AMERICAN UNIVERSITY OF BEIRUT

MODULATION OF CYTOKINES IN TNBS INDUCED COLITIS TREATED WITH ESTROGEN

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
August 6, 2012

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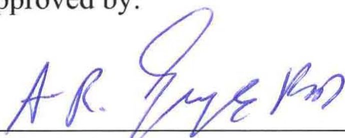

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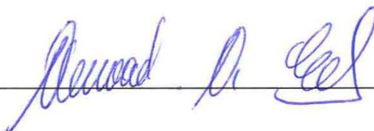

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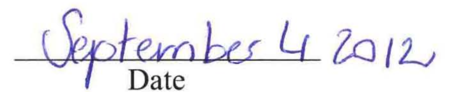

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ACKNOWLEDGMENTS

I would like to thank all of those who have helped me directly or indirectly for the past two years.
Dr. Ado Jurjus thank you for your guidance and support. Dr. Inaya Abdallah Hajj Hussein thank you for your precious advice and help. Rony Maksoud, thank you for being part of this journey.
I would like to extend my gratitude to Dr. Assaad Eid and Dr. Kassem Barada for their thorough remarks.
I would also like to thank Dr. Joseph Ghafari and all the Department of Orthodontics and Dentofacial Orthopedics in AUBMC, as well as Dr Nayef Saade and the Department of Anatomy, Cell Biology and Physiology for their encouragement and assistance.
My sincere gratitude goes to my parents and sisters for their unlimited support.

AN ABSTRACT OF THE THESIS OF

Stephanie S. Jambart

Title: Modulation of cytokines in TNBS induced colitis treated with estrogen

Background: Epidemiological studies showed that pregnant women and women under birth-control pills experienced less inflammation than other women. Even if estrogens were thought to have more pro- than anti-inflammatory-like reaction (because of their inflammatory-like reaction on the ovarian follicle maturation) there are now numerous experimental models where the lack of estrogens facilitates the onset of inflammation **Objectives:** This study investigated the anti-inflammatory effect of estrogen in TNBS induced colitis models, focusing specifically on the morphological changes, the activity of mast cells and the expression of cytokines (Interleukin 6 (IL-6), Tumor Necrosis Factor-α (TNF-α)), extra cellular matrix (fibronectin and collagen IV) as well as reactive oxidative species (ROS). **Materials and Methods:** 120 adult male Sprague-Dawley rats (n=120), weighing 250 to 300g, were divided into 4 groups: Group I: induced with colitis, not receiving treatment, Group II induced with colitis and treated with estrogen (17β-estradiol), Group III receiving estrogen only, Group IV not provided with anything. In groups I and II colitis was induced according to previously established procedures: 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) (n=20 for each group) and Dextran Sulfate Sodium Salt (DSS) (n=20 for each group). Rats were observed daily where scores was given to signs and symptoms. Biopsies of the colon, jejunum, liver and kidney were extracted from the rats on days 7, 14, 28 and 56, where macroscopic, microscopic and molecular evaluations were performed. **Results:** The rats from Group II receiving estrogen treatment, expressed statistically reduced clinical scores by approximately 10%. The gross morphologic inflammation alterations showed statistically significant amelioration when the rats were treated with estrogen by about 20 %. Estrogen reduced significantly the expression of collagen IV and fibronectin protein expressions as well as IL-6, TNF-α, and fibronectin gene expression by 20 %. ROS expression tested with the dihydroethidine (DHE) staining was significantly decreased by 30 %. **Conclusions:** Estrogen in experimental colitis depicted an overall preventive and or protective role. Rats treated with estrogen showed less inflammation at all time points, less necrosis and less production of ROS compared to non-estrogen treated animals.

Keywords: Inflammatory Bowel diseases – Estrogen – TNF-α – IL-6 –Fibronectin-Collagen IV- Mast Cells- DHE

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I- INTRODUCTION

1 Estrogen

Estrogen is a primary female sex hormone. Natural estrogens (i.e. estradiol) are steroid hormones, while the synthetic ones are non-steroidal. Steroids consist of a special kind of fat molecule with a four-ringed, carbon atom core, like their cholesterol predecessor. The main sources of estrogen in the body are the ovaries and the placenta. In humans, they are also formed in the adrenal cortex and testis. 17-beta estradiol is the most abundant and potent natural estrogen in all vertebrates. Estrone and estriol are the other types (Ryan KJ, 1982).

1.1 General effects

Estrogens have a wide-spectrum of effects in females ranging from puberty to menopause. At puberty, their actions guide the appearance of secondary sex characteristics, such as reproductive tract development, mammary gland and nipple growth, and fat distribution. The adolescent bone-growth spurt is fueled, and then halted, by estrogens. During the menstrual cycle, they act on the female genitalia to produce an environment suitable for fertilization, implantation, and nutrition of the early embryo (Kassi et al., 2001).

Their signals also affect blood fat levels, enzyme production, water and salt balance, bone density and strength, skin and blood vessel elasticity, heart muscle, brain functions such as memory, and sexual and maternal behavior (Nelson LR et al., 2001).

Males need estrogens, too. These hormones influence fertility through their effects on the prostate, testis, and other sex tissue, and by controlling fluid absorption around the maturing sperm in the epididymis (Hess et al., 1997). At puberty, estradiol regulates growth hormone and determines final height, as in females, by shutting off bone growth at the growing ends, or ephiphysis, of arm and leg bones (Smith et al., 1994).

1.2 Side effects

Estrogens can also harm people and animals. Long-term exposure to the hormones can increase the risks of breast, endometrial, and vaginal cancers in women (Nelson HD. et al. 2012). Too much estrogen aggravates endometriosis, while too little estrogen leads to osteoporosis. Males exposed at certain times to estrogens can develop female features and organs or have diminished male characteristics that might lead to testicular cancer, reduced sperm health, or genital defects, such as a malformed penis (hypospadias) or undescended testicles (cryptorchidism) (Grumbach and Conte, 1998).

1.3 Role during pregnancy and birth control

Birth control pill were shown to have anti-inflammatory effects. They protected against pelvic inflammatory disease, and reduced the risk of symptomatic endometriosis. Epidemiological studies demonstrated that increased levels of sex hormones during pregnancy could lead to a significantly reduced severity of multiple sclerosis, whereas clinical symptoms often exacerbate postpartum, when sex hormone levels were remarkably reduced. Pregnancy dramatically improved the clinical and pathological phenotype of a late-onset chronic form of genetic leukodistrophy and keeping estrogen supplementation substantially duplicated the protective effect of pregnancy (Matsuda et al., 2001). Estrogen, at periovulatory to pregnancy serum levels, was able to stimulate

antibody secretion under healthy conditions and also in autoimmune diseases, whereas similar serum levels of estrogen could lead to a suppression of pro inflammatory cytokines. At pregnancy levels, in human and mice, it was shown that estrogen inhibited important proinflammatory pathways such as TNF-α, IL-6, IL-1β, Monocyte Chemotactic Protein (MCP), iNOS expression, production of matrix metalloproteinases (MMPs), and activity of natural killer cells, whereas estrogen at the same concentration stimulated anti-inflammatory pathways such as IL-4, IL-10, TGF-β, tissue inhibitor of metalloproteinases, and osteoprotegerin. At lower concentrations, estrogen stimulated TNF-α, IFN-γ, IL-1β, and activity of natural killer cells (Straub, 2007). Even though women do show changes in their immune response during pregnancy when estrogen levels are high (Nelson, 1998; Wilder, 1998), there appears to be no significant difference between the course of Crohn's disease or ulcerative colitis during pregnancy (Mogdam et al., 1981). Understanding the anti-inflammatory effect of the estrogen may be beneficial for the treatment of IBD patients.

1.4 Anti-inflammatory effect

Estrogens were thought to have more pro- than anti-inflammatory-like role because of their well-known effect on ovarian follicle maturation (an inflammatory-like reaction). There are now numerous experimental models where the lack of estrogens facilitates the onset of inflammation (Cuzzocera, 2000; Miyamoto, 1999; Jansson, 1994). In all of these models estradiol clearly opposed the inflammatory process. Studies showed that in ovariectomized rats, the severity of inflammation in the pancreatic islets was increased compared to naive females, and this was suppressed by weekly subcutaneous injections of estrogen for twenty weeks, suggesting that estrogen played a pivotal role in the occurrence of islet lesions (Shinohara et al. 2005).

Different biological mechanisms underlying estrogen anti-inflammatory activity have been proposed:

1.4.1 Estradiol restricted the adhesion and migration of leukocytes through the endothelium of the vascular system, which caused a decrease in the expression of adhesion molecules (such as E-selectin, cadherins and cell adhesion molecules) and chemokines (such as the monocyte chemoattractant protein-1, MCP-1) (Zang et al., 2002; Kanda et al., 2003).

1.4.2 Estrogen inhibited the secretion of proteases specifically secreted by macrophages (cathepsin K and L involved in bone resorption or metalloproteinase-9 implicated in matrix homeostasis). They were inhibited by estrogen receptor activation. In addition, a reduced synthesis in proteolytic enzymes might have also accounted for estrogen reduction of tissue degradation and inflammation (Vegeto et al 2002).

In addition, estrogens were widely studied for their action in bone remodeling. It was demonstrated that estrogens possessed a protective role in inflammatory processes by blocking resident inflammatory cell production of proinflammatory cytokines (IL-1, IL-6 and TNF-α), hematopoietic growth factors and cell differentiation agents (receptor activator of NF-κB ligand, RANKL). Inhibition of the synthesis of these inflammatory mediators had been proposed to explain the detrimental effects of estrogen deficiency on the human skeletal system at menopause. Other researches have also reported that estradiol opposed the inflammatory reaction by blocking the synthesis of proinflammatory mediators, such as IL-6, TNF-α and iNOS. Such data came up from

several cellular and biological systems suggesting a primary role of estrogens as anti-inflammatory agents (Hue, 1988; Dodel, 1999; Polan, 1988; Straub, 2007).

As reported, estrogen at periovulatory to pregnancy levels inhibits NF-κB activation, membrane expression of adhesion molecules, cell adhesion to endothelial cells, important proinflammatory cytokines like IL-6 and TNF-α. This must be viewed as an anti-inflammatory signal. Nevertheless, low estrogen concentrations were demonstrated to have no or even stimulatory effects. On the other hand, at the same level, estrogen stimulates the secretion of IL-4, IL-10, and TGF-β, cytokines assuming to exert antiinflammatory effects as long as tissue-specific autoimmune diseases are considered (Straub, 2007). This renders a woman in the postmenopausal phase to a more proinflammatory situation, which might well contribute to the manifestation of chronic inflammatory diseases after the menopause (Straub, 2007)

Regarding the nitric oxide, estrogen exerts quite different effects depending on absence or presence of additional inflammatory stimuli such LPS or IL-1β. In the absence of inflammatory stimuli, estrogen increases NO production by stimulating the expression and activity of different isoforms of NOS. However, in the presence of an inflammatory stimulus, estrogen at pregnancy levels typically inhibits NO production elicited by cytokine-stimulated iNOS activity. In the latter situation, this might reflect the direct effect of estrogen on NF-κB activation and proinflammatory cytokine secretion (Wen et al. 2004). For the ROS formation, the overwhelming majority of studies demonstrated that estrogen at proestrus to pregnancy levels inhibits ROS formation while it stimulates it below periovulatory/proestrus to pregnancy levels, which lead to a more proinflammatory situation at low concentrations of estrogen in the postmenopausal state (Wagner et al., 2001; Sumi et al., 2001)

It was also suggested that, since macrophages play an essential role in the inflammatory process, the anti-inflammatory effect of estrogen could be mediated by suppressing monocyte recruitment and/or macrophage activation at the inflammatory site (L'aszl'o et al., 1993).

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1.5 Effect on the gastrointestinal function

Several clinical observations and animal experiments depicted a range of effects of sex steroids on gastrointestinal function. Some showed that estrogen therapy, significantly reduced the need for blood transfusion and that it could be a symptomatic therapy for severe gastrointestinal bleeding seen in the elderly, or in chronic radiation colitis (Wurzer et al., 1998; Cacoub et al., 2000). Furthermore, in humans, peptic ulcer disease was reported to be more frequent in men than in women (Michaletz Onody, 1992; Heitkemper et al 1988) and that during pregnancy, hemorrhage or perforation from gastroduodenal ulceration has shown to be rare compared to its higher incidence in postpartum period (Michaletz Onody, 1992), suggesting anti-inflammatory effects of estrogens on the gastrointestinal tract. Similarly, symptoms such as abdominal pain, stomach pain, and diarrhea have been reported to increase during the premenstrual and menses phases (Gué et al., 1997). The clinical observations showing that gastric mucosal erosions were enhanced in male rats in comparison with females following oral administration of ethanol (Aston et al., 1991; L'aszl'o et al 1992).

2 Inflammatory Bowel Diseases (IBD's):

2.1 Definition

Inflammatory bowel diseases include a range of intestinal disorders among which two major forms: ulcerative colitis (UC) and Crohn's disease (CD).

CD and UC are distinct entities. The main difference between them is their location and the nature of inflammation: UC is limited to the colon, it begins in the rectum (always involved in UC) and extends proximally in an uninterrupted fashion to the proximal colon, eventually involving the entire length of the large intestine. CD can involve any part of the gastrointestinal tract from the mouth to the anus (although it most commonly affects the small intestine and/or the colon) in a segmented fashion (normal area of the bowel interspersed with diseased areas). Microscopically, UC affects the mucosa and the submucosa of the gut only while CD affects the whole bowel wall (Fiocchi C, 1998; Rowe, 2011).

2.2 Etiology

UC and CD's etiologies remain unclear. A recent hypothesis suggested that persons with IBD have a genetic predisposition for the disease (Mathew, 2004): *NOD2* gene (now called *CARD15*) was discovered on chromosome 16, as the first gene clearly associated with IBD (as a susceptibility gene for CD). Strong support for IBD susceptibility genes was also found on chromosomes 5 (5q31) and 6 (6p21 and 19p) (Hugot JP et al, 1996, 2001).

People with IBD have altered immune responses and regulations and autoimmune reactions (Podolsky, 1991; Elson et al., 1995; Farrell and Peppercorn, 2002). Some

agents or a combination of agents could trigger the body's immune system to produce an inflammatory reaction in the GI tract. It could be an infectious agent such as bacteria or viruses or an antigen such as a protein from cow milk or an autoimmune process. (Rowe, 2011).

2.3 Epidemiology

Epidemiological data including ethnic and racial differences, geographic location, familial aggregations and twin studies suggest evidence favoring a genetic contribution to the pathogenesis of IBD (Karlinger et al., 2000). Population based studies showed that 5% to 10% of affected individuals report with a positive family history suggesting that genetic factors play an important role in determining the susceptibility to IBD (Bonen et al., 2003). However, the strongest evidence for genetic predisposition comes from twin studies. Monozygotic twins show 42-58% concordance in the incidence of CD and about 15% concordance in the incidence of UC whereas dizygotic twin concordance is not significantly different from that of all siblings (Lakatos et al., 2006). The lack of 100% concordance in monozygotic twins shows that nongenetic factors, such as environment, are involved in the pathogenesis of the disease (Bonen et al., 2003).

CD and UC are most commonly diagnosed in late adolescence and early adulthood, but the diagnosis may occur at all ages. CD affects mainly adolescents and young adults between 15 and 25 years of age, with a second onset between 50 and 80 years of age. In North America, the mean age at diagnosis ranges from 33.4 years to 45 years (Loftus EV et al., 2002; Blanchard JF et al., 2001), whereas the median age at diagnosis is 29.5 years (Loftus EV et al., 1998).

Both genders are affected in similar proportions, with a preference for the Caucasian

race (Sandler and Golden, 1986). This disease is common in the Western countries; it represents in the USA the second most common chronic inflammatory disorder after rheumatoid arthritis (Sandler and Golden, 1986; Amit-Romach et al., 2006).
The incidence of IBD varies within different geographic areas, with the highest rates in developed countries (northern Europe, United Kingdom and North America) and the lowest in developing regions. Colder climate regions and urban areas have a greater rate of IBD than those of warmer climates and rural areas (Sedlack et al., 1980; Binder V et al., 1982; Loftus CG et al., 2007). In North America, the incidence of IBD is approximately 2.2-14.3 cases per 100,000 person-years for UC and 3.1-14.6 cases per 100,000 person-years for CD. The combined incidence for IBD is 10 cases per 100,000 annually (Loftus EV et al 2000, 2004).
In general, there is a slight female predominance in CD, which suggests that hormonal factors may play a role in disease expression (especially among women in late adolescence and early adulthood). On the other hand, if there is a slight gender predominance in UC, it rests with males (Loftus et al 2000). In brief, these diseases affect males and females equally and most of the diagnoses are made before the age of 30 (Russel at al, 1996).

2.4 Signs and symptoms

The systemic signs and symptoms described in the literature are loss of appetite, weight loss, bloody diarrhea, rectal bleeding, nausea, fever, abdominal painful cramps and pain, severe urgency to have a bowel movement and iron deficiency anemia due to blood loss (Podolsky, 1991; Fiocchi, 1998; Head et al., 2003).
The inflammation can spread to other organs. Extraintestinal manifestations of IBD

include iritis, episcleritis, arthritis, skin involvement as well as pericholangitis and sclerosing cholangitis (Rowe, 2011).

The disease is characterized by phases of relapses and remissions.

2.5 Complications and risks

Researchers found that children and adults with IBD were more than twice as likely to develop a deep vein thrombosis, or pulmonary embolism. The risk generally increased with age (Warner J., 2011).

Complications of CD may be related or unrelated to the inflammation within the intestine. Intestinal complications of CD include obstruction and perforation of the small intestine, abscesses, fistulae, and intestinal bleeding, megacolon, and rupture of the intestine.

Patients with UC are at increased risk of colorectal cancer (Dashwood, 1999; Lashner, 2002; Vagefi et al., 2005; Delaunoit et al., 2006). This risk increases by 0.5-1.0% per year beyond 8-10 years after diagnosis (Rowe, 2011). The severity of inflammation may also be an important marker of risk for colorectal cancer and that the expression of CDX2, a cancer suppressor gene, could be considered an important surveillance marker for monitoring the development of the colorectal cancer (Moskaluk et al., 2003). Intestinal cancer may become a more important long-term complication in patients with CD because of longer survival (Rowe, 2011).

2.6 Treatment

Treatment of IBD can be either medical, surgical or a combination of both. It generally follows a step-wise approach to medication therapy, with progression of the medical

regimen until a response is achieved, using more aggressive drugs until the symptoms are relieved. The first step in medication therapy for IBD is usually aminosalicylates. They are useful for treating flares of IBD and for maintaining remission. (Jurjus A. et al., 2004). If the patient's condition fails to respond to aminosalicylates, the second step is corticosteroids. They usually provide rapid relief of symptoms and significant decrease in inflammation (Carter et al., 2004). To treat flares of IBD, high doses of corticosteroids are used. Once a clinical response is obtained, the dose is tapered. To prevent relapse, most patients can only occasionally tolerate a relatively rapid taper and a very prolonged steroid taper is necessary. If relapse occurs or if corticosteroids, are required for prolonged periods, the use of alternative drugs (immune modifiers, anti-tumor necrosis factor therapy or anti-integrin therapy) could be considered (Rowe, 2011). They work by causing a reduction in the lymphocyte count, and because of their steroid-sparing action in persons with refractory disease (Rowe, 2011).

3 Inflammatory mediators in IBD

Many inflammatory agents have been identified in IBD among which neutrophils, macrophages, mast cells, fibroblasts, cytokines, nitric oxyde, peroxinitrite and $CD4^+$ cells. Considerable additional evidence suggested that these mediators played an important role in the pathologic and clinical characteristics of these disorders (Baumgart D. et al., 2007)

3.1 Neutrophils

Neutrophils (60% of all circulating white blood cells) constitute the largest cellular component of the human immune system. They are part of the granulocytes, and their infiltration into colonic mucosa is the first sign of inflammation (Geboes, 1994). Neutrophils are produced in the bone marrow from granulocyte-monocyte progenitor cells and they are called polymorphonuclear cells (PMNs). They are the first cells to reach the site of inflammation by leaving the blood vessel through extravasation through the endothelium into the tissue. This recruitment is mediated by specific adhesion molecules, which control the whole process of migration (Lazaris et al., 1999; Vainer and Nielsen, 2000; Vainer et al., 2000; Umehara et al., 2006). Neutrophils are the most effective cells in killing ingested microorganisms through different pathways (Srivastava et al., 1989; Kanamori et al., 1997; D'Odorico et al., 1999; Leik and Walsh, 2006). Once the microorganisms are killed, the involved neutrophils self-destruct (apoptosis).

The possible anti-inflammatory role of the estrogen has been reported by Garcıa-Duran et al. (1999) and Chiang et al. (2004); they proved that estrogen stimulated neuronal nitric oxide synthase (NOS) protein, generated by neutrophils as part of the human immune response, and activated by tumor necrosis factor.

3.2 Macrophages

Produced by division of monocytes, macrophages are large white blood inflammatory cells with a great amount of intracellular lysosomes. Their role is to phagocytose cellular debris and pathogens, and to stimulate lymphocytes and other immune cells to respond to the pathogens.

Monocytes are normally attracted to a damaged site by chemical substances through chemotaxis, triggered by numerous stimuli: damaged cells, pathogens and cytokines released by macrophages already at the site... Macrophages, usually inactive, are activated by cytokines like INF-γ or lipopolysaccharide during inflammatory response (Mosser, 2003; Tanner et al., 1984). After activation, macrophages target and kill microorganisms with the removal of toxic inflammatory products (Nathan, 1991). Additionally, macrophages secrete a wide range of enzymes and other factors that intensify the inflammatory response (Allison *et al*., 1978; Reinecker *et al*., 1993; Romagnani, 1999). The imbalance among these activities contributes to the pathogenesis of inflammatory diseases and may damage tissue in a non-specific manner (Allison *et al*., 1978). Macrophages survive in the body up to a maximum of several months. Literature showed that estrogen effects on the monocyte-macrophage system were primarily repressive. Most of these effects were mediated by repression of expression of genes for cytokines or modulation of other inflammatory mediators by the estrogen receptor (ER)-dependent or nongenomic pathways (Harkonen et al., 2006).

3.3 Mast cells

A mast cell is a resident cell of several types of tissues and contains many granules rich in histamine and heparin. Although best known for their role in allergy and anaphylaxis, mast cells play an important protective role as well, being intimately involved in wound healing and defense against pathogens. When activated, a mast cell releases its characteristic granules and various hormonal mediators into the interstitium. Mast cell (MC) hyperplasia is a feature of the chronic inflammatory bowel diseases. Mast cells are often seen to be degranulated in areas of active disease, suggesting that the

inflammatory mediators released from these cells contribute to the pathophysiology of these disorders (Dvorak et al 1978, 1980). Nicovanie et al., (2001) demonstrated that estrogen receptors are detected primarily in arterial MCs of fertile women. MCs respond to 17-β estradiol mainly by a moderate increment in NOS mRNA and nitric oxide and a reduction in TNFα cytotoxicity. It was shown that estrogen exerted its activity in part through modulation of mast cell activity. It was demonstrated that cell-specific protease genes were decreased in the estrogen-treated rat colons compared with vehicle in their study of estrogen action in HLA-B27 transgenic rats (Harnish et al., 2003)

3.4 Fibroblasts

Fibroblasts are identified by their morphology, ability to adhere to plastic, production of extracellular matrix (ECM), and lack of epithelial, vascular, and leukocyte lineage markers. Fibroblasts are primarily responsible for the synthesis and remodeling of ECM in tissues. They are important participants in inflammation. In addition to playing an important role in structuring the microenvironment, fibroblasts through their production of cytokines and chemokines play a key role in the development of immune responses in lymphoid tissues (NIH American government). In vitro studies have shown that estrogen influenced processes critical to wound repair, such as cellular proliferation and cytokine production (Piccini et al., 1995; Ashcroft et al., 1997; Emmerson et al. 2009) Furthermore, there was evidence that estrogen replacement substantially accelerated healing in both aged humans and estrogen-depleted animal models (Ashcroft et al, 1997). These studies suggested that estrogen provided an indirect effect on fibroblasts by stimulating responses in other cell types invading the wound site (Kanda et al., 2005).

3.5 Cytokines

Reactive oxygen species induce various kinds of inflammatory mediators, among which TNF-α, considered to be the most potent (Kohler et al., 1999; Pupe et al., 2003). TNF-α is produced and secreted from a range of inflammatory cells including macrophages, lymphocytes and polymorphonuclear neutrophils (Mazlam and Hodgson, 1992; Neurath et al., 1997; Reinecker et al., 1993). It triggers the cytokine and chemokine inflammatory cascades. Oxidative stress and TNF-α induce the production of NF-κB, which exists in its inactive form in the cytoplasm of unstimulated under normal conditions. Once activated, NF-κB translocates to the nucleus and activates the proinflammatory genes including TNF-α, IL-6, IL-2 and IL-8 (Baeuerle and Henkel, 1994; Baldwin, 2001; Rogler et al., 1998). Both, TNF-α and NF-κB are responsible for the activation of macrophages, either directly or indirectly, and thus play predominant roles in inflammation (Neurath et al., 1997; Neurath and Pettersson, 1997). The main difference between ulcerative colitis and Crohn's disease in the immune response pattern is the type of lymphocytes involved in inflammation activation and their subsequent inflammatory cascades. In Crohn's disease, CD4+ lymphocytes, type 1 helpers (Th1), are involved, and INF-γ, IL-2 and TNF-α are the characteristic cytokines (Butcher and Picker, 1996; Romagnani, 1999; Cottrez *et al*., 2000; Papadakis and Targan, 2000). By contrast, the mucosal response in ulcerative colitis patients is dominated by type 2 helpers (Th2) with the production of TGF-β and IL-5 (Fiocchi, 1998). Literature showed that estrogen inhibited important proinflammatory factors such as TNF, IL-1β, IL-6, MCP-1, iNOS expression, production of MMPs, and activity of natural killer cells. However, at lower concentrations, estrogen stimulates TNF, IFN-γ, IL-1β, and activity of natural killer cells (Straub, 2007).

4 Aim of the study

As estrogen has been shown to have an anti-inflamamtory effect we investigated in this study the possible effect of estrogen in the management of intestinal inflammation in induced colitis models with Trinitrobenzenesulfonic acid solution (TNBS), and Dextran sulfate sodium salt (DSS). We chose those two models as they have different ways of induction: one intrarectally, the other intraorally. We aimed to compare the role of estrogens on inflammation in the two models. We focused specifically on the morphological changes, the activity of mast cells, and the modulation of TNF-α and IL-6 as well as collagen IV and fibronectin in addition to the expression of reactive oxygen species.

II- Material and methods

1 Experimental animals

A total of 120 adult male Sprague-Dawley rats (Dieleman LA et al. 1940), weighing 250-300g, were used in this experimental procedure 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. Animals were housed in rack mounted cages, 5 rats per cage, and kept on a 12 hours light/dark cycle in a controlled temperature and humidity room. Standard laboratory pelleted formula and tap water were provided ad libitum.

2 Methods:

The rats were divided into 4 groups: 2 major groups of 40 rats, Group I and II, and 2 minor groups: Group III and IV of 20 rats each. The rats in Group I were induced with colitis while the rats in Group II were induced with colitis and injected with estrogen as a potential treatment. The rats in Group III were injected with estrogen (17ß-estradiol, Sigma-Aldrich, E8875) only while the rats in Group IV served as control, they were neither induced with colitis nor received estrogen.

Groups I and II were subdivided into 2 subgroups of 20 rats. Colitis was induced in these groups according to previously established procedures using rectal injection of Trinitrobenzene Sulfonic acid (TNBS) (Sigma-Aldrich, 92822-1ML) in 40 rats (Morris

et al., 1989) and using drinking water mixed with the Dextran Sulfate Sodium Salt (DSS) (Sigma-Aldrich, D4911-1G) in the other 40 rats (Writz et al., 2007)

Rats were weighed and their health conditions were checked on a daily basis. Signs and symptoms of the colitis (weight loss, diarrhea, mucus with the stools, blood in stools, fur aspect) were monitored. Scores were given by two different observers for the severity of the signs and symptoms. Averages of the two scores of each category were calculated (Ho-Lam et al., 2007; Yasu-Taka et al., 2010).

Table 1: Criteria for scores given on the severity of the signs and symptoms.

Signs and symptoms	Scores		
Weight loss	0= none	1= 1 to 5%	2= 5 to 10%
Diarrhea	0= normal	2= loose stool	4= Diarrhea
Blood in stool	0= normal	2= slight bleeding	4= gross bleeding
Total clinical score	0= healthy	10= maximum clinical score	

2.1 Preparation of the solutions

<u>Estrogen solution:</u>

50 µg of 17β-estradiol was added to every 0.5ml of twice-filtered corn oil and stored away from light. (Imakoa et al., 2009).

<u>TNBS solution (5%):</u>

The solution contained a mixture of 1 volume of 5% (w/v) in H_2O TNBS solution with 1 volume of absolute ethanol. It was stored in the refrigerator.

The starting solution of TNBS was prepared by diluting the TNBS solution 6 times into distilled water to obtain a 30% solution of TNBS (Fan et al., 2009; Wirtz et al., 2007).

DSS solution (2%):

The DSS solution was prepared by dissolving 10 g DSS powder in 500ml of autoclaved drinking water. The solution was stored until use at 4°C (Writz et al., 2007).

2.2 Injection of estrogen

Estrogen: the rats in the groups II and III were injected with 500µl of the estrogen solution subcutaneously, consistent with the dosage level reported by Shinohora et al (2005), in the interscapular region once per week for two weeks preceding the beginning of the experiment and for the following 8 weeks, till the end of the experiment (Imakoa et al., 2009) (figure 1).

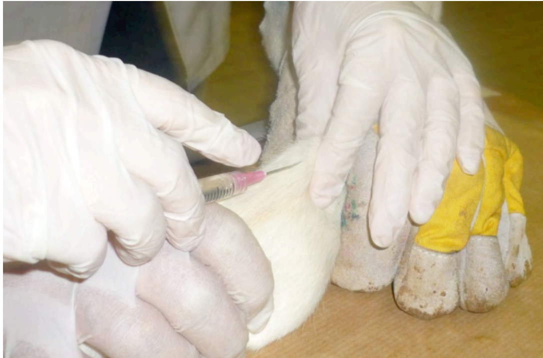


Figure 1: Subcutaneous interscapular injection of estrogen done on a weekly basis in the rats of Group II and III

2.3 Induction of colitis

TNBS: 100µl of TNBS solution was inoculated intrarectally 5 cm proximal to the anal verge using 2 mm diameter polyethylene tubing on days 1, 8, 15, 22, 36 (Writz et al., 2007; Fan et al., 2009).

DSS: Rats were provided with two cycles of a mixture of drinking water and DSS (2%): 5ml/rat/day (7 days DSS, 14 days autoclaved water). The mixture was replaced 1 time every 2 days (Writz et al., 2007).

2.4 Sacrifice:

Following the first injection, 5 rats of each subgroup of groups I and II, and 5 rats of group III and IV were sacrificed on days 7, 14, 28 and 56.

The animals were anesthetized by intraperitoneal injection of Ketamine (75-100mg/kg) and xylazine (10mg/kg). From xyphoid to pubic symphysis, a midline incision was made, followed by a longitudinal cut till the sternum, reflecting laterally the skin and the abdominal muscles.

The abdominal cavity was exposed and the uncovered viscera were examined for any sign of swelling or redness. Next, the jejunal loops were reflected then dissected using tweezers in order to expose the descending segment of the colon (DC). Macroscopic assessment of the inflammatory index was performed according to a previously described scale (Table 2).

The descending colon was excised. Biopsies from the colon were taken; a 1cm piece was immersed in 4% parafolmaldehyde labeled container for light microscope, and a 6 cm piece for mucosal scraping. The lumen of the large piece was cleaned with syringe filled with cold phosphate buffer saline (PBS), at pH 7.4. After cutting transversally the DC on ice, the mucosa and submucosa layers were scrapped by using two sterilized slides (figure 2). The extracted materials were transferred into labeled aliquots, snap-frozen in liquid nitrogen, and kept at -80°C for more molecular analysis. Biopsies from the jejunum were also extracted and treated in the same fashion as the colon, while

kidneys and liver biopsies were fixed in 4% parafolmaldehyde or snap-frozen and stored at -80°C until further processing.

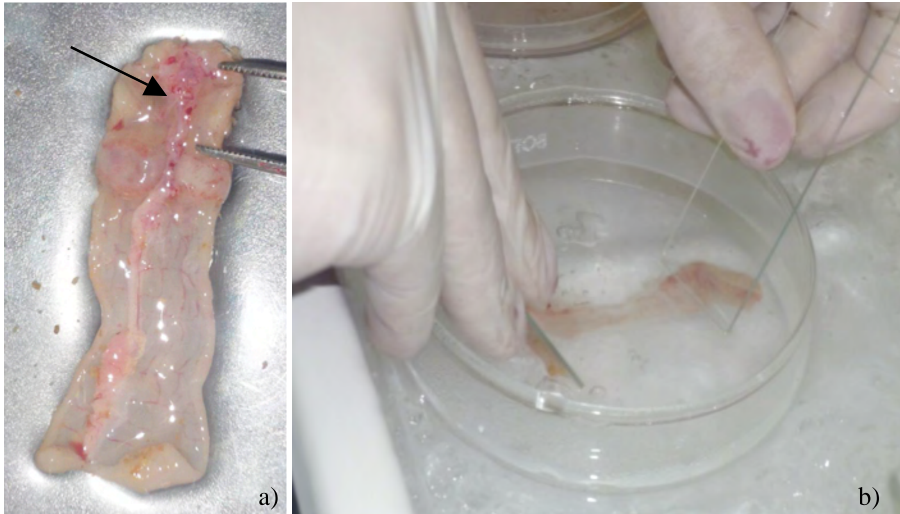


Figure 2: figure a: Spread of inflamed colon from the group I TNBS subgroup where a thickening of the wall and vasodilation can be noticed (arrow), figure b: Scrapping of the mucosa of the descending colon of the DSS subgroup of group I. The wall of this organ was less thickened than the one of TNBS (figure a).

3 Macroscopic assessment

3.1 Daily observations:

On a daily basis, the animals were checked for body weight change, a strong indicator of health or sickness, diarrhea, soft stools, bloody stools, weakness and other criteria Each observation day, such criteria were given a score ranging from 0 to 4, a null grade describing a normal healthy state and a score of 4 portraying a sever sickness feature (Table 1). Two different observers were scoring. The averages of the two provided scores for each category were calculated with the corresponding standard deviation. A total clinical score was used to be able to assess the degree of sickness of animals in

each group (Miller FJ Jr at al., 1998; Loher et al. 2004; Azuma et al., 2010; Carter M., 2011).

3.2 Scoring the gross morphologic inflammatory changes:

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 (Table 2). Averages of their findings on each described parameter were calculated, providing a descriptive progression of the inflammation through the experimental period. Final composite scores (over 18) were calculated to compare differences between the animals of various groups (Bartevello et al., 2005; Sachar et al., 2011) (table 2) (figure3).

Table 2: Macroscopic assessment of abdominal structures on sacrifice time point.

Signs	**Scores**			
Size of affected area	0= not shown	1= less than 1 cm	2= 1 cm	3= more than 1 cm
Morphology of DC	0=normal	1= pink mucosa	2= red mucosa	3=blackish mucosa
Mushroom-like structures/ 10 cm	0=absence	1= 1 or 2	2= 3 or 4	3= 5 or more
Adhesions	0=no adhesion	1= 1 organ	2= 2 organs	3= more than 2 organs
Wall of the DC	0=normal	1= thin	2= thin/thick	3= thick
Vasodilation	0=normal	1= at the inoculation site	2= through the DC	3= on the colon or jejunum

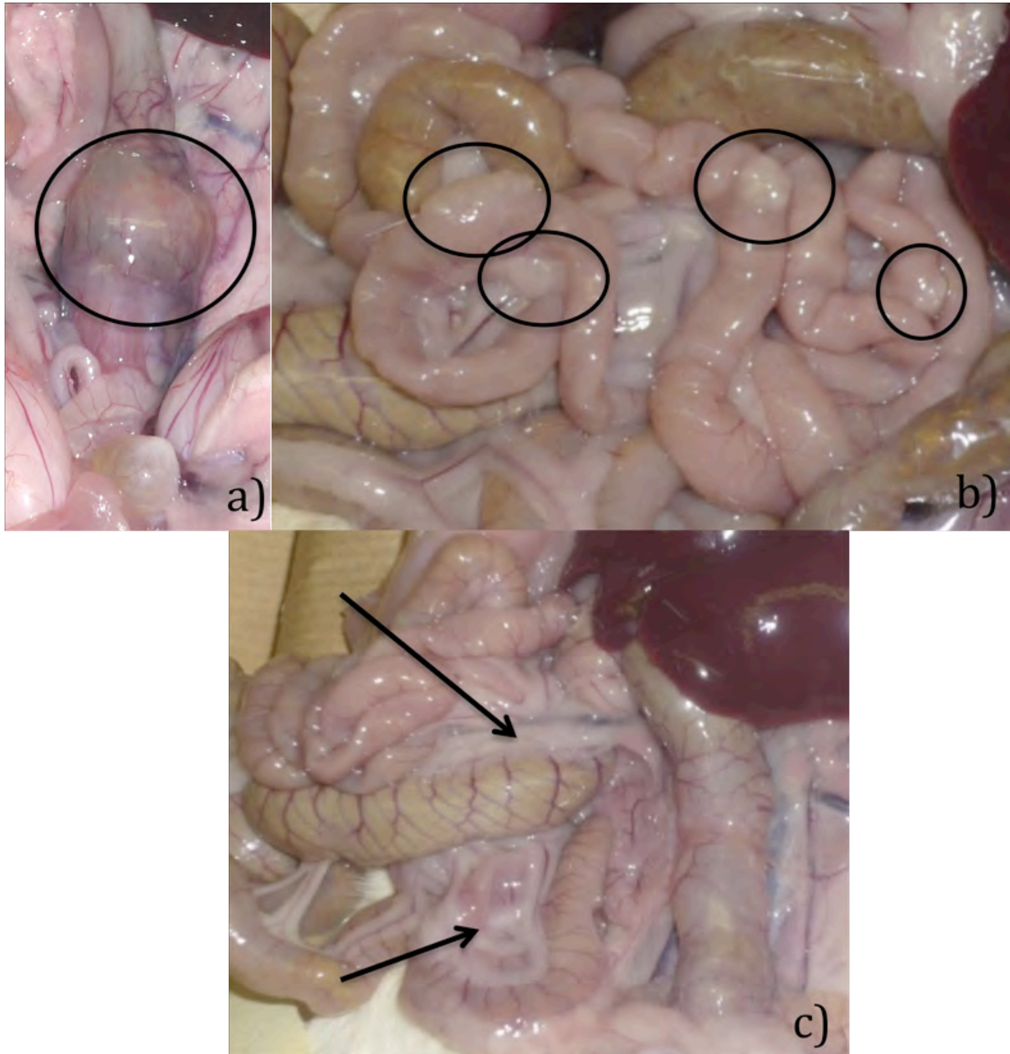


Figure 3: Observations of abdominal structures during the sacrifice days seen in the TNBS subgroups: a) vasodilation, b) mushroom-like structures in the serosa of the jejunum, c) adhesions between different organs.

4 Histological evaluation

Already established histological protocols in the lab were performed on the biopsies fixed with 4% parafolmaldehyde for 24 hours. Blocks were sectioned on a microtome, 5 µm-thick sections and mounted on slides pre-treated with tissue adhesive solution sta-On©. Ten slides of each biopsy were prepared, one slide was stained with water-soluble

hematoxylin and eosin (H&E), six other (consecutively cut) were stained with toluidine blue (TB) for mast cell counting and the rest were kept for immunohistochemistry.

The H&E stained slides of jejunum and DC biopsies were examined under the light microscope at different magnifications. As criteria to assess the degree of tissue damage in the descending colon, the following points were studied: interruption of intestinal epithelium, the dilation of glandular crypts, the depletion and loss of goblet cells, inflammatory cells infiltration, edema, the crypt abscesses and the dysplasia. A scheme modified from Gaudio et al. (1999) and Obermeier et al. (1999) was used to determine the extent of inflammatory reaction in colonic tissue. Slides at 400x magnification under the light microscope were photographed, evaluated and scored for each time point (days 7, 14, 28 and 56). The scores of two observers were averaged. Histological grades ranging from "0 to 18" represented the numerical sum of scoring criteria (Table 3).

Table 3: Scores given according to the histological criteria.

Histological parameters	**Score**			
Architecture of the epithelium	0= morphologically normal	1= focal destruction	2= zonal destruction	3= diffuse destruction
Glandular crypt architecture	0= normal aspect	1=Mild atrophy	2= atrophy+ branching of the crypts	3= Atrophy+ branching of the crypts
Loss of goblet cells	0= no loss	1= slight loss	2= moderate loss	3= diffuse loss
Edema	0= absent	1= mild	2= moderate	3= extensive
Crypt abcesses	0= absent	1= focal	2= zonal	3= extensive
Inflammatory cells infiltration	0= absent	1= only in the mucosa	2= extends to muscularis mucosa	3= from mucosa to submucosa

The slides stained with TB were observed by the two different observers. They counted the amount of mast cells on each slides, summed them up and averaged them (La et al 2004). Loss of intracellular granules, with stained material dispersed diffusely within the lamina propria, was taken as an evidence of MC degranulation (degranulation meaning activation of MC).

The values were computed and represented with matching standard error of the mean using PRISM5 software.

5 Molecular evaluation:

To study the effect of estrogen on the protein and the gene expression, qRTPCR, western blot and dihydroethidine staining were performed on the collected scrapped mucosal tissues from the different colitis models.

5.1 RNA extraction and qRTPCR for the expression of I-L6, TNF-α and fibronectin:

RNA extraction was performed from the scrapped tissues collected on sacrificed days using TRIzol® reagent (Bio-Rad) according to the supplier’s protocol. After following the protocol and having diluted 1µl of RNA with 25µl of DEPC water, the final RNA concentrations were determined with the Nanodrop knowing that A_{260}/A_{280} should be between 1.8-2.0. Next, Genomic DNA elimination mixture was prepared using 2µl of GE (5x genomic DNA elimination buffer) for every µg of RNA. To that, RNase free water was added to reach a volume of 10µl. 10 µl of reverse transcriptase cocktail was

added to each sample (4µl of BC3, 1µl of P2, 2µl of RE, 3µl of RNAse free water). The plates were prepared using 23µl of the RTPCR mixture (12.5µl RT^2qPCR Master Mix, 1µl of forward primer, 1µl of reverse primer and 8.5µl of RNAse free water) with 2µl of cDNA, then sealed.

5.2 Protein extraction and Western Blot:

Proteins were extracted from the scrapped mucosal tissues of the descending colon biopsies using RIPA buffer (for each rat of each group at each time point). The protein assay was performed using Bio-Rad assay kit based on the Lawry's method. Briefly, the standard curve was done using different concentration of Bovine Serum Albumin (BSA). 22µl of double distilled water was added to 3 µl of the samples in tubes. The quantification was read on the spectrophotometer under the absorbance of 750nm.
The final value of protein content is calculated as µg protein per ml on an excel sheet.
Then Western Blot was performed (using Bio-Rad kit) loading equal amount of proteins for each sample.
For each gel, one sample of each group at the same time point was chosen. Gels were run first with housekeeping genes as secondary antibodies for proper interpretation of Western Blot. Then they were run with fibronectin and collagen IV, known to be over expressed with presence of inflammation.

5.3 Dihydroethidine staining:

Dihydroethidium (DHE) is a cell-permeable compound which by its ability to freely penetrate cell membranes is used extensively to monitor superoxide production (Bindokas et al., 1996; Rivera et al., 2005). Reactive oxidative species (ROS) are

chemically reactive molecules containing superoxide anion $O2^{-}$. They are biologically quite toxic and deployed by the immune system to kill invading microorganisms (Miller et al 1998). They increase during time of stress. DHE is oxidized by reaction with $O2^{-}$ to ethidium bromide, or oxyethidium (Zhao et al., 2003), which binds to DNA in the nucleus and fluoresces in red detectable qualitatively by a fluorescent microscopy. Frozen 10 μm sections were cut from the collected frozen biopsies were cut in the cryostat and placed on slides (2 slides/ biopsy). The slides were incubated in DHE (10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. They were checked by the two observers using confocal microscopy and the relative mean intensity of the red fluorescence was calculated.

6 Statistical analysis

Statistical significance comparing the treatment with estrogen versus no treatment was evaluated by Student's t test. P value of less than 0.05 indicates statistically significant difference. The significance while comparing all the groups was assessed with the ANOVA test.

III-RESULTS

1 Macroscopic results

1.1 Daily observations:

Group I animals showed all signs and symptoms of an induced ulcerative colitis (UC), more in the TNBS treated rats compared to the DSS. On the other hand, Group II, treated with estrogen, showed lower clinical scores, thus less inflammation compared to Group I. The subgroups in Group II showed a reduced total score by 0.5/10 compared to their corresponding subgroups in Group I.

The total clinical score of the TNBS induced colitis was the highest, the most severe inflammation (≈6.5/10), while that of the DSS was a much lower score (≈3/10), almost 50% less during the whole experiment. The score increased after each intra-rectal induction of the colitis with TNBS, and after each cycle of DSS. For TNBS, after day 28, the clinical scores gradually decreased till day 56 but never reached the control baseline levels. It is important to note that on day 36, where the last induction of colitis took place, another peak of inflammation was observed, however, it was less in severity than the previous peaks by about 30 % (figures 4 and 5).

Figure 4: Observation of diarrhea in the TNBS subgroup of group I. A: On the day of inoculation no stain was noticeable. B: two days after inoculation, the stools were watery. C: 6 days after inoculation, the stains were less noticeable than in figure B but mucus was seen as well as blood.

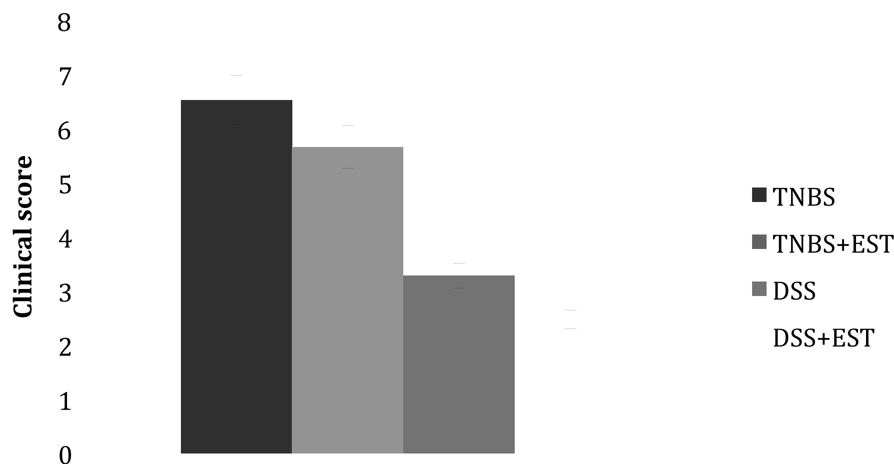


Figure 5: Total clinical colitis scores of group I and II. The score was a composite of the different parameters: weight loss, diarrhea stains, presence of mucus or blood within the stool, althrough the experiment. Note that in estrogen treated rats the scores decreased. No statistical significance was noticeable between the TNBS and the DSS subgroups with their concordant estrogen treated subgroups. However, there was a statistically significant difference when comparing the TNBS model to the DSS model. The groups III and IV constitute the baseline

1.2 Observations at the sacrifice days and scoring the gross morphologic alterations:

The TNBS model revealed the highest score on each sacrifice day (day 7=13.5/18, day 14=15.5/18, day 28=10/18, day 56=6.5/18). It showed a peak score on day 14 and then it tapered down till the end of the experiment, day 56, but always above controls. On the other hand, DSS showed its highest score on day 7 (7/18), on day 28 (6/18), then it tapered down till day 56. Comparing the models to their treatment subgroups, a statistically significant difference was present at each end point for the TNBS model (decreasing by approximately 20%) noting that the least difference was present on day

56. For the DSS model, the statistically significant difference was present on days 7 and 56 (figure 6).

The estrogen and the control groups showed close and similar inflammatory scores all along the experiment.

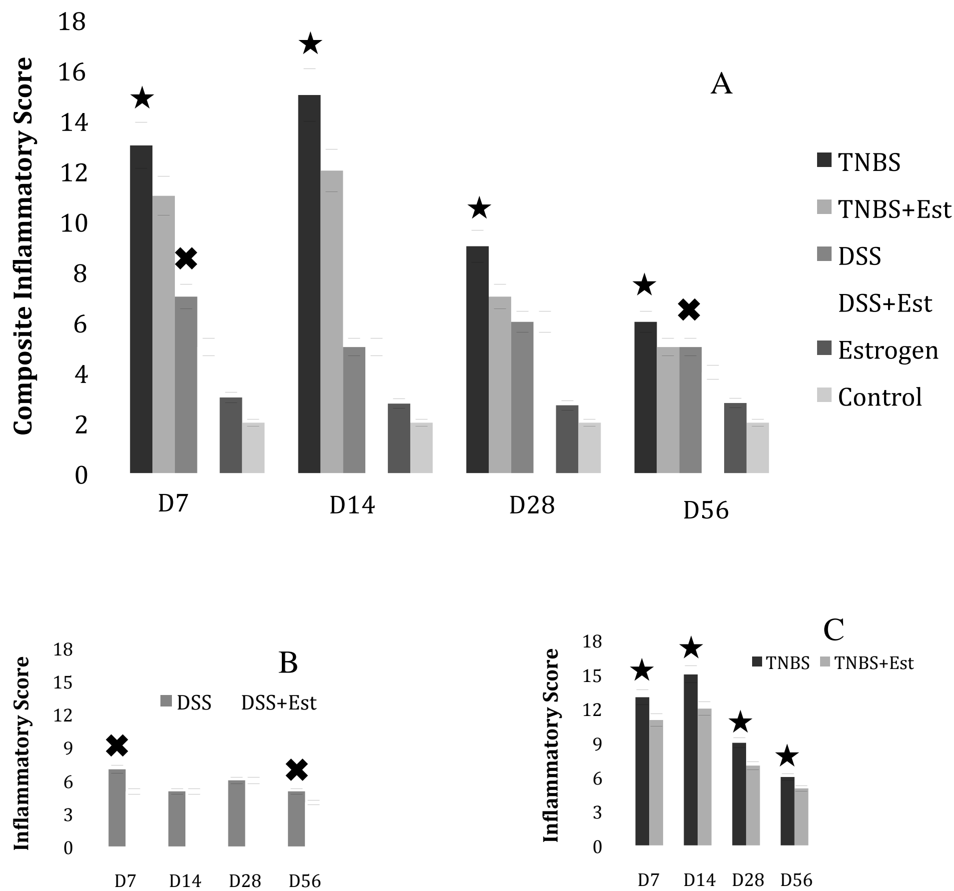


Figure 6: Composite inflammatory scores at the different time points (Average ± SEM). Values represented the average score between parameters such as: Damage site, morphology of DC, mushroom-like structures per 10 cm, adhesions, vasodilatations and thickness of DC. The score of the TNBS subgroup was statistically significant with its concordant treatment subgroup at days 7, 14, and 28. A shows all the groups during the different times points. B and C focus on the DSS and TNBS treated groups, respectively. The data collected for the DSS subgroup was statistically significantly higher than the DSS+Estrogen subgroup at day 7. ★ $p<0.05$, TNBS vs TNBS+Est. ✖ $p<0.05$, DSS vs DSS+Est.

2 Histological alterations

Studying the biopsies by light microscopy, they revealed that the signs of inflammation were more frequent in Group I where the rats did not received any treatment. The TNBS treated groups showed a higher histological score in the colon while the DSS group showed a higher histological score in the jejunum (probably because of the way DSS was provided to the rats). As reported before, in the TNBS induced colitis, there was alteration in the overall architecture of the overall architecture of the colon with severe infiltration of inflammatory cells through the mucosa and submucosa, exaggerated blood diffusions, goblet cells depletion and zonal disruption of the mucosal epithelium, explaining their average score of 9.5/18. On the other hand, the rats treated with DSS presented histological parameters that were not much increased (average score of 5.5/18); only dilated crypts and mild edemas were noticeable.

There was a significant decrease in the histological score of the groups treated with estrogen (score decreased of almost 2 points/18) (figures 7. 8). The morphological alterations were less and sometimes more localized. Inflammatory cellular infiltrate was less extended and limited more to the mucosa layer with distinct edema limited to the perivascular region. In addition, the goblets cells were similar to the non-treated animals with less inflammation in the crypts (figure 8).

Rats treated with estrogen alone did not depict any histological changes except for a mild vasodilation, they were similar to the normal controls (2.8 for group III and 2.2 for the control).

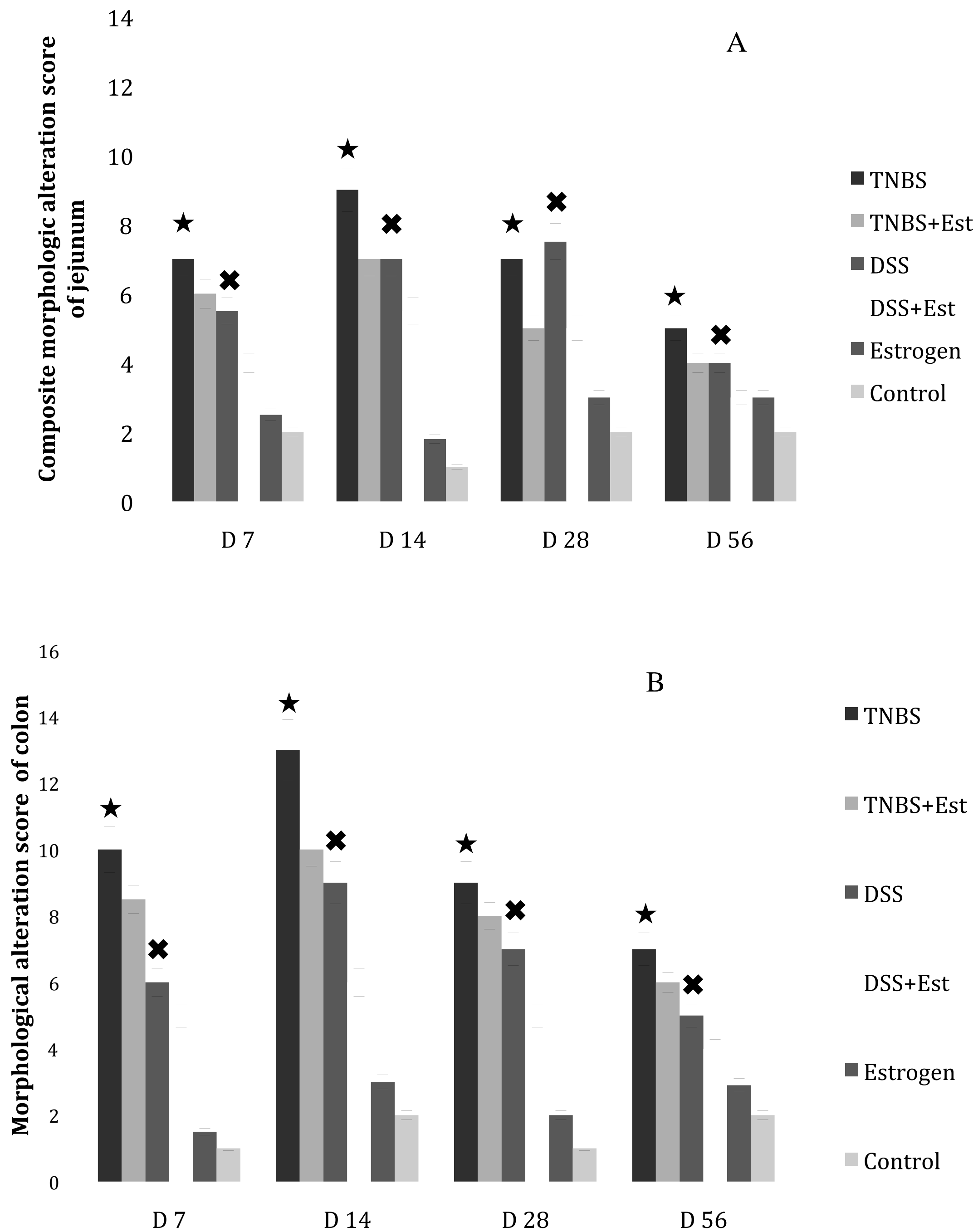


Figure 7: Composite histological scores per subgroup and per time point (Average ± SEM). A: in the jejunum. B: in the colon. Score representing the sum of each parameter score (mucosal architecture, crypt morphology, loss of goblets cells, edema, crypt abscesses, cell filtration). ★ $p<0.05$, TNBS vs TNBS+Est. ✖ $p<0.05$, DSS vs DSS+Est

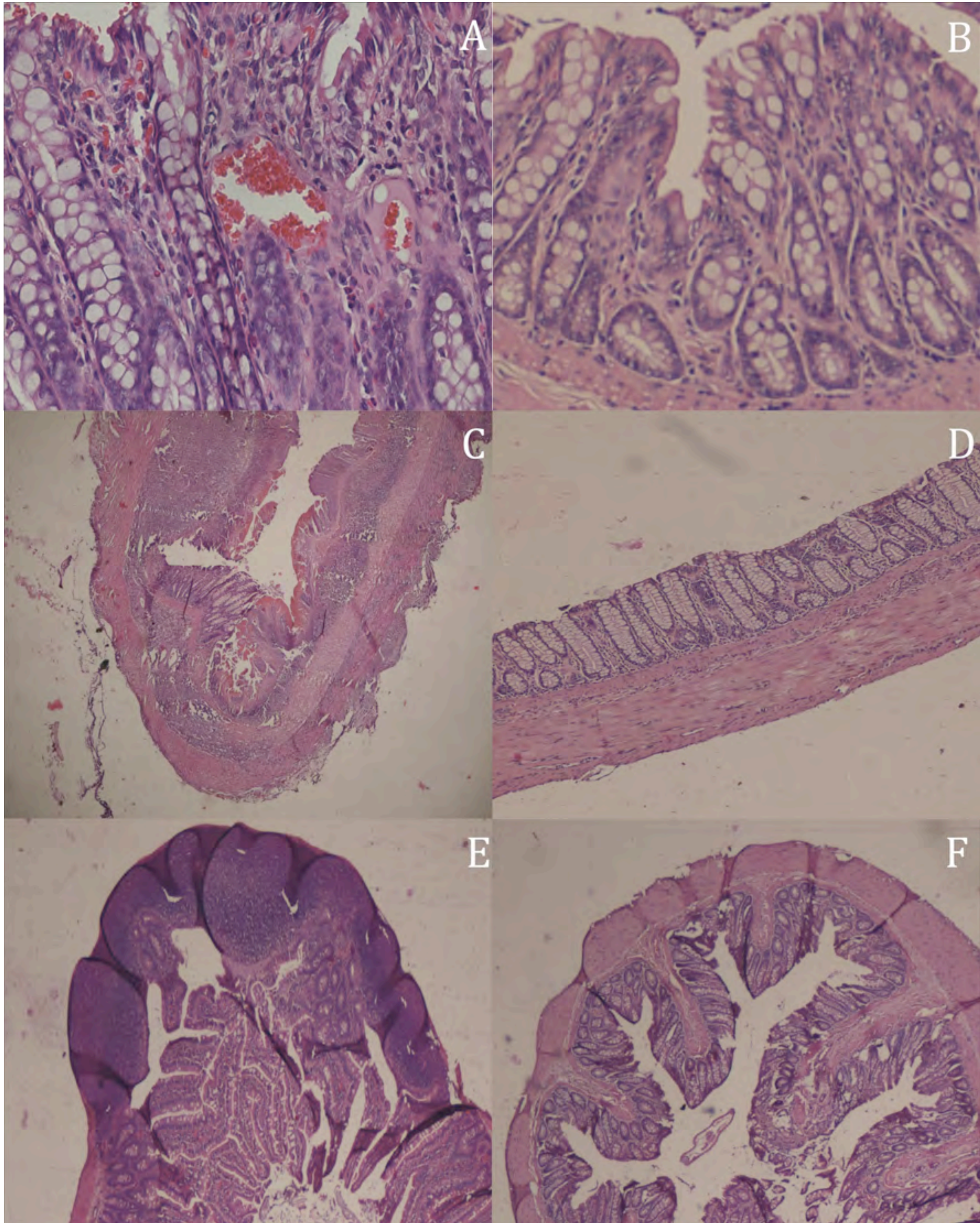


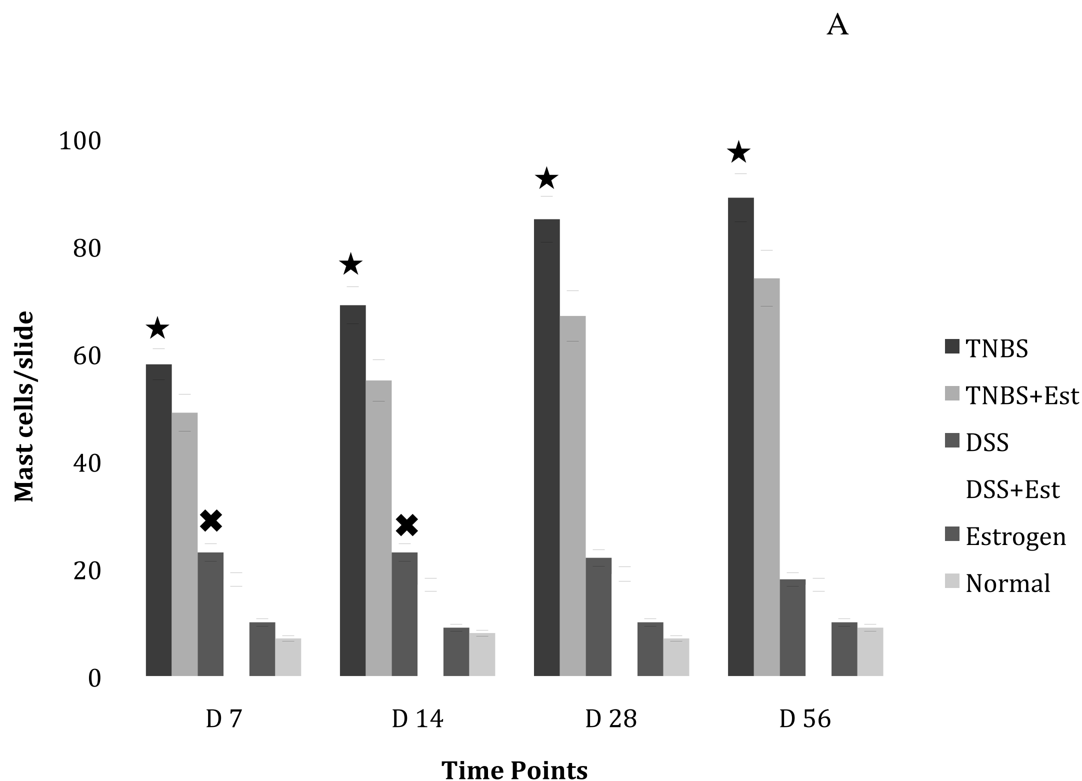
Figure 8: Upon treatment with estrogen, microscopic alteration in the colon decreases. B. Note decreased edema in the colonic submucosa of estrogen treated rats, compared to non-treated rats in A. In C. the sloughed epithelium in colitis animals is significant compared to D where estrogen was used (100X). In E. note the infiltration of inflammatory cells and extensive lymphoid follicles in non-treated colons compared to those of treated in F whereby the alteration were less.

With the induced colitis, there was an overall marked increase in the MC count in TNBS (60 mast cells/slide). Such an increase was time dependent; it was significantly increased with more duration of the inflammation. As the inflammation became chronic, the mast cells, usually predominant in the submucosa and around blood vessels, invaded the various intestinal layers. Rats treated with TNBS showed aggregates of mast cells diffused throughout the colonic layer (figure 10 A-B-C-D).

Mast cells were counted and described according to their granulation or degranulation status. About 80% of the mast cells found in TNBS were degranulated, meaning that they were activated and secreting their granules to the extracellular matrix.

In the DSS model, the MC counts were significantly heigher than the ones in the group III and IV (almost 4 times more than in the contol). They showed a significant decrease with the treatment on the day 7 and 14 only. The presence of estrogen reduced significantly the MC counts in all categories (in average from 60 to 49 mast cells/slide in the TNBS model and from 23.5 to 17 mast cells/slide in the DSS model). The possible anti-inflammatory ability of the estrogen decreased the attraction of the MCs in the affected area and consequently lowering the inflammatory response in the rats. The effect of estrogen led to a decrease in the number across the board and in all groups almost 20% less in the TNBS subgroup. Throughout the experiment, the difference in the mast cell count between the groups I and II decreased, as well as the amount of mast cells present on each time point (figures 9, 10).

A



B

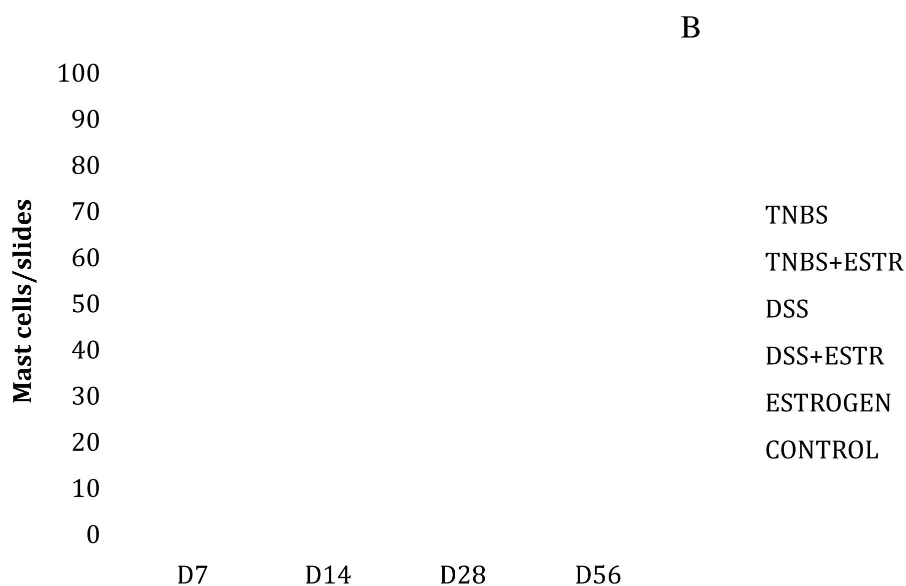


Figure 9: Mast cells count on TB stained colonic sections. A and B showed an increase in the mast cells count throughout the experiment in the TNBS subgroups. The DSS subgroups and group III showed scores that were close to the control. Scores represent average number of mast cells found in each subgroup at each time point (Average ± SEM point) t-test ★p<0.05, TNBS vs TNBS+Est.✖ p<0.05, DSS vs DSS+Est

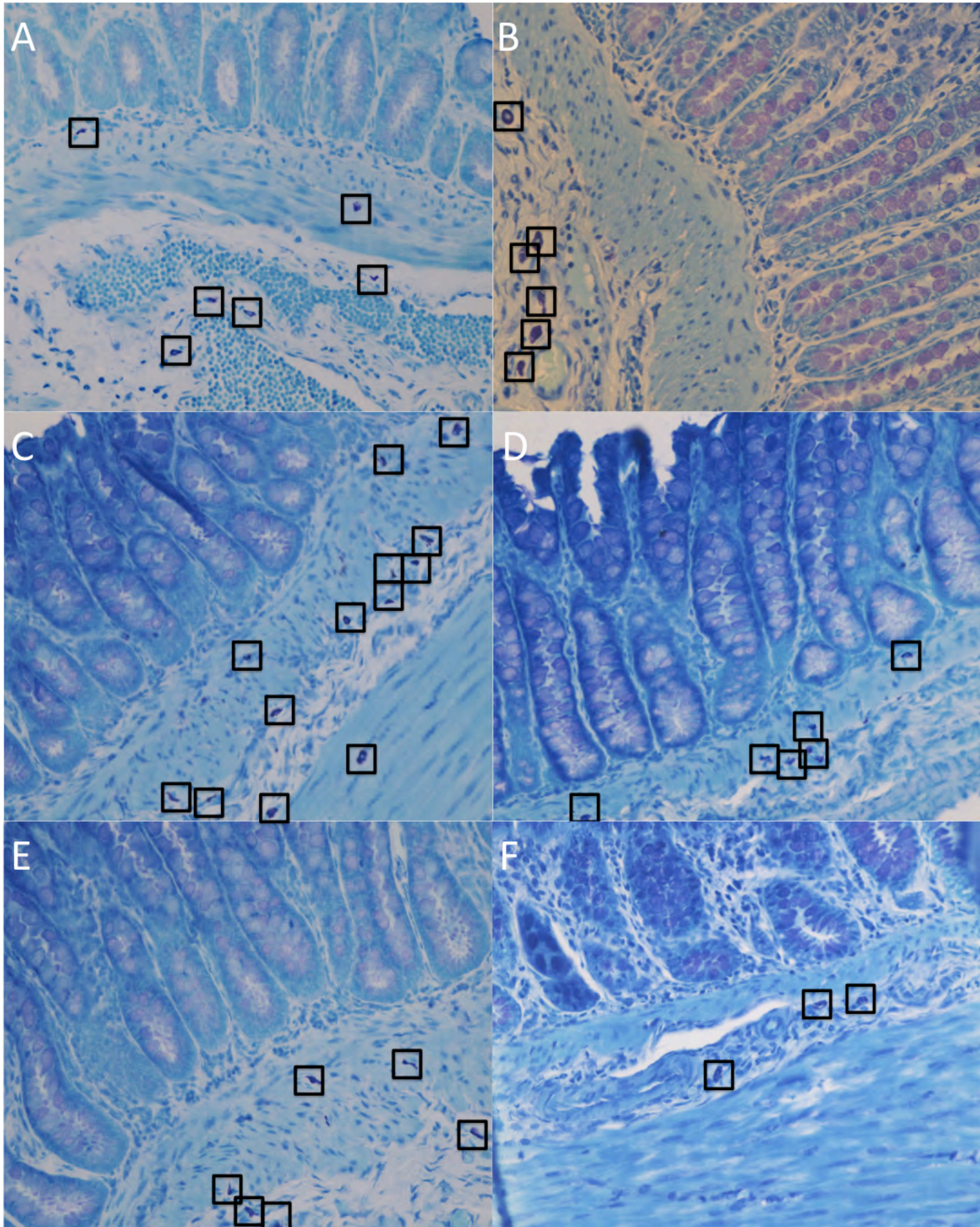


Figure 10: Mast cells infiltrating the colonic layer increased with the progression of inflammation and decreased with estrogen treatment. A: Microscopic observation of mast cells in colon of rats treated with the TNBS at 400 X. The number of mast cells increased along with the progression of colitis from day 7 (A), day 14 (B), day 28 (C) and day 56 (D). Colonic mast cells decreased after estrogen treatment (E) but it did not reach control level (F).

3 Molecular results:

3.1 Western Blot:

As expected, all the samples (n=5 for each group/time point) showed expression of the housekeeping genes. Fibronectin and collagen IV were expressed in all samples. However, they were over expressed in the TNBS subgroups especially at day 14 (fibronectin/GAPDH (ADU)= 3.8, collagen IV/GAPDH (ADU)= 2). They were less expressed in the DSS subgroup. When treated with estrogen, the expression of fibronectin and collagen IV decreased (figures 12 and 13). This decrease was the most significant on day 14 were it reached 50% less of the original expression in the TNBS model for both fibronectin and collagen IV.

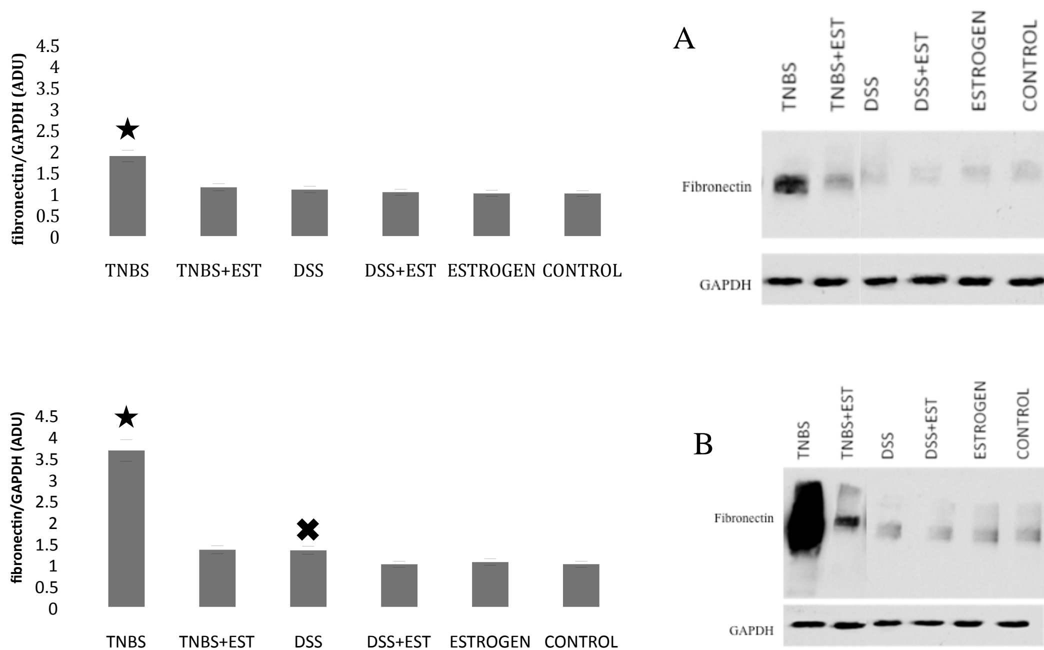


Figure 11: Treatment with estrogen decreases protein expression of fibronectin on day 7 (A) and 14 (B). The values represent the relative induction as measured by western blotting relative to GAPDH proteins levels. They highest values were found on the TNBS models on day 14. The values decreased when the rats were treated with estrogen. For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est. ✖ $p<0.05$, DSS vs DSS+Est

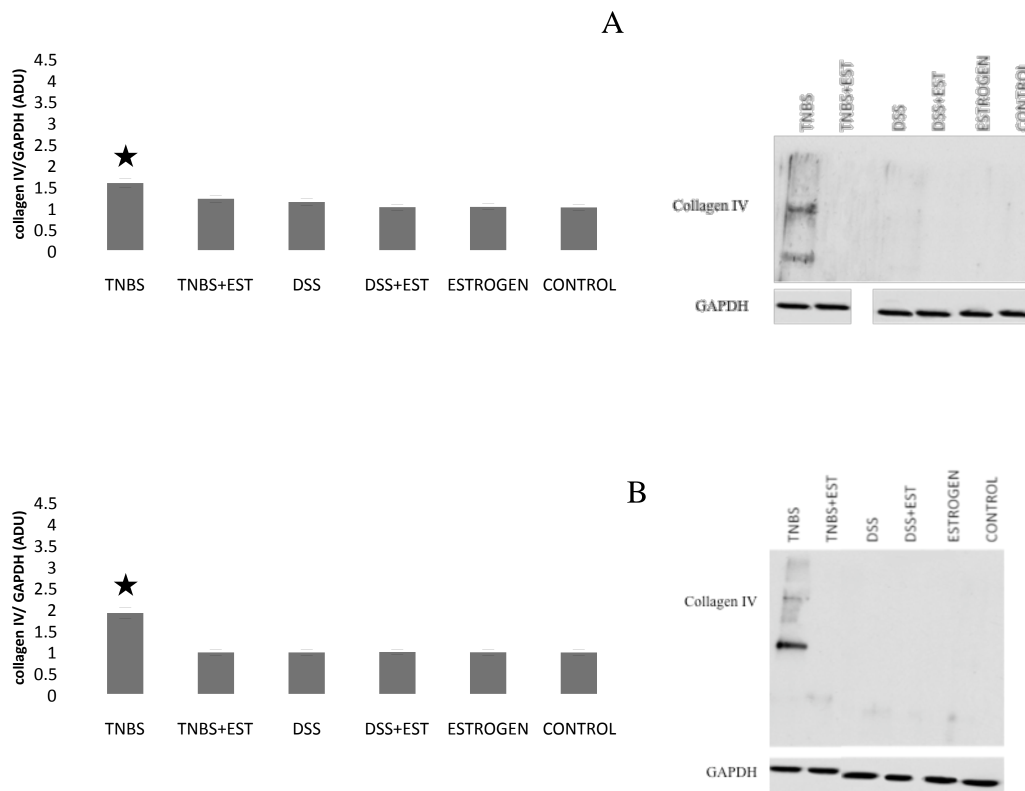


Figure 12: Treatment with estrogen decreases protein expression of Collagen IV on day 7 (A) and 14 (B). The values represent the relative induction as measured by western blotting relative to GAPDH proteins levels. They highest values were found on the TNBS models on day 14. The values decreased when the rats were treated with estrogen. No statistical difference was found in the DSS subgroups. For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est.

3.2 qRTPCR:

Fibronectin, IL-6 and TNF-α were expressed in every group of the study, however, they were more expressed in the TNBS model (IL-6/mRNA= 5.3, TNF-α/mRNA= 6.4, fibronectin/mRNA= 4.4) compared to the DSS model (IL-6/mRNA= 3, TNF-α/mRNA= 3.7, fibronectin/mRNA= 2.3). The values in the DSS model were less by the average of 1.5 than the ones of the TNBS model. Groups III (estrogen) and IV (normal) showed similar results. The group II, treated with estrogen, showed significantly less expression of fibronectin, IL-6 and TNF-α compared to the group not treated with estrogen. The

TNBS model showed statistically decreased value in the expression of fibronectin, IL-6 and TNF-α when treated with estrogen (by ± 20%) while the DSS model showed statistically significant difference in those values on days 7, 28 56 for IL-6, and on days 7,14, 28 for TNF-α (by ±10 %) and no significant difference was noted with the fibronectin expression (figures 14, 15, 16).

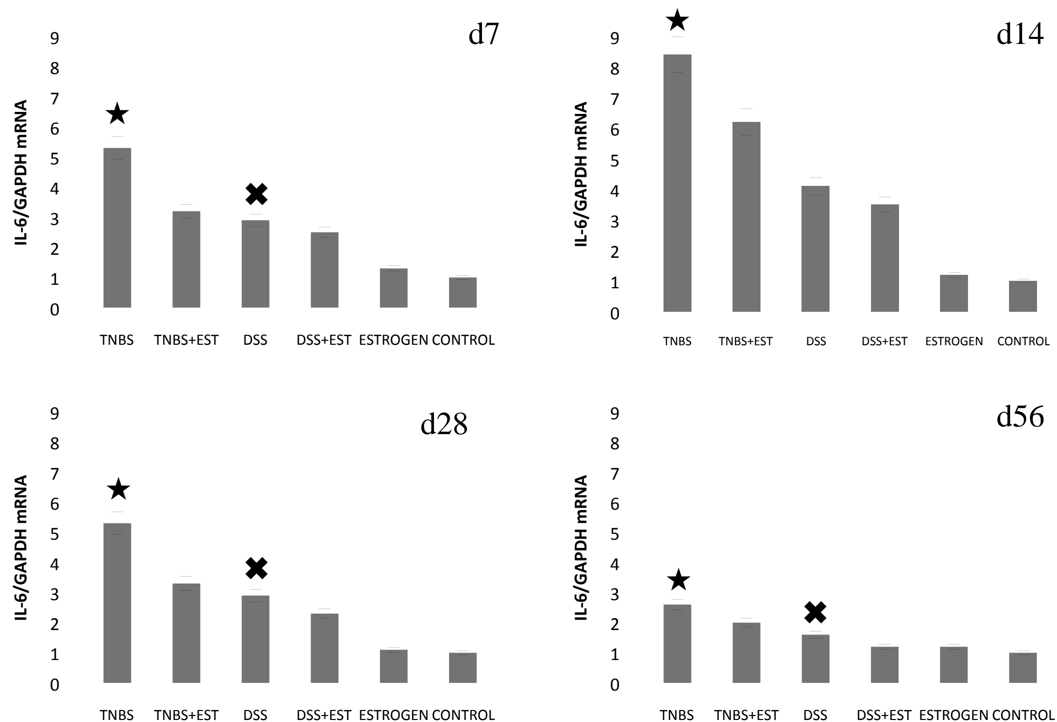


Figure 13: IL-6 gene expression decreases with estrogen treatment (n=5 for each group/time point) . The values represent amplification of IL-6 relative to GAPDH mRNA levels. The values reach a peak on day 14. The highest value was always found on the TNBS model on the 4 time points. They decreased when the rats were treated with estrogen For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est.✖ $p<0.05$, DSS vs DSS+Est.

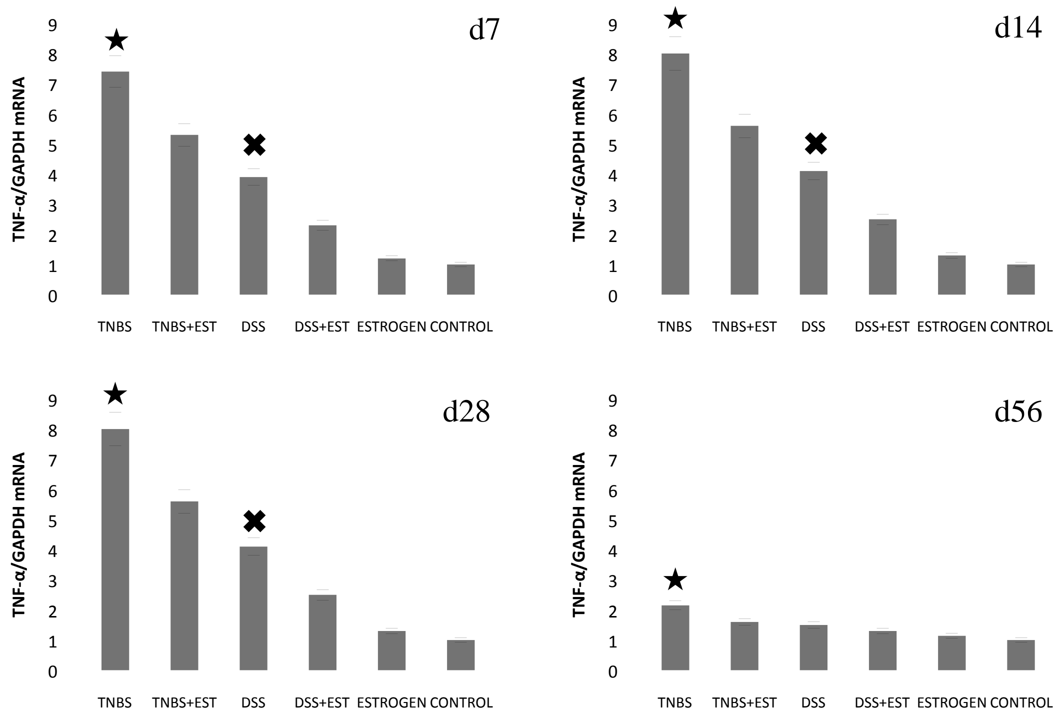


Figure 14: TNF-α gene expression decreases with estrogen treatment (n=5 for each group/time point). The values represent amplification of TNF-α relative to GAPDH mRNA levels. The values on days 7, 14, and 28 were mostly equal, then they decreased by more than half on day 56. The highest value was always found on the TNBS model on the 4 time points. They decreased when the rats were treated with estrogen. For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est. ✖ $p<0.05$, DSS vs DSS+Est

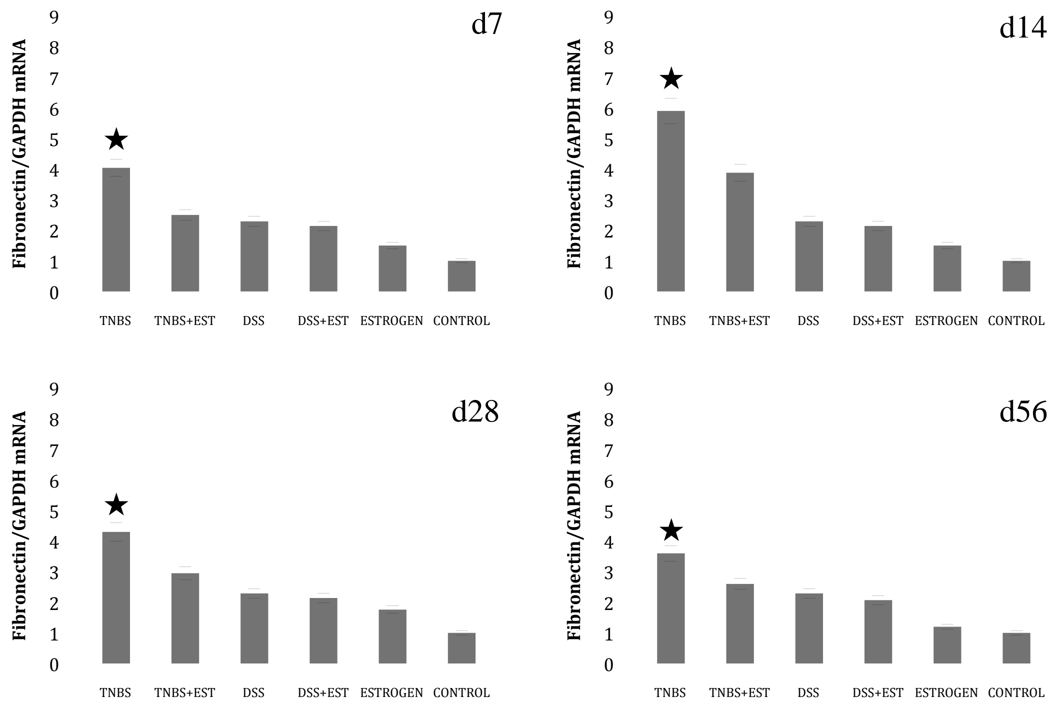


Figure 15: Fibronectin gene expression decreases with estrogen treatment (n=5 for each group/time point). The values represent amplification of fibronectin relative to GAPDH mRNA levels. The values reach a peak on day 14. The highest value was always the ones of the TNBS model on the 4 time points. They decreased when the rats were treated with estrogen. No statistical difference was found in the DSS subgroup. For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est.

3.3 Dihydroethidine staining

Using dihydroethidine staining, the fluorescence was detected mostly in the TNBS model on day 7 (Relative mean of intensity= 20 for jejunum, 14 for the kidney and 14.5 for the liver). Such expressions decreased significantly ($P<0.05$) consequent to the use of estrogen treatment (by more than 30%). The fluorescence intensity was correlated directly with the duration of the inflammation, the longer the duration, the more discrete was the staining intensity (figures 17 and 18). 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). The fact that ROS was expressed in the liver and kidney proved that the intestinal inflammation expanded to the neighboring organs.

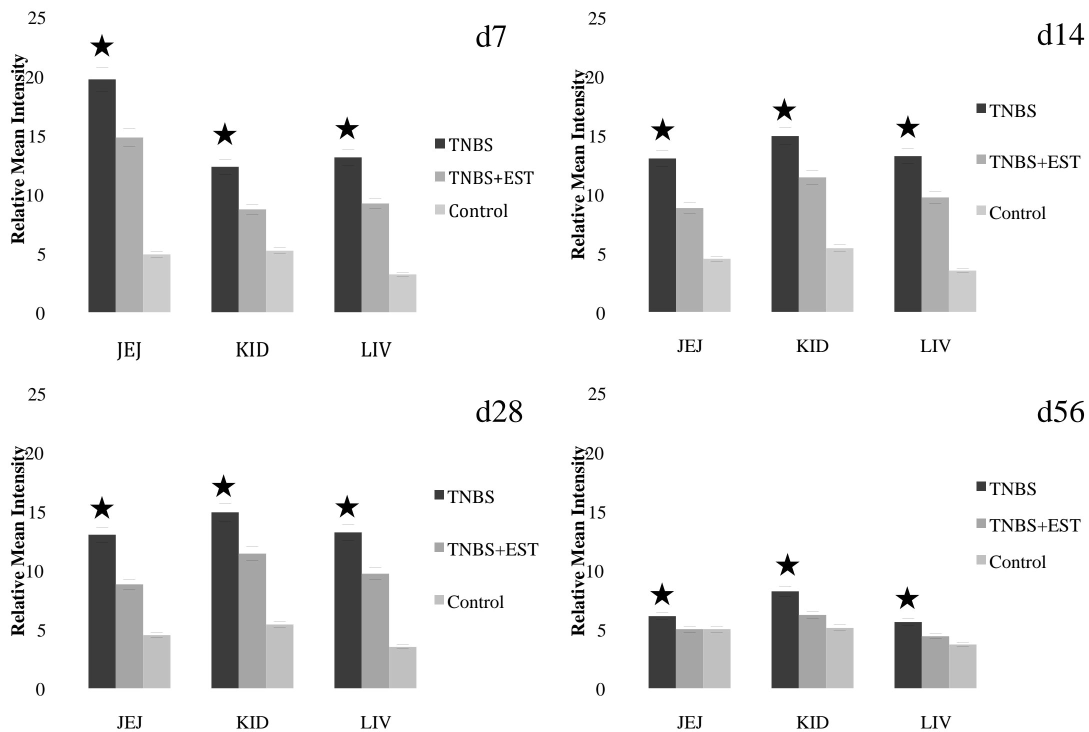


Figure 16: Relative mean intensity of the red fluorescence in the rats' jejunum kidney and liver of the TNBS treated rats model on days 7, 14, 28, 56 (n=5 for each group/time point). It is more intense on day 7 followed by days 14, 28 then 56. The estrogen treatment reduces this intensity significantly. For the statistical analysis, values are the means ± SEM. t-test ★ $p<0.05$, TNBS vs TNBS+Est. $p<0.05$ (figures 17 and 18).

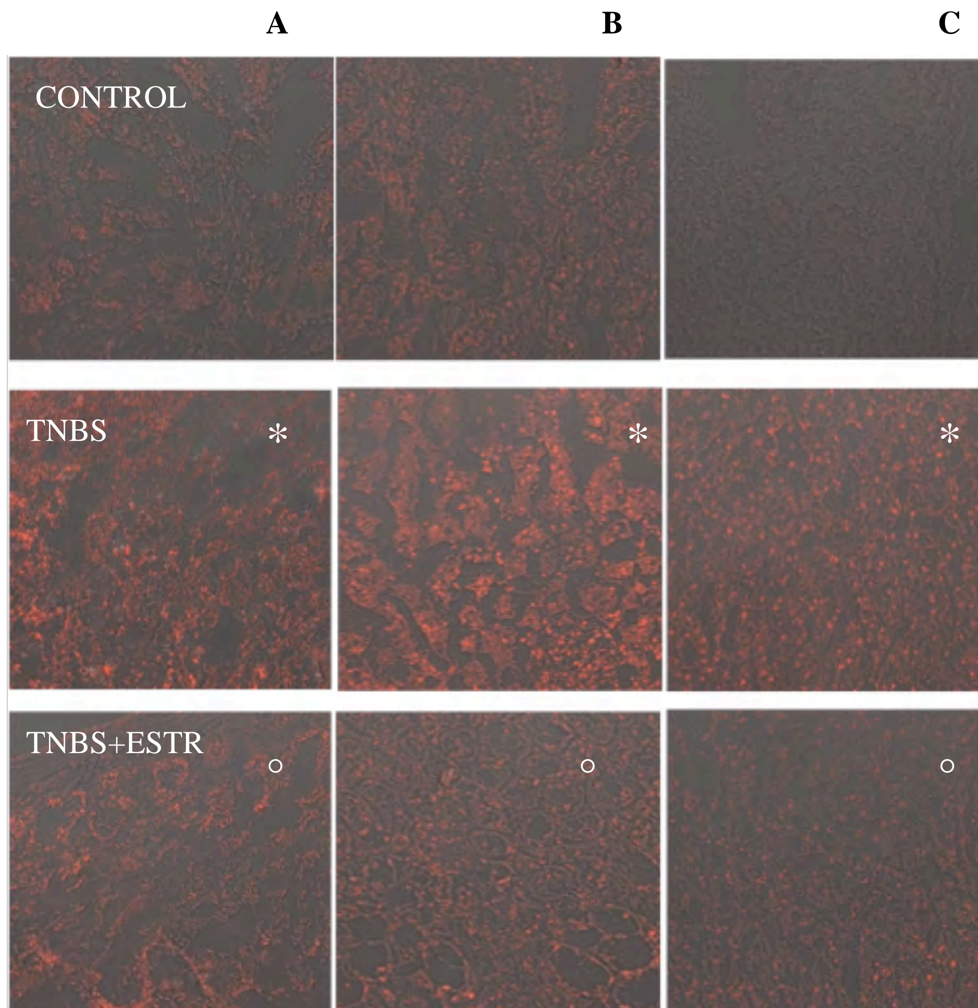


Figure 17: DHE staining of tissues on day 7: jejunum (A), kidney (B), and liver (C) of rats treated with TNBS, TNBS+ Estrogen and control. Note the increase of fluorescence intensity in the TNBS treated animals in all tissues (*). Such intensity decreased when estrogen was injected (°).

IV- DISCUSSION:

It has been clinically noted that females of all ages experience significantly lower rates of inflammation, a decrease in humoral immune function and regulation of the concentration of some pro-inflammatory cytokines like IL-6 (Grossman, 1985; Olsen, 1996).

There is a paradox regarding the role played by estrogen in inflammation, as it can be pro or anti-inflammatory. It was shown that during pregnancy in human and mice, the high estrogen levels inhibit important proinflammatory factors such as TNF, IL-1β, IL-6, MCP-1, iNOS expression, production of MMPs, and activity of natural killer cells. On the other hand, at low concentrations, estrogen stimulates TNF-α, IFN-γ, IL-1β, and activity of natural killer cells (Straub, 2007).

Only a few studies related IBD to estrogen concentration. Pregnancy or menstruation have been reported to increase the severity of symptoms associated with IBDs, while other reports have noted a protective role for pregnancy (Kane et al., 1998; Ferguson et al., 2008). The effect of pregnancy on the course of disease depends on the state of disease at the time of conception. Patients with active disease at conception often develop more severe inflammation throughout the pregnancy, while patients in remission at conception tend to remain disease free throughout pregnancy (Riis et al., 2006; Ferguson et al., 2008; Et-Tawil, 2008). Along with natural production of estrogen; exogenous estrogen treatment has also been shown to have effects on IBDs severity. Pregnancy levels of estrogen suppress many cytotoxic and innate immune responses, whereas antibody production, neoangiogenesis, and growth associated phenomena can be stimulated as shown in previous reports by Doria et al. (2006). One

of the most important immunological modifications during pregnancy is the increase of B cell responses due to the progressive increase of progesterone and estrogens, which reach their peak level in the third trimester of gestation (Doet el at, 2006).

Estrogen effects regarding IL-1 and IL-6 are only observed at midfollicular to pregnancy levels. Previous studies mentionned that estrogen at postmenopause did not lead to marked effects or even exert stimulatory effects on IL-1 and IL-6 (Hue, 1988; Polan, 1988; Dodel, 1999). Proinflammatory cytokines, such as IL-17, TNF-α and IL-6, activate the NFκB signaling pathway and increase iNOS levels. In turn, iNOS is involved in initiating inflammation and inducing DNA damage that could activate β-catenin. Both NFκB and β-catenin signaling pathways enhance the progression of inflammation. Gilmore (1997) and Correale (1998) made a distinction whereby stimulation of TNF-α appears only at lower levels of estrogen, whereas high pregnancy levels inhibit TNF-α secretion. Such a situation renders a woman in the postmenopausal phase to be more in a proinflammatory status, which might contribute to the manifestation of chronic inflammatory diseases after the menopause.

Two estrogen receptors have been identified, ERα and ERβ. Both are present in most tissues, including the intestine (Campbell-Thompson 1997, 1999) and cells of the immune system (Tornwall et al., 1999; Kassi et al., 2001; Rider et al., 2001).

Kurebayashi (2001) and Stoner (2002) reported that inflammatory conditions, reduced expression of Estrogen Receptor α (ERα) and oxidative stress increased the expression of Estrogen Receptor β (ERβ) (Tamir et al., 2002). Such data support the concept of up-regulation of ERβ relative to ERα under hypoxic conditions, which might lead to a preponderance of signaling through ERβ pathways.

A recent study by Saleiro et al. (2012) demonstrated that ERβ deficiency increased the

incidence of precancerous lesions in the colon. It supported the findings by Campbell-Thompson (2001) and Konstantinopoulos (2003) saying that ERβ, the predominant functional estrogen receptor (ER) subtype expressed in human colonic epithelium, has its levels reduced in colorectal tumors compared to normal colonic tissues. This suggests that the protective role of estrogen in the colon may be exerted via ERβ signaling. Similarly, the use of an ERβ-selective agonist was shown to prevent intestinal tumorgenesis and exert beneficial effects by suppressing NFκB signaling and inhibiting iNOS production. In parallel, ERβ over expression in colon cancer cell lines was shown to downregulate the expression of pro-inflammatory molecules (Edvardsson, 2011). In addition, Saleiro et al. (2012) demonstrated the upregulation of IL-6 expression in the absence of ERβ signaling in vivo, as well as an enhanced expression of TNF-α and IL-17. Such data offered mechanistic insights into the protective role of ERβ against the development of colorectal cancer. On the other hand, in support of a further protective effect of ERβ in IBD, Looijer-van Langen et al. (2011) demonstrated that ERβ mRNA levels were reduced in animal models of colitis and colonic biopsies from IBD patients. Based on such data, this work aimed to investigate the role of estrogen in previously established colitis models focusing on the differential immune and genetic response. The two models chosen for this study were TNBS (Morris et al., 1989) and DSS (Wirtz et al., 2007). Even though we recognize that there is no perfect model mimicking human IBD, TNBS inflammation mimics several prominent clinical and morphological features of Ulcerative Colitis (Elson et al., 1995), whereas DSS inflammation mimics similar features of Crohn's Disease (Gaudio et al., 1999; Strober et al., 2002). The TNBS was administered with ethanol. It resulted in breaking the mucosal barrier leading to a transmural infiltrative disease. It is believed that TNBS reacted as an

antigenic compound with microbiota proteins in order to stimulate an immune response (Neurath et al., 1995). On the other hand, DSS was toxic to epithelial cells of the basal crypts and affected the integrity of the mucosal barrier. It disrupted the epithelial cell barrier and therefore promoted increased cellular exposure to normal mucosal microflora. One possible consequence of this change in barrier function was that mucosal phagocytes became subject to activation by substances in the mucosal flora and this, in turn, led to an antigen nonspecific release of pro-inflammatory cytokines and inflammation (Kitajima et al., 1999). The TNBS model showed a higher degree of inflammation than the DSS model. Comparing the total clinical score of the two models, TNBS showed the most severe inflammation (≈6.5/10), followed by DSS (≈3/10). TNBS showed also a higher inflammatory score on each sacrifice day (day 7= 13/18, day 14=15/18, day 28= 9/18, day 56= 6/18) as well as a higher histological score (day 7=10/18, day14= 13/18, day 28= 9/18, day 56= 7/18)

The results from the current study supported an anti-inflammatory effect for the estrogen on the TNBS and DSS colitis models. The clinical, macroscopic and microscopic data resulting from this study were in conformity with previously reported work both on TNBS (Morris et al., 1989) and DSS (Wirtz et al., 2007), showing different degrees of severity of the clinical signs and symptoms expressed in the composite clinical scores. However, much lower inflammatory scores were attributed to the DSS model. The use of estrogen has significantly decreased the signs and symptoms of the colitis. These results were concordant with the findings of Verdu et al. (2002) demonstrating anti-inflammatory effects of 17beta-estradiol in TNBS models of experimental colitis leading to healthier animals. In fact, estrogen lowered the severity of the clinical scores significantly from 6.5/10 to 5.6/10 in the TNBS model and from

3.2/10 to 2.5/10 in the DSS model. Estrogen seemed to have partly inhibited the inflammatory reaction. It actually expressed a protective effect as expected from clinical observations.

Another parameter was checked, namely the presence and activity of Mast cells. Those cells produce growth factors, interleukins and other cytokines in addition to various vasoactive substances and immune mediators like histamine, proteases and eicosanoids (Jun-Ho et al., 2004). As for estrogen, Zaitsu et al. (2007) found that low concentrations of environmental estrogens bound to ER-α caused a rapid mast cell degranulation, using release of β-hex from human mast cells as a marker for degranulation and release of allergic mediators. Exposing human MCs' to a low dose of estrogen had an additive effect on degranulation. As a possible mechanism, they suggested that at least part of the degranulating activity of environmental estrogens on mast cells was mediated through ERα. They were based on previous data reporting that bone marrow derived mast cells deficient in ERα expression had significantly reduced responses to some concentrations of environmental estrogens (Harnish, 2003)

As expected, our results showed that there was an overall marked increase in the Mast Cells (MC) count in TNBS, (from 58 to 89 Mast cells/slide) going in parallel with the severity of the disease (Harnish et al., 2004).

Further evaluation of the inflammation process was carried out with molecular studies, analyzing the protein expression of fibronectin and collagen IV, and mRNA expressions of fibronectin, TNF-α and IL-6. These parameters were checked knowing that the interaction between antigen-presenting cells and the local bacterial flora contributes to an uncontrolled activation of mucosal CD4+ T lymphocytes with the consecutive release of proinflammatory cytokines such as TNF-α, IL-6. In addition, increased

concentrations of pro-inflammatory cytokines in inflamed mucosa appear to be responsible for the increase of MMPs in inflammatory diseases of the gut (Pender et al.,1997). The results from western blotting and qRT-PCR showed an over expression of fibronectin, collagen IV, TNF-α and IL-6 upon induction of colitis in TNBS treated rats. The DSS models showed relatively a lower degree of inflammation that is always above the values of the control. The high expression of fibronectin protein and mRNA, found in the TNBS model especially on day 14 of the experiment, could be linked to an over activity of fibroblasts and fibrosis of the intestinal tissue. As mentioned by Childow et al. (2007), it could be associated with necrosis or with the onset of early tumor growth, or at least with pathological polyps and masses following standing inflammation. The over expression of collagen IV, found primarily in the basal lamina could explain a chronic mucosal fibrosis, which could stimulate the acute phase reaction of inflammation characterized by an over production of other cytokines, including the IL-6, leading to an inflammatory and immune cell migration and further phagocytic reactions.

As previously reported by Satoh et al. (1997), the destruction of the mucosal barrier and the constant interaction of the luminal bacterial antigen with the isolated cellular compartment of the colon launched the inflammatory action of macrophages, fibroblasts, mast cells and leukocytes to secrete a variety of proinflammatory cytokines, including TNF-α and IL-6. Estrogen treated animals showed a marked decrease in the secretion of such proinflammatory cytokines (about 30%) and less of collagen and fibronectin (about 20%).

CD and UC, primarily involve the bowel. However, multiple other organ systems can be affected in IBD, including the bones and joints, skin, eyes, hepatobiliary

system, lungs, and kidneys. The overall prevalence of any of these extraintestinal manifestations in IBD patients ranges from 21%–40% (Lakatos et al. 2003; Ricart et al., 2004). Although management typically involves control of the active disease, there are treatment options for those patients who continue to be symptomatic despite treatment of their bowel disease. This was concordant with our findings showing that the induced inflammation was spread to organs and tissues other than the colon, specifically in the TNBS treated animals. The reaction was somehow diffused or passed through blood to liver, kidney and jejunum. The amount of ROS increased significantly with the increase in duration of the experiment (figure 16). However, the estrogen exhibited, one more time, a significant lowering in the intensity of fluorescence for DHE in all tissues tested. The estrogen could limit the amount of ROS production and exhibited a protective effect for the intestinal tissue.

In the macroscopic, microscopic and molecular results, the effect of estrogen was expressed across the board; reducing the inflammation scores macroscopically and microscopically and reducing the expression of the fibronectin, collagen IV, TNF-α and IL-6. However, it was noted that wherever the inflammation was more sever the effect of estrogen was more significant. Our results came in line with the previous studies of Gunal et al. (2003), Straub (2007) and Harnish et al. (2011). They reported that estrogens ameliorated the conditions of patients and animals with IBDs and demonstrated that estrogen has complex effects on intestinal inflammation (Gunal et al., 2003; Straub, 2007; Harnish et al., 2011).

Considering the available data and laboratory experience gained in this study, it will be important to draw a few conclusions:

-Estrogens decreased the production of TNF-α and IL-6.

-Estrogens can impact directly on mast cells, and this may in part be responsible for blocking recruitment of neutrophils and the improvement in the clinical and histological scores observed on all models.

-Estrogens played an overall role in prevention and or protection in all models and at various time points.

-The necrosis of the intestinal tissues was much less in estrogen treated rats as illustrated by lower levels of collagen IV and fibronectin.

-Estrogens had an antioxidant effect, the amount of ROS decreased in all tested tissues and at all time points tested.

This study stimulated some important questions for future research:

1. Repeat the same experiment using female rats.
2. Do a dose response curve of estrogen using multiple physiological and pharmacological doses.
3. In the DSS model, it could be pertinent to increase the dose level of DSS that might show higher inflammation scores and consequently more significant effects of the estrogen in this model.
4. As inflammations are known for their flair out and quiet zones of activity, it could be pertinent to increase the duration of the experiment and extend it beyond day 56.
5. Compare ROS production in the various parts of the gastrointestinal tract.
6. Use transgenic mice predisposed to IBDs when available.

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