11 and Fig. 12).

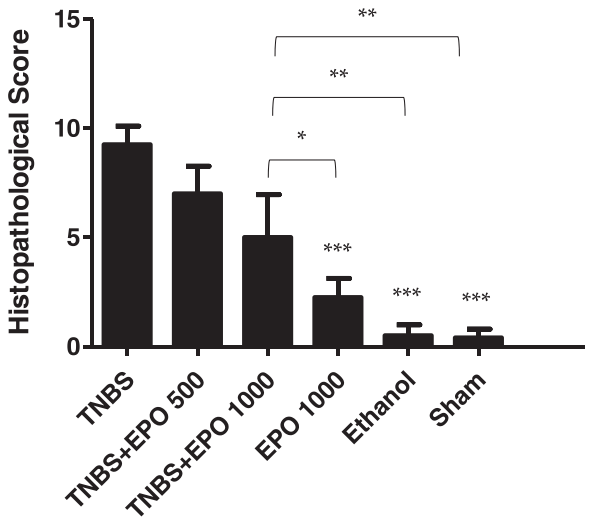

Fig. 9. – Histopathological score between the groups. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$; * *$p < 0.01$; * **$p < 0.001$; compared with TNBS group and between the other groups. Experimental data: TNBS group (n = 10), 9.25 ± 2.44; TNBS + EPO500 group (n = 8), 7.00 ± 2.83; TNBS + EPO1000 group (n = 8), 5.00 ± 4.42; EPO1000 group (n = 3), 2.25 ± 2.49; Ethanol (n = 6), 0.5 ± 1.23; Sham group (n = 6), 0.4 ± 0.89.

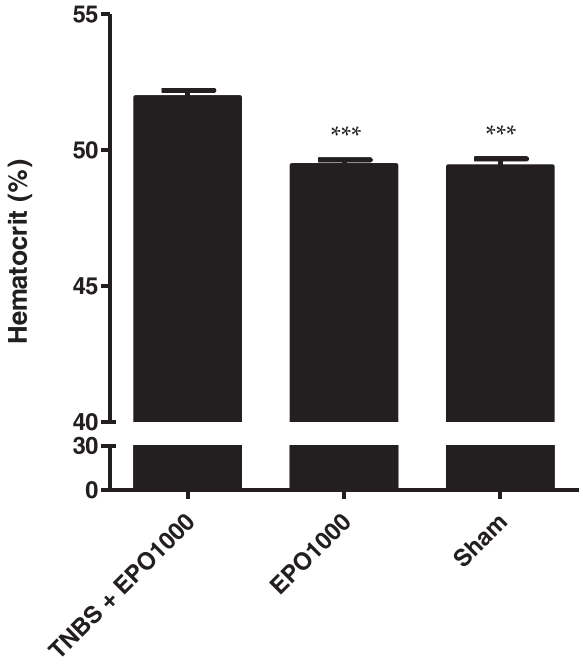

Fig. 10. – Analysis of the hematocrit (%) levels. Legend: One-way ANOVA and Tuckey's post hoc test. * **$p < 0.001$; compared with TNBS + EPO1000 group. Experimental data: TNBS + EPO1000 group (n = 8), 51.95 ± 0.52 %; EPO1000 group (n = 3), 49.45 ± 0.40 %; Sham group (n = 6), 49.40 ± 0.58 %.

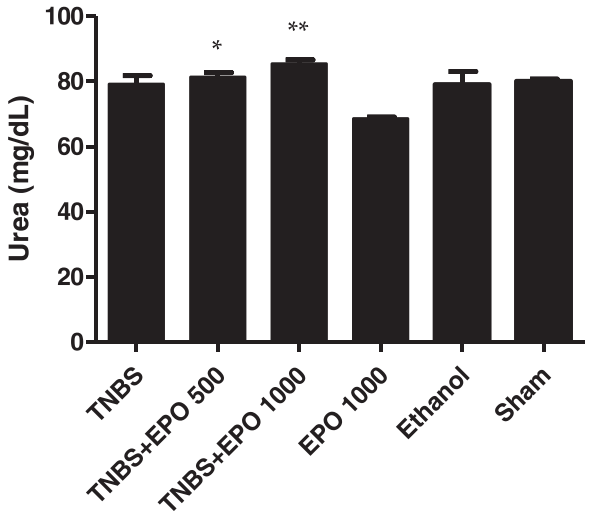

Fig. 11. - Urea concentration during the experiment. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$ and * *$p < 0.01$ compared with EPO 1000 group. Experimental data: TNBS group (n = 8), 79.13 ± 7.50 mg/dl; TNBS + EPO500 group (n = 8), 81.25 ± 4.23 mg/dl; TNBS + EPO1000 group (n = 8), 85.38 ± 3.58 mg/dl; EPO1000 group (n = 2), 68.50 ± 0.71 mg/dl; Ethanol (n = 2), 79.00 ± 5.66 mg/dl; Sham group (n = 5), 80.00 ± 1.58 mg/dl.

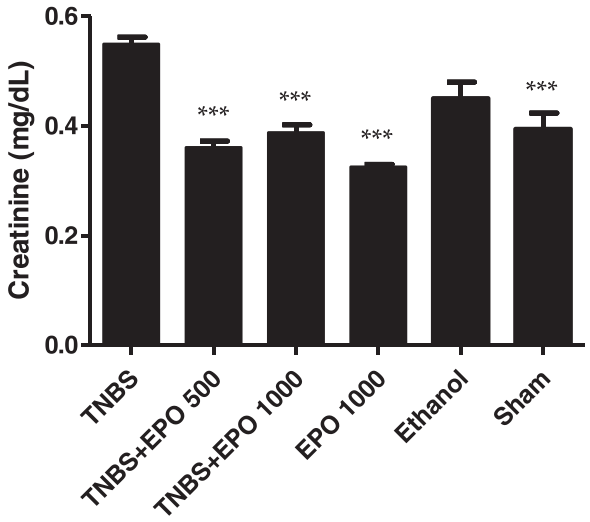

Fig. 12. - Creatinine concentration between groups. Legend: One-way ANOVA and Tuckey's post hoc test. * **$p < 0.001$ compared with TNBS group. Experimental data: TNBS group (n = 8), 0.55 ± 0.04 mg/dl; TNBS + EPO500 group (n = 8), 0.36 ± 0.03 mg/dl; TNBS + EPO1000 group (n = 8), 0.39 ± 0.04 mg/dl; EPO1000 group (n = 2), 0.32 ± 0.00 mg/dl; Ethanol (n = 2), 0.45 ± 0.04 mg/dl; Sham group (n = 5), 0.32 ± 0.00 mg/dl.The quantification of ALT levels allows to evaluate the hepatic function and its comparation between the groups permits an evaluation of the possible effect of EPO in this same parameter (Fig. 13). The TNBS group showed statistically significant differences with the TNBS+EPO 500 group and sham group (30.88 ± 4.64 U/L vs 21.38 ± 3.42 U/L vs 19.00 ± 1.58 U/L, $p < 0.001$) and with TNBS+EPO1000 (30.88 ± 4.64 U/L vs 23.00 ± 4.78 U/L, $p < 0.01$) and EPO 1000 group (30.88 ± 4.64 U/L vs 20.00 ± 1.41 U/L, $p < 0.05$). On the other hand, it was possible to observe a reduction in ALT levels at ethanol group (22.00 ± 4.24 U/L), however it was not statistically significant when compared to the TNBS group. In terms of treatment with EPO, it was a lower decrease in TNBS+EPO 1000 compared to TNBS+EPO 500 but without statistically significance between doses.

Firstly, in terms of urea quantification, there were not statistically significant differences between TNBS (79.13 ± 7.50 mg/dl) and treated groups, namely TNBS + EPO500 (81.25 ± 4.23 mg/dl) and TNBS + EPO1000 (85.38 ± 3.58 mg/dl) groups. In fact, the TNBS+EPO 1000 group presented the highest value for this marker. In addition, the EPO 1000 group presented the lowest value (68.50 ± 0.71 mg/dl), but without statistically significant in comparison to the TNBS group. The ethanol and sham control groups presented similar results with 79.00 ± 5.66 mg/dl and 80.00 ± 1.58 mg/dl, respectively. Considering the quantification and comparison of creatinine levels between all groups, there were noticed statistically significant differences, more specifically, between the TNBS group and TNBS+EPO 500 group (0.55 ± 0.04 mg/dl vs 0.36 ± 0.03 mg/dl, $p < 0.001$) and TNBS+EPO 1000 group (0.55 ± 0.04 mg/dl vs 0.39 ± 0.04 mg/dl, $p < 0.001$). Additionally, there were also statistically significant differences between TNBS group and the EPO 1000 and the sham group (0.55 ± 0.04 mg/dl vs 0.32 ± 0.00 mg/dl vs 0.39 ± 0.06 mg/dl, $p < 0.001$). Although the fact that TNBS+EPO 500 and TNBS+EPO1000 groups exhibited lower levels of creatinine in comparison to the TNBS group, demonstrating a possible protective effect of EPO, there were not statistically significant differences. The ethanol group revealed relatively high levels of creatinine (0.45 ± 0.04 mg/dl), which resulted in the absence of statistically significant differences in comparison to the TNBS group. Fig. 13.

12. Discussion

As previously described, EPO is commonly used in clinical practice in anemic patients with chronic renal insufficiency due to its erythropoietic effects, but has also shown other functions, including the anti-inflammatory effect. Indeed, there is already some evidence about its beneficial effect on inflammatory diseases, including IBD, taking into account the existent preclinical studies [7,29]. The binding of EPO with the heterodimeric receptor EPOR/CD131 has demonstrated the inhibition of the expression of NF-kB pathway and to reduce the expression of pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-1β, through via STAT3/5 signaling [7,22–24]. Concretely, the data available about the utilization of EPO in IBD have shown a positive influence, which is demonstrated by the reduction of the expression of pro-inflammatory cytokines, the increase of an anti-inflammatory cytokine, which is IL-10, a diminished severity of the disease through a histopathological

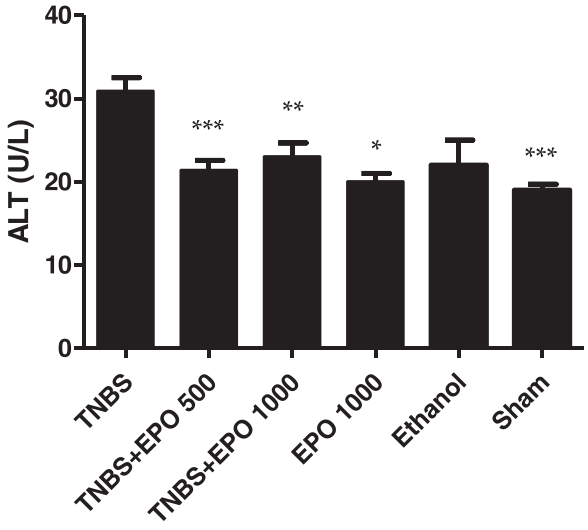

Fig. 13. - Alanine aminotransferase concentration between the groups. Legend: One-way ANOVA and Tuckey's post hoc test. *$p < 0.05$; * *$p < 0.01$; * **$p < 0.001$; compared with TNBS group. Experimental data: TNBS group (n = 8), 30.88 ± 4.64 U/L; TNBS + EPO500 group (n = 8), 21.38 ± 3.42 U/L; TNBS + EPO1000 group (n = 8), 23.00 ± 4.78 U/L; EPO1000 group (n = 2), 20.00 ± 1.41 U/L; Ethanol (n = 2), 22.00 ± 4.24 U/L; Sham group (n = 5), 19.00 ± 1.58 U/L.

evaluation and from the alleviation of typical clinical signs, such as the body weight, stool consistency, and annus appearance [7]. Since the preclinical study that evaluated the effect of EPO in IBD took 4 days in total, it would be interesting to evaluate the same molecule, at the same dosages, but now in a chronic animal model of IBD, which is the objective of this experiment.

Concerning the efficacy of EPO in a chronic model of colitis, it was evaluated several parameters, as previously referred. Firstly, in terms of the manifestation of the disease by the evaluation of clinical signs, such as stool consistency and annus appearance, the disease control group, also denominated as the TNBS group, has shown the worst prognosis with the presence of diarrhea and/or softer stools and severe edema on the anus. These results are compatible with the correct induction of experimental colitis with TNBS. The treatment with EPO demonstrated the alleviation of these signs, but contrariwise to what was expected and demonstrated at other non-clinical study, it did not show a significant effect on the body weight, where only the lower dosage used was associated with a slight increase on this parameter [7].

In order to evaluate the influence of EPO in the inflammatory response, it was measured TNF-α and IL-10, which are a pro-inflammatory and an anti-inflammatory cytokines, respectively. Regarding to TNF-α, EPO showed a beneficial effect at both dosages used, where the TNBS + EPO500 and TNBS + EPO1000 demonstrated reduced levels of this cytokine in comparison to TNBS group ($p < 0.001$). In addition, the values obtained in TNBS + EPO500 and TNBS + EPO1000 were similar, demonstrating that there is not an increased effect of EPO, when administrated at a higher dosage, on the expression of this pro-inflammatory cytokine. Indeed, the values obtained also show that EPO has the capability to significantly attenuate the inflammatory response, reaching similar results to the control groups, such as ethanol and sham groups. The data obtained are consistent with another preclinical study developed and to the mechanism of action of EPO, by its binding to the heterodimeric receptor EPOR/CD131, which results in the blockage of NF-kB activation and the reduction of the concentration of TNF-α through STAT3/5 signaling [7, 22–24]. After measuring IL-10, it was noticed that the TNBS group presented the highest value, which can be attributed to the adaptation of the immune system to the chronic inflammation, producing a higher concentration of this anti-inflammatory cytokine. EPO demonstrated the capability to significantly reduce the concentration of this anti-inflammatory cytokine, at both dosages, however without a dose-dependent manner ($p < 0.001$). Generally, there were not significant differences between the EPO-treated groups and the control groups, excluding between TNBS + EPO1000 and EPO1000 groups ($p < 0.05$). These results might be explained by the fact that EPO reduced the inflammatory response, throughout the 14 days of administration, and at the end of the experiment, the concentration of this anti-inflammatory cytokine was significantly lower, in comparison to the TNBS group. Taking in account these results, EPO have shown a beneficial effect on the inflammatory response, being consistent to another preclinical studies developed [7].

The determination of fecal hemoglobin allows to evaluate the intensity of the hemorrhagic focus, being useful in the detection of colorectal diseases and other possible lesions which are accompanied by bleeding [31] The treatment with EPO had a positive influence on decreasing the levels of fecal hemoglobin, in both dosages, in comparison to the disease control group ($p < 0.001$). Indeed, the values obtained in EPO-treated groups were similar to the control groups, such as ethanol and sham groups, demonstrating the beneficial effect of EPO on the hemorrhagic focus. These results are partially in accordance with the literature since in other preclinical studies, the treatment with EPO was capable of reducing the concentration of fecal hemoglobin, but the final values were not similar to the controls [7].

The measurement of fecal calprotectin permit the evaluation of the accumulation of leukocyte in stool, which is considered as one of the most sensitive non-invasive marker in the distinction of IBD from other functional disorders [28,32] According to the results obtained, it is possible to conclude that the treatment with EPO was able to significantly reduce the concentration of this marker, in comparison to the TNBS group ($p < 0.001$), reaching similar values to the control groups. On the other hand, since there was not identified a dose-dependent effect by EPO, there is not an increased beneficial role from this molecule, when administrated in higher dosages. In the literature, it is possible to observe that this marker is not considered in several preclinical studies, concerning a IBD context, however, as demonstrated in this experiment, it can be a valuable parameter to be evaluated since its relatively easy to determine and reveals a high sensitivity.

The expression of ALP in the intestine is mediated by the enterocytes and it is responsible for the mucosal defense [7,10] As expected, the TNBS control group presented the highest concentration of ALP, due to the inflammation caused by the administration of this chemical [7] Additionally, to sustain this affirmation, the EPO1000, ethanol, and sham control groups presented significantly lower levels of this marker in comparison to the TNBS group ($p < 0.001$, $p < 0.05$ and $p < 0.001$, respectively). The administration of EPO showed a beneficial effect on this parameter taking in account the fact that was observed a significantly reduction on ALP levels in both dosages used ($p < 0.001$). However, there was not observed a dose-dependent effect since there were not significant differences between both EPO-treated groups. Indeed, the results obtained in both EPO-treated groups were similar to the control groups. These results demonstrate an anti-inflammatory effect of EPO, which was expected taking into account the preclinical evidence existent [7].

Regarding the macroscopical evaluation of the colonic tissue, it is recognized as an important approach for the analysis of the damage present on the tissue itself, taking in account the development of the disease and the treatment applied [25] Indeed, the induction of colitis through the administration of TNBS is associated with the shortening of the colon, being one of the macroscopical factors to be aware of in the end of the experiment [7,10,30] The treatment with EPO did not demonstrate a potential beneficial effect since it was observed a reduction of the length of colonic tissue in the animals treated with both dosages, which was not expected according to the preclinical evidence existent [7]. Additionally, contrariwise to what was expected, it is also possible to notice a reduction of the colon length in animals which were not subjected to the induction of the disease. Indeed, the animals that presented the lowest values were inserted at the sham and ethanol groups. In this sense, although the fact that the length of colon tissue represents an important marker to be aware of, it can be influenced by other external factors.

On the other hand, throughout the microscopical evaluation, it was observed a potential beneficial effect by EPO, specially at 1000 IU/Kg, translated in the reduction of the histopathological score on the EPO-treated groups in comparison to the TNBS group, however it is not possible to clearly confirm it since there were not significant differences. Indeed, according to the data available on the literature, EPO had demonstrated to be capable of significantly attenuate the acute inflammatory response, concerning the histopathological evaluation, but in a longer spectrum of time, it was not possible to clearly ascertain it, as shown in this experiment [7].

EPO is recognized a potent stimulator of erythropoiesis, which by itself can be a major risk factor for cardiovascular events, taking in account its capability of increasing the hematocrit(%) [7,34,35] Concerning the results obtained, it is not possible to identify a significant effect of EPO in the increase of this marker since the drug control group, EPO1000 group, had similar results to the sham control group, which was not subjected to the treatment with this molecule. Indeed, these results are in accordance with another non-clinical study, which used the same dosages but was developed in 4 days, proving that the administration of EPO was not capable of significantly increase the hematocrit, concerning the treatment designs addressed.[7].

The evaluation of renal and hepatic functions is essential due to the

characteristic extra-intestinal manifestations of IBD and to analyze the safety profile of EPO in terms of its influence on the kidneys and liver [33]. Regarding to urea concentration, there were only significant differences between EPO1000 group and TNBS + EPO500 and TNBS + EPO1000 groups ($p < 0.05$ and $p < 0.01$, respectively). Contrariwise to what was expected, the values of urea reached in the TNBS group were similar to the control groups, such as ethanol and sham groups. Indeed, the highest values obtained were in TNBS + EPO500 and TNBS + EPO1000, which was also not expected [7]. In this sense, although the fact that the increase on the concentration of urea in EPO-treated groups, in comparison to TNBS group, did not demonstrate statistical significance, it might indicate a possible negative influence of EPO in this biomarker. On the other hand, after quantifying creatinine, it was possible to observe that TNBS group demonstrated the highest concentration of this marker. In addition, the treatment with EPO, at both dosages, demonstrated the capability to significantly reduce the concentration of creatinine ($p < 0.001$), which is in accordance to the literature [7]. Indeed, the values obtained in TNBS + EPO500 and TNBS + EPO1000 groups were lower than those observed in ethanol and sham control groups, however without statistical significance. Considering both markers, the treatment with EPO demonstrated to have a positive influence on the renal function, especially according to the values obtained in creatinine.

In order to evaluate the influence of IBD and EPO on hepatic function, ALT was measured and compared, and the results showed that the group whose presented the highest value for this marker was the TNBS group, which was expected at the beginning [7,30,33]. Interestingly, the use of EPO, at both dosages, demonstrated a beneficial effect in the concentration of this biomarker, since the values obtained in both TNBS + EPO500 and TNBS + EPO1000 groups were significantly lower than in TNBS group ($p < 0.001$ and $p < 0.01$, respectively). Indeed, the values reached in EPO-treated groups were similar to the control groups, showing that EPO had a beneficial effect on the hepatic function. Additionally, it is also observed that the administration of EPO, at the highest dosage, do not increase the concentration of ALT, revealing that it is not prejudicial in terms of hepatic function. These results are in line to the current preclinical evidence existent concerning the administration of EPO, at the same dosages, in a IBD context [7].

According to the data obtained in this experiment, the treatment with EPO demonstrated to have a beneficial effect in the chronic inflammatory response induced with TNBS, taking in account the several parameters evaluated and referred previously. Additionally, it was also possible to conclude a beneficial role of this molecule on extraintestinal manifestations, including the renal and hepatic functions, with the absence of significant adverse effects.

13. Conclusion

The treatment with EPO demonstrated to be effective in a IBD context, which can be explained by its capability of significantly attenuate the inflammatory response, without any significant side effect to be noticed, considering for example, the hematocrit. These results can ascertain the presence of an anti-inflammatory effect of EPO, upon its binding to the heterodimeric receptor EPOR/CD131, and its further influence on JAK/STAT signaling and NF-kB expression. In this sense, according to the fact that EPO is commonly used in clinical practice, there is already information regarding its safety profile, which can highlight an opportunity of drug repositioning for the future. Indeed, according to the literature and the data obtained in this experiment, it can be emphasized a new possible role from this molecule in the clinical practice, making it as an interesting future pharmacological approach for the management of inflammatory-related disorders, including IBD. In fact, considering the safety profile of the current drugs normally applied in IBD, EPO could arise as a new safer option, used in combination or not with the standard treatment approaches. In this sense, it would promote the reduction of the exposition to the current drugs used in IBD patients, improving their overall quality of life. Additionally, the production of similar molecules to EPO, such as ARA 290 and carbamylated EPO, which have a higher sensitivity to the heterodimeric receptor EPOR/CD131, and consecutively, do not present any hematopoietic activity, can be also recognized as interesting pharmacological tools for further investigation.

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Informed Consent Statement

Not applicable.

Conflicts of interest statement

The authors declare no conflicts of interest.

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