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ORIGINAL ARTICLE

Effect of female Sex hormones on irritable bowel syndrome induced by water avoidance stress in experimental rat model of menopause

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ABSTRACT

Background: Recently there are contradictory reports on the effects of sex hormones on pathogenesis of IBS, so many researches were held to clarify the relation between the level of sex hormones and IBS .
Aim of the study: to examine the effect of female sex hormones on the inflammatory signs present in serum and changes in GIT associated with chronic IBS stress in rats.
Material, Methods:60 healthy female of local strain group were divided equally into 6 groups: Group1:OVX Sham group , Group2:OVX sham group With IBS, Group3: OVX IBS group, Group4 OVX IBS Estrogen treated group , Group5: OVX IBS Progestin treated group , Group6: OVX IBS estrogen &Progestin treated group . after OVX hormones were given for continues 14 days and rats were submitted to chronic stress exercise for 10 days ,and after the last dose of replacement hormones serum histamine, serotonin and IL8 levels were determined , distal colon was processe d for histopathology.
Results :There was a significant increase in serum IL8, histamine with marked drop in serotonin level in both IBS and IBS OVX groups compared to control and hormonal treated groups. additionally, there was a marked disturbance in colonic mucosal structure with elevated claudin 1 protein level in both IBS and OVX IBS groups compared to other groups.
Conclusion: female replacement hormones were found to attenuate inflammatory markers and mucosal disturbance associated with IBS.

Keywords: IBS , OVX , female hormones , WAS

INTRODUCTION

In the World Gastroenterology Organization statement , Irritable bowel syndrome (IBS) has been defined as "functional bowel disorder associated with abdominal pain , discomfort and changes in bowel habit associated with structure abnormalities **[1].**The underlying pathogenesis of IBS remains still unclear. Its etiology may include dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, neuroendocrine with visceral hypersensitivity alterations, microbiota changes and permeability disorders of the colonic mucosal barrier **[2].** However, most of the advanced reports emphasized that dysregulation in immune and inflammatory pathways are considered as important pathophysiological mechanisms implicate in the occurrence of IBS. It was found that immune cell populations e.g. mast cells and T-lymphocytes act as major presented cells in IBS **[3-5].**A meta-analysis had found that the world-wide prevalence of IBS was between 10 and 25.0% with great risk to develop colorectal cancer by 1% when compared to the general population **[6]**. Studies from Middle East countries showed a raise in the burden of IBS in the Arab world **[7]** and the prevalence was ranged from 8.9% to 31.8% **[8, 9]**. The prevalence of IBS in women was 67% higher than men world widely and gender related differences in gastrointestinal and somatic symptoms are most prominent in postmenopausal women **[10]**. Exacerbation of IBS symptoms occurs around time of luteal phase , menses , premenopausal and menopause (at times of low ovarian hormones) suggesting that modulation of the disease controlled

by estrogen and progesterone withdrawal directly or indirectly **[11]**.

Additionally they modulate regulatory mechanisms of the brain-gut axis involved in pathophysiology of gastrointestinal tract leading to alterations in visceral sensitivity, permeability, motility with immune activation in intestinal mucosa **[12& 13]**. Up to date, there are insufficient and conflict data determining the exact effect of hormonal replacement therapy (HRT) on IBS symptoms during menopause **[14&15]**. women with IBS taking oral contraceptives (OCs) , containing estrogen and progestin , was found to had a reduced levels of abdominal symptoms compared to women not taking OCs with IBS **[16].** However many report had found that HRT may increase the prevalence of IBS in postmenopausal women, also it may prolongs IBS symptoms to a later age or even induce disturbance in gastrointestinal function in females not affected before **[17]**.

AIM OF THE WORK

This study was designed to evaluate the modulatory influences of ovarian sex hormones on experimentally induced IBS of menopausal rat model.

METHODS

Sixty adult female albino rats of local strain (200-240g) were purchased from the Animal House of Faculty of Medicine, Zagazig University. They were housed in steel wire cages (50cm X 60cm X 60cm) (5 rats per cage) at comfortable room temperature, light cycle (12h dark and 12h light) and hygienic conditions. All rats had a free access to water and fed standard rat chow. Animals were left for one week prior to the beginning of the experiments for adaptation to laboratory conditions. The experimental protocol was approved by local medical ethics committee and physiology department in Faculty of Medicine of Zagazig University (The Institutional Animal Care and Use Committee, Zagazig University, ZU-IACUC) approval number: ZU-IACUC/3/F/166/2019.Experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. Rats randomly divided into six equal groups (n = 10 rats for each). **Group1**: controlled Sham ovariectomized group (sham OVX) , **Group 2:** sham ovariectomized group With IBS (sham OVX/ IBS), **Group 3:** ovariectomized IBS group (OVX /IBS), **Group 4:** Ovariectomized IBS / Estrogen treated group (OVX/IBS+E). **Group5**: Ovariectomized IBS/ Progestin treated group (OVX/IBS+P). **Group 6:** Ovariectomized IBS Combined estrogen &Progestin treated group (OVX/IBS+E&P).

Ovariectomy was performed in rats to induce experimental menopause; rats were anesthetized with ketamine (100 mg/kg, ip) (*Sigma Chemicals*) and underwent bilateral ovariectomy. between the last rib and pelvis a transversal dorsolateral incision was made in skin , muscle was dissected to expose the abdominal cavity. The ovaries were located in a fat pad beneath the muscles. The fallopian tube was crushed and ligated, and the ovaries were removed by cutting above the clamped area. The muscle and the skin incision were closed with nylon suture **[18]**. In **SHAM surgery**, the same incisions were made to rats , the ovaries and fallopian tubes were exposed and then put back in the abdominal cavity and the muscle and skin were closed. At the end, garamycin cream was put over the closed incision and covered with sterilized gauze.

Determination of sexual cycle: Vaginal secretions were obtained by inserting the tip of a plastic pipette filled with 10mL of NaCl 0.9% into the vagina of the non-ovariectomized rats. Then the collected material was observed under a light microscope without staining, cycles with duration of 4-5 days were considered regular According to **Marcondes et al.(2002) [19]**.

Hormonal replacement method: Two weeks after ovariectomy the rats received daily subcutaneous injections of 17β-estradiol (10µg /kg/ day) (*Misr CO. pharm. Ind. S.A.A. Materia. Cairo-A.R.E.)* dissolved in 0.1 ml sesame oil as vehicle (ADWIC Laboratory Chemicals, Egypt), subcutaneous injections of progesterone (10mg/kg/day) (*Misr CO. pharm. Ind. S.A.A. Materia. Cairo-A.R.E.)* dissolved in 0.1 ml sesame oil for 14 days .OVX and sham operated groups were injected with 0.1 ml sesame oil for 14 days**[20-21]**.

Induction of IBS by water avoidance stress (WAS) technique: after 2 weeks of ovariectomy, each rat was placed on the top of a cube (6 x 6 x 9.5 cm^3) which placed in the middle of the water tank (25 x50 x 25 cm^3) for 1 hour every day for 10 consecutive days. The water (at room temperature) was filled up to 2 cm away from the top. The control rat was handled and kept in its housing cage in the experimental room for 1 hour**[22].**

Blood sampling: Blood samples were collected from non-ovariectomized groups in the afternoon of estrous day of their cycles after an overnight fasting

to avoid circadian rhythm of serum hormonal levels. After The end of all experiments rats were anesthetized by Urethane (1200mg/kg) (Sigma St. Louis, MO) then scarified by decapitation, blood were collected into clean centrifuge tubes and allowed for clotting, and then serum was separated by centrifugation of blood for 10 minutes at 3000rpm and stored at-20ºC until assayed for :

A- serum histamine level using histamine ELISA kit, (My bio source Southern California San Diego (USA) according to **Lv et al**. **[23]**

B- serum Interleukin 8(IL-8) using IL-8 kit (My bio source Southern California, San Diego (USA) according to **Xia et al.[24]**

C- serum serotonin level using rat ELISA kit (My bio source Southern California, San Diego (USA) according to **Liu et al. [25]**

Histopathological Examination:

Specimens from the distal colon were fixed in 10% buffered formalin. Paraffin-embedded serial sections were cut at 5-7 µm thickness and were subjected to:

•Hematoxylin and Eosin stain**[26].**

•Immunohistochemical stain for claudin 1**[26].**

Steps of Immunohistochemical staining

A rabbit polyclonal antibody against human claudin-1 (RB-9209-P, Labvision, Fremont, California) was diluted 1:300 in antibody diluent (Dako Co., Mississauga, ON, Canada) and applied to 5-um-thick sections from formalin-fixed, paraffin-embedded tissue specimens, using the avidin-biotin peroxidase method (Vectastatin Elite ABC kit, Vector Laboratories, Burlingame, California), following the manufacturer's instructions. manually at room temperature The immunohistochemical (IHC) stain was performed. Negative controls were used with omission of primary antibody. For test optimization and run validation ,a separate positive controls of normal skin were used **[27]**.

Statistical analysis

Data were expressed as ± mean and standard deviation (SD) for the quantitative variable. statistical package SPSS version 16 (SPSS Inc., Chicago, USA) was used to analyze the Data . ANOVA (analysis of variance) was used to make a Comparisons between groups followed by post hoc test for multiple comparisons between each 2 groups. The results were considered significant when $p<0.05$.

RESULTS

OVX IBS rats showed a significant rise ($p<0.001$) in the mean values of serum levels of histamine and IL-8 while a significant reduction ($p<0.001$) in the mean values of serum serotonin levels and Claudin 1 mean area percent versus other groups (sham OVX, sham OVX IBS, OVX/IBS+E, OVX/IBS+P, OVX/IBS+E&P) . However, in OVX/IBS rats treated with E &P, all parameters are shown to return near their normal levels compared to sham OVX (no significant difference; $p>0.05$) (**table 1**). Moreover, there was insignificant difference between OVX/IBS+E group and OVX/IBS+P group in all parameters measured ($P>0.05$).

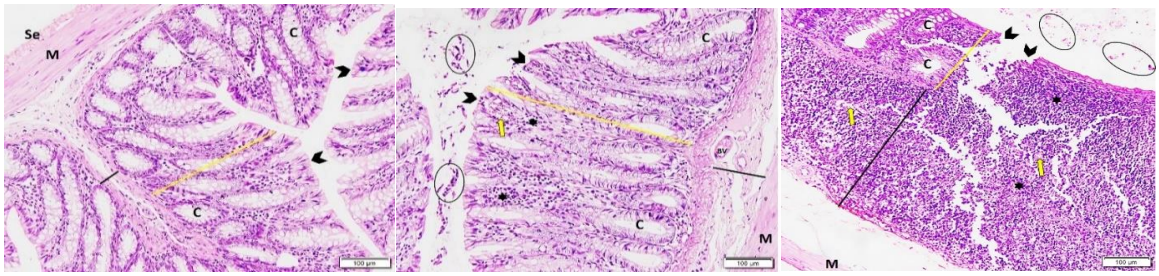
Regarding histopathological changes using H&E , **Group 1** showed normal colonic structure in which the mucosa was folded and covered by continuous simple columnar epithelium and has regularly arranged tubular crypts. **Group 2** showed mild inflammatory infiltrate, congested capillaries, absence of mucosal folding, loss of surface epithelium and widening of submucosa. **Group 3** showed marked inflammatory infiltrate, congested blood vessels, distorted mucosa, absent mucosal folding, loss of surface epithelium, distorted or absent crypts and marked widening of submucosa. **Group 4** showed mild inflammatory infiltrate, absent mucosal folding, loss of few surface epithelial cells, few distorted crypts and apparently normal submucosa. **Group 5** showed absent mucosal folding, loss of few surface epithelial cells, apparently normal crypts, and mild widening of submucosa. **Group 6** showed apparently normal structure without inflammatory infiltration or congestion**. (figure 1).**

By using immunohistochemistry, **group 1,6** showed mild immunoreactivity in mucosal epithelial cells**. group 2 , 3** displayed widespread immunoreactivities in mucosal epithelial cells and in cells of the connective tissue while **group 4 , 5** exhibited moderate immunoreactivity (**figure 2).**

Table 1. Serum histamine, serum il8 ,serum serotonin and colon claudin-1protien in all groups

groups	sham OVX	sham OVX IBS	OVX IBS	OVX/IBS+E	OVX/IBS+P	OVX/IBS+E&P
Serum Histamine (ng/ml)						

groups	sham OVX	sham OVX IBS	OVX IBS	OVX/IBS+E	OVX/IBS+P	OVX/IBS+E&P
Mean± SD P value	3.68±1.04	9.07±1.62 p<.001 [a]	12.16±1.45 p< .001 [a] p< .001 [b]	6.57±1.34 p<.001 [a] p<.001 [b] p<.001 [c]	7.24±1.08 p< .001 [a] p< .01[b] p< .001 [c] N.S [d]	3.67±1.02 N.S [a] p< .001[b] p< .001[c] p< .001[d] p< .001[e]
Serum IL8 (pg/ml) Mean± SD P value	29.6 ±6.13	70.6 ±10.07 p< .001 [a]	102.8 ±12.28 p< .001 [a] p< .001 [b]	44.5 ±7.65 p< .001 [a] p< .001 [b] p< .001 [c]	43.9 ±6.60 p< .001 [a] p< .01[b] p< .001 [c] N.S [d]	32.9 ±5.60 N.S [a] p< .001[b] p< .001[c] p< .01[d] p< .01[e]
Serum Serotonin (ng/ml) Mean± SD P value	173.56 ±12.3	133.56 ±7.3 p< .001 [a]	115.27 ±9.6 p< .001 [a] p< .001 [b]	145.24 ±10.4 p< .001 [a] p< .05 [b] p<.001 [c]	151.0 ±9.4 p< .001 [a] p< .01[b] p< .001 [c] N.S [d]	167.1 ±13.4 N.S [a] p< .001[b] p< .001[c] p< .001[d] p< .01[e]
colon claudin-1 area percent Mean± SD P value	17.6±1.04	35.5±1.2 p< .001 [a]	39.5±0.9 p< .001 [a] p< .001 [b]	28.1±0.8 p< .001 [a] p< .001 [b] p< .001 [c]	27.9±1.3 p< .001 [a] p< .01[b] p< .001 [c] N.S [d]	18.3±1.4 N.S [a] p< .001[b] p< .001[c] p< .001[d] p< .001[e]



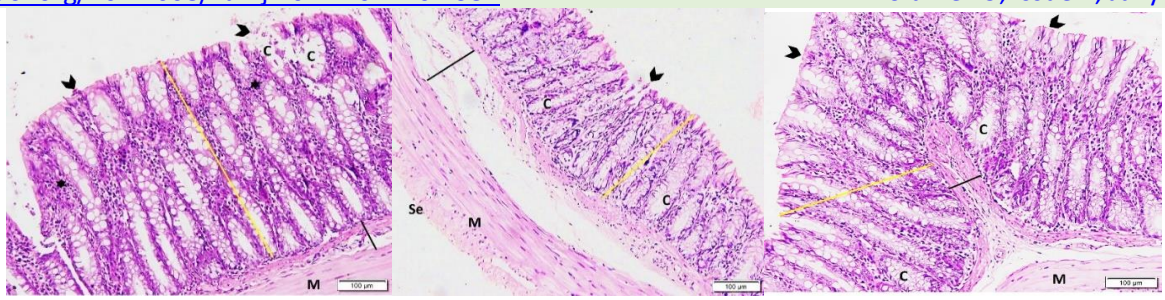


Figure 1. H&E staining of colon in all studied groups

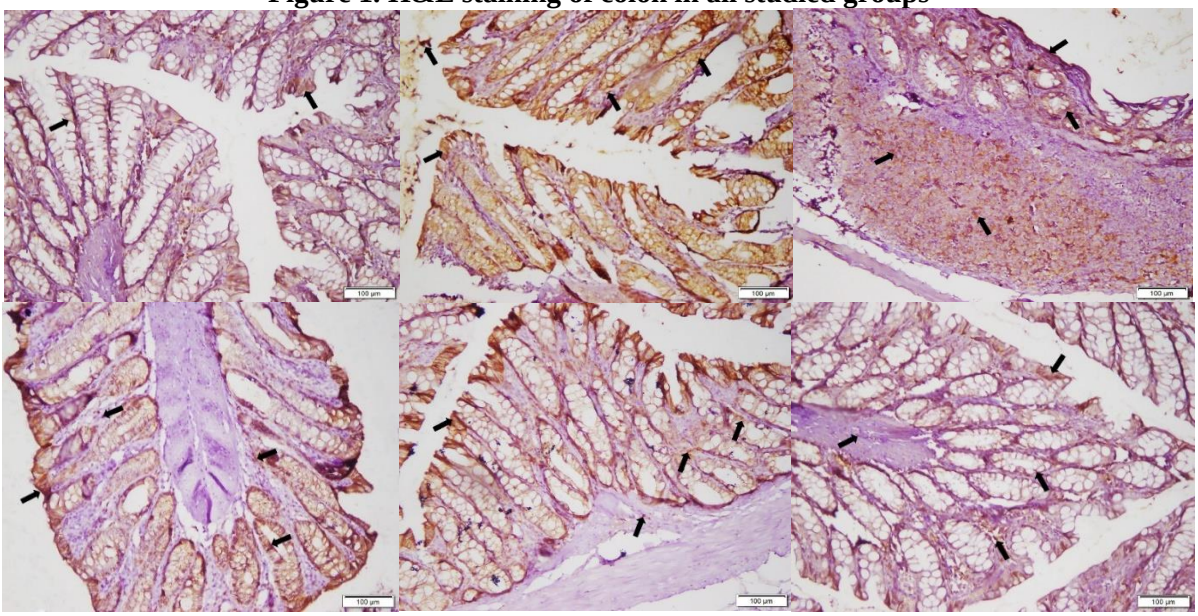


Figure 2. Claudin- I staining of colon in all studied groups

DISCUSSION

Ovariectomy is a standard surgical procedure to induce menopause in experimental animals and ovariectomized females show a dramatic cessation of ovarian function [28].IBS model was successfully confirmed by significant elevated serum levels of stress inflammatory markers as; histamine which is a potential biomarker for IBS [29] also IL8[30] and decreased serum levels of serotonin [31],besides histopathological data revealed a marked inflammatory infiltration , disturbed mucosal epithelial with villi lost [32] , increased mucosal permeability and disturbed barrier protein claudin-1in colonic mucosa [33].

As IL8 is one of the inflammatory cytokines that is thought to play a significant role in IBS etiology [30], results of this work revealed that postmenopausal rats with IBS(OVX /IBS) had significantly the most increased serum levels of IL8 as compared to all groups , also IBS rats had significant increased serum IL8 when compared to controls and these data were confirmed by Lee et al.[34]who found an a marked increase in serum level of pro inflammatory cytokines IL8, TNFA and IL1β in rat model by using WAS method to induce colonic microinflammation. Low grade inflammation in IBS activates HPA axis [35] leading to elevations of inflammatory cytokines, such as IL6, IL1β, and TNFA [36].

These cytokines also stimulate the HPA axis by activating nociceptive, visceral, and somatosensory afferents [37] .These inflammatory cytokines markedly released in diarrheal type of IBS (IBS-D) patients and may be associated with other symptoms like anxiety.

Changes in ovarian hormone level may modify the IBS symptoms status[38] and previous researches suggested that estrogen and progesterone have anti-inflammatory immune-modulatory effects by dcreasing inflammatory cytokines [39& 40] .Correspondingly, in current study treating IBS OVX rats with 17 b estradiol or progesterone significantly reduce serum IL8 .

Animal studies concerning sex hormones on IBS have shown conflicting data as **Verdú et al.**[41]

found that supra physiological dose of 17β-estradiol in dextran sodium sulfate (DSS) murine had an anti-inflammatory effect on colitis model , while it had a pro-inflammatory effect in a dinitrobenzene sulfonic acid (DNB) on colitis model, making a suggestion that the effect of estradiol on bowel symptoms may be context dependent. Also, progesterone was found to significantly decrease oxidative damage in the colonic mucosa In rats with trinitrobenzene sulphonic acid (TNBS)-induced colitis [42].

Additionally, **van der Giessen et al.** [43] mentioned that adding of estrogen or progesterone had suppressed IL8 in female patients with inflammatory bowel disease by attenuating endoplasmic reticulum stress which is thought to induce inflammatory responses, and to contributes the rise of pro-inflammatory cytokines [44].

In IBS There is a link between reduced estrogen level in menstrual cycle and increase gastrointestinal symptoms also , during pregnancy the gastrointestinal complaints was attenuated giving a suggestion upon the positive effect of pregnancy hormones on intestinal health In [45]. Moreover, **Jacenik et al.**[46**]** found that α and G protein-coupled estrogen receptor expression were up-regulated in IBS and estrogen signaling was associated with alterations in pro-inflammatory and anti-inflammatory cytokines expression besides changes in microRNAs that regulate genes found in the immune response.

Concerning the serotonin (5HT) which is a neurochemical transmitter involved in initiating peristaltic, secretory, vasodilatory, vagal and nociceptive reflexes in gastrointestinal tract [47], results of current work revealed that OVX /IBS rats had significantly the most reduced serum levels of serotonin, also IBS rats had significant decreased serum serotonin as compared to controls and these findings were in agreement with **Shi et al**.[48] who revealed low 5-HT levels in IBS rats , conversely serum serotonin levels were high in diarrheal type of IBS[49&50]**.**

In healthy individuals, enterochromaffin(EC) cells is the main source of 5-HT in the gastrointestinal tract and it is inactivated through serotonin transporter (SERT) reuptake from the mucosa into nerve fibers[51]. Changes in SERT expression and function in IBS could result in abdominal hypersensitivity and abnormal colonic motility [52]. A Previous study found that ulcerative colitis, IBS-D, and IBS with constipation (IBS-C) were associated with decrease in mucosal 5-HT and SERT [52]. However, **Kerckhoffs et al.[**53**]** reported that IBS-C patients have increased EC cell numbers and mucosal 5-HT concentration, on the other side IBS-D patients have reduced mucosal 5-HT concentration.

Also, IBS is associated with low 5-HT level may be due to decreased expression of the rate-limiting enzyme (TpH-1) which is the biosynthesis of 5-HT[48].

Interestingly, OVX/ IBS rats which treated with 17 b estradiol or progesterone significantly had elevated serum serotonin levels relative to non-treated OVX IBS rats ,these data were in accordance with **Paredes et al.** [54] who found that progesterone /estrogen had a stimulatory effect on the serotonin release and facilitate its activity by enhance binding to its receptor that can modulate IBS symptoms ,they also revealed that estrogen increases the density of 5-HT2A receptors, which stimulates release of intracellular calcium (Ca) and activate protein kinase C (PKC), with cell-dependent effects. PKC produce -ve feedback and uncoupling of the 5-HT1A auto-receptors, lead to decrease the number and effect of these receptors, also it increase the serotonin concentration through shutdown the system to produce a -ve feedback. **Meleine and Matricon.** [55] showed that in female patient colonic muscle the progesterone administration had increased the 5-HT levels by decreasing the level of SERT which participates in 5-HT reuptake, monoamine oxidase mRNA expression, and increasing the availability of 5-HT precursor, tryptophan.

In this work, IBS rats with sham ovariectomy had significant increased serum histamine when compared to controls ,but OVX /IBS rats had the highest serum level of histamine as compared to all groups, these data were matched with **Barbara et al.** [56] who investigated high levels of histamine in supernatants from IBS colonic samples. Recently, **Xu et al.** [57] showed an increase mast cells (MCs) number, MC tryptase expression and histamine level in the colonic tissue of stressed female rats. Activated MCs spontaneously secreting higher amounts of histamine in close proximity to colonic nerves correlated with severity and frequency of abdominal pain in IBS patients[58]. Additionally, **Fabisiak et al.** [59] reviewed that chronic IBS patients exacerbate mast cell degranulation and produce histamine that induced murine jejunal afferent firing and excited primary sensory neurons,

also it enhanced the pronociceptive effect through H1R expressed on sensory afferents**.**

Administration of 17 b estradiol or progesterone to IBS OVX rats significantly decreased serum histamine levels ,these results were supported by **Harnish et al.**[60] who found that 17βestradiol attenuated MC and prevent its activation and histamine release through inhibiting MC protease in rat colon , in contrast, **Xu et al.**[57] found that 17β-estradiol treatment had increased the visceral hypersensitivity and activation of MCs lead to accelerate the synthesis of allergic factors leaded to increase histamine level in the colon of OVX stress rat model . This difference in our results and **Xu's** study may be attributed to duration of stress induced or the injected dose of estrogen used. Moreover, **Vasiadi et al.** [61] found that progesterone inhibited histamine secretion from purified rat peritoneal MCs .

As chronic stress was proved to enhance mucosal permeability leading to increase passage of antigen and bacteria that produce mucosal injury and inflammation [32]. Microscopic data of distal colon of IBS rats in current work were confirmed by **Zhang et al.** [32] who stated that WAS was associated with hyperpermeability with obvious intestinal inflammation in the mucosa of colon and follicle associated epithelium besides lymphocytes infiltration. Moreover, sex hormones treated rats had apparently nearly normal mucosal architecture with minimal inflammatory infiltrate.

These data were supported with **van der Giessen et al.** [62] who found that Progesterone and estrogen improved wound healing and epithelial barrier function in intestinal epithelial cells by upregulation of tight junction proteins. Furthermore, these sex hormones attenuated significantly endoplasm reticulum ER- stress and pro-inflammatory cytokine production in intestinal epithelium.

Claudins are junctional proteins that mainly located in the intestinal epithelia; many types of claudins are responsible for forming a network of strands in tight junction(TJ) plaques within the intercellular space of neighboring epithelial cells and build selective channels between adjunct cells, while the others act as signaling proteins and modulate cell behaviors. thus, claudins dysfunction occurs in enterocytes may associated with disturbance in epithelia permeation and multiple intestinal diseases [63]. We found a widespread area of claudin-1 immunoreactivity in colon in both IBS and OVX /IBS groups ,these results were in line with **D'Antongiovanni et al.** [33]who revealed that Claudin-1 was increased in IBS associated with constipation while decreased in IBS with diarrhea ,in addition , **Zhu et al.** [63] had found that experimental colitis in mice was associated with higher level of claudin-1, delay epithelial recovery, sustained inflammation, and crypt hyperplasia. The elevated level of claudin-1 may be explained by endoplasmic reticulum stress that associated with different colonic inflammatory disorders [64].

Sex hormones treatment markedly attenuated the expression of cludin-1 protein in our work. And these were in controversy with result of **van der Giessen et al.** [43] who revealed that estrogen and progesterone treatment elevated the level of claudin -1 that helped to maintain gut barrier during examination of an intestinal biopsy from ulcerative colitis patient.

CONCLUSION

WAS induced IBS in a model of menopause (ovariectomized) rats associated with increased inflammatory serum IL-8 , serum histamine and expression of claudin-1 proteins in colon besides decline in serotonin level . Replacement OVX rats with sex hormones attenuated the inflammation (decrease serum IL-8, histamine) and elevated the serotonin level , enhanced the mucosal repair by reducing expression of claudin-1 level which may help to modulate the signs of IBS .

Conflicts of interest**:** no conflicts of interest

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