The effect of progesterone in the prevention of the chemically induced experimental colitis in rats

Efeito da progesterona na prevenção de colite experimental induzida quimicamente em ratos

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ABSTRACT

PURPOSE: To study the effects of progesterone on an experimental colitis model.

METHODS: Wistar albino rats were treated subcutaneously with 2mg/kg once a day during seven days. Colitis was induced by intrarectal administration of 5mg trinitrobenzene sulfonic acid (TNBS). Disease activities, macroscopic and microscopic scores were evaluated. To determine the response provoked by progesterone we measured Colonic malondialdehyde (MDA), TNF alfa, IL-6 and Nitric oxide (NO) levels in addition to the MPO (Myeloperoxidase) and caspase-3 activities.

RESULTS: Progesterone ameliorated significantly the macroscopic and microscopic scores. TNBS-induced colitis significantly increased the colonic MDA levels and caspase-3 activities in group 2 in comparison to the control group. The results of the study revealed a decline in MDA, NO, IL6 and TNF-α levels in the colon tissue and in blood due to progesterone therapy in group 3 when compared to the group 2, a significant improvement. Progesterone treatment was associated with decreased MDA, MPO, TNF alfa and caspase-3 activity.

CONCLUSION: Progesterone therapy decreased oxidative damage in the colonic mucosa.

Key words: Progesterone. Colitis. Inflammation. Colon. Rats.

RESUMO

OBJETIVO: Investigar os efeitos da progesterona em um modelo de colite experimental.

MÉTODOS: Ratos albinos Wistar foram tratados subcutaneamente com 2mg/kg por dia durante sete dias. A colite foi induzida por administração intraretal de 5mg ácido sulfônico trinitrobenzeno (TNBS). Foram avaliadas as atividades da doença, escores macroscópicos e microscópicos. Para determinar a resposta provocada pela progesterona foi medida no cólon os níveis de malondialdeído (MDA), TNF alfa, IL-6 e óxido nítrico (NO), além da atividade da MPO (Myeloperoxidase) e caspase-3.

RESULTADOS: A progesterona melhorou significativamente os escores macroscópicos e microscópicos. A colite induzida pelo TNBS significativamente aumentou os níveis colônicos de MDA e a atividade da caspase-3 no grupo 2 em comparação com o grupo controle. Os resultados do estudo revelaram um declínio nos níveis de MDA, NO, IL6 e TNF-α no tecido colônico e no sangue devido à terapia com a progesterona no grupo 3 quando comparado ao grupo 2. O tratamento com a progesterona foi associado com decréscimo do MDA, MPO, TNF alfa e atividade da caspase-3.

CONCLUSÃO: A terapia com progesterona decresce o dano oxidativo na mucosa do cólon.

Introduction

The term inflammatory bowel disease (IBD) covers a group of disorders characterized by intestinal inflammation and mucosal damage and that involves any part of the gastrointestinal tract. The incidence of IBD is on the rise1. Although many clinical and experimental studies have focused on IBD the optimum treatment strategy has not yet been defined. Basically the aim of the experimental studies in the literature is to decrease tissue inflammation and to determine the agents that could minimize systemic effects by hampering extraintestinal findings.

One candidate agent is progesterone which was found to depress the inflammatory response and reduce the cell membrane damage caused by lipid peroxidation and development of free radicals2-4. Immune modulation was demonstrated by experimental studies that localized membrane progesterone receptors (mPRs) on CD4 and CD8 T-lymphocytes3. Moreover it has been experimentally confirmed that progesterone inhibits the development of oxygen-centered free radicals and prevents cell damage after development of benzene induced toxicity as shown from the levels of lipid peroxidation (malondialdehit), glutathione (GSH) and cytochrome P450 2E1 in the liver and kidney tissues4. By these mechanisms, progesterone could prevent cellular apoptosis also in brain and heart tissues5-9. Building on these findings we performed a study aimed at evaluating the protective effect of treatment with progesterone in the TNBS-induced colitis rat model.

Methods

Twenty-eight male Wistar-albino rats weighing 250-300g were used in the study. All animals were housed in wire-mesh bottomed cages in 12 hours light/12 hours dark cycle at a constant room temperature of 22 ± 2 ºC. They were fed a standard chow diet and water. The study was approved by the local ethics review board.

Induction of colitis

Rats were lightly anesthetized with ether following a 24-h fast. A 5-French polyurethane cannula was inserted into the anus and the tip was advanced to 8 cm distal to the anal verge. TNBS (Sigma, USA) dissolved in 50% ethanol was instilled into the colon through the cannula (10 mg in a volume of 0.25 ml to induce colitis). Next, the rats were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillation.

Three equally numbered groups were created as follows:

**Group 1:** Control (sham) group (n=8). After catheterization across the rectum anesthetized rats were substituted with 1 ml of 0.9% NaCl.

**Group 2:** Colitis group (n=8). After catheterization across the rectum colitis was induced in anesthetized rats by administering 30 mg/0.1 ml TNBS and 0.5 ml 50% of ethanol mixture.

**Group 3:** Treatment group (n=8). After catheterization colitis was induced by intrarectal administration of TNBS (30 mg/0.1 ml and 0.5 ml 50% of ethanol) in anesthetized rats. Next rats received 2mg/kg of progesterone (Progynex ampoule, 50mg/ml, FARMAKO) injected subcutaneously.

The rats were restrained in a supine position until the end of anesthesia to prevent leakage of the administered material. All rats were fed standard industrial rat food for seven days.

Surgical procedure

The surgical procedure was carried out under sterile conditions on the 7th day of the therapy received by the experiment group. All rats were anesthetized by intramuscular administration of 80 mg/kg of ketamine hydrochloride (Ketalar, Eczacıbası) and 8 mg/kg of xylazin (Rompun, Bayer). Thus a general anesthesia and spontaneous respiration were maintained. The abdominal region was cleansed with povidone iodine. The colitis-induced colonic segment was transected distal to the rectum as low as possible and an approximately 10 cm long colonic segment was removed. The segment was opened longitudinally and the fecal content was rinsed with 0.9 % saline solution. 5cc of blood was drawn into an appropriate sample tube suitable for the investigation of biochemical parameters. Tissue samples were obtained for biochemical and histopathological investigations and transferred into 0.9% NaCl solution or into 10% formaldehyde solution. Blood samples were centrifugated and preserved at −80 ºC for biochemical investigations. Finally rats were sacrificed via administration of lethal dose of thiopental sodium.

Histopathological evaluation

Macroscopic scoring

Macroscopic changes in the colonic mucosa were classified into 5 levels as described10.

0 - No colonic damage;
1 - Hyperemia but no ulcer;
2 - Linear ulcer but no colonic wall thickening;
3 - Linear ulcer and colonic wall thickening at one area;
4 - Colonic ulcer at multiple areas;
5 - Major ulcer and perforation.
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Microscopic scoring

The colon after the macroscopic evaluation of the mucosa was divided into two pieces for pathologic evaluation and biochemical studies. Histopathological examination was performed in the Department of Pathology in the Okmeydanı Training and Research Hospital, Istanbul, Turkey. The colon tissues were cut into 6-mm-thick sections and fixed in 10% formaldehyde. Analysis was according to Yamamoto et al.11. In brief, 5 micron-thick sections prepared from colon after the formation of paraffin blocks were stained with hematoxylin-eosine and assessed under the light microscope. The sections were examined for the presence of edema, necrosis, inflammatory infiltration, apoptosis, vascular congestion and thrombosis and severity and prevalence of partial separation in apical epithelial cells. The histopathologist was blinded for group allocation and performed all analyses and graded microscopic changes in the colonic mucosa from 0 to 3⁹.¹⁰

Biochemical evaluation

Biochemical evaluation of the tissue and blood

Blood and tissue samples (terminal ileum) were collected from the rats for biochemical examination. Blood samples were drawn by inserting injectors with sterile-needles by intracardiac punction. Then blood samples were collected into silicon tubes, centrifuged and separated into serums. MPO, MDA, NO, Caspase-3, IL-6 and TNF-α were analyzed. The tissues were rinsed with serum physiologic for the biochemical analysis and then weighted and homogenized using the method by previously described12. Tissue samples (distal colon) were obtained from the rats for biochemical examination. MPO, TNF-α and IL-6 were used to determine inflammation in the tissues. Nitric oxide (NO) and malondialdehyde (MDA) to establish oxidative damage and Caspase 3 activity was examined to find the rate of apoptosis.

MPO measurements

The measurements of MPO activity were performed by the method used by Kruidener et al.13. The tissue was homogenized and incubated in 0.5% hexadecyl-trimethylammonium bromide (pH 5.5) and 0.026% ortho-dianisidinedihydrochloride and plus 0.018% H₂O₂ was added. The reaction period was 30 minutes. The reaction was specifically confirmed by sodium azide (0.1mM). All analyses were performed and controlled twice¹².

MDA measurements

MDA and thiobarbituric acid (TBA) were analyzed by spectrophotometric method. 0.2 ml of serum was mixed with 0.8 ml of tamponated phosphate and 25 µl of butylated hydroxytoluene (ph 7.4). 0.5ml of 30 % trichloroacetic hydro barbituric acid was added to the prepared solution and kept on ice for 2 hours and then was centrifuged at 2000g for 15 minutes at 25°C. After the process of centrifuge ethylene diamine, tetra acetic thiobarbituric acid (EDTA) 0.075 mm and 1% thiobarbituric acid 0.25 ml of 0.1 mol/l was prepared for every ml. The supernatant was kept in boiled water for 15 minutes and left cooling at room temperature. Spectrometric measurement was performed on the most recently prepared supernatant at 532nm wave length. Results were recorded as nmol/ml.

NO measurements

Serum NO levels were measured by Griess reagent according to the method of Moshage13. In the first stage nitrate reductase reaction was performed in order to convert nitrate in the serum. Components from nitrogen purple in color were developed by Griess reagent in the second step. Zinc sulphate was added in this formation and was centrifuged at 10.000 g for 5 minutes. Measurements were performed using azo chromatographic spectrometer at 450 nm wave length. The measured values were recorded as mmol/l.

Caspase - 3 measurements

The enzymatic activities of tissue samples in Caspase 3 were measured by the method defined by Jonges et al.¹⁴. Five 10 µm-thick tissue sections were lysed in buffer containing the following: 10 mmol/L HEPES (pH 7.0), 40 mmol/L β-glycerol phosphate, 50 mmol/L NaCl, 2 mmol/L MgCl₂ and 5 mmol/L EGTA. Then they were placed on ice for 10 minutes. Following this procedure, the solution for bestowing cellular decomposition was frozen at -80° C and melted 4 times within 10 hours. Protein concentration for example was determined as described by Bradford at the next stage. Enzyme caspase-3 activity was determined with 2.5 nmol DEVD-AMC. 15 µg of protein were incubated in 100 mmol/L HEPES enzyme subtract containing 10% sucrose tampon (pH 7.25), 0.1% (v/v) NP40 and 10 mmol/L. DTT at 37° C Caspase activity level obtained fluorescent signal released from AMC at wave lengths of 360 and 460 nm, measured by a FLUO star Optima. Tissue levels were indicated in pmolAMC/min/mg protein¹³.

TNF-α and IL-6 measurements

TNF-α and IL-6 serum levels were measured using immune enzymatic ELISA technique (Quantikine High Sensitivity Human by R&D Systems, USA). The minimum detectable values were determined as 0.12 pg/ml for TNF-α and 0.03 pg/ml for IL-6.
Statistical evaluation

The results were assessed as mean and standard deviation. The data for the groups were analyzed by Mann Whitney-U tests and the multiple variable data between the groups were analyzed by post-hoc test (Tukey’s HSD test). P<0.05 was accepted as statistically significant. General assessment of the data was performed by the computer program Statistical Package for the Social Sciences for Windows (SPSS for windows 16.0 SPSS, Chicago, Il; USA).

Results

Macroscopic colitis score

In all the TNBS induced rats, macroscopic score was found to be increased significantly compared to sham group (p<0.001). Macroscopic damage of the colon on the seventh day after administration of TNBS showed ulceration, thickening of the colonic wall, hyperemia and severe adhesions between the colon and other organs. Treatment with progesterone for TNBS significantly decreased the score compared to group 2 (p<0.001) (Figure 1).

![Figure 1](image1.png)

FIGURE 1 - Mean±SD colonic macroscopic score in the groups. Values with different letters have significance according to ANOVA test.

Microscopic colitis score

In all the TNBS induced rats microscopic pathological score was found to be significantly higher when compared to sham group (p<0.001). Treatment with progesterone with TNBS significantly decreased the pathological scores compared to group 1 (p<0.001) Administration of progesterone presented less mucosal damage and ameliorated mucosal structure and epithelial integrity (Figures 2 and 3).

![Figure 2](image2.png)

FIGURE 2 – A. X 200, H&E, normal colonic mucosa. B. Mild inflammatory cell infiltration in group 3.

![Figure 3](image3.png)

FIGURE 3 – A. X100, grade 2 inflammatory cell infiltration in the treatment of the progesterone. B. X200, grade 3 inflammatory cell infiltration and ulceration on the control group.
Biochemical results

The parameters for the oxidative injury, NO and MDA levels between groups were compared. The levels of group1 was significantly different when compared with group 2 and 3 (p=0.001). Both blood and tissue levels of NO and MDA was significantly decreased in progesterone treated group than group 2 (blood NO p=0.001, tissue NO p=0.001, blood MDA p=0.001, tissue MDA p=0.001). The comparison of oxidative injury results were shown at Table 1.

For inflammatory parameters we examined the blood and tissue IL-6 and TNF-α levels. The results of group 2 and 3 were much more significant when it compared with sham group (Group 1) (p=0.001). In treatment group the results was markedly decreased than group 2 (p=0.001). The other parameter for inflammatory results was tissue MPO levels. In groups 2 and 3 MPO levels were not statistically different (p=0.878). The results for inflammatory injury were shown at Table 1.

TABLE 1 - Colonic tissue, inflammatory and oxidative damage parameters.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Article</th>
<th>Group 2</th>
<th>Group 3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td>0.770 ± 0.035</td>
<td>0.376 ± 0.038</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>0.575 ± 0.074</td>
<td>0.348 ± 0.051</td>
<td>0.001</td>
</tr>
<tr>
<td>MDA</td>
<td></td>
<td>2.462 ± 0.292</td>
<td>1.287 ± 0.258</td>
<td>0.001</td>
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<tr>
<td>NO</td>
<td></td>
<td>134.500 ± 7.425</td>
<td>101.000 ± 4.780</td>
<td>0.001</td>
</tr>
<tr>
<td>MPO</td>
<td></td>
<td>0.430 ± 0.141</td>
<td>0.430 ± 0.107</td>
<td>0.878</td>
</tr>
<tr>
<td>Caspase 3</td>
<td></td>
<td>31.525 ± 3.094</td>
<td>27.552 ± 2.36</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Discussion

We investigated the progesterone and validated that it could be a protective agent for IBD. All inflammatory parameters, including macroscopic, and microscopic scores in the progesterone+TNBS group were significantly lower than those in the TNBS group. Progesterone could effectively attenuate the degree of damage and colonic inflammation caused by TNBS, showing its ability to prevent colitis.

Over the past several years, a variety of IBD animal models have been developed and investigated, with the ultimate goal of understanding the causes of CD and UC. Several mechanisms were investigated in the pathogenesis of IBD and among them are the nitric oxide, eicosanoids and free oxygen radicals (FOR) that generate tissue injury in the colonic mucosa by the way of cytokine release by lymphocytes, macrophages or neutrophils. An increase in FOR release from stimulated cells, have been exhibited in the colonic biopsy samples collected from patients with inflammatory bowel disease. Intracellular or extra cellular FOR in the colonic mucosa causes lipid peroxidation and development of peroxide radicals. There has been a balance between FOR and antioxidant mechanisms under physiological conditions. A significant reduction in mucosal antioxidant activity has been observed in the experimental colitis model. However, the exact role of FOR in colonic inflammation remains still controversial and is the subject of intense investigation. A substantial number of the experimental studies demonstrated an improvement of colitis with FOR inhibition46. There are also several reports showing an exacerbation of colitis with application of FOR inhibitors37. It is known that FOR expression is regulated by several proinflammatory cytokines and FOR function to generate high levels of NO via oxidative metabolism of L-arginine. NO seems to have a dual role in colitis leading to opposite results in IBD models. On one hand NO leads to vasodilatation and inhibition of neutrophil activation and platelet aggregation. On the other hand NO is a highly reactive free radical and may rapidly react with active oxygen to generate peroxynitrate, which causes severe detrimental effects on colonic mucosa. Clinical and experimental studies concerning the therapy of ulcerative colitis are generally related to these antioxidant defense mechanisms. No single agent that effectively participates in the therapy of IBD was found, despite numerous agents used in practice.

Progesterone, known as a sex steroid, is a cholesterol derivative. In recent years, progesterone has been reported to suppress especially inflammatory responses to reduce lipid peroxidation and FOR-related cell membrane damage in many numerous clinical and experimental studies3. Some autoimmune diseases enter a remission period during pregnancy. That is also true for ulcerative colitis. In experimental studies progesterone was reported to suppress the oxidative damage mechanisms on cardiac and brain tissues and mitochondrial cell membrane as well45. Moreover, progesterone was observed to exhibit immune modulatory effect via the membrane progesterone receptors on CD4/CD8 T lymphocytes2. Verma et al.13 reported that progesterone lessened liver and kidney injury following benzene toxicity and positively affected the levels of lipid peroxide, glutathione and cytochrome P450 in their study. With the help of these findings FOR-related cell damage was suppressed. Sayeed et al.4 reported that progesterone reduced cell apoptosis in brain tissue. However, the potential role of progesterone in colitis has not been studied extensively. In the present study, colonic MDA, NO, IL6 and TNF alfa levels were found to be significantly higher in the TNBS treated
rats when compared to control group. In addition, demonstration of colonic mucosal damage by histopathological examination in the TNBS treated rats confirmed colitis-induced inflammation and oxidative damage. This increase exhibits a statistically significant difference in progesterone-treated rats than the control group.

In addition to the increasing number of incidents with oxidative damage and inflammatory processes, enhanced programmed cell death in IBH was also exhibited in the literature.5-11. Programmed cell death is defined as apoptosis. Apoptosis is basically a physiological event. It is the process of programmed cell suicide due to irreparable tissue damage. In contrast to the inflammatory event programmed cell death is not associated with cytokine, FOR or eicosanoid release or coagulation cascade that will also affect the surrounding tissue. Excessive or inadequate apoptosis response during the inflammatory process, leads to different pathological events. Even though many enzymatic structures are appointed, the most important activators of apoptosis that promote DNA-damage are enzymes acting on caspase-3 activity on programmed cell death. In the present study we also observed that caspase-3 levels were significantly higher in the colonic tissues of colitis-induced control group rats. Caspase-3 activity on the colonic progesterone treated rats was significantly higher than those of the control group and programmed cell death in the colonic mucosa was also decreased.

There are studies in the literature that reports inflammatory cytokines such as TNF-α and IL-1 play role in the etiology of IBD.11-14. TNF-α is the most important cytokine in inflammatory process. In mice studies, TNF-α administration resulted in vasoconstriction and increased leucocyte migration and tissue damage that led to severe alterations in the gastrointestinal mucosa.15-17. Moreover, Hassan et al.18 reported that when oxidative and inflammatory damages were reduced, colonic injury also was reduced. We observed in our study that both TNF-α and IL-6 levels in the colon and blood specimens obtained from TNBS-colitis induced rats were significantly higher than those of the controls. This increase exhibits a statistically significant difference in progesterone-treated rats than the control group.

Conclusions

Our study demonstrates that progesterone treatment exerts a beneficial anti-inflammatory effect on the experimental colitis and results in acceleration of colonic healing. The amelioration of experimental colitis by progesterone is at least partly due to the decreased release of NO, inhibition of IL 6 and TNF alfa and depends on anti apoptotic effects.

References


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