ORIGINAL ARTICLE

Effects of Progesterone and Estradiol on the Inflammatory and Apoptotic Markers of Ovariectomized rats Challenged with Acute Septic Systemic Inflammation

SINAN SUBHI FARHAN[1], SAMIR SAAD MAHGOUB[2], SAADABDULRAHMAN HUSSAIN[3], RASHA ABDULATEEF ABDULHUSSEIN[4]

[1]*Faculty of Pharmacy, Al-Rafidain University College, Baghdad, Iraq*

[2]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Al-Minia University, Al-Minia, Egypt*

[3] *Faculty of Pharmacy, Al-Rafidain University College, Baghdad, Iraq.*

[4] *Ministry of Health, Iraq*

Correspondence to Dr. Sinan S. Farhan ; Department of Pharmacology, Al-Rafidain University College, 10052 Al-Mustansiriya, Q. 506, St. 75, Baghdad, Iraq; E-mail: sinansubhi@gmail.com ; Tel: 07711424735

ABSTRACT

Background: The inflammatory responses during septic systemic inflammation were affected by the differential role of progesterone and estrogen that demonstrated pro-inflammatory and anti-inflammatory roles.

Aim: The present study was designed to evaluate the differential effects of estradioland progesterone supplementation on the inflammatory and apoptotic responses in an ovariectomized rat model of acute systemic septic inflammation (SSI).

Methods: The present study was conducted on 60 female Wistar rats. 40mg/kg estradiol and 5 mg/kg progesterone were given s.c. to ovariectomized (OVX) rats after induction of systemic septic inflammation (SSI) through caecum puncture with a 21-gauge needle.

Results: TNF-α, CRP, ALT, estradiol, and progesterone were evaluated in sera; additionally, iNOS, COX-II, and caspase-3 were evacuated in liver tissues homogenates using ELISA method. In OVX rats challenged with SSI, serum TNF-α, CRP and ALT levels were significantly increased associated with a decrease in serum estradiol levels. They also showed overexpression of the iNOSand increased activity of COX-II and caspase-3 in the livercompared to non-OVX rats subjected to SSI. Supplementation with estradiol significantly decreases all serum and liver tissuemarkers of inflammation and decreased apoptosis. In contrast, OVX rats supplemented with progesterone,SSI resulted in a significant increase of the studied markers.

Conclusion: supplementation of estradiolin OVX rats challenged with SSI significantly attenuated the systemic andliver inflammatory and apoptotic markers. Meanwhile, supplementation with progesterone exacerbates the effects of the inflammatory markers and increases the tendency of apoptosis in the liver tissue.

Keywords: Septicsystemic inflammation, liver, estradiol, progesterone, apoptosis

INTRODUCTION

The inflammatory response during sepsis was associated with over-expression of different inflammatory markers including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) to be involved early in the pathogenesis of septic shock and the associated apoptosis and tissue damage[1-3]. During the advanced stage of septic shock, the increase in hepatic functions results in excessive hepatocellular apoptosis, a critical step in acute hepatic failure that complicates systemic sepsis[4]. Estradiol and progesterone have various immunomodulatory impacts that may be effectively involved in the pathophysiology of sepsis[5]. However, the effects of serum levels of both hormones in the prediction of the severity and outcomes of end-organ damage, especially in acute hepatic injury, during septic shock remains controversial[6,7]. Several studies have investigated the differential effects of estradiol and progesterone on the systemic inflammatory responses of various organs and tissues. It has been reported that estrogen therapy in postmenopausal females increases hepatic production of C-reactive protein[8] and the expression of IL-6, suggesting a pro-inflammatory role[9]. Meanwhile, other investigators demonstrated contradictory results for estradiol in this regard[10,11]. Moreover, the effects of progesterone in systemic inflammation represent a lot of controversies. In a previous study, progesterone augments the outcome of experimental stroke[12], while another study indicates that progesterone resolves oxidative stress and reduces production of IL-6 and TNF-α and ameliorates the sepsis syndrome[13]. The present study was designed to evaluate the differential effects of estradiol and progesterone supplementation on the inflammatory and apoptotic responses in an ovariectomized rat model of acute septic inflammation.

MATERIALSAND METHODS

Animals: Sixty female Wistar rats (200-250 gm) were used in the study. The animals were housed in the animal house, Faculty of Medicine, Mu'tah University, under standardized conditions of temperature and humidity with 12:12 hr light/dark cycle and fed standard rodent chow and tap water *ad libitum*. The study protocol was approved by the local Committee of Research Ethics in compliance with the international standard care of experimental animals reported elsewhere.

Study design: The rats were randomly allocated to six groups (10 rats each) as follow: group I, served as a negative control group and subjected to Sham ovariectomy procedure (Sh-OVX); group II, a positive control where the Sh-OVX was followed by the induction of a septic systemic

inflammation (SSI) two weeks later; group III, exposed to OVX followed by the induction of SSI two weeks later[14]; group IV, after OVX each rat was administered a daily s.c. estradiol dose of 40 mg/kg body weight followed by induction of SSI two weeks later; group V, after OVX each rat received 5 mg/kg/day progesterone s.c.followed by SSI induction two weeks later[15]; group VI, OVX is followed by the administration of a combination of estradiol and progesterone doses (as mentioned previously) followed by SSI induction two weeks later.

Surgical intervention and induction of septic systemic inflammation: After short anesthesia with diethyl ether, the rats in groups I and II were subjected to Sham OVX, while the rats in groups I-IV were subjected to bilateral ventral OVX and followed by the hormonal supplementation (as mentioned above). In all rats (except group I), SSI was induced after two weeks. The induction of inflammation was performed by ileocaecal ligation and puncture using a 21-gauge needle. After 24 hr of sepsis induction, a blood sample was obtained through direct cardiac puncture under mild anesthesia; then, the animals were euthanized to extract livers for the assay of the tissue markers of inflammation and apoptosis according to standard procedures[13].

Measurements of markers: The obtained blood samples were kept in plain tubes and left to clot; the collected sera were used for the assay of estradiol, progesterone, TNF-α[16], ALT activity, and C-reactive protein levels[17] using ready-made kits (Biomatic, Ontario, Canada). The rate of expression of iNOS and the levels of Caspase-3 and COX-II in the liver tissue homogenates were analyzed using ELISA kits (My Biosource, USA) according to the specifications of the manufacturer.

Statistical analysis: The results were expressed as mean±SD; unpaired Student's t-test and ANOVA confirmed with Bonferroni's post hoc test were utilized to evaluate the differences between groups. A p-value <0.05 was considered for significant differences.

RESULTS

Table 1 shows that serum levels of progesterone were significantly increased in groups IV, V and VI (72%, 225%, and 268%, respectively) compared to the corresponding levels in group I, while progesterone levels were not significantly changed in animal groups II and III (P>0.05) versus controls. Moreover, serum levels of estradiol in groups III and V were found to be significantly lower than the obtained resultsin group I (79% and 43%); meanwhile, group VI demonstrates a significantly higher level of serum estradiol compared to all other groups (*P*<0.05). Additionally, table 1 shows that the level of TNF-α was increased in groups II, III, V, and VI (636%, 726%, 795%, and 213%, respectively) compared to group I; however, serum TNF-α level in group IVwas not significantly changed versus the controls (*P*<0.05) and found to be significantly lower than that reported in other groups. A similar pattern of changes was reported in serum CRP levels, where groups IV and VI demonstrated significantly lower levels compared to the other groups but they are still significantly higher than the levels in the control group (43% and 36%, respectively). In Figure 1, serum ALT activity was significantly elevated in all test groups compared to the controls. However, serum ALT levels in groups IV and VIwere found to be significantly lower than the other test groups (II, III, and V). Figure 2 indicates that the liver tissues, the activity of caspase-3 was significantly elevated in all test groups compared with the sham-operated without SSI group of rats (*P*<0.05). The highest levels of caspase-3 were reported in groups II and III, and the lowest degree of elevation in caspase-3 level was reported in group III. Therewas no significant difference in the liver tissue level of caspase-3 between groups V and VI(*P*>0.05). Regarding the influence of estradiol and progesterone supplementation to OVX rats challenged with SSI on the expression of COX-II in the liver, Figure 3 shows that the liver tissues, the levelof COX-II were significantly elevated in all test groups compared to the control group, and the highest levels of expression were reported in groups II and III. Meanwhile, the levels of COX-II expression in groups V and VI were found non-significantly different (*P*>0.05). Additionally, the expression of iNOS in the liver tissue was significantly elevated in groups II, III and V versus the controls (Figure 4), while the levels of expression in groups IV and VI were not significantly different from that reported in thecontrol group and found to be comparable when compared with each other (*P*>0.05).

Table 1: Effects of estradiol and progesterone supplementation on their serum levels and the concentrations of CRP and TNF-α in ovariectomized rat challenged with systemic septicinflammation

Marker	Animal group (*n*=10)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
S. Progesterone (ng/ml)	11.6±1.4^{a}	11.5±1.8^{a}	10.1±1.1^{a}	19.9±3.6*^{b}	37.7±5.1*^{c}	38.4±5.0*^{c}
S. Estrogen (*p*g/ml)	49.6±5.8^{a}	41.7±6.8^{a}	10.5±1.4*^{b}	40.5±5.7^{a}	28.3±3.2*^{c}	60.2±7.9*^{d}
S. CRP (µg/ml)	1.4±0.4^{a}	2.6±0.3*^{b}	3.9±0.5*^{c}	2.0±0.22*^{b}	4.9±0.3*^{c}	1.9±0.3*^{b}
S. TNF-α (*p*g/ml)	11.5±1.2^{a}	84.6±4.1*^{b}	95.5±4.2*^{c}	14.5±0.8^{a}	103.5±6.0*^{d}	36.1±2.8*^{e}

Values are expressed as mean±SD; n: number of rats in each group; * significantly different compared to the controls (unpaired *t*-test, *P*<0.05); values with non-identical superscripts (a,b,c,d,e) among different groups are significantly different (ANOVA, *P*<0.05)

Fig.1: Effects of estradiol and progesterone supplementation on serum ALT levels in ovariectomized rat challenged with systemic septic inflammation. Number of rats: 10 in each group; values with non-identical letters (a,b,c,d,e) are significantly different (ANOVA, *P*<0.05).

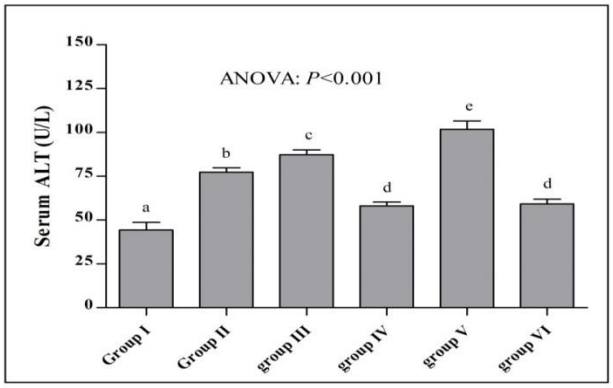


Fig. 2: Effects of estradiol and progesterone supplementation on liver tissue caspase-3 activity in ovariectomized rat challenged with systemic septic inflammation. Number of rats: 10 in each group; values with non-identical letters (a,b,c,d,e) are significantly different (ANOVA, *P*<0.05).

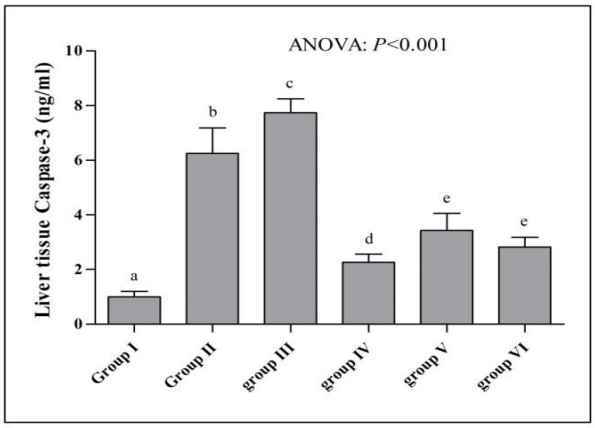


Fig. 3: Effects of estradiol and progesterone supplementation on liver tissue COX-II levels in ovariectomized rat challenged with systemic septic inflammation. Number of rats: 10 in each group; values with non-identical letters (a,b,c,d) are significantly different (ANOVA, *P*<0.05).

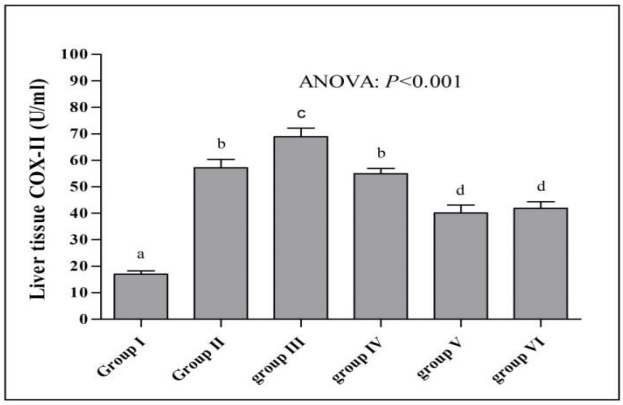
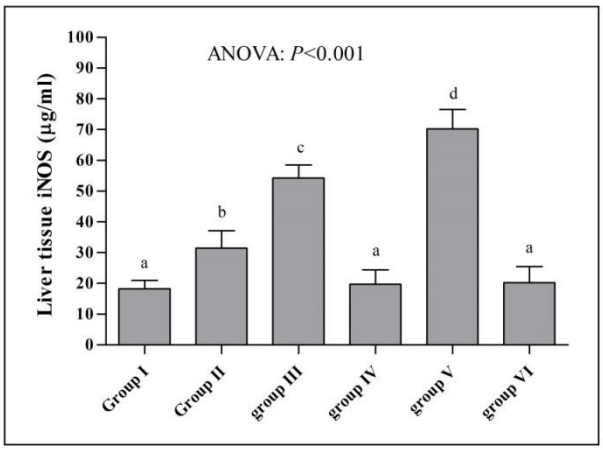


Fig. 4: Effects of estradiol and progesterone supplementation on liver tissue iNOS levels in ovariectomized rat challenged with systemic septic inflammation. Number of rats: 10 in each group; values with non-identical letters (a,b,c,d) are significantly different (ANOVA, *P*<0.05).



DISCUSSION

In the present study, table 1 shows that SSI induction was associated with significant reduction in serum estradiol level in group III, which can be attributed to the excessive production of NO that leads to direct inhibition of GnRH synthesis[9]. Moreover, previous reports suggested that the pro-inflammatory mediators directly inhibited the process of steroidogenesis in the ovaries[18], which may explain the decrease in serum estradiol level in ovariectomized rats challenged with SSI compared with negative and positive control groups. Supplementation of the OVX rats with daily doses of estradiol and progesterone (group VI) resulted in increased serum levels of both hormones that may achieve the range of the diestrus/proestrus levels found in normal cycling rats[19]. However, the reported higher concentrations than that reported in our control groups may explain the positive effect of estradiol supplementation on the peripheral mechanisms that control progesterone synthesis[19].

The present study reported highest levels of TNF-α, CRP, ALT andiNOS expression in the ovariectomized rats treated with progesterone and challenged with septic inflammation (group V), while the highest caspase-3 and COX-II expressionwas reported in group III compared to the control group (group I). Meanwhile, the groups treated with estradiol or its combination with progesterone (groups IV and VI) showed a marked reduction of these parameters compared to both the control and the other groups. It has been reported that progesterone enhances both the susceptibility to septic challenge and the associated inflammatory responses that may lead to severe infections and aggravation of the inflammatory changes[20]. However, the combination of progesterone with estradiol in the present study revealed the predominant anti-inflammatory effects of the estradiol in thismodel. Conflicting data with our findings was observed where Jiang et al[21] reported the decrease of TNF-α expression by progesterone in a rat model of brain injury; mean while, Roof et al[22] revealed that progesterone may also decrease the oxidative stress by its

membrane-stabilizing effect. This might be attributed to the differences in the experimental model and methods of analysis. Additionally, estradiol protects the CNS against neurotoxic stimuli and decreases the TNF-α expression in female rats treated with a combination of progesterone and estradiol[23]. The degree of liver damage in rats was found to be influenced by the gender, where serum ALT was reported to be lower in female rats with hepatotoxicity versus the males of the same model[24], this came in tune with our finding of the role of supplementation of estradiol on the severity of liver damage due to a septic shock. Moreover, Yin et al [25] reported that estradiol replacement decreases the severity of hepatic damage in ovariectomized rats compared to those with intact ovaries.This finding does not agreewith ours, whereserum ALT levels were high in group II and III and peaked in group V, while lower levels were obtained in groups IV and VI supporting the assumption of theanti-inflammatory role of estradiol[26]. The expression of iNOS was enhanced in ovariectomized rats 2-6 weeks post operation compared with sham-operated rats[27], and the result of the present study was in tune with this finding, where liver tissue expression of iNOS was significantly higher than that reported in the sham-operated rats. In this regard, administration of 17-β estradiol inhibits the expression of iNOS mRNA in ovariectomized rats challenged with lipopolysaccharide[28]; meanwhile, Yilmaz et al[29] reported that iNOS expression can be inhibited by estradiol supplementation. The differential effects of progesterone and estradiol on the expression of iNOS in different tissues seem to be controversial according to many studies. Ogando et al[30] mentioned that both progesterone and estradiol enhanced iNOS expression supporting the involvement of those hormones in the regulation of iNOS expression. Our finding in this respect was in tune with the reports of Hassouna et al[31] and Buhimschi et al[32] regarding the negative effect of progesterone on the hepatic NO levels, whereas Al-Hijji et al[33] reported the stimulatory effect of progesterone administration on uterine NOS activity. Those variable effects on iNOS expression could be attributed to the use of different types of cell preparations or animal models.In experimental animals, previous data indicated that the administration of estradiol to ovariectomized ratscauses overexpression of uterine COX-II mRNA compared to the use of progesterone alone[34]. Regarding the impacts on COX-II, the presented data were in tune with the previous finding, wherelowest COX-II expressionwasobserved in group V supplemented with progesterone alone and a little bit higher in group VI after supplementation with estradiol/progesterone combination. However, both findings were in conflict with that reported by Hassouna et al[31], they suggested a generalized decrease in COX-IIexpression in the liver tissues during estradiol supplementation in physiological doses. The present study revealed the reduction of liver tissues caspase-3 expression in the groups treated with estradiol, progesterone or their combination. A similar finding was reported by Karatepe et al[35], where treatment with progesterone was associated with the decreased caspase-3 activity. Moreover, Da et alshowed that estradiol down-regulates the expression of caspase-3 on the lacrimal and submandibular glands of ovariectomized rats which is due to the anti-apoptotic and antioxidant effects[36]. In this regard, Xue et al[37]attributed the progesterone-inducedreduction ofcaspase-3 expression tothe inhibition of the NF-κB pathway in the ovariectomized rats. In Conclusion, supplementation of estradiol in ovariectomized rats challenged with systemic septic inflammation significantly attenuated the systemic and liver inflammatory and apoptotic markers of acute systemic inflammation. Meanwhile, supplementation with progesterone doses exacerbates the effects of the inflammatory mediators and increases the tendency of apoptosis in the liver tissues.

Significance statement: The present study discovered the differential effects of estradiol and progesterone during septic systemic inflammation that can be beneficial to aid the use of these hormones or their derivatives as replacement therapy during conditions of septic inflammation. The finding of the study may trace the modulatory effects of sex hormones on the pathophysiology of sepsis and support the previous ideas in this field.

Acknowledgement: The authors thank Al-Rafidain University College for supporting the project.

Competing Interest: The authors have declared that no competing interest exists.

REFERENCES

1. Bohannon, J., Guo, Y., Sherwood E. R. The role of natural killer cells in the pathogenesis of sepsis: the ongoing enigma. *Crit. Care*.2012; 16(6):185.
2. Jedynak, M.,Siemiątkowski, A.,Rygasiewicz K. Molecular basics of sepsis development. *Anaesthesiol.Intensive Ther*.2012; 44(4):221-225.
3. Lowes, D.A., Webster N. R., Murphy, M. P., Galley,H. F. Antioxidants that protect mitochondria reduces interleukin-6 and oxidative stress improves mitochondrial function and reduces biochemical markers of organ dysfunction in a rat model of acute sepsis. *Br. J.Anaesth*. 2013; 110(3):472-480.
4. Marshall, J.C. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. *Crit. Care Med*.2001; 29:99-106.
5. Dellinger, R.P., Levy, M. M.,Carlet, J. M.,Bion, J., Parker, M. M.,*et al*. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit. Care Med*.2008; 36:296-327.
6. Angstwurm, M.W.,Gaertner, R.,Schopohl,J. Outcome in elderly patients with severe infection is influenced by sex hormones but not gender. *Crit. Care Med.*2005; 33:2786-2793.
7. Szalay, L., Shimizu, T., Suzuki, T., Hsieh, Y. C., Choudhry, M. A.,*et al*. Androstenediol administration after trauma-hemorrhage attenuates inflammatory response, reduces organ damage, and improves survival following sepsis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2006; 291(2):G260-266.
8. Cushman, M.,Legault, C., Barrett-Conner, E., Stefanick, M. L., Kessler, C.,*et al*. Effect of postmenopausal hormones on inflammation-sensitive proteins: the postmenopausal Estrogen/Progestin Interventions (PEPI) study. *Circulation*1999; 100:717-722.
9. Herrington, D.M.,Brosnihan, K. B.,Pusser, B. E.,Seely, E. W.,Ridker, P. M.,*et al*. Differential effects of E and droloxifene on C-reactive protein and other markers of inflammation in healthy postmenopausal women. *J. Clin.Endocrinol.Metab.*2001; 86:4216-4222.
10. Wakatsuki, A.,Ikenoue, N., Shinohara, K., Watanabe, K.,Fukaya, T. Effect of lower dosage of oral conjugated equine estrogen on inflammatory markers and endothelial

function in healthy postmenopausal women. *Arterioscler.Thromb.Vasc. Biol.* 2004; 24:571.
11. Sunday, L., Tran, M. M.,Krause, D. N.,Duckles, S. P. Estrogen and progestagens differentially modulate vascular proinflammatory factors. *Am. J. Physiol. Endocrinol.Metab*.2006; 291:E261-E267.
12. Rosano, G.M., Webb, C. M.,Chierchia, S.,Morgani, G. L.,Gabraele, M.,*et al*. Natural progesterone, but not medroxyprogesterone acetate, enhances the beneficial effect of estrogen on exercise-induced myocardial ischemia in postmenopausal women. *J. Am. Coll.Cardiol*. 2000; 3(6):2154-2159.
13. Aksoy, A.N.,Toker, A.,Celik, M.,Aksoy, M.,Halici, Z.,Aksoy, H. The effect of progesterone on systemic inflammation and oxidative stress in the rat model of sepsis. *Indian J. Pharmacol*.2014; 46(6):622-626.
14. Fink, M.P., Heard, S. O. Laboratory models of sepsis and septic shock. *Surg. Res*.1990; 49(2):186-196.
15. Tsai, H.W.,Legan, S. J. Loss of luteinizing hormone surges induced by chronic estradiol is associated with decreased activation of gonadotropin-releasing hormone neurons. *Biol. Reprod*.2002; 66:1104-1110.
16. Mizutani, A.,Okajima, K.,Uchiba, M.,Isobe, M., Harada, N.,*et al*.Anti-thrombin reduces ischemia-reperfusion-induced renal injury in rats inhibiting leucocyte activation through promotion of prostacyclin production. *Blood*2003; 101(8):3029-3036.
17. Gewurz, H., Siegel, J.,Fiedle, B. C-reactive protein and the acute phase response. *Adv. Intern.Med*.1982; 27:345-372.
18. Son, D.S., Roby, K. F. Interleukin-1alpha-induced chemokines in mouse granulosa cells: impact on keratinocyte chemo-attractant chemokine, a CXC subfamily. *Mol. Endocrinol*.2006; 20:2999-3013.
19. Sunday, L., Tran, M. M., Krause, D. N.,Duckles, S. P. Estrogen and progestagens differentially modulate vascular pro-inflammatory factors. *Am. J. Physiol. Endocrinol.Metab*.2006; 291:261-267.
20. Kautschic, C., Zhou, F.,Murdin, A. D.,Wira, C. R. Effects of estradiol and progesterone on susceptibility and early immune responses to Chlamydia trachomatis infection in the female reproductive tract. *Infect. Immun*.2000; 68(7):4207-4216.
21. Jiang, C., Wang, J., Li, X., Liu, C., Chen, N.,Hao,Y. Progesterone exerts neuroprotective effects by inhibiting inflammatory response after stroke. *Inflamm. Res*.2009; 58(9):619-624.
22. Roof, R.L., Hall, E. D. Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. *J.Neurotrauma*.2000; 17(5):367-388.
23. Vegeto, E.,Benedusi, V., Maggi, A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. *Front.Neuroendocrinol*.2008; 29(4):507-519.
24. El-Toukhy, S.E.,Alnahdi, H.,Ayaz, N., Hamza, A. H. Hormonal modulation of ginseng against hepatotoxicity induced in experimental animals. *Int. J. Acad. Res*. 2012; 4(6):174-180.
25. Yin, M.,Ikejima, K., Wheeler, M. D., Bradford, B. U.,Seabra, V.,*et al*.Estrogen is involved in early alcohol-induced liver injury in a rat enteral feeding model. *Hepatology*2000; 31(1):117-123.
26. 26.Shivers, K.Y., Amador, N., Abrams, L., Hunter, D.,Jenab, S.,Quiñones-Jenab, V. Estrogen alters baseline and inflammatory-induced cytokine levels independent from hypothalamic-pituitary-adrenal axis activity. *Cytokine*2015; 72(2):121-129.
27. Lee, Y.M., Cheng, P. Y., Hong, S. F., Chen, S. Y., Lam, K. K.,*et al*. Oxidative stress induces vascular heme oxygenase-1 expression in ovariectomized rats. *Free Radic. Biol. Med*. 2005; 39(1):108-117.
28. Yallampalli, C., Dong, Y. L. Estradiol-17b inhibits nitric oxide synthase (NOS)-II and stimulates NOS-III gene expression in the rat uterus. *Biol. Reprod*.2000; 63:34-41.
29. Yilmaz, O.,Yagci, A.,Ulutas, E.,Sevimli, A.,Altunbas, K.,*et al*.The effect of 17β estradiol on the expression of eNOS and iNOS in ovariectomized rat uterus.*Eurasian J. Vet. Sci*. 2013; 29(2):65-69.
30. Ogando, D., Farina, M., Ribeiro, M. L., Perez Martinez, S.,Cella, M.,*et al*. Steroid hormones augment nitric oxide synthase activity and expression in rat uterus. *Reprod.Fertil. Dev*. 2003; 15(5):269-274.
31. Hassouna, A.,Obaia, E.,Marzouk, S.,Rateb, M.,Haidara, M.The role of sex hormones in induced-systemic inflammation in female albino rats.*Acta. Physiol. Hung*. 2014; 101(1):112-127.
32. Buhimschi, I.A.,Yallampalli, C.,Buhimschi, C. S.,Saade, G. R., Garfield, R. E. Distinct regulation of nitric oxide and cyclic guanosine monophosphate production by steroid hormones in the rat uterus. *Mol. Hum. Reprod*. 2000; 6:404-414.
33. Al-Hijji, J., Larsson, I.,Batra, S. Effect of ovarian steroids on nitric oxide synthase in the rat uterus, cervix and vagina. *Life Sci*. 2001; 69:1133-1142.
34. Engstrøm, T. The regulation by ovarian steroids of prostaglandin synthesis and prostaglandin-induced contractility in non-pregnant rat myometrium. Modulating effects of isoproterenol. *J. Endocrinol*. 2001; 169(1):33-41.
35. Karatepe, O.,Altiok, M.,Battal, M.,Kamali, G.,Kemik, A.,*et al*.The effect of progesterone in the prevention of the chemically induced experimental colitis in rats.*Acta Cir. Bras*.2012; 27(1): 23-29.
36. Da, Y.,Niu, K., Wang, K., Cui, G., Wang, W.,*et al*. A comparison of the effects of estrogen and *Cimicifuga racemosa* on the lacrimal gland and submandibular gland in ovariectomized rats. *PLoS One*2015; 10(3):e0121470.
37. Xue, X.T., Kou, X. X., Li, C. S., Bi, R. Y., Meng, Z.,*et al*. Progesterone attenuates temporomandibular joint inflammation through inhibition of NF-κB pathway in ovariectomized rats. *Sci. Rep*. 2017; 7(1):15334.