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Short- and long-term effects of lipopolysaccharide-induced endotoxemia on mice ovarian tissue: histomorphometrical evaluation

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ABSTRACT

Endotoxemia is an acute systemic reaction of the body caused by the presence of endotoxins such as bacterial lipopolysaccharide in the blood, which is associated with clinical signs. Lipopolysaccharide is a part of the outer membrane of the cell wall of gram-negative bacteria. The aim of the present study was to determine the effect of E-coli lipopolysaccharide administration on histomorphometric changes in mice ovarian tissue. Adult female mice were randomly divided into the control and treatment groups, with ten mice in each. The treatment group received 6750 µg/kg of lipopolysaccharide intraperitoneally and in the control group, normal saline was injected at the same dosage. Five mice from each experimental group were euthanized on days 3 & 30 after the beginning of the treatment, then the right ovary was removed, fixed and serially sectioned for histomorphometric evaluation. The results obtained showed that 3 days after lipopolysaccharide injection the estimated ovarian parameters, including primary follicles, secondary follicles and corpora lutea, had decreased significantly ($P \leq 0.05$). The mean number of antral follicles was not influenced by lipopolysaccharide injection on days 3 and 30. The mean number of primary follicles on day 30 (53.4 ± 4.6) showed a significant increase in comparison with the treatment group on day 3 (42.1 ± 4.1) which was close to its values in the control group. The mean number of secondary follicles and corpora lutea on day 30 (36 ± 4.8 and 34.2 ± 10.8, respectively) showed a relative improvement, however it was still lower than the control group counterparts (49.5 ± 5.0 and 39.2 ± 3.9, respectively). According to our results, endotoxemia induced by lipopolysaccharide has short-time deleterious effects on ovarian follicles but they recovered somewhat after a short time equal to a folliculogenesis cycle, from primordial follicle to preovulatory antral follicle.

Key words: lipopolysaccharide; endotoxemia; ovary; mice; histopathology

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List of abbreviations:
ANOVA - analysis of variance; BW - body weight; *E. coli* - *Escherchia coli*; H&E - hematoxylin and eosin; IL - interleukin; LH - luteinizing hormone; kDa - kilo Dalton; LPS - lipopolysaccharide; LSD - least significant deference; PIP3 - phosphatidylinositol 3,4,5-triphosphate; PTEN - phosphatase and tensin homolog; TNF - tumor necrosis factor; TGF - tumor growth factor; TLR - toll like receptor

Introduction

The most frequent consequence of infection with gram negative bacteria may be endotoxemia. Endotoxemia is an acute systemic immune system response to lipopolysaccharide (LPS), a large molecule that is present in the gram negative bacteria's outer membrane (RAETZ and WHITFIELD, 2002). There is a great deal of concern about the effect of an acute invasion of endotoxemia on the next generation if the infection occurs at any stage of the reproductive cycle in males and females. Several studies have demonstrated that an LPS challenge affects reproductive function profoundly. Treatment with LPS in male rats induced oxidative stress damage to mitochondria in the testicles and eventually led to impairment of spermatogenesis (ALY et al., 2012). In our previous work, we demonstrated that endotoxemia has long-term destructive effects on spermatogonia and the later stages of germ cells (JAFARI et al., 2018).

Reproduction in female animals has a distinct aspect whereby there is a mass of germ cells in the ovary without any proliferation during the lifetime. Any perilous insult after birth could create a permanent pathological change in the female gametes. The female ovarian cycle includes a complex process of growth and differentiation, phagocytosis, apoptosis, and atresia. Some of these structural ovarian procedures, such as ovulation and luteolysis, involve leukocytes (BOWEN et al., 1999; BUKULMEZ and ARICI, 2000) Interleukin-1β, interleukin-6, interferon-γ, and TNFα, which are examples of pro-inflammatory cytokines are released from leukocytes, as a result of LPS binding to its receptors. This phenomenon initiates an unregulated immune cascade that can cause detrimental pathological changes in multiple organs (ZHOU et al., 2011) and this can change the normal function of the hypothalamic-hypophyseal-ovarian axis. LPS may affect sex steroid hormone secretion from both theca-interstitial and granulosa cells (TAYLOR and TERRANOVA, 1996: MAGATA et al., 2014) and thus, it may induce ovarian dysfunction by affecting the functions of the follicular cells. LPS treatment during pro-estrus significantly increases atresia and apoptosis in the follicles of rats (BESNARD et al., 2001). However, few studies have investigated the effects of bacterial LPS on adult mice ovarian tissue. Therefore, the aim of the present study was to determine the short and long-term effects of bacterial LPS administration on histomorphometric changes in mice ovarian tissues.

Materials and methods

Animals. A total of 20 healthy adult female NMRI mice (aged 6-8 weeks, 27-31 g) were housed under controlled conditions of light (lights on 06.00 - 18.00 hours) and temperature (22 ± 2 °C) and had free access to food (pellet form), Javaneh Khorasan Co., Mashhad, Iran) and water at the Laboratory Animal House of the Veterinary Faculty of Shahid Bahonar University of Kerman, Iran. All the investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals.

Study design. The mice were randomly assigned to either the control or treatment group with ten mice in each group. To evaluate the short and long-term effects of LPS on ovarian tissue changes, ten mice from the treatment group were intraperitoneally inoculated with 6750 µg/kg BW of LPS based on our previous study, that showed reversible endotoxemia without killing the animals at this dose (JAFARI et al., 2018). Saline-administered mice served as the control group. Five mice from each experimental group were sacrificed at 3 and 30 days following LPS inoculation, and their right ovaries were removed and used for histopathological evaluations.

Histopathological procedures. All ovarian specimens were fixed in Bouin's solution, embedded in paraffin wax, serially sectioned at 5 µm thicknesses, stained with hematoxylin and eosin (H&E), and studied by a light microscope blindly by an expert pathologist (Nikon, Digital Sight DS-Fi2, Japan). The differential structural count was gathered from every 20th section to provide a 5% sample selection per ovary (BUCCI et al., 1997). Each ovary yielded approximately 600 sections and approximately 30 sections per ovary were counted. The number of different classes of follicles and corpora lutea in each sample was recorded. Types of follicle were based on the JUNQUEIRA et al. classification (MESCHER, 2013). Briefly: "Primary" follicles showed a single layer of cuboidal granulosa cells; "Secondary" follicles were surrounded by more than one layer of cuboidal granulosa cells and accumulations of follicular fluid appeared between the granulosa cells. "Antral" follicles were characterized by a central oocyte enriched by a fluid-filled space and bordered by hundreds of layered granulosa cells. In addition, structural normality (follicle and stromal cell morphology, even distribution of granulosa cells, intact theca and appearance of oocytes) was evaluated.

Statistical analysis. Data were subjected to analysis by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All data were tested for homogeneity of variances by the Levene static test. Evaluation of the significance of differences between the experimental groups was performed using one-way analysis of variance (one-way ANOVA) followed by the least significant difference test (LSD) for multiple comparisons when the variances were homogeneous, otherwise Tamhane's test was used as *post hoc*. Values were expressed as mean ± SEM. The significance considered level was $P<0.05$.

Results

Morphometry findings. The mean number of different types of follicles and corpus luteum in each experimental group on days 3 and 30 after LPS-induced endotoxemia are presented in Table 1. The short-term response to endotoxemia on day 3 following the injection of LPS reduced all of the evaluated ovarian parameters except for antral follicles. The number of primary follicles was significantly decreased on day 3 following LPS inoculation in comparison to the control group (26.8 ± 3.3 *vs.* 42.1 ± 4.1, P = 0.021, respectively) but it completely recovered 30 days after endotoxemia (53.4 ± 4.6 *vs.* 42.1 ± 4.1, respectively). Similar results were observed for secondary follicles (29 ± 1.9 *vs.* 49.5 ± 5, P = 0.012, respectively) and corpora lutea (17.6 ± 4.3 *vs.* 39.2 ± 3.9, P = 0.027, respectively) on day 3 after LPS injection with complete recovery on the 30th day.

Table 1. The mean ± SEM number of different classes of follicles and corpus luteum in the control and treated groups on days 3 and 30 following endotoxemia

Experimental groups	No. of mice	Primary follicle	Secondary follicle	Antral follicle	Corpus luteum
Control	10	42.1 ± 4.1^{a}	49.5 ± 5.0^{a}	41.5 ± 7.5^{a}	39.2 ± 3.9^{a}
LPS 3	5	26.8 ± 3.3^{b}	29 ± 1.9^{b}	36.2 ± 5.9^{a}	17.6 ± 4.3^{b}
LPS 30	5	53.4 ± 4.6^{a}	36 ± 4.8^{ab}	42.8 ± 4.8^{a}	34.2 ± 10.8^{ab}

Control - Specimens from the control group obtained on days 3 and 30. LPS 3 - Specimens from LPS-treated mice obtained on the day 3. LPS 30 - Specimens from LPS-treated mice obtained on the day 30. $^{a, b}$ Different alphabetic letters in each row, show a significant difference (P<0.05) between the control and treatment groups for each individual day.

Histopathological findings. Normal ovarian structures, including primary, secondary and antral follicles with corpora lutea were observed in the control group (Fig. 1).

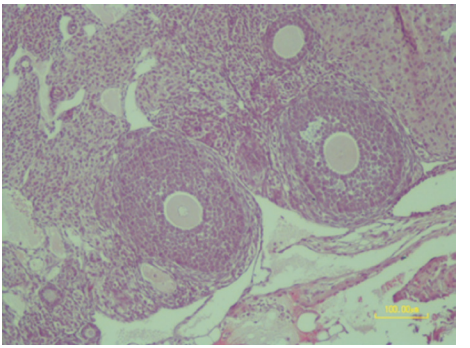

Fig. 1. Control group: Normal structure of ovary with primary follicles, secondary follicles, and corpora lutea

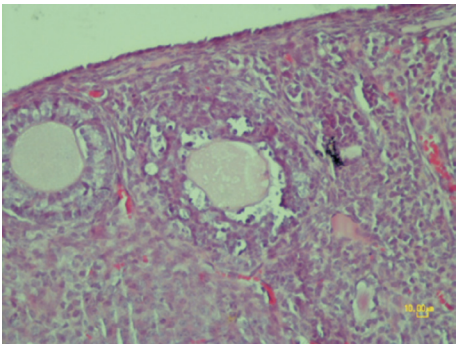

Fig. 2. Three days after LPS injection, secondary follicles show degenerative changes including oocyte shrinkage and some spaces between granulosa cells

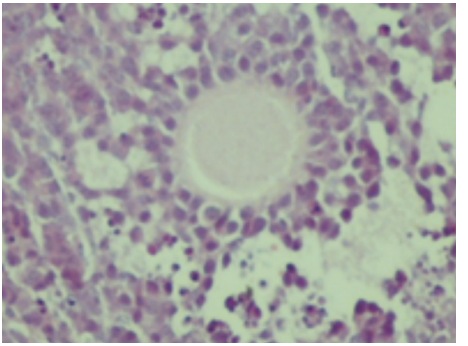

Fig. 3. Three days after LPS injection, apoptotic granulosa cells are seen in the spaces between granulosa layers

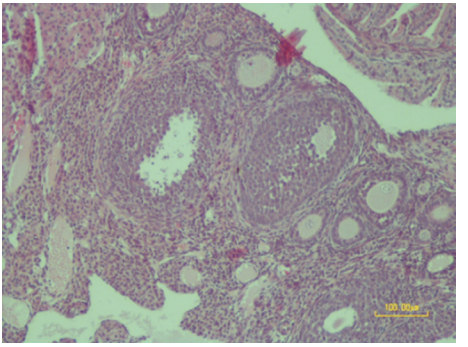

Fig. 4. 30 days after LPS injection. An atretic follicle with apoptotic granulosa cells and lack of oocyte is present. Other follicles have normal structure

After 3 days from the LPS injection, the number of all follicles decreased. Several atretic follicles were dispersed in the ovarian parenchyma and their nuclei had degenerated. Detached granulosa cells were seen in the antrum of follicles. Some hollow spaces were created between the granulosa layers, so dead granulosa cells were seen within these spaces. Some granulosa cells showed apoptotic changes (Fig. 2 and 3). Luteal cells in corpora lutea were vacuolated.

Thirteen days post LPS injection, the ovarian damage improved and degenerative changes were seen to a lesser extent than on day 3. A few atretic follicles, as well as different types of normal follicles, were present (Fig. 4). The cellular content of the corpora lutea was normal at 30 days after LPS injection.

Discussion

The present study has prompted to investigate the short and long-term effect of LPS-induced endotoxemia on ovarian tissue structure. The effect of LPS on female reproductive system has been evaluated by several studies (BESNARD et al., 2001; WILLIAMS et al., 2008; BROMFIELD and SHELDON, 2013; MAGATA et al., 2014; YOO and LEE, 2016; SHIMIZU et al., 2017), but none of them have assessed the impact of LPS on histopathological changes to the ovary and folliculogenesis procedure in mice. The dose of LPS used for the induction of endotoxemia was an important point and differed in our study. Based on our previous study we used a dose of 6750 µg/kg for induction of reversible endotoxemia without killing the animals (JAFARI et al., 2018). BROMFIELD and SHELDON (2013), BESNARD et al. (2001), DEB et al. (2004), YOO and LEE (2016) used doses of 3000, 400, 250 and 50 µg/kg, respectively, in their reports. However, we found endotoxemia induced by LPS at a dose of 6750 µg/kg is similar to that occurring naturally so our evaluation of ovarian changes would be more reliable.

In the present study, LPS treatment led to a significant decrease in the number of primary follicles on day 3 following endotoxemia. This result may be due to a disturbance in the process of the growth of the primordial follicles into primary follicles, or the degeneration of the ongoing primordial follicles. In the female gonads of mammals and rodents, all gametes or oocytes are stored in the ovaries as inactive primordial follicles (RODGERS and IRVING-RODGERS, 2010). Although some reports have demonstrated follicular renewal in postnatal and adult ovaries which occurs in some strains of female mice (KERR et al., 2006), primordial follicles may be affected by environmental insults and this may lead to future reproductive disorders. It is well known that good quality oocyte production and subsequent good fertility depends on exact primordial follicle activation and development, along with the true regulation of the oocyte, and granulosa cell communication and proliferation (MATZUK et al., 2002). In ruminants, the process of converting primordial follicles to antral follicles takes approximately 150 to 200 days (SCARAMUZZI et al., 2011). So, events around the time of follicular development,

including severe endotoxemia, might impact preantral follicle development, which would reduce the likelihood of conception several weeks later. Follicular development from primordial follicles to preovulatory antral follicle takes less than 30 days in rodents (PETERS, 1969). Therefore, this would be a useful opportunity for investigating the effect of detrimental factors such as endotoxemia on the complete follicular growth process. BROMFIELD and SHELDON (2013) demonstrated that the presence of LPS in the tissue culture medium of the bovine ovarian cortex is able to activate primordial follicle development, which enhances the number of primary follicles. To activate primordial follicles, the concentration of phosphatidylinositol 3,4,5-triphosphate (PIP3) should be increased, until this phenomenon leads to Akt/phosphokinase-B (AKT/PKB) pathway activation (KALOUS et al., 2006). Phosphatase and Tensin homolog (PTEN) and the FOXO3 transcription factor are the major regulators of the AKT/PKB pathway (CASTRILLON et al., 2003; JAGARLAMUDI et al., 2009). In the mouse, PTEN maintains reduced levels of PIP3 in the follicles and restrains the follicles in a quiescent state, so effectively preventing the activation of the follicles (REDDY et al., 2008). Administration of LPS reduces the expression of PTEN protein genes, which increases the amount of PIP3 and eventually activates the primordial follicles (BROMFIELD and SHELDON, 2013). Also, previous *in vitro* reports have shown that primordial follicle activation is enhanced by decreasing intracellular amounts of FOXO3 following addition of LPS to the culture medium including bovine ovarian tissue (BAO et al., 2011; BROMFIELD and SHELDON, 2013). However, the findings from these studies contradict the results obtained in the present study, which may be due to the differences in *in vitro* and *in vivo* conditions.

In the present study, 3 days after the induction of endotoxemia by LPS, the number of secondary follicles also decreased significantly. This result may be due to the continuing disorder in the process of folliculogenesis and the conversion of primary follicles to secondary follicles. Regarding the fact that the number of antral follicles did not significantly differ between the treatment groups involved in acute endotoxemia and the control group, it may be concluded that the process of development of secondary follicles into antral follicles can be continued in the presence of high levels of LPS. BROMFIELD and SHELDON (2013) demonstrated that preantral follicles can undergo normal follicular growth in a medium containing LPS without any pathological changes and are in fact resistant to the effects of LPS.

In the present study, the number of corpus luteum in endotoxemic mice on day 3 after LPS injection had decreased. This result was due to the reduced rate of ovulation in the LPS treated groups compared with the control group. SUZUKI et al. (2001) showed that administration of LPS in heifers during the period of proestrus induces a delay in ovulation by reducing the amount of estradiol, followed by a decrease in LH pulses. It is well-known that LPS reduces the production of estradiol by granulosa cells in the antral

follicles, and the positive feedback of estradiol is the main prerequisite for the secretion of LH and subsequent ovulation (HERATH et al., 2007). LPS also has negative effects on the hypothalamus-pituitary-ovary axis which are at the level of neurons in the brain (HERMAN et al., 2010). In the brain, LPS reduces GnRH secretion by releasing suppressor factors of GnRH-releasing neurons, such as opioid and GABA (YOO and LEE, 2016). It has been shown that LPS administration in rats blocks the increase of LH levels due to the administration of naloxone (opioid antagonist)(HE et al., 2003). In another study, it was shown that the administration of LPS blocks the LH surge in response to the positive feedback of estradiol by increasing the glutamic-decarboxylase-67 enzyme (AKEMA et al., 2005). KARSCH et al. (2002) found that LPS administration in ovariectomized ewes can reduce the concentration of GnRH in the hypothalamic-pituitary portal vein, and also suppress the response of pituitary gonadotrophic cells to the GnRH.

Administration of LPS leads to an acute inflammatory reaction due to activation of the immune system, resulting from binding of LPS to its receptors on leukocytes. Following this reaction, a large amount of pro-inflammatory cytokines, such as interleukin-6, interlukin1β, TNFα, interferon-γ and TGFβ, are released (ZHOU et al., 2011). One of the main receptors that the LPS through its attachment initiate a cascade process of inflammatory mediators release within immune cells, is a TLR4 receptor (BEUTLER, 2004). A basement membrane surrounds the granulosa cells and blocks the leukocytes' entry into these cells (PETROVSKÁ et al., 1996). However, molecules with molecular weights less than 850 kDa, including LPS, have the ability to cross the block and enter the follicle (RODGERS et al., 1999). On the other hand, it has been shown that TLR4 receptors exist on granulosa cells, and LPS binding to these surface receptors may deteriorate follicular growth and reduce estradiol secretion by granulosa cells (SHELDON et al., 2002). Inflammatory mediators, such as IL1β and TNFα that are released into the systemic blood circulation as a response to binding LPS to their receptors on the surface of the immune cells, interfere in the ovulation process and also decrease the aromatase gene expression in granulosa cells and thus reduce the concentrations of estradiol (GHERSEVICH et al., 2001). HERATH et al. (2007) observed that LPS binding to its receptors on the surface of granulosa cells did not increase the inflammatory mediators of IL1α and TNFα, and therefore concluded that LPS directly disturbed the production of estradiol in granulosa cells. They also found that addition of LPS to the antral follicle culture did not affect the granulosa cell count. This is contrary to the results observed in other studies, which showed that administration of LPS strongly stimulated programmed cell death in granulosa cells (PEREZ and TILLY, 1997; BESNARD et al., 2001). BROMFIELD and SHELDON (2011) observed that addition of LPS to a culture medium containing follicles induced an inflammatory response in granulosa cells by increasing the levels of IL-8 and IL-6. IL-6 is one of the first regulators of an inflammatory response in the body, thus it plays a crucial role in the ovaries (GERARD et al. 2004). A temporary increase in IL-6 in the follicular phase regulates the maturity of the relationship between

oocytes and cumulus cells, oocyte maturation, ovulation, and the formation of corpus luteum (LIU et al., 2009). Aggregation of leukocytes at the site of the infection is caused by IL-8 and although its role in the ovary is not well known, it seems to have a role in the formation and function of corpus luteum through the accumulation of leukocytes in the ovulation site (ARICI et al. 1996; MURAYAMA et al., 2010). It seems that LPS, by increasing IL-8 to a level higher than that observed in the normal follicular phase causes an adverse inflammatory reaction in the ovarian tissue, and also the increase in IL-6 levels may disrupt oocyte maturation. In the present study, on the third day after LPS administration, evidence was observed of cell death in granulosa cells, accompanied by the onset of atresia in some antral follicles, which, based on the above, could be due to inflammatory mediator secretion induced by LPS injection, and/or the direct effects of LPS binding to their receptors on granulosa cells.

Conclusion

In this study, we were able to prove that the destructive effects of acute LPS induced endotoxemia on the process of folliculogenesis and ovulation, which was clearly visible on the third day after LPS induction, could be restored to a normal level over a period of time. This suggests that an acceptable source of primordial follicles resistant to the effects of acute endotoxemia is present in the ovary, and guarantees the continuation of the process of folliculogenesis. To the best of our knowledge, there are no studies that have considered the long-term effects of LPS on folliculogenesis in mice.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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SAŽETAK

Endotoksemija je akutna sistemska reakcija tijela uzrokovana prisutnošću endotoksina poput bakterijskih lipolisaharida u krvi, a popraćena je kliničkim znakovima. lipopolisaharidi su dio vanjske membrane gram-negativnih bakterija. Cilj ovoga istraživanja bio je odrediti učinak primjene lipopolisaharida *E. coli* na histomorfometrijske promjene u tkivu jajnika u miša. Odrasle ženke miša nasumično su podijeljene u kontrolnu i pokusnu skupinu od po deset jedinki. Životinje u pokusnoj skupini primile su 6750 µg/kg lipopolisaharida intraperitonealno, dok je kontrolnoj skupini na isti način aplicirana fiziološka otopina. Pet miševa iz svake skupine eutanazirano je 3. i 30. dana od početka pokusa, nakon čega im je uklonjen desni jajnik koji je fiksiran i izrezan za histomorfometrijsku procjenu. Rezultati su pokazali da su tri dana nakon injekcije lipopolisaharida promatrani pokazatelji jajnika – primarni i sekundarni folikuli te žuto tijelo – znakovito smanjeni ($P \leq 0,05$). Injekcija lipopolisaharida nije utjecala na prosječan broj antralnih folikula 3. i 30. dan. Prosječan broj primarnih folikula 30. dan (53,4 ± 4,6) pokazao je znakovit porast u usporedbi s pokusnom skupinom 3. dan (42,1 ± 4,1) što je približno vrijednostima u kontrolnoj skupini. Prosječan broj sekundarnih folikula (36 ± 4,8) i žutoga tijela (34,2 ± 10,8) 30. dana pokazao je relativno poboljšanje, no vrijednosti su ipak niže nego u kontrolnoj skupini (49,5 ± 5,0 i 39,2 ± 3,9). Prema našim rezultatima endotoksemija uzrokovana lipopolisaharidima ima kratkoročni štetan učinak na folikule jajnika što se ubrzo ispravlja u ciklusu folikulogeneze iz primordijalnog folikula u predovulacijski antralni folikul.

Ključne riječi: lipopolisaharidi; endotoksemija; jajnik; miševi; patohistologija