IJBMS

Original article

The Impact of Gender on the Inflammatory Parameters and Angiogenesis in the Rat Air Pouch Model of Inflammation

Tahereh Eteraf Oskouei*[1, 2], Nasrin Maleki-dizaji [3], Moslem Najafi[3, 4]

Abstract

Objective(s)

Air pouch is a well-established inflammatory model in which fluid extravasations; leukocyte migration, angiogenesis and other parameters involved in the inflammatory response can be measured. In this study, the influence of gender on inflammatory parameters has been examined in the air pouch model.

Materials and Methods

To induce air pouch, adult male and female Wistar rats were anesthetized, then 20 ml and 10 ml of sterile air were injected subcutaneously on the back on day 0 and day 3, respectively. On day 6, inflammation was induced by injection of 1 ml of carrageenan 1% into pouches. After 6 and 72 hr, the rats were sacrificed, pouch fluid was collected in order to determine exudates volume and the accumulated cells were counted using a hemocytometer. Pouches were dissected out and weighed. Angiogenesis of granulomatous tissue was assayed using hemoglobin kit.

Results

Analysis of our data demonstrated a sexually dimorphic pattern in inflammation parameters both in acute and chronic forms ($P<0.05$). The value of angiogenesis in the air pouch model in male rat was higher than that female rats ($P<0.001$).

Conclusion

The degree of inflammation and angiogenesis induced in Wistar rat air pouch model is gender-dependent, suggesting that gender may be a key consideration in the design of inflammation experiments.

Keywords: Air Pouch, Angiogenesis, Gender, Inflammation

1- School of Health and Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran
*Corresponding author: Tel: +98-411-3357580 - 83; Fax: +98-411-3340634; email: eteraf_t@yahoo.com
2- Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
3- Department of Pharmacology and Toxicology, School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
4- Research Center for Pharmaceutical Nanotechnology, School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

Introduction

In humans, a marked sexual dimorphism in the immune system is well established and is reflected in different immunological vulnerabilities of men and women (1). Recently, gender-specific differences in severity have been reported in a number of murine models of inflammation. Such findings have direct implications on the design and interpretation of experiments, and may also prove useful for investigating the nature of similar phenomena observed in patients (2).

The air pouch model has been extensively used for the study of various types of inflammation and inflammatory processes. This model has distinct advantages over other models of inflammation because of the ability to perform biochemical analysis of both the exudates and inflammatory cells together with the histological and angiogenesis analysis of the air pouch lining (3). The volume of fluid, cellular components and granulation tissue weight as well as angiogenesis in this animal model were hypothesized to have different sex-based patterns of development. Thus the current study was undertaken to examine the role of gender dimorphism in inflammatory response in the Wistar rats.

Materials and Methods

Animals

Fifty albino Wistar rats (200-250 g, Razi, Iran) were divided into 8 groups of at least 6 animals each. Rats in each experiment group were matched in age and weight. Rats were housed at constant temperature (20 ± 1.8 $^\circ$C) and relative humidity (50 ± 10 %) in standard polypropylene cages, six per cage, under a 12L: 12D schedule, and were allowed food and water freely. The rats were acclimatized to the laboratory for at least 1 hr before testing. All the procedures involving animals and their care were conducted in conformity with Tabriz University of Medical Sciences laws and policies.

Induction of air-pouch type inflammation by carrageenan in rats

To induce an air pouch, rats were lightly anesthetized with halothane, their back were shaved and swabbed with 70% ethanol, and 20 ml of sterile air was injected into the subcutaneous tissue of the back in the region of the clavicles to make an oval-shaped air pouch. After 2 days, the pouches were re-inflated with 10 ml of air. On day 6, inflammation was induced by injection of 1 ml of a 1% (w/v) suspension of Type1 Lambda carrageenan (Sigma, St. Louis, MO, USA) in saline into the air pouch under light halothane anesthesia (4). The carrageenan solution had been sterilized by autoclaving at 121 °C for 15 min and supplemented with antibiotics [0.1 mg of penicillin G potassium (Jaber Ebn-e-Hayyan, Iran) and 0.1 mg streptomycin sulfate (Jaber Ebn-e-Hayyan, Iran) per milliliter of the solution] after cooling to 40-45 °C (5).

Determination of pouch fluid volume, leukocyte infiltration, and granulation tissue weight

Six hr and three days after the injection of carrageenan solution, the rats were sacrificed by halothane overdose. The pouches were flushed with 3 ml of PBS, pH=7.4, and vigorously massaged for 30 sec. The pouches then were opened with a small incision and the exudates were collected and their volumes were measured. The leukocytes in the fluid were counted using a hemocytometer, and the formed granulation tissue was dissected and weighed (6).

Determination of angiogenesis in granulation tissue

Measurement of angiogenesis in granulation tissue was carried out according to the methods described by Ghosh *et al* (7) with slight modifications. The granulation tissue (formed, three days after the injection of carrageenan solution) was dissected and weighed. The dissected granulation tissue was washed in PBS, pH 7.4, and cut into small pieces with scissors before being homogenized in Drabkin reagent (Ziestchem Diagnostics, Iran) using a homogenizer (HO4 AP-Edmund Bühler, B. Braun, Germany) for 4 min at scale 40 on an ice bed. The tissue homogenate was centrifuged at 10,000 g and 4 °C for 30 min. The supernatants were filtered through a 0.22 μm filter (Millipore). The hemoglobin concentration in the supernatant was then

determined spectrophotometrically by measuring absorbance at 540 nm using a hemoglobin assay kit (Hemoglobin Colorimetric-method, Ziestchem Diagnostics, Iran). The amount of hemoglobin in the granulation tissue was expressed as mg hemoglobin/100 g wet tissue.

Statistical analysis

All results were expressed as mean±SEM. The statistical significant of the results was analyzed by the unpaired t-test with a *P*-value of <0.05 considered significant.

Results

Time changes of pouch fluid volume, number of Infiltrating leukocytes and granulation tissue weight

After injection of the carrageenan solution into the air pouch, the pouch fluid volume increased significantly after 6 hr and then markedly for the next 72 hr in female and male rats (Figure 1A and Figure 2A).
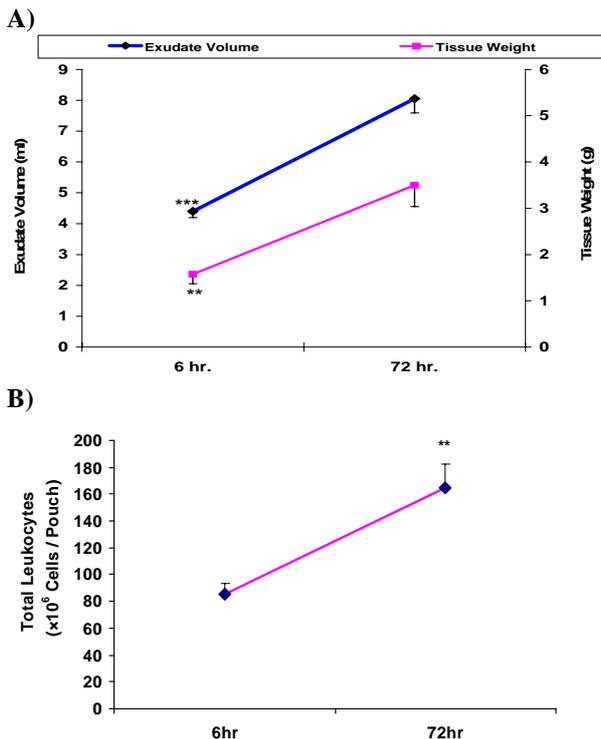
A)

B)

Figure 1. Time course of pouch fluid accumulation, total number of infiltrating leukocytes in the pouch fluid and granulation tissue weight in female rats. One milliliter of a 1% (w/v) carrageenan solution in saline was injected into the air-pouch. The pouch fluid volume, granulation tissue weight (A), the total number of leukocytes in the pouch fluid (B), were determined 6 and 72 hr after carrageenan injection. Values are the mean with SEM shown by vertical bars from six rats. **P<0.01, ***P<0.001.

The total number of infiltrating leukocytes in the pouch fluid was highest at hr 72 in female rats (Figure 1B). At the time of carrageenan injection, a very thin granulation tissue was formed in the subcutaneous tissue which was difficult to dissect quantitatively. Granulation tissue was dissectible at hr 6 and the wet weight was further increased at 72 hr later in female and male rats (Figure 1A and Figure 2A).
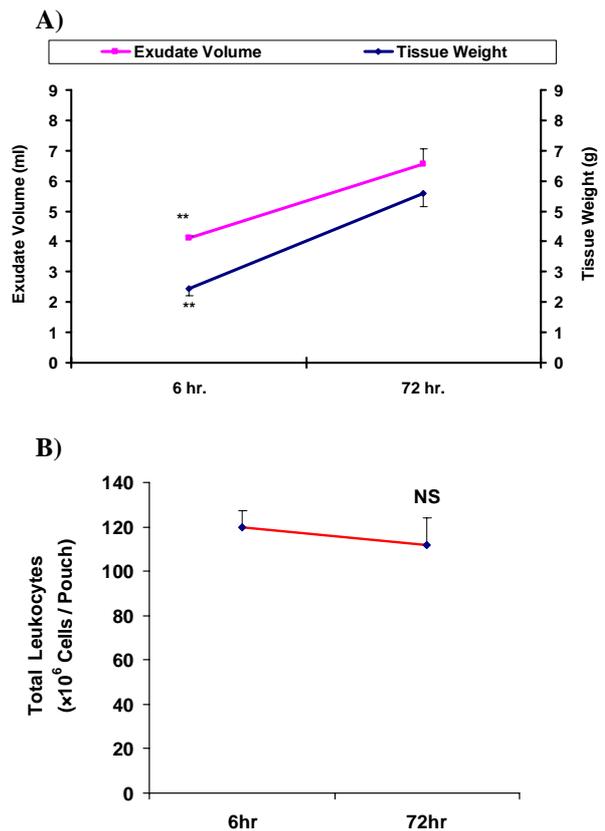
A)

B)

Figure 2. Time course of pouch fluid accumulation, total number of infiltrating leukocytes in the pouch fluid and granulation tissue weight in male rats. One milliliter of a 1% (w/v) carrageenan solution in saline was injected into the air-pouch. The pouch fluid volume, granulation tissue weight (A), the total number of leukocytes in the pouch fluid (B), were determined 6 and 72 hr after carrageenan injection. Values are the mean with SEM shown by vertical bars from six rats. NS *P*>0.05, **P<0.01, ***P<0.001.

Effects of gender on pouch fluid volume, number of infiltrating leukocytes and granulation tissue weight 6 hr after carrageenan injection

As shown in Figure 3A, 6 hr after carrageenan injection, the male rats had greater numbers (*P*<0.05) of leukocytes (119.64±7.8, n=6) than female rats (85.56±8.1, n=6). However, the

exudates volume of female rats was significantly ($P<0.01$) greater than male rats (Figure 3A). Figure 3B shows that the granulation tissue weight of male rats (2.64 ± 0.253 g, n=6) is significantly ($P<0.05$) greater than female rats (1.58 ± 0.2 g, n=6).
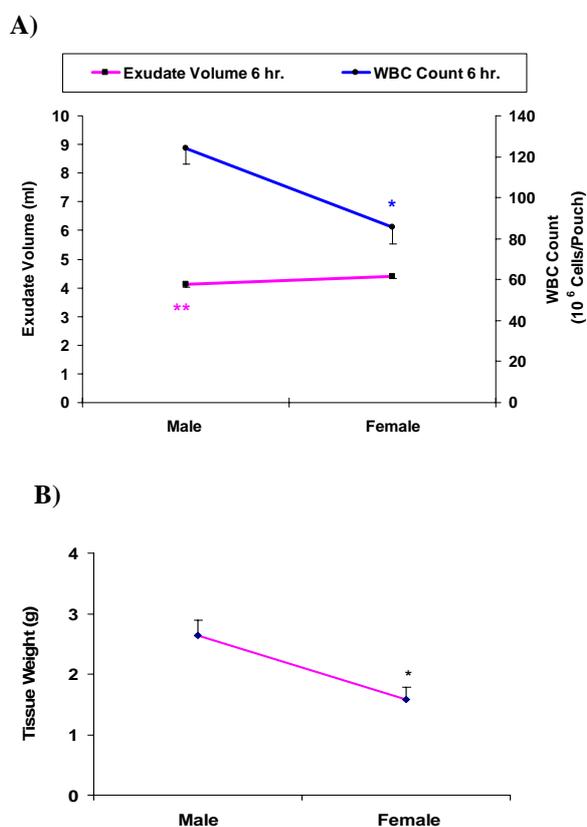
A)

B)

Figure 3. Effect of gender on pouch fluid accumulation, total number of infiltrating leukocytes in the pouch fluid and granulation tissue weight 6 hr after carrageenan injection. The total number of leukocytes, pouch fluid (A), and the granulation tissue weight (B) were determined 6 hr after carrageenan injection. Values are the mean with SEM shown by vertical bars from six rats. * $P<0 .05$, ** $P<0. 01$.

Effects of gender on pouch fluid volume, number of infiltrating leukocytes, granulation tissue weight and angiogenesis 72 hr after carrageenan injection

In three days postcarrageenan challenge, as shown in Figure 4, the female rats had greater numbers of leukocytes ($P<0.05$). The exudates volume of female rats (8.05 ± 0.45 ml, n=8) was significantly ($P<0.05$) greater than male rats (6.83 ± 0.44 ml, n=9). Referring to Figure 4B the granulation tissue weight of male rats (5.08 ± 0.43 g, n=9) was significantly ($P<0.05$)

higher than female rats (3.5 ± 0.46 g, n=6). The levels of hemoglobin in the granulation tissue of female rats were significantly ($P<0.001$) less than those in male rats 72 hr after carrageenan injection (Figure 4 B).
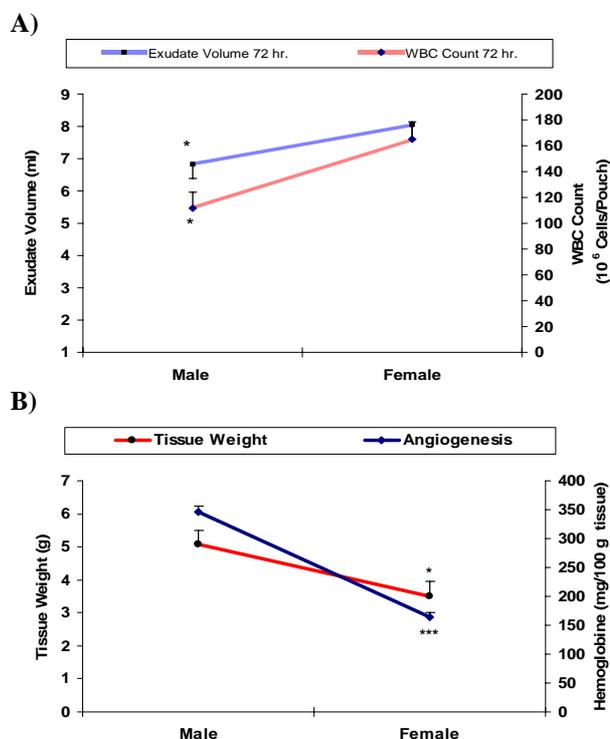
A)

B)

Figure 4. Effect of gender on pouch fluid accumulation, total number of infiltrating leukocytes in the pouch fluid, granulation tissue weight and hemoglobin value in granulation tissue 72 hr after carrageenan injection. The total numbers of leukocytes, the pouch fluid (A), the granulation tissue weight and hemoglobin values (B) were determined 72 hr after carrageenan injection. Values are the mean with SEM shown by vertical bars from six rats. *$P<0.05$, ***$P<0.001$.

Discussion

The results of this study indicated that after injection of the carrageenan solution into the air pouch, the pouch fluid volume, the total number of infiltrating leukocytes in the pouch fluid, distinct granulation of the pouch wall lining and angiogenesis increased significantly after 6 hr and then markedly for the next 72 hr. The inflammatory response of the air pouch has been shown to change dramatically due to the age of the air pouch (8). In Wistar rat we observed differences in the degree of inflammation induced in males compare to females. These findings may be in part consistent with those of Joe *et al* (2), who also reported intra-strain gender differences in

some, but not all tested strains in the volume of inflammatory exudates accumulated in air pouches of rats. Delano *et al* (9) reported that distinct sets of genetic loci are potentially associated with the degree of inflammation reached in male versus female mice. Based on these reports, the gender–related genetic loci controlling carrageenan-induced inflammatory responses may exist.

Crockett *et al* (10) reported that during the diestrous and metestrous stages, there is a marked increase in the number of leukocytes in the vaginal stroma and lumen of mice, which denotes the presence of an inflammatory response. If the mice were at either of these stages of their estrous cycle, it would account for the difference observed between males and females. These differences may be due to circulating estrogen in females, although the exact mechanisms by which estrogen exerts its effects are unknown. In addition, estrogen has been observed to enhance wound healing through an anti-inflammatory effect by limiting neutrophil and macrophage infiltration and suppressing the production of numerous cytokines including Tumor necrosis factor alpha (TNF-α), and macrophage migration inhibitory factor (11). Testosterone has been shown to increase TNF-α induced expression of E-selectin which regulates the extravasation and migration of neutrophils (12).

In addition, results from this study showed that both in 6 hr and 72 hr after inflammation induction the granulation tissue weight was greater in male rats than female rats. Hermes *et al* have shown that Sprague-Dawley male rats produced granulomas 48% larger by weight than those produced by females (13). The granulomatous tissue weight differences between sexes may be due to circulating sex hormones. If the progestogen had been deposited into the granuloma pouch at the same time as the croton oil or on the previous day, the membrane, compared with that induced solely by the croton oil would have been much thinner. Injected progestogen into the air sac produced no granulomatous membrane at all (14). In the chronic phase of a granuloma response to carrageenan, Interleukin-1 (IL-1) plays a primary role in

maintaining permeability of the vascular endothelium as well as in promoting wound healing through its effects on angiogenesis, fibroblast proliferation, and chemotactic effects on immune cells. Thus the difference between male and female rats can be explained in part by the significantly reduced IL-1 secretion present in the exudates, suggesting a link between sex dimorphism and the production of proinflammatory cytokines (13).

Angiogenesis contributes to the development and progression of various pathological conditions such as inflammatory diseases. Down-regulation of angiogenesis has been considered to be advantageous for the prevention of neoplastic growth and inflammation. Using an air pouch-type carrageenan-induced inflammation model in rats, we demonstrated that gender may influence angiogenesis in the developing chronic granulation tissue. The decrease in hemoglobin content in granulation tissue seemed to correlate with the decrease in the capillary density (7). Vascular endothelial growth factor, VEGF, has been shown to be a key mediating factor in the underlying cascade of chemical events leading to angiogenesis, which makes it a very important precursor molecule. There is evidence that VEGF baseline levels appear to be dependant upon the gender of the animal (15).

Taken together, these findings are consistent with our data, which demonstrate clear gender-dependence in the air pouch model of acute inflammation.

Conclusion

The results of this study clearly showed the degree of inflammation and angiogenesis induced in Wistar rat air pouch model is gender-dependent. It seems that gender may be considered as an important factor in the design of inflammation experiments. A better understanding of gender disparities may ultimately lead to new pharmacological targets for the treatment of inflammatory disorders.

Acknowledgment

References

1. Da Silva JA. Sex hormones and glucocorticoids: interactions with the immune system. Ann NY Acad Sci 1999; 876:102–117.
2. Joe B, Garrett MR, Dene H, Remmers EF, Meng H. Genetic susceptibility to carrageenan-induced innate inflammatory response in inbred strains of rats. Eur J Immunogenet 2003; 30:243–247.
3. Sedgwick AD, Moore AR, Al-Duaij A Y, Edwards JC, Willoughby DA. The mmune response to pertussis in the 6-day air pouch: a model of chronic synovitis. Br J Exp Pathol 1985; 4:455–464.
4. Gambero A, Becker TL, Gurgueira SA, Benvengo YHB, Ribeiro ML, Mendonca S, *et al*. Acute inflammatory response induced by Helicobacter pylori in the rat air pouch. FEMS Immunol Med Microbiol 2003; 38:193-198.
5. Ghosh AK,Hirasawa N,Niki H,Ohuchi K.Cyclooxygenase-2-mediated angiogenesis in carrageenin-induced granulation tissue in rats. J Pharmacol Exp Ther 20002; 95:802-809.
6. Colville-Nash P, Lawrence T. Air-pouch models of inflammation and modifications for the study of granuloma-mediated cartilage degradation. In: Winyard PG,Willoughby DA.Methods in Molecular Biology. London: The William Harvey Research Institute; 2003.p.181-189.
7. Ghosh AK, Hirasawa N, Ohtsu H. Defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice. J Exp Med 2002; 195: 973-982.
8. Martin SW, Stevens AJ, Brennan BS, Davies D, Rowland M, Houston JB. The six-day-old rat air pouch model of inflammation: characterization of the inflammatory response to carrageenan, J Pharmacol Toxicol Methods 1994; 32:139-147.
9. Delano Dl, Montesinos MC, D'Eustachio P, Wiltshire T, Cronstein BN. An interaction between genetic factors and gender determines the magnitude of the inflammatory response in the mouse air pouch model of acute inflammation. Inflammation 2005; 29:1.
10. Crockett ET, Spielman W, Dowlatshahi Sh , He J. Sex differences in inflammatory cytokine production in hepatic ischemia-reperfusion. J Inflamm 2006; 3:16.
11. Sinha I, Cho BS, Roelofs KJ, Stanley JC, Henke PK, Upchurch GR. Female gender attenuates cytokine and chemokine expression and leukocyte recruitment in experimental rodent abdominal aortic aneurysms. Ann N Y Acad Sci 2006; 1085:367-379.
12. Barker LA, Dazin PF, Levine JD, Green PG. Sympathoadrenal-dependent sexually dimorphic effect of nonhabituating stress on in vivo neutrophil recruitment in the rat. Br J Pharmacol 2005; 145:872-879.
13. Hermes GL, Rosenthal L, Montag A, McClintock MK. Social isolation and the inflammatory response: sex differences in the enduring effects of a prior stressor. Am J Physiol Regul Integr Comp Physiol 2006; 290:R273-282.
14. Lindhe J, Sonesson B.The effect of sex hormones on inflammation. I. The effect of a progestogen. J Periodontal Res 1966; 1:212-217.
15. Kemp SW, Reynolds AJ, Duffy LK. Gender differences in baseline levels of vascular endothelial growth factor in the plasma of alaskan sled dogs American. J Biochem and Biotech 2005; 2:111-114.