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Comparative gender differences in local and systemic concentrations of pro-inflammatory cytokines in rats with experimental periodontitis

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Background and Objective: There have been few studies of gender differences in response to periodontitis. Thus, we compared gender-specific differences in systemic cytokine concentrations in rats with and without ligature-induced periodontitis.

Material and Methods: Experimental periodontal disease was initiated in Sprague–Dawley rats by placing a ligature around the crowns of the second right maxillary molar tooth. Sham-operated control groups were also created. Two weeks later, the right and left maxillary quadrants of teeth, liver and serum were collected from all the rats, and uterine horns were collected from the female rats. Liver and uterine samples were ground in phosphate-buffered saline (10 mg of tissue/mL of phosphate-buffered saline + protease inhibitor) containing a protease inhibitor, and cytokine concentrations were determined by enzyme-linked immunosorbent assay. Digital radiographs were made of maxillary quadrants, and the distance from cemento–enamel junction to alveolar crest was measured using image analysis software. Data were compared by factorial analysis of variance and a post-hoc Tukey test.

Results: Female rats with ligatures had greater, but not significantly different, alveolar bone loss than males with ligatures. However, they had higher serum concentrations of interleukin-6, tumor necrosis factor-α and C-reactive protein, and liver C-reactive protein ($p < 0.05$). These females also had higher interleukin-6, tumor necrosis factor-α and vascular endothelial growth factor concentrations within the uterine horn, compared to female controls ($p < 0.05$). Male animals with ligatures had lower serum concentrations of C-reactive protein and higher interleukin-6 and tumor necrosis factor-α concentrations within serum, compared to male controls ($p < 0.05$).

Conclusion: Our study suggests that females with periodontal disease have a greater risk for inflammatory-based systemic diseases than males.

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There is general agreement that immune mediators originating from a site of either infection or severe trauma activate acute-phase protein synthesis and secretion by hepatocytes, which are then released into the systemic circulation (1). Most acute-phase proteins are produced by hepatocytes; however, some are synthesized by other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes (1). The release of acute-phase proteins into the systemic circulation is termed the 'acute phase response', and is the usual initial reaction to a bacterial infection (1–3). Interleukin-6 is an important mediator of inflammation and is an important stimulus for the acute phase response (4,5). Interleukin-6 induces the hepatic synthesis of C-reactive protein, a potent pro-inflammatory mediator. The acute phase response is a risk factor for several systemic diseases, including cardiovascular disease, and there is a reported association of interleukin-6, C-reactive protein and cardiovascular disease among healthy men and women (6–9).

There is substantial evidence that sites of gingivitis and periodontitis elicit an acute phase response (10–26), as these sites are a port of entry for oral microorganisms, which initiate the release of pro-inflammatory cytokines into the surrounding tissues and into the systemic circulation. The serum concentrations of both interleukin-6 and C-reactive protein show a dose-dependent response to the severity of periodontal inflammation; that is, their concentrations become higher with more extensive disease (16,23,25,27–29). These events increase the risk for cardiovascular disease (30–32).

Inflammation has been reported to affect men and women differently. In general, women produce a more vigorous cellular and humeral immune response to antigens than men, whereas men produce a more intense inflammatory response to a microbial stimulus (33). However, information concerning the specific gender response to inflammation is conflicting. Recent studies of the gender-specific response to inflammation have utilized a sterile endotoxin challenge. One human study reports that women have a greater increase than men in serum levels of C-reactive protein, tumor necrosis factor-α and interleukin-1β, and no differences in interleukin-6 and interleukin-10 (34). Another study, using identical methodology, reported no differences between men and women in the serum concentrations of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-10 following challenge by sterile endotoxins (35). Similar studies in animals report that females have lower serum levels of these inflammatory cytokines than males (33,36,37).

There is little information available concerning gender differences in serum pro-inflammatory cytokine concentrations in persons with periodontal disease. Gender differences in the incidence and severity of inflammatory disease have been reported, suggesting that estrogens may modulate the inflammatory response (38).

Women have a 30% lower innate immune response (33) and female animals are more likely to develop a T helper 1-type response after exposure to an infectious agent (39). There are reports of gender differences in the relationship among periodontal disease, tooth loss and atherosclerosis, with men having more evidence of periodontal infections and atherosclerosis (40–42).

Therefore, there is little information concerning the biological mechanisms for gender-specific differences in the acute phase response to periodontal disease. Rats have been used in periodontal disease studies because the periodontium of their molar teeth is similar to that of humans (43). Periodontitis can be initiated in these animals using a ligature that acts as a gingival irritant and a site for bacterial colonization (44) and results in gingival inflammation (and subsequent alveolar bone loss) at that site. Animal experiments have reported significant circulating levels of C-reactive protein following ligature-induced periodontitis (19), which was down-regulated by the administration of anti-inflammatory cytokines, including interleukin-10, interleukin-4 and transforming growth factor-α (2). However, there is little information concerning gender-specific differences in other aspects of the acute phase response coincident to periodontitis. The objective of this study was to compare these potential differences using the ligature model of periodontal disease in male and female rats.

Material and methods

This study was approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

Induction of inflammatory periodontal disease

Age-matched male and female Sprague–Dawley rats were weighed and then anesthetized with a 4:1 solution of ketamine/xylazine at a dose of 0.15 mL/100 g body weight. Then, a ligature was placed around the cervix of the right second maxillary molar

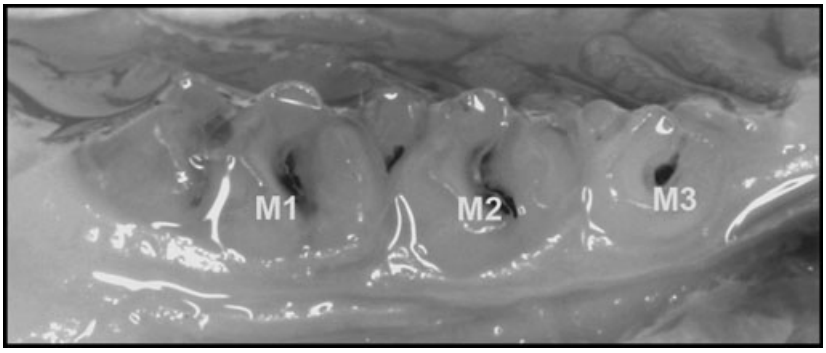

Fig. 1. Right quadrant of the maxillary molar teeth before treatment with ligatures (M1 is the first molar, M2 is the second molar and M3 is the third molar).

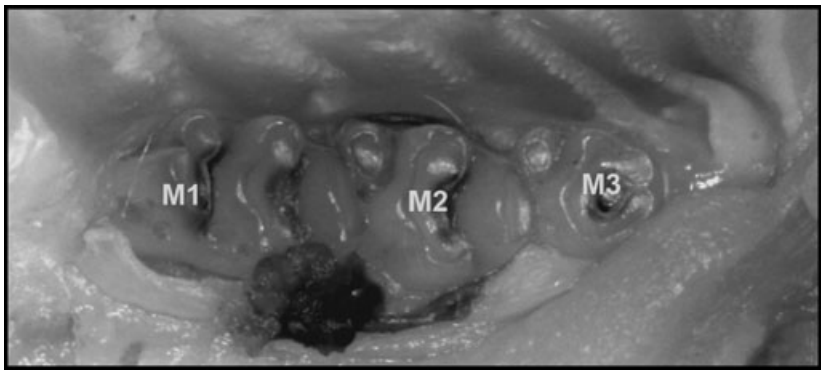

Fig. 2. Right quadrant of the maxillary molar teeth with the ligature in place. The ligature compresses the interdental gingival papilla between the first molar (M1) and the second molar (M2) and between M2 and the third molar (M3).

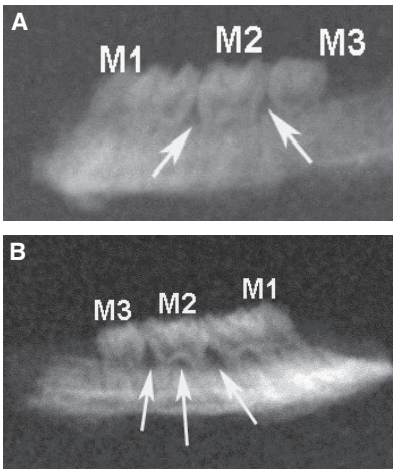

Fig 3. Digital radiograph of the right maxillary molar quadrant in female rats: (A) sham control and (B) experimental. Arrows indicate the crest of the interdental septum between the first maxillary molar tooth (M1) and the second maxillary molar tooth (M2) and between M2 and the third maxillary molar tooth (M3).

tooth (Figs 1 and 2). The ligature was knotted on the buccal side of the tooth. The contralateral left side was untreated and served as an unligated control. In addition, following general anesthesia, sham-operated male and female control groups were created by briefly passing the ligature between the mesial and distal contacts of the second right maxillary molar and then removing it. The rats were divided into four groups: females with ligatures ($n = 9$); sham-operated control females ($n = 8$); males with ligatures ($n = 10$); and sham-operated control males ($n = 10$). A power analysis of preliminary data indicated that our assays could detect significant differences between groups if each group had a minimum of seven animals. Rat chow and water were available to all animals *ad libitum*.

Tissue and serum collection

Two weeks later, the animals were killed and the right and left maxillary quadrants containing the molar teeth, the liver and serum were collected from all the rats. In addition, both uterine horns were collected from the female rats. The maxillary quadrants, the right uterine horn and the right lobe of the liver were fixed in Zamboni's fixative (45); the remaining tissues and serum were immediately frozen in liquid nitrogen and maintained at −80°C until analysis. The frozen liver and uterine horn samples were thawed and then ground in phosphate-buffered saline [containing a protease inhibitor (10 mg of tissue/mL of phosphate-buffered saline + protease inhibitor)] (Sigma Chemical Company, St Louis, MO, USA).

Protein assay

A standard bicinchoinic acid assay (Pierce Chemical Company, Rockford, IL, USA) was used for protein determination. The absorbance was read in a microplate spectrophotometer at 570 nm, and protein concentrations were calculated from a standard curve. Data were expressed as mg/mL.

Enzyme-linked immunosorbent assays

C-reactive protein, interleukin-6, vascular endothelial growth factor and tumor necrosis factor-α concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (C-reactive protein: ALPCO, Salem, NH, USA; and others: R & D Systems, Minneapolis, MN, USA). The absorbance of each well was read in a microplate spectrophotometer at 450 nm and the cytokine concentrations were calculated by reference to the standard curve included with each kit. Appropriate positive and negative controls were included with each test. Data were expressed as pg of cytokine/mg of protein.

Measurement of alveolar bone loss

Digital radiographs were made of each maxillary quadrant using electronic sensors (Schick Technologies, Long Island City, NY, USA) (Fig. 3). The maxillae were exposed at 65 kV and 10 mA at 12 impulses/s. The source to film distance was 50 cm, and an aluminum wedge was incorporated within each field to provide a radiographic linear standard.

Radiographs were analyzed by computerized histomorphometry using SIGMA SCAN PRO software (Systat, Chicago, IL). Measurements of alveolar bone loss were made using our previous techniques (46), without filters or other methods of image enhancement (except enhancement of the grey level). Measurements were performed between the molar M1 and M2 teeth and between the M2 and M3 teeth and the deepest extension of the bony defect on each side of the interdental septum by a blinded examiner. Projection errors were assessed for each radiograph by comparing the dimensions of the image of the density wedge on each radiograph with those of the actual wedge. An elongation, or foreshortening, factor was calculated for each radiograph and used to adjust the raw data.

Statistical analysis

Outcome data were compared by factorial analysis of variance and the

post-hoc Tukey test using SPSS v15 (SPSS, Chicago, IL, USA). Differences in group means were considered to be significant when the p-value was < 0.05.

Results

After 2 wk, both female and male rats with ligatures showed significantly greater alveolar bone loss at all sites examined compared with the sham control groups ($p < 0.05$) (Figs 4 and 5). Although alveolar bone loss in female rats was greater than in male rats at most sites, there were no significant differences in the alveolar bone loss between male and female rats with ligatures.

Female rats with ligatures had significantly higher serum concentrations of tumor necrosis factor-α, interleukin-6 and C-reactive protein (Fig. 6) and higher concentrations of liver C-reactive protein (Fig. 7), than female sham control rats and all male rats. The concentrations of these biomarkers were significantly higher within both groups of female rats than within both groups of male rats. Female rats with ligatures had significantly higher concentrations of interleukin-6, tumor necrosis factor-α and vascular endothelial growth factor within the uterine horn compared with sham controls (Fig. 8).

Male rats with ligatures had lower concentrations of C-reactive protein within the serum compared with sham controls (Fig. 6). Male rats with ligatures also had higher concentrations of serum interleukin-6 and tumor necrosis factor-α compared with sham controls (Fig. 6). There was no significant difference in the concentration of liver C-reactive protein in male rats between ligature and sham-operated groups (Fig. 7).

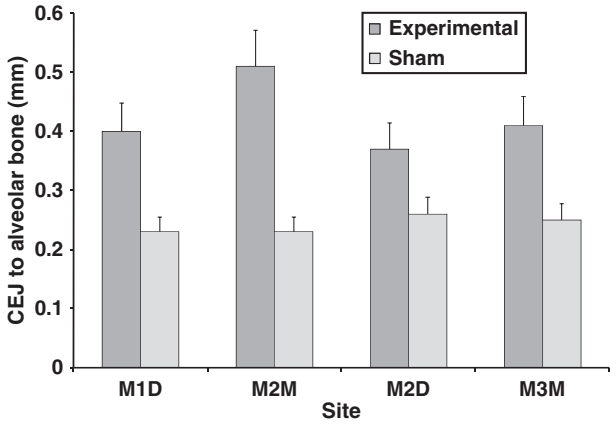

Fig. 4. Distance from the cemento–enamel junction (CEJ) to the deepest bone defect of the interdental septum in male rats 2 wk following either placement of ligatures (experimental) or sham-operation (mean ± standard error of the mean). Sites [molar teeth = the first molar (M1), the second molar (M2) and the third molar (M3); surface = M (mesial) or D (distal)] were all significantly greater in experimental rats than in sham-treated rats, $p < 0.05$.

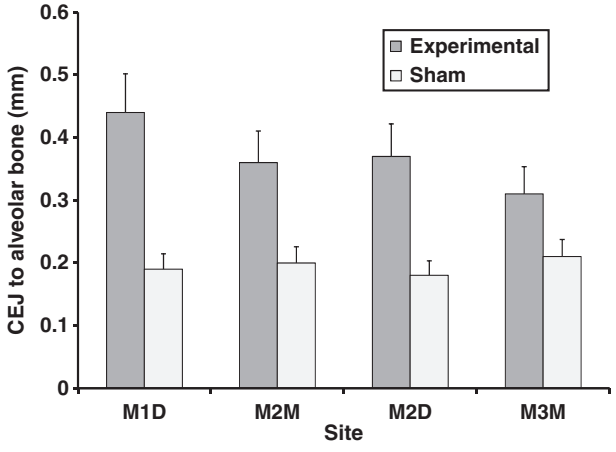

Fig. 5. Distance from the cemento–enamel junction (CEJ) to the deepest bone defect of the interdental septum in female rats 2 wk following either placement of ligatures (experimental) or sham-operation (mean ± standard error of the mean). Sites [molar teeth = the first molar (M1), the second molar (M2) and the third molar (M3); surface = M (mesial) or D (distal)] were all significantly greater in experimental rats than in sham-treated rats, $p < 0.05$.

Discussion

Our study indicated that all rats with ligatures had significantly greater alveolar bone loss and greater serum concentrations of C-reactive protein than controls, supporting the results of previous studies (19). Because no significant difference was found in alveolar bone loss between our male and female rats with ligatures, we assumed that the periodontal disease produced by the ligatures was of similar severity. However, we reported significant gender differences in the acute phase response in rats with experimental periodontitis, extending the results of previous studies.

Our data indicated that experimental periodontitis in female rats resulted in higher serum concentrations of C-reactive protein, interleukin-6 and tumor necrosis factor-α than experimental periodontitis in male rats. Thus, the female rats seem to have experienced a greater acute phase response to the periodontal inflammation than the male rats. These conclusions were supported by higher liver concentrations of C-reactive protein in female rats than in male rats.

Recent studies of the gender-specific response to inflammation have utilized a methodology for sterile endotoxin challenge, rather than evaluation of a

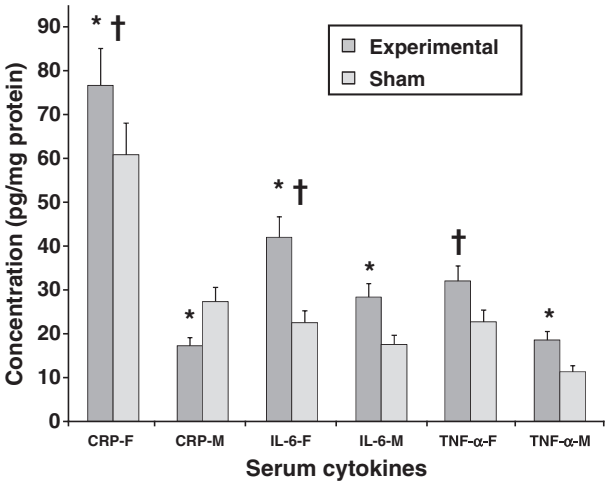

Fig. 6. Gender differences (M, male; F, female) in serum biomarker concentrations (pg/mg of protein, mean ± standard error of the mean) from rats with ligatures (experimental) and sham-operated controls. *Significantly different from sham controls, $p < 0.05$; †significantly different from male rats, $p < 0.05$. CRP, C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

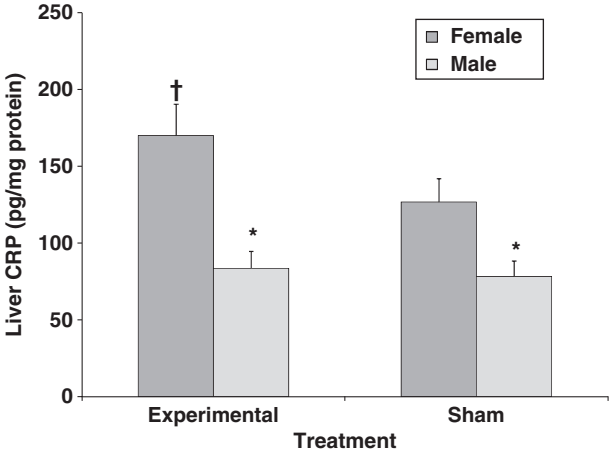

Fig. 7. Gender differences in liver C-reactive protein (CRP) concentrations (pg/mg of protein, mean ± standard error of the mean) within rats with ligatures and sham-operated controls. †Significantly different from sham controls, $p < 0.05$; *significantly different from female rats, $p < 0.05$.

chronic inflammation. Our data contradict several previous studies of gender differences in the acute phase response to infection, utilizing a challenge model in human subjects, which reported a greater male acute phase response compared with the female acute phase response (33,35,37). However, our data do support another study utilizing that model which reported a greater acute phase response to infection by women than by men (34). Similar studies utilizing this model in animals report that females have lower serum levels of these inflammatory cytokines than males (33,36,37). Differences between the outcome data from these studies and ours is difficult to reconcile, but possibly resulted from differences in the duration of the challenge to the immune system. Our study produced a localized, chronic inflammation that could have affected the acute phase response differently from the acute challenge produced by injection of endotoxins.

The female rats with ligatures in our study had greater tissue concentrations of uterine pro-inflammatory cytokines, suggesting that animals with periodontal inflammation could have a concurrent uterine inflammation. Pro-inflammatory cytokines have been reported to be harmful and destructive to a successful pregnancy because they contribute to placental inflammation (47,48). Elevated placental and serum concentrations of tumor necrosis factor-α, interleukin-6 and C-reactive protein have been associated with preterm birth, low birthweight and developmental defects (17,48–62). Our study extends previous studies by suggesting an association between the female rat acute phase response, from periodontal inflammation and inflammation of the uterus. This association could provide a biological mechanism for the adverse effects of periodontal inflammation on pregnancy outcomes.

Our study provides a hypothesis for the association between periodontal inflammation and adverse pregnancy outcomes that has been reported by others. However, the biological mechanism for this association has not been defined. The maternal immune response is essential for a successful pregnancy (63) because it protects the developing embryo from fetal immune rejection (64,65) and facilitates placental development (66). Angiogenesis is essential for embryo implantation, formation of a placenta and maintaining a pregnancy. Defective angiogenesis may result in miscarriage or placental problems leading to either pre-eclampsia or fetal growth retardation. Endothelial cells are major components of the uterine stroma. These cells respond to vascular endothelial growth factor by proliferation and sprouting, thus increasing the size of the vascular bed (67). These sprouts have been reported to be damaged by pro-inflammatory cytokines, preventing successful implantation of the embryo (68,69). Thus, we propose that elevation of the concentrations of circulating pro-inflammatory cytokines coincident to periodontal inflammation could adversely affect the pregnancy by damaging the vascular growth that had been stimulated by elevated tissue concentrations of vascular endothelial growth factor. This expansion of the

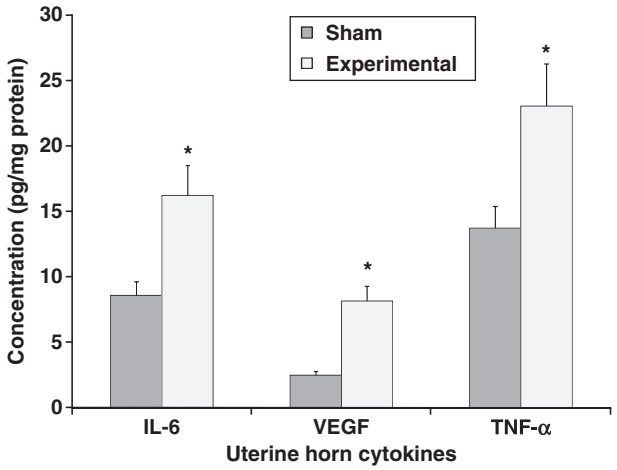

Fig. 8. Uterine horn cytokine concentrations (pg/mg of protein; mean ± standard error of the mean) from female rats with ligatures and sham-operated controls. *Significantly greater than control, $p < 0.01$. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

vasculature would increase the possibility for interactions with pro-inflammatory cytokines. Thus, elevated tissue concentrations of pro-inflammatory cytokines could produce unfavorable pregnancy outcomes by inhibiting angiogenesis. This situation could shorten the duration of the pregnancy and inhibit fetal growth, which are the outcomes from several studies (48,49,51–55,58,59).

Our study suggested that female rats had a greater acute phase response to periodontal lesions than male rats with lesions featuring similar clinical attachment loss. These differences suggest a mechanism for gender disparities in the incidence of systemic diseases with chronic inflammation as a risk factor, such as cardiovascular disease, diabetes mellitus and obesity (33,70–72), as female rats experienced significantly greater serum concentrations of tumor necrosis factor-α, interleukin-6 and C-reactive protein, and of liver C-reactive protein concentrations, than male rats. In addition, female animals with periodontal disease could become more susceptible to adverse pregnancy outcomes as a result of elevated concentrations of interleukin-6, tumor necrosis factor-α and vascular endothelial growth factor within the uterine tissue, which could adversely affect angiogenesis and vascular expansion. The latter situation could damage the attachment and growth of the fetus within the uterus, producing the adverse pregnancy outcomes described by others. Further studies of the biological mechanisms for adverse pregnancy outcomes in women with periodontal inflammation are necessary to confirm our preliminary hypotheses.

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