Effects of budesonide and probiotics enemas on the systemic inflammatory response of rats with experimental colitis[1]

Efeito de enemas contendo budesonida e probióticos na resposta inflamatória sistêmica de ratos com colite experimental

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ABSTRACT

Purpose: The aim of this study was to investigate the effect of enemas containing probiotics and budesonide on the systemic inflammatory response in experimental colitis. **Methods**: Fifty male Wistar rats with experimental colitis induced by 10% acetic acid enema were randomized to five groups (10 rats each) according to the treatment: group 1 - saline solution, group 2 - budesonide (0.75 mg/kg/day), group 3 - probiotics (1mg/day), group 4 - probiotics plus budesonide, and group 5 - control, with not-treated rats. The following variables were studied: body weight, serum levels of albumin, C-reactive protein and interleucine-6 (IL-6). **Results**: All animals lost weight between the beginning and the end of the experiment (280$\pm$ 16 mg versus 249$\pm$21 mg, $p< 0.001$). There was a significant decrease in the serum albumin between the normal pre-induction level (3.45 + 0.49mg/dL) and the 1st day after colitis induction (1.61$\pm$051mg/dL, $p< 0.001$) in all treated groups when compared to the control group. C- reactive protein increased after induction and diminished on the 7th day in all groups. In the control group there was an increase in the IL-6 after colitis induction. None of the treated groups significantly differed from IL-6 pre-colitis status ($p>0.05$). Only probiotic rats presented a significant decrease of IL-6 than controls (0,30±0,08 mg/dL vs. 0,19±0,03 mg/dL; $p<0.01$). **Conclusion**: Probiotic associated with budesonida Probiotics are effective to diminished inflammatory status mediated by IL-6 in experimental colitis.

Key words: Ulcerative colitis. Budesonide. Probiotics. IL-6. Mucosa. Colon. Rats.

RESUMO

Objetivo: Investigar o efeito da administração retal de probióticos e budesonida na resposta inflamatória de ratos com colite experimental. **Métodos**: Cinqüenta ratos Wistar com colite experimental induzida pelo acido acético à 10% foram randomizados em 5 grupos (n=10 por grupo) para diferentes tratamentos: grupo 1 - solução fisiológica; grupo 2 – budesonida (0,75mg/kg/dia); grupo 3 - probióticos (1 g/dia); grupo 4 - probióticos associados a budesonida; e finalmente grupo 5 - controle, composto por ratos sem tratamento. As seguintes variáveis foram estudadas: peso corporal, dosagens séricas de albumina, proteína C reativa (PCR) e interleucina-6 (IL-6). **Resultados:** Todos os animais perderam peso entre o inicio e o fim do experimento (280±16 vs 249±21g; $p<0.001$). Ocorreu uma queda significativa da albumina sérica entre o normal (3,45±0,49g/dL) e o 1º dia de colite (grupo 5 = 1,61±0,51 g/dL; $p <0.001$) em todos grupos com tratamento em relação ao grupo controle. A PCR aumentou após a indução da colite, diminuindo no sétimo dia de colite em todos os grupos. No grupo controle houve um aumento da IL-6 após a indução da colite. Nenhum dos grupos de tratamento diferiu significantemente dos valores de IL-6 antes da indução a colite ($p>0.05$). As comparações entre o grupo controle (0,30±0,08 mg/dL) e outros mostraram que houve uma queda significante nos níveis de IL-6 apenas no grupo probiótico (0,19±0,03 mg/dL; $p<0.01$). **Conclusão**: Probióticos são efetivos na diminuição do estado inflamatório mediado pela IL-6 na colite experimental.

Descritores: Colite ulcerative. Budesonida. Probióticos. Mucosa. Colon. IL-6 Proteina C reativa. Ratos.

Introduction

The medical treatment for ulcerative colitis is limited by its enigmatic ethiopathogeny, incomplete comprehension on both subjacent immunological and inflammatory events and by the lack of a gold standard method to measure the activity of the illness[1]. Thus, the therapeutic intervention is concentrated mainly on both the consequences and systemic repercussions of the immunological and inflammatory cascades.[2]. The most used drugs in the treatment of ulcerative colitis are sulfasalazine and its derivatives, corticosteroids, and immunosuppressants. However, the biological treatment with anti-TNF α (infliximab) and other drugs such as transdermic nicotine, heparin, short chain triglycerides, probiotics and antibiotics may benefit the patient[3,4]. Corticosteroids constitute the most effective substances for the treatment of active ulcerative colitis. However, they are not used to maintain remission due to side effects and lack of evidence of its effectiveness for this purpose. The corticosteroids most frequently used in the treatment of ulcerative colitis are hydrocortisone, prednisone, prednisolone and metil-prednisolone. However, these traditional corticosteroids are being gradually substituted by new compounds with less side-effects[4]. A recent metanalysis on the use of budesonide enemas for ulcerative colitis showed similar benefits when compared to conventional glucocorticosteroids[5]. Probiotics have been used for various clinical and surgical conditions. There is substantial evidence showing that probiotics are widely beneficial[6,7]. The mechanisms by which probiotic bacteria might exert their beneficial effects and protect against colitis include direct effects on local bacteria and stimulation of protective immune responses. In fact, some clinical and experimental studies have shown that probiotics are beneficial in the treatment of diarrhea and ulcerative colitis. [3,8-10]. The lack of knowledge of the etiology, etiopathogeny and definitive medical treatment of ulcerative colitis encourages experimental studies and thus a number animal model of colitis have been proposed [11]. MacPherson and Pfeiffer[12] proposed an experimental model of colitis induced by acetic acid in rats. The colon and rectum lesions produced by this method are reproducible in 100% of the animals and total mucosal recovery occurs 60 days after the induction. Moraes[13] carried out a similar study and concluded that 10% acetic acid is useful as an inducing agent of the inflammatory process of the colonic mucosa. There is little information in the literature on the use of probiotics in experimental colitis. Furthermore, as far we could search in the literature, the concomitant use of both probiotocs and budesonide in ulcerative colitis has never been investigated. We were not able to found any studies in the literature which examined the combined action of these products in the mucosal recovery in experimental colitis. Because of the antiinflamatory action associated with corticoids and the diverse beneficial effects associated with probiotocs[14], the combined use of these products may diminish the systemic inflammatory reaction that enhances after mucosal injury. Thus, an experimental study to investigate the use of budesonide and probiotics in experimental colitis might contribute to the comprehension of inflammation in colitis. The aim of this study was to investigate the effects of retal administration of probiotics and budesonide on the systemic inflammatory response in experimental acute colitis.

Methods

Fifty male Wistar rats (250-300g) entered the experiment. The animals were obtained from the Central Animal Laboratory of the Federal University of Mato Grosso. The experiment follows the COBEA (Brazilian Committee on Experimental Animal Care) guidelines. Throughout the whole experiment the animals were kept in appropriate environments inside metabolic cages. They remained for 3 days in the laboratory prior to the experiment for accommodation in 12 hour cycles of light/dark receiving rat chow and water *ad libitum*. The animals were randomized after induction of colitis into five treatment groups as follows: Group 1 (saline, n=10) treated with 1ml of saline solution (0.9%); Group 2 (budesonida, n=10) treated with 1ml (equivalent to 0.25mg) of budesonide (Entcort, Astra Zeneca, Socertalje, Sweden); Group3 (probiotics, n=10) treated with 1g of probiotics (L.acidophillus $3.33x10^6$ UFC/g; B. bifidum $3,33x10^6$ UFC/g; E. faecium $3.3x10^6$; Probiotico Vetnil, Vetnil Laboratory, São Paulo); Group 4 (budesonida+probiotics, n=10) treated with 1ml (0.25mg) of budesonide plus 1 g of the same probiotic as used in the previous group; and Group 5 (colitis, n=10) consisting of rats with colitis and not treated. Blood samples were collect of animals of this group before colitis induction for a normal standard control (normal group).

Induction of colitis and data collection

The method of colitis induction was the same as that used by Mac-Pherson and Pfeiffer[12] and standardized by Moraes[13]. After fasting for 12 hours the animals underwent mechanical preparation of the colon with enemas containing 20ml of 0.9% saline solution in bolus. Polyethylene catheters 6Fr and 20 mL syringes were used in the colonic lavage. Thirty minutes after mechanical cleansing each animal was placed in a glass chamber and anaesthetized by inhalation of ethylic ether. Following this, they received an enema containing 0.5 mL of 10% acetic acid. Polyethylene catheters 6Fr similar to those used for the colonic cleansing were used attached to 1mL syringes. After the instillation of the acid, the

animals were suspended by the tail for 30 seconds. A pilot study with four animals showed that both the cleansing and the colitis induction provided excellent quality of the desired effect. The treatment was started on the first day after colitis induction with the exception of group 5 and was maintained on a daily basis up to the sixth day after the colitis. Levels of albumin (negative acute phase protein), C-reactive protein (CRP, positive acute phase protein) and interleukin 6 (IL-6) in the animals were determined. In the animals of the treatment groups (groups 1 to 4) the levels of both CRP and albumin were assayed on the first day and sixth day after the beginning of the treatment (second and seventh days after induction), and IL-6 was determined only on the seventh day after the treatment. Blood samples were collected under anesthesia with inhalatory ethylic ether either from the tail vein (first day samples) or the portal vein by laparotomy (seventh day samples). All the animals were killed by means of inhalation of a lethal dose of ethylic ether. Animals of group 5 were killed on the day after colitis induction and those in groups 1 to 4 on the sev.

Laboratory assays

CRP assay (g/dL) done by turbidimetric method carried out on Targa 3000 equipment using the CRP latex kit (Turbitest AA, Winer laboratórios S.A.I.C, Rosario, Argentina. IL-6 levels (pg/mL) were determined using the Elisa method on the Funrise model of the Tecam apparatus using the Immunoassay Kit (BioSource International, California, USA.

Statistical method

The comparison of the groups was carried out using either the ANOVA or the Kruskall-Wallis test. Data that need comparisons within group (within-subject analysis) and between groups (between-subject analysis) were analyzed by the repeated measures ANOVA. *Post-hoc* comparisons between groups, when necessary, were carried out using the Tukey test. In the text, the data was presented as mean and standard deviation or mean and variation according to distribution. In graphics data represents the mean and standard error mean (SEM). The statistical significance was set at 5% ($p<0.05$). The tests were carried out using the statistics package SPSS 8.0.

Results

Four animals of the budesonide group and two animals of each of the other groups of treatment died during the experiment resting 40 animals to complete the study ($p>0.05$). All the animals that died underwent an autopsy and all presented colonic perforation. During evolution all rats developed diarrhea with blood.

Progress of weight variation

There was a significant weight loss between the beginning and end of the experiment (280±16 vs 249±21g; $p<0.001$) in all the groups. Comparatively the weight loss was greatest in the budesonide group when compared with the group treated with saline solution ($p = 0.03$). The other comparisons were not significant (Table 1).

TABLE 1 - Weight evolution (g) among the groups. Data are mean and standard deviation.

GROUP	Weight before	Weight after
Saline	287 ± 11	268 ± 13†
Budesonide	269 ± 15	241 ± 6 †‡
Probiotics	287 ± 16	238 ± 30†
Budesonide + Probiotics	279 ± 17	247 ± 15†
Colitis control*	276 ± 16	
Overall mean	280 ± 16	249 ± 21†

* Animals killed on the 1st day of colitis. †, $p < 0.01$ versus Weight Before. ‡, $p = 0.03$ versus saline ANOVA with repeated measures.

Albumin

2nd day of colitis

There was a significant decrease of seric albumin between the normal (3.45±0.49g/dL) and the 2nd day of colitis (group 5 = 1.61±0.51 g/dL; $p <0.001$). This fall from the normal values was significant in all other treatment groups (saline=2.82±0.48g/dL; $p=0.01$, probiotics = 2.92±0.41 g/dL; $p=0.02$; and budesonide+probiotics = 2.89±0.34 g/dL; $p=0.02$) except for budesonide group (2.97±0.52 g/dL; $p=0.10$). Compared with non-treated group at the 2nd day of colitis, there was a recovery of seric albumin in all the treatment groups ($p < 0.01$) without difference among them ($p = 0.94$). These findings can be seen in figure 1.

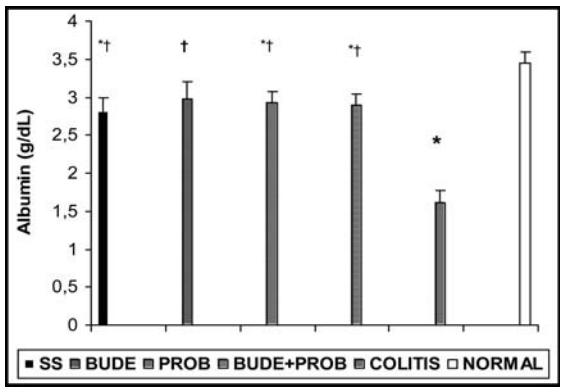

FIGURE 1 - Albumin on the 2nd day of colitis according to groups of treatment. *, $p<0.01$ vs. Normal ; †, $p < 0.001$ vs. Colitis. Data represent the mean and SEM. SS= saline solution, group 1; BUDE = budesonide, group 2; PROB = probiotic, group 3; BUDE+PROB = Budesonide+probiotic, group 4; COLITIS = control group, group 5; NORMAL = group 5 before colitis induction.

7th day of colitis

The findings on the 7th day can be seen in figure 2. The comparisons between the normal (3.45±0.49g/dL) and the other groups showed that albumin levels continued at significantly lower levels ($p<0.01$) in all the groups on the 7th day of colitis (SS = 2.54±0.44 g/dL; budesonide = 2.45±0.32 g/dL; probiotic = 2.48±0.34 g/dL; and budesonide+probiotics = 2.31±0.38 g/dL; $p<0.01$). Similarly, all the groups improved ($p<0.01$) in relation to the 1st day of colitis without treatment (group 5 = 1.61±0.51 g/dL). There was no significant difference between the four treatment groups ($p=0.74$).

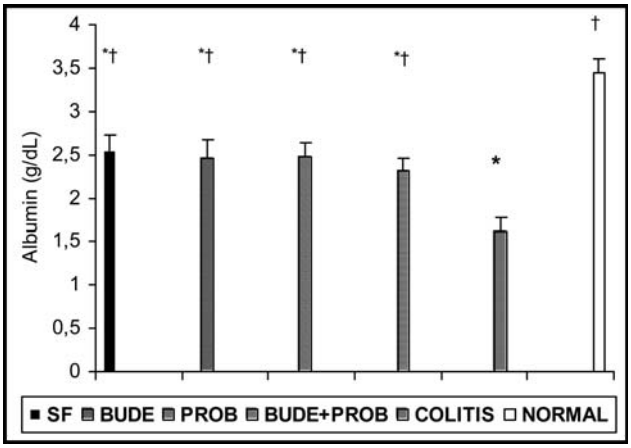

FIGURE 2 - Albumin on the 7th day of colitis according to the group of treatment. *, $p<0.01$ vs. Normal ; †, $p < 0.001$ vs. Colitis. Data represent the mean and SEM. SS= saline solution, group 1; BUDE = budesonide, group 2; PROB = probiotic, group 3; BUDE+PROB = Budesonide+probiotic, group 4; COLITIS = controls, group 5; NORMAL = group 5 before colitis induction.

C-Reactive Protein

2nd day of colitis

CRP significantly increased after induction of colitis (normal = 0.07±0.02 mg/dL versus colitis = 0.37±0.28 mg/dL; $p<0.01$). Compared with colitic values (group 4), a significant fall in serum CRP occurred in both saline ($p=0.04$) and budesonide+probiotics groups ($p=0.04$). There was no significant decrease of CRP with either budesonide ($p=0.16$) or probiotics ($p=0.04$) given individually (Figure 3). The decrease of CRP levels in groups 1 (saline, 0.10±0.10 mg/d; $p=0.49$); 2 (budesonide=0.16±0.17 mg/d; $p=0.34$) and 4 (budesonide+probiotics = 0.09±0.08 mg/d; $p=0.64$) reached similar normal values. Probiotic given associated to budesonide promoted significant fall of serum CRP when compared to probiotic alone (0.09±0.08 mg/d vs 0.29±0.16; $p=0.01$).

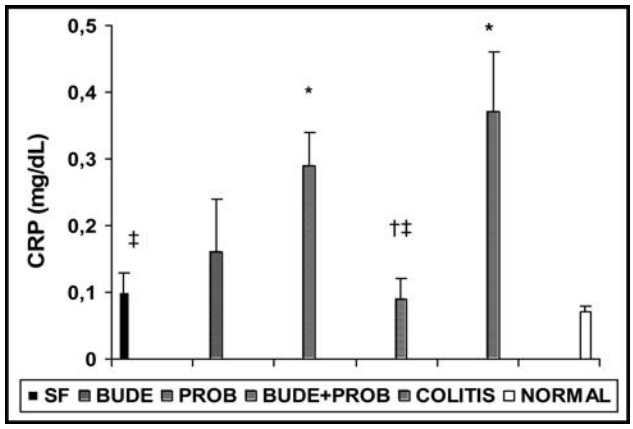

FIGURE 3 - Serum C-reactive protein (CRP) on the 2nd day of colitis according to the groups of treatment. *, $p<0.01$ vs. Normal ; †, $p = 0.03$ vs. Probiotics; ‡, $p = 0.04$ vs. Colitis. Data represent the mean and SEM. SS= saline solution, group 1; BUDE = budesonide, group 2; PROB = probiotics, group 3; BUDE+PROB = Budesonide+probiotics, group 4; COLITIS = control group, group 5; NORMAL = group 5 before colitis induction.

7th Day of colitis

On the 7th day of colitis (after 6 days of treatment), serum CRP in all the treatment groups diminished and did not significantly differ in the four treatment groups from pre-colitis values (saline=0.10±0.06 mg/d; $p=0.16$; budesonide group = 0.19±0.14 mg/d; $p=0.14$; probiotics group = 0.18±0.14 mg/d; $p=0.17$ and the budesonide+probiotics group = 0.16±0.16 mg/d; $p=0.25$). There was also a decrease in the mean values of all treatment groups ($p<0.001$) compared with colitic rats without treatment. No difference occurred between the four treatment groups when compared ($p=0.55$) (Figure 4).

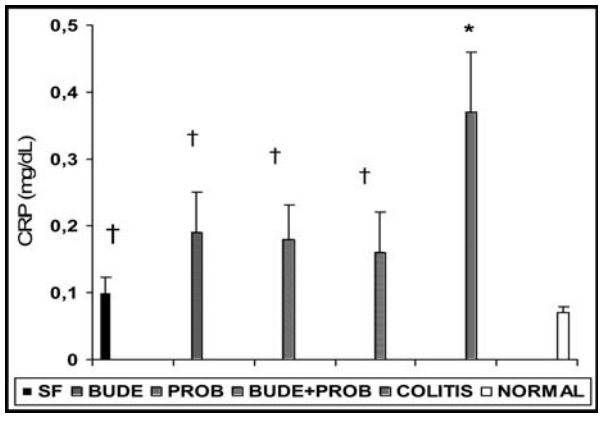

FIGURE 4 - CRP results on the 7th day of colitis according to the treatment applied. *, $p<0.01$ vs. Normal. †, $p<0.01$ versus colitis. Data represent the mean and SEM. SS= saline solution, group 1; BUDE = budesonide, group 2; PROB = probiotics, group 3; BUDE+PROB = Budesonide+probiotics, group 4; COLITIS = control group, group 5; NORMAL = group 5 before colitis induction.

Interleukin-6

None of the groups significantly differed from the normal values (0.24±0.09 mg/dL; p>0.05). Compared with colitic animals without any treatment (group 5 = 0.30±0.08 mg/dL), only animals receiving enemas containing probiotics (0.19±0.03 mg/dL; p<0.01) presented a significant decrease in serum IL-6 levels. In the comparison between the four treatment groups probiotics rats presented a lower level of serum IL-6 than both saline (0.38±0.19 mg/dL; p=0.03) and budesonide+probiotics rats (0.41±0.24 mg/dL; p=0.01). These results can be seen in figure 5.

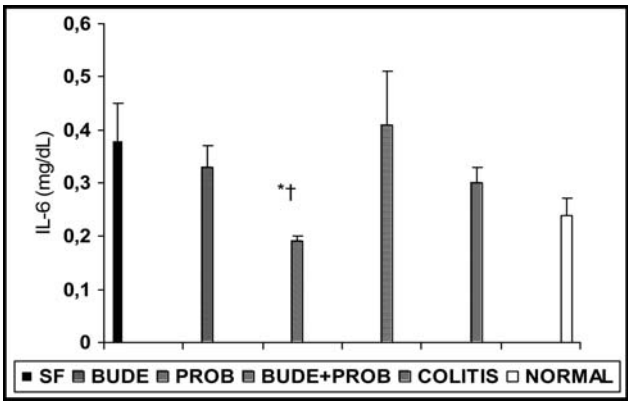

FIGURE 5 - Serum IL-6 on the 7th day of colitis according to the groups of treatment. *, p<0.01 vs. Colitis; †, p = 0.03 vs. SS and p = 0.01 vs. Budesonide+Probiotics. Data represent the mean and SEM. SS= saline solution, group 1; BUDE = budesonide, group 2; PROB = probiotics, group 3; BUDE+PROB = Budesonide+probiotics, group 4; COLITIS = control group, group 5; NORMAL = group 5 before colitis induction.

Discussion

The pilot study carried out with four animals showed that both the enema and the colitis induced by the 10% acetic acid were excellent using the applied method. This pilot study showed that the enema treatment with both probiotics and budesonida was easily carried out. Other authors have proven the efficacy of acetic acid as an inducer of experimental colitis as well as the tendency for spontaneous healing. [12,13]. The daily clinical assessments showed that animals, in all the groups, developed diarrhea with blood. All animals that died before the end of the experiment were necropsied and in all cases colon perforations were found. Albumin is a serum negative acute phase protein which tends to diminish during inflammation. This occurs due to the inhibition of its synthesis by pro-inflammatory cytokines and by the increase in vascular permeability. It is the most abundant plasmatic protein representing 50% of the total proteins found in human serum.[15,16] In this study, experimental colitis induced a fall of serum albumin in all treatment groups followed by a recovery in the normal levels without difference between any of the studied treatment. This pattern continues and was seen by the seventh day of colitis all the groups. C-reactive protein is a serum positive acute phase protein which begins to be secreted 6 hours after wither trauma or inflammatory stimulus and reaches its peak level around 50 hours after its start and has a mean life span of 19 hours. It is synthesized exclusively in the liver.[17,18] There was an increase in CRP level after the induction of colitis followed by a drop on the 7th day. The increase in early days of colitis was lesser in rats treated with budesonida associated to probiotics. This result suggests that the use of probiotics associated with corticoids may promote an anti-inflammatory action in this model of colitis. However, the use of either probiotics or budesonida alone was less efficient. On the 7th day all the actions were the same. Interleukin-6 is a protein produced by lymphocytes, fibroblasts, monocytes, macrophages, dendritic cells, endothelial cells and T cells. IL-6 is related to the production and production of acute phase proteins (C-reactive protein) from the hepatocytes in situations of trauma, infection and sepsis.[19] It was detected in human serum after trauma surgery, burns and patients with sepsis.[17] After a stimulus, IL-6 is released and can be detected in the circulatory system within 60 minutes with seric levels reaching peak in 2 hours, and remains detectable for up to 20 hours.[20] The increase of IL-6 in this study was particularly visible in the control group after colitis induction. Only animals treated with probiotics showed a significant drop on day one of colitis. Apart from this, probiotics group was the only group where the IL-6 reduced significantly more than the group which received saline solution. This result is relevant and suggests that the topical use of probiotics leads to a reduction of inflammatory reaction in experimental colitis. This result support another study that previously showed that probiotics can reduce the inflammatory response of experimental colitis by reducing the cytokines response.[21] Therefore, probiotics, by blocking the IL-6 receptor animal colitis models[21,22], may play an important weapon in the treatment of colitis. The overall results allow to conclude that the association of probiotics and budesonide in colonic enemas may reduce systemic inflammation in experimental colitis. Topical probiotics seems to have a potential anti-inflammatory response by reducing cytokines response. The results allow for the conclusion that in the experimental model studied, probiotics and budesonide are effective in the treatment of ulcerative colitis.

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