ESTROGENS CONTROL INFLAMMATION IN EXPERIMENTAL COLITIS

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There is now a wealth of experimental evidence indicating that the deficit in endogenous estrogen facilitates the onset of inflammation that can be antagonized by estrogen replacement therapy. This work investigated the role of estrogen in the control of intestinal inflammation in a panel of colitis models, focusing on the morphological changes, the activity of mast cells, the expression of cytokines (IL-1β, IL-6, and TNF-α), fibronectin and reactive oxygen species. Two hundred adult male rats were divided into 4 groups: colitis was induced in Group I and Group II but only the latter was treated with estrogen; Group III received estrogen only, and Group IV saline. Colitis was induced in 4 models using: iodoacetamide; iodoacetamide + enteropathogenic *E. coli*; 2,4,6-Trinitrobenzene sulfonic acid; and dextran sulfate sodium salt. Macroscopic and microscopic evaluations of abdominal structures as well as molecular analysis were made on days 7, 14, 28 and 56. There was a significant improvement in the health condition of the estrogen-treated rats: the inflammation scores were reduced by at least 10-15%, the number of mast cells in the colon decreased by 30%, fibronectin expression was only 50% and reactive oxygen species decreased by 30%. In addition, there was a significant decrease in TNF-α, IL-6 and IL-1β expression by about 25%. In conclusion, there was an improvement in the inflammatory status in all estrogen-treated groups through the duration of the experiment at all-time points. In addition, there was less tissue necrosis as depicted by less fibronectin and a marked antioxidant effect.

Estrogens (Es) have been proven to modulate the immune system and to have anti-inflammatory as well as pro-inflammatory effects (1). In addition, it has been well documented that young females, in general, experience significantly lower rates of infection while women in menopause show enhanced production of the pro-inflammatory cytokines (1, 2). Es have also been known to possess a strong inhibitory effect on leukocyte migration in inflamed areas, particularly neutrophils and monocytes, leading to less severe inflammation (3).

At pregnancy levels or higher, Es inhibit pro-inflammatory pathways. They mediate the inhibition of TNF-α, decrease IL-2 production in mature dendritic cells, inhibit IL-1 and IL-6, decrease reactive oxygen species (ROS), and down-regulate

Key words: inflammatory bowel diseases, estrogen, colitis, TNF-α, IL-1β, fibronectin, mast cells

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adhesion molecules (4-7). On the other hand, post-menopausal levels of Es increase the expression of adhesion molecules (5), and render women to a more pro-inflammatory situation, thus contributing to the manifestation of chronic inflammatory diseases after menopause (1).

The role of Es in human inflammatory bowel diseases (IBDs), both Crohn's disease (CD) and ulcerative colitis (UC) has not been fully investigated. Some experimental studies using dinitrobenzene sulfonic acid colitis (DNCB) showed that Es, at pregnancy level, reduced macroscopic and histological alterations, as well as myeloperoxidase levels and ICAM-1 expression (8). In another model of chronic inflammation in HLA-27 transgenic rats, Es treatment also improved the histological scores, decreased inflammatory cell infiltration, fibrosis, and mast cell proteases 1, 3 and 4 (9-11). On the other hand, Es treatment of the dextran sodium sulfate (DSS) colitis increased the macroscopic and histological scores (9). In brief, earliest studies demonstrated anti-inflammatory and pro-inflammatory effects of Es in 3 different models out of more than sixty reported models of inducible colitis (10-12).

This article reexamined the anti-inflammatory effect of Es on a panel of induced colitis including: trinitrobenzene sulfonic acid (TNBS), dextran sodium sulfate (DSS), as well as iodoacetamide (IA) alone or plus enteropathogenic *E. coli* bacteria (IA+B). The parameters assessed included: clinical signs and symptoms, microscopic alterations, TNF-α, interleukins IL-1β, IL-6, fibronectin, and ROS.

MATERIALS AND METHODS

Experimental animals

A total of 200 adult male Sprague-Dawley rats, weighing 250-300 g, were used in accordance with the criteria set for care and use of animals by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut. The animals were housed in rack mounted cages, 5 rats per cage, and kept on a 12-h light/dark cycle in a controlled temperature and humidity room. Standard laboratory pelleted formula and tap water were provided *ad libitum*.

Colitis induction

The rats were divided into 4 groups: 2 major groups, GI and GII, of 80 rats each, and 2 minor groups, GIII and GIV of 20 rats each. Colitis was induced in GI and GII but only GII was treated by weekly interscapular injections of estrogen (250 µg) starting one week before the induction. The rats in GIII received subcutaneous injections with estrogen (E) at the same dose and schedule as in GII (17ß-estradiol, Sigma-Aldrich, E8875); while the rats in GIV served as control (C), they were neither induced with colitis nor received estrogen (13).

Groups I and II were further subdivided into 4 subgroups of 20 rats each. Colitis was induced in 40 rats according to previously established procedures using rectal injection with 100 µl of 4% Iodoacetamide in 1% methyl cellulose (IA) (Sigma Aldrich, St. Louis, MO, USA), according to Satoh et al. (14). Another 40 rats received IA plus 200 µl containing 4×10^8 colony factor unit of Enteropathogenic *E.coli* (EPEC) according to Hajj Hussein et al. (11). In addition, another 40 rats were subjected to 100 µl TNBS solution (Sigma-Aldrich, 92822-1ML) according to Morris et al. (15), and Fan et al. (16), and 40 rats used drinking water mixed with 2% Dextran Sulfate Sodium Salt (DSS) (Sigma-Aldrich, D4911-1G), 5 ml per rat per day, according to Writz et al. (17).

Daily observations

Rats were weighed and their health condition was checked daily. Signs and symptoms of colitis (weight loss, diarrhea, mucus and/or blood in stools) were monitored. Scores were given by two different observers and averages of the two scores of each category were calculated (18, 19).

Macroscopic assessment

The rats were sacrificed on days 7, 14, 28 and 56. Abdominal viscera were checked for inflammatory parameters, and biopsies were removed and fixed in 10% formaldehyde for routine microscopy, or frozen at -80°C for immunohistochemistry and RNA testing. The tissues included the descending colon, the jejunum, the kidney and the liver. Parameters, such as the size of the affected area due to inoculation, the descending colon morphology, number of mushroom-like structures in the serosa, vasodilatations, the presence of adhesions, were observed and scored by two different observers (11).

Histological evaluations

The H&E stained slides of jejunum and descending colon biopsies were examined under a light microscope at different magnifications. A previously reported scheme was used to determine the extent of inflammatory reaction and included: interruption of intestinal epithelium, dilation of glandular crypts, depletion and loss of goblet cells, inflammatory cells infiltration, edema, crypt abscesses and dysplasia (11). Slides were evaluated and scored by

PROOF

two different observers for each time point (days 7, 14, 28 and 56).

Analysis of cytokine expression

Molecular parameters, also assessed at various time points by qRTPCR, included TNF-α, IL-1β, IL-6, and Fibronectin. RNA extraction was performed from the scrap tissues collected on sacrifice days using Trizol reagent (Bio-Rad) according to the supplier's protocol. After following the protocol and having diluted 1 µl of RNA with 25 µl of DEPC water, the final RNA concentrations were determined with the Nanodrop knowing that A260/A280 should be between 1.8-2.0. Next, Genomic DNA elimination mixture was prepared using 2 µl of GE (5x genomic DNA elimination buffer) for every µg of RNA. To that, RNase free water was added to reach a volume of 10 µl. 10 µl of reverse transcriptase cocktail was added to each sample (4 µl of BC3, 1 µl of P2, 2 µl of RE, 3 µl of RNAse free water). The plates were prepared using 23 µl of the RTPCR mixture (12.5 µl RT2qPCR Master Mix, 1 µl of forward primer, 1 µl of reverse primer and 8.5 µl of RNAse free water) with 2 µl of cDNA, then sealed. All tests were performed according to procedures previously reported (22).

Dihydroethidine staining

Dihydroethidine (DHE) for ROS was carried out on 10 µm frozen sections using confocal microscopy (20, 21). DHE is a cell-permeable compound which, due its ability to freely penetrate cell membranes, is used extensively to monitor superoxide production (20, 21). Reactive oxygen species (ROS) are chemically reactive molecules containing superoxide anion O2−. They are biologically toxic and deployed by the immune system to kill invading microorganisms (23). They increase during time of stress. DHE is oxidized by reaction with O2- to ethidium bromide, or oxyethidium (24), which binds to DNA in the nucleus and fluoresces in red, detectable qualitatively by a fluorescent microscopy.

Statistical analysis

The histological scores were computed and represented with matching standard error of the mean using PRISMS software. Statistical significance comparing the treatment with estrogen versus no treatment was evaluated by Student's *t*-test and ANOVA test. P value of less than 0.05 indicates a statistically significant difference.

RESULTS

Estrogens improved the clinical picture in the rats

Animals in GI showed to various degrees, all signs and symptoms of an induced active ulcerative colitis (UC), more severe in the IA+B treated rats compared to the others. On the other hand, GII rats, treated with estrogen, showed lower clinical scores, thus less inflammation compared to GI. In general, the subgroups in GII showed a significantly reduced spectrum of total scores, compared to their corresponding subgroups in Group I, $p < 0.05$ (Fig. 1).

The total clinical score of the IA+B induced colitis was the highest, the most severe inflammation (7.6/10), followed by the TNBS (6.5/10), the IA (5.5/10) then the DSS (3/10). The colitis score increased after each intra-rectal induction with IA+B, IA, and TNBS, and after each cycle of DSS. For the IA+B, IA and TNBS, after day 28, the clinical scores gradually decreased till day 56 but never reached the control baseline levels. However, it is important to note that on day 36, when the last induction of colitis took place, the inflammation peaked but it was 30% lower than the previous peaks. Therefore, the inflammation was present after one month and it was maintained at a lower scale till the end of the experiment time; and estrogen decreased inflammation significantly as depicted in Fig. 1.

Estrogens decreased inflammation in the abdominal structures at inspection

The IA+B model had the highest scores of alterations in abdominal viscera on each sacrifice day (day 7=15/18, day 14=17/18, day 28=14/18, and day 56=7/18) (Fig. 1). Comparing the models to their treatment subgroups, a statistically significant difference was present at each end point for the IA, IA+B and TNBS models, decreasing by approximately 20% compared to the non-treated group. A similar trend was observed with the DSS but the difference was not significant on days 7 and 56.

On the other hand, the estrogen treated rats and the non-treated controls showed low and similar inflammatory scores all along the experiment (Fig. 1).

Histological alterations

The IA+B, IA and TNBS-treated groups showed higher colonic histological scores than the controls or those treated with estrogen alone. However, the

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DSS group showed a higher histological score in the jejunum than in the colon, probably because the DSS was provided orally to the rats. All four models exhibited inflammatory changes to various extents in the colon and jejunum. They were most severe in IA+B-treated groups at all time points, while relatively less in IA, TNBS, and DSS, respectively (Figs. 1 and 2).

The estrogen treatment decreased significantly all these changes, in all groups and at all time points. However, these alterations were never completely resolved by estrogen to reach the normal non treated or estrogen alone treated groups (Fig. 3).

Estrogens decreased the activity of mast cells in the colon

Mast cells responded to the induction of colitis by increasing significantly in number in all models compared to controls. The highest numbers were in the IA+B group at all time points and the lowest were in the DSS group (Figs. 1 and 4). The number of mast cells increased with the duration of the inflammation in each group, particularly in the IA+B group from an average of 73.2 on day 7, to 80 on day 14, to 91 on day 28 and 120 on day 56 compared with 8.3 and 7.5 in the group that received only estrogen and the non-treated group. In brief, estrogen treatment did show a

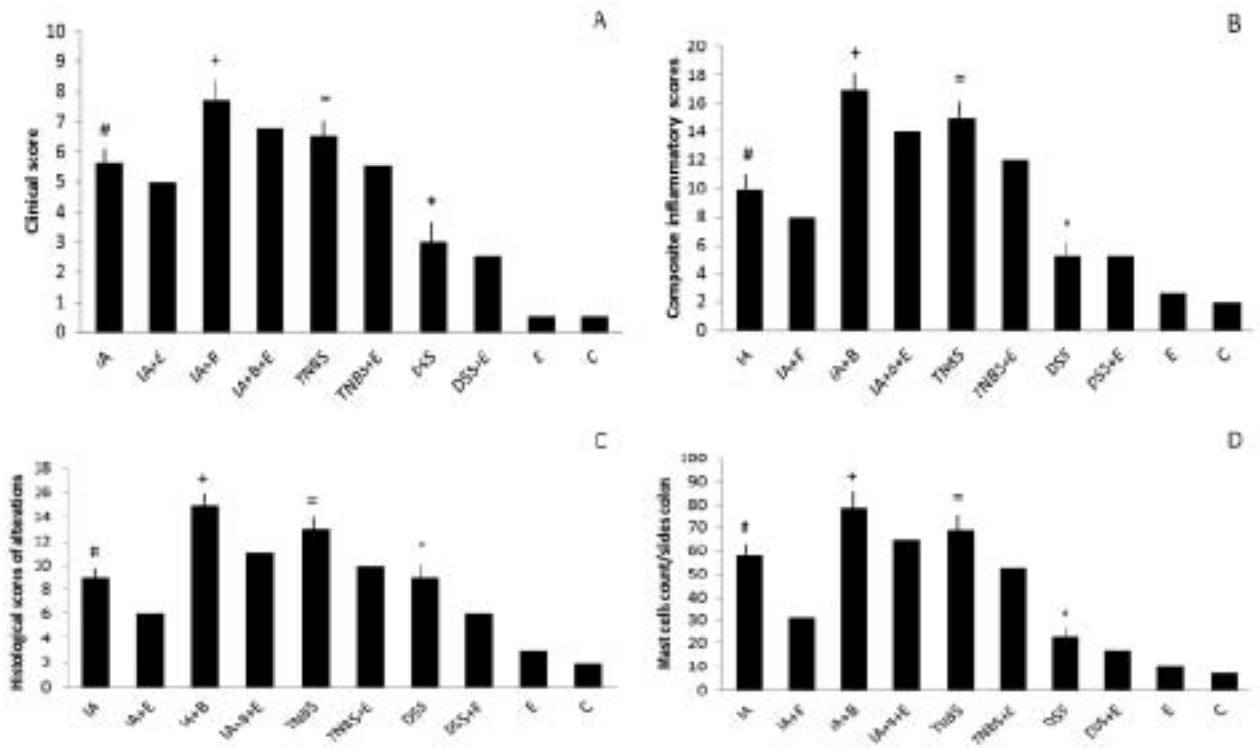

Fig. 1. *Clinical, macroscopic and histological assessment. **A)** Total clinical score on day 14. Note that in estrogen-treated rats, the scores were markedly lower. **B)** Composite inflammatory scores of abdominal viscera on day 14. Values represented the average scores of parameters including: damage site, morphology of Descending Colon (DC), mushroom-like structures per 10 cm, adhesions, vasodilatations and thickness of DC. The scores of the IA, IA+B, and TNBS subgroup were statistically significant when compared to their concordant treatment subgroups. **C)** Average composite histological scores of alterations in colon on day 14. The improvement of inflammatory reaction parameters was approximately 20%. Each score represents the average of total changes in mucosal architecture, in crypts, goblet cells, inflammatory cells infiltrate and edema. These alterations decreased significantly with estrogen. **D)** Mast cell count in the colon on day 14. Note that the count increased throughout the experiment in the IA, IA+B, and TNBS subgroups while in the DSS subgroups, the counts were much lower and constant; however, they were relatively two-fold more than the estrogen alone or normal. Similar results were seen on days 7, 28 and 56 but to a lesser extent (data not shown). T-test $p<0.05$; #$p<0.05$: IA vs IA+E groups; +$p<0.05$: IA+B vs IA+B+E groups; =$p<0.05$: TNBS vs TNBS+E groups; *$p<0.05$: DSS vs DSS+E groups.*

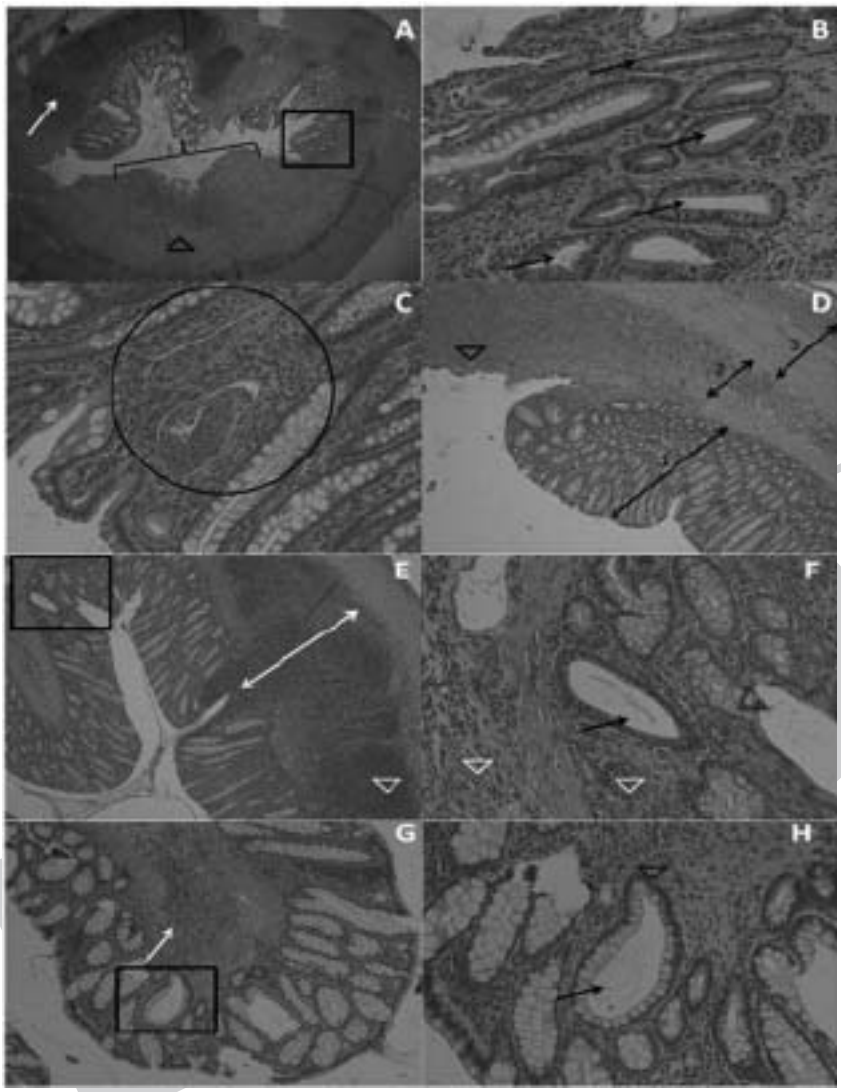

Fig. 2. *Induction of colitis led to microscopic changes in colonic structures.* ***A****) Disruption of colonic mucosal epithelium (parenthesis 100X) and Colonic Payer's patch (A-white arrow).* ***B****) Dilated crypts, circular and less loaded goblet cells (B-black arrow).* ***C****) Abscess in the mucosa crypts (C-black circle 400X) with invasion of the mucosa by inflammatory cells (A-D: black triangle 400X).* ***D****) Altered architecture of the colon layers in colon of rats treated with IA+EPEC (D-1: mucosa, 2: submucosa and 3: musculosa 100X).* ***E****) Altered architecture of the colon layers of the IA-treated rats (E-two headed white arrow 100X) with lymphoid tissue expansion.* ***F****) Dilated crypts, circular and less loaded goblet cells (F-black arrow 400X). Note the invasion of mucosa by inflammatory cells (E-F-white triangle).* ***G****) Cellular mucosal infiltration of the colon of DSS-treated rats (G-white arrow 100X).* ***H****) Dilated lumen of crypt (H-black arrow) with inflammatory aggregations (H-black triangle 400X).*

marked reduction in the number of mast cells across the board in all groups and at all time points. These reductions were significant in all models $p < 0.05$, except the DSS model (Figs. 1 and 4).

Modulation of TNF-α, IL-1β and IL-6 by estrogen

All 3 parameters showed a similar trend of expression. They all increased to various extents as a result of colitis induction throughout the experiment. The highest relative increase was in the IA+B, followed by TNBS and IA. This increase in expression in the colon was relatively the least with the DSS model.

As expected, TNF-α increased significantly

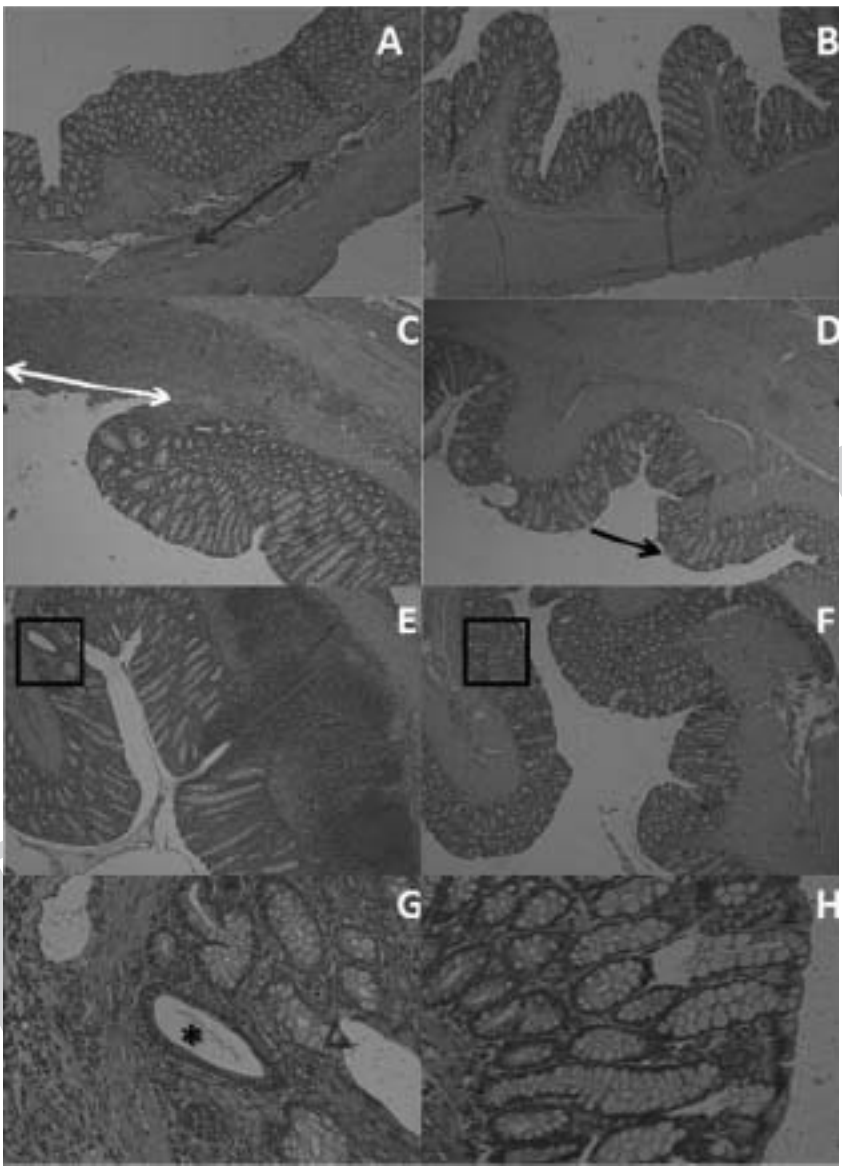

Fig. 3. *Microscopic alteration in the colon decreases upon treatment with estrogen. **A)** Note decreased edema in the colonic submucosa of estrogen-treated rats (gray doubled-headed arrow, 100X), compared to non-treated rats in (**B**). **C)** The sloughed epithelium in colitis animals (white double-headed arrow) is significant compared to (**D**) where estrogen was used (100X). **E)** Note the infiltration of inflammatory cells and extensive lymphoid follicles in non-treated colons compared to those of treated in (**F**) whereby the alterations were less. **G)** Higher magnification of the changes (400 X) compared to treated animals in (**H**) whereby alterations were less.*

compared to control levels in all models and at all time points. However, such increases were much lower on day 56 when the inflammatory reaction was sustained, and it was significantly higher than the controls in all groups except in the DSS (Fig. 5 A).

The high expressions of TNF-α, IL-1β and IL-6 were significantly reduced to various extents ($p < 0.05$) in the estrogen-treated groups at all time points (Fig. 5 B).

IL-B gene expression was similar to that of IL-6. Rats treated with estrogen showed marked and significant decreases in the levels of expression at all

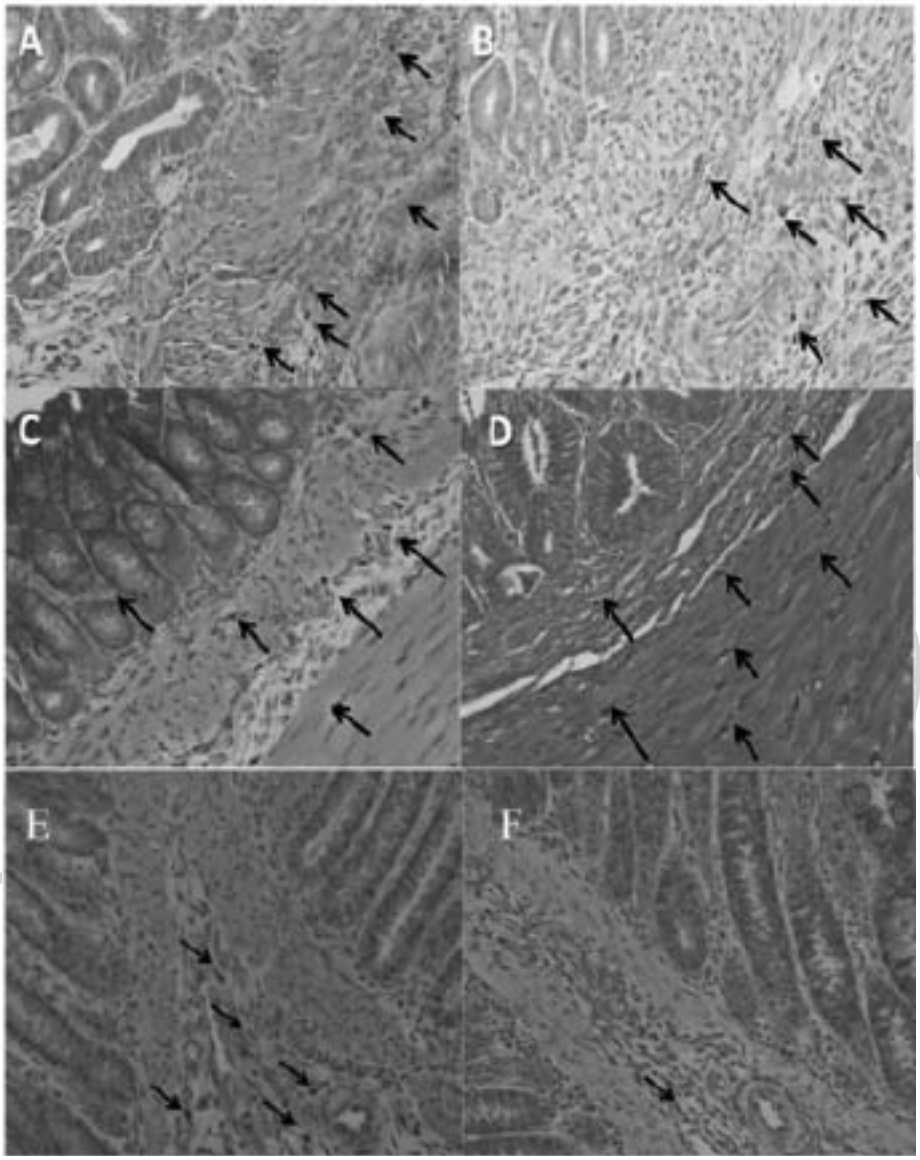

Fig. 4. *Number of mast cells infiltrating the colonic layer increases with the progression of inflammation and decreases with estrogen treatment. **A)** Microscopic observation of mast cells (black arrows) in colon of rats treated with IA+B at 400 X. The number of mast cells increases along with the progression of colitis from day 7 (**A**), day 14 (**B**), day 28 (**C**) and day 56 (**D**). In exacerbated cases, mast cells were diffused throughout the colonic layers (C-D). Animals treated with estrogen (**F**) had a decreased number of infiltrating mast cells in the colon compared with non-treated (**E**).*

time points (Fig. 5 C).

Fibronectin decreased by estrogen treatment

Fibronectin RNA expression increased significantly as a result of the induction of inflammation. Such an increase was maintained in the various groups compared to controls throughout the experiment. However, it decreased significantly in the tissues removed from the estrogen-treated rats at all time points (Fig. 5 D).

Dihydroethidine staining

Using DHE staining, the fluorescence was detected mostly in the IA + B model at day 14

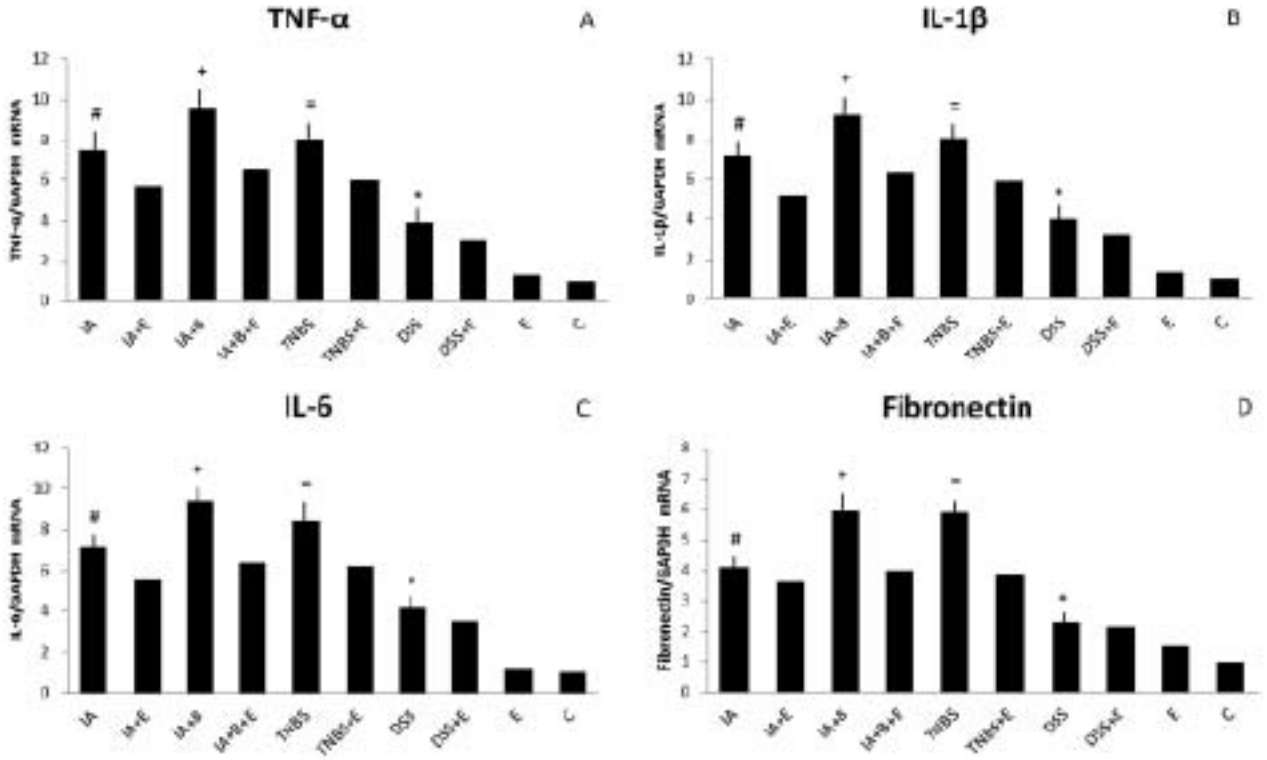

Fig. 5. *TNF-α, IL-1β, IL-6 and fibronectin expression on day 14. **A)** TNF-α gene expression decreased with estrogen treatment. The values represent amplification of TNF-α relative to GAPDH mRNA levels. The values on days 7, 14, and 28 were mostly equal, and then they decreased by more than half on day 56. The highest value was always found on the IA+B model at the 4 time points. They decreased significantly when the rats were treated with estrogen, except for DSS. **B)** IL-1β gene expression decreased with estrogen treatment. **C)** IL-1β gene expression decreased with estrogen treatment. **D)** Fibronectin gene expression decreased with estrogen treatment. In all these figures, the highest value was always found on the IA+B model at the 4 time points (data not shown). They decreased significantly when the rats were treated with estrogen. Similar results were seen on days 7, 28 and 56 but to a lower extent (data not shown). T-test p<0.05; #p<0.05: IA vs IA+E groups; +p<0.05: IA+B vs IA+B+E groups; =p<0.05: TNBS vs TNBS+E groups; *p<0.05: DSS vs DSS+E groups.*

(Relative mean of intensity= 27 for jejunum, 19 for the kidney and 24 for the liver). Such expressions decreased significantly by more than 30% (P<0.05) following estrogen treatment (Figs. 6, 7). There was a positive correlation between the fluorescence intensity and the duration of the inflammation. The DSS model showed fluorescence intensity that was very close to normal (average of 5 for the intensity in the control and 5.5 in the DSS model).

DISCUSSION

The beneficial effects of estrogen treatment in experimental models of inflammatory bowel disease are well documented (3, 8, 9, 25). A significant reduction of colonic TNF-α level in rats with experimentally-induced colitis was previously reported (9, 10). The ability of estrogen to improve disease progression has been attributed to its interference with NF-KB activity. This interference is mediated by the ER receptors, and is not a direct antioxidant effect of estrogen (26). In addition, mast cells numbers and degranulation decreased in the estrogen-treated rat colons compared with non-treated rats. This fact suggests that estrogen may exert its effect partly through modulation of mast cell activity (22, 27). Understanding the basic mechanism of estrogen action in the various colitis models might provide new pathways for the treatment of inflammatory bowel disease.

To date, no consensus has been reached regarding

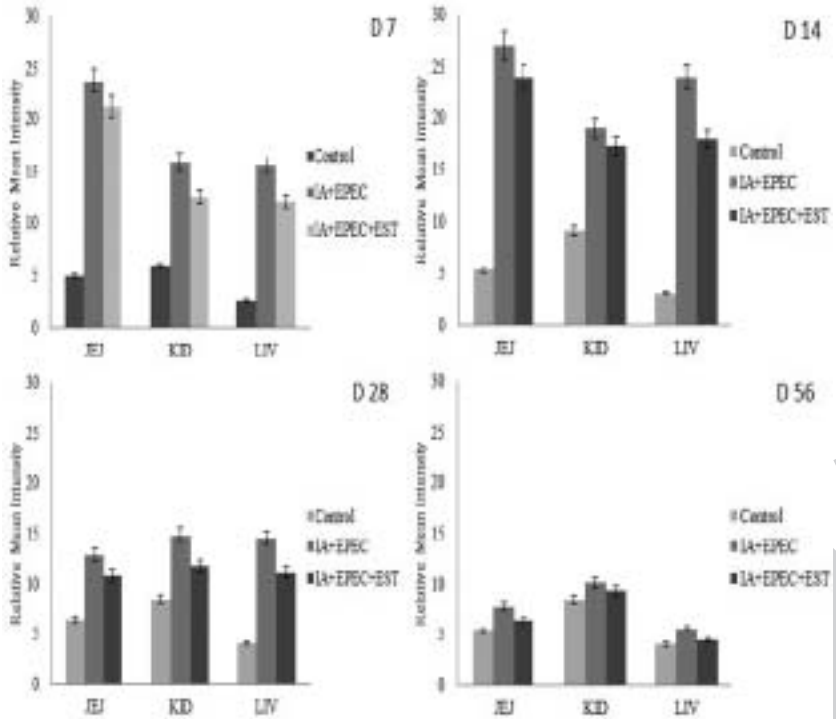

Fig. 6. *Relative mean intensity of the red fluorescence in the jejunum, kidney, and liver of the IA+B-treated rat models on days 7, 14, 28, 56. It is more intense on day 14 followed by days 7, 28 then 56. The estrogen treatment reduces this intensity significantly. T-test p<0.05; + p<0.05: IA+B vs IA+B+E groups.*

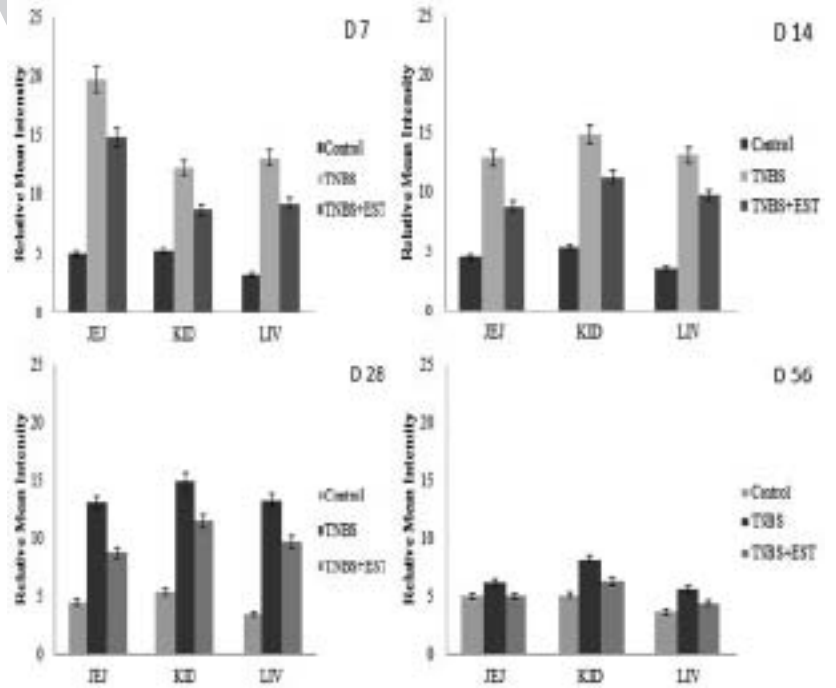

Fig. 7. *Relative mean intensity of the red fluorescence in the jejunum, kidney, and liver of the TNBS-treated rats model on days 7, 14, 28, 56. It is more intense on day 7 followed by days 14, 28 then 56. The estrogen treatment reduced this intensity significantly.T-test p<0.05; = p<0.05: TNBS vs TNBS+E groups.*

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the role of estrogen in IBD. Multiple studies have shown that estrogen suppresses chronic inflammation in several animal models of chronic inflammatory diseases. On the other hand, other studies have reported that estrogen has a pro-inflammatory effect in some chronic autoimmune diseases in humans as an initiating or perpetuating factor.

The present data show that estrogen possesses an anti-inflammatory role in experimental colitis. Estrogen improved experimental colitis both clinically and histologically. The subgroup that showed the least expression of such parameters and the lowest scores was the DSS, while the one that showed the most and the highest inflammation scores was the iodacetamide plus bacteria, seconded by the iodoacetamide alone and TNBS groups. Such results go in concordance with findings reported by Verdu et al. (9). Such an effect of estrogen was expressed across the board; however, where the inflammation was more severe the effect of estrogen was more significant (Fig. 1). In addition, treatment with estrogen significantly attenuated the damage in morphology associated with all the panel models of induced colitis (Figs. 2, 3).

The mast cells, which are important players in this inflammatory process, have been affected. Data showed that the number of mast cells increased markedly in the various IBD models upon induction compared to controls. Previous data, reported by Harnish et al. (8), as well as our own, showed that such an increase of mast cells correlated significantly with the severity and duration of the disease found in rat models of experimental colitis (22). In our experiment, the numbers were highest in the IA+B group seconded by the IA and TNBS then the DSS (Figs. 1, 4). It is important also to note that the numbers were associated with the chronicity of the inflammation in each group and not only the total number of mast cells increased but also the number of degranulating (activated) cells. It seems as if the estrogen is inhibiting the activity of mast cells or even their proliferation through the decreased expression of cytokines from nearby cells. Thus, leading to a decrease in the secretion of TNF-α, IL-1β and other factors or through the ERβ down regulation (1, 28).

Previous work from our group (11, 22) also reported increased levels of mucosal proinflammatory cytokines in IBD's, such as Il-1β, IL-6, TNF-α and others, which have been shown to play an important role in sustained inflammatory responses. It was also reported that expression of these immunomodulatory agents seems to be regulated mainly by the transcription factor NFkB. Preventing NFkB activation and cytokine production might result in the prevention of mucosal inflammation (28). Compounds like estrogen which down regulate the production of cytokines as IL-1β, IL-6 and TNF-α, might be acting through the deactivation or prevention of activation of NFkB, thus proving to be beneficial in the treatment of IBD. The use of estrogen as a treatment for IBD has alleviated inflammation and reduced symptoms but has not provided a cure or completely prevented complications. In brief, estrogen shows the potential as a proper effective molecule which might induce protective responses by inhibition of the production of proinflammatory cytokines. Estrogen has also decreased intestinal fibrosis, a dynamic multifactorial process which results from the reaction of intestinal tissue to the damage inflicted by chronic inflammation. Intestinal fibrosis can occur in both forms of IBD and is a common complication that follows inflammation. Estrogen treatment decreased fibronectin significantly in all tested models of colitis. Consequently, the process of fibrosis which is commonly recognized as unidirectional and irreversible could be improved in light of the data emanating from these experiments (29, 30). The effect is probably through decreasing the proliferation and activity of fibroblasts secreting such extracellular matrix molecules.

In conclusion, rats with IBD improved clinically and histologically with the use of estrogen. Based on this observation, further studies are needed to elucidate estrogen mechanism of action and possible side effects. The results we present suggest that this protective effect is mediated by limiting the inflammatory reaction, decreasing mucosal mast cell activity as well as the secretion of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 and decreased necrosis through its effect on fibronectin and ROS.

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