Effect of estrogens level on the fracture healing of tibia bone after ovariectomy and ovariohysterectomy in female dogs

O.A. Bader1 and H.H. Nahi2

1Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Mosul, Mosul, 2Department of Surgery and Obstetrics, College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq

Abstract

This study aimed to find out how estrogen affects fracture healing. Thirty adult bitches were employed, and they were put into three groups at random: First, second, and third. A mixture of ketamine and xylazine has been used to anesthetize experimental animals. Electrical drilling in the distal part of the tibial bone with a 10 mm diameter was done on the first group to create a hole in the tibia. The Tibia hole was created after ovariectomy in the second group but after ovariohysterectomy in the third group. Clinical, estrogen assay and histopathological characteristics were assessed in all groups. During the first week after surgery, clinical results showed that symptoms of inflammation in the surgical region were substantial in the first group, while the inflammation symptoms in the second group were similar to those in the first group; however, it was milder in the third group. In the second group, estrogen levels reduced modestly; however, in the third group, estrogen levels declined significantly in the fourth week after surgery. According to histopathological findings, the proliferation of osteoclasts and osteoblasts at 15 and 30 days after surgery is much more pronounced in the first and second groups than in the third groups. Osteocyes are also more common in the first group than in the second and third groups. According to the results of this study, fracture healing in the third group is much less quantitative and qualitative than in the other groups.

Keywords: Ovariectomy, Ovariohysterectomy, Estrogen, Fracture healing

Correspondence: O.A. Bader
omaralbader2003@uomosul.edu.iq

Introduction

After an injury, fracture healing entails cellular activities that restore the usual bone architecture and function (1). Cellular division and maturation, chemotaxis, and extracellular matrix formation are all examples of biological processes (2). Complications seem relatively common in open fractures involving severe soft tissue injury. Common complaints include long healing times, infections, osteomyelitis, compartment syndrome, wound collapse, and non-union (3). Most of these disorders necessitate prolonged inpatient care and repeated operations with lengthy rehabilitation procedures and substantial expenditures (4). Aside from clinical consequences, disability, failure to return to work, and persistent pain are prevalent. Following orthopedic trauma, these negative consequences frequently last a year or longer (5). Many biological and biomechanical (hormonal, cellular) elements affect the healing process in a fracture. Bone metabolism is a complicated procedure requiring delicate stability among osteoclast bone creation and osteoblast bone formation (6). Both systemic and local factors are thought to play a role in fracture repair. The main hazardous factor for postmenopausal is ovarian hormone insufficiency (1). Eliminating sex hormones, such as estrogen, causes an imbalance in skeletal turnover, resulting in osteoporosis since they are essential regulators of bone mass (7,8). This elimination of female hormones significantly impacts bones in hip fractures due to osteoporosis worldwide.
One of every three women over 50 will have hormone-related osteoporosis leading to bone breakdown (9). Estrogen affects osteoblastic and osteoclastic elements of the remodeling process; estrogen has both anabolic and anticatabolic effects, according to morphologic and metabolic research (10,11). The hard callus remodeling back to the matured lamellar bone is crucial in fracture healing. Estrogen is identified to affect the bone remodeling mechanism, at least to some extent, suggesting that estrogen could probably play a role in fracture healing (12). In postmenopausal osteoporotic and ovariecetomized patients, altered bone production during fracture healing is frequently seen, which suggests that the endocrine status is also relevant (8). Estrogen has already been shown to play a role in cartilage homeostasis, growth, and maturation. Estrogen influences epiphyseal plate closure and growth during pubertal growth, which spurt by affecting the release of cytokines and growth hormones (12). Understanding the molecular processes that drive the healing of bone through estrogen response might be crucial for future treatment of fracture developments, particularly for elderly females (13). Female sex hormones play a critical role in achieving peak bone density in both females and males and mostly in maintaining bone density in adults (14). Estrogen's effects on bone metabolism and its beneficial effects on bone mineral density seem well known. However, its impact on fracture healing is still being studied. The more significant risk factor for postmenopausal osteoporosis is ovarian hormone insufficiency (15,16).

As a result, we determined to investigate the effect of estrogen on distal tibia fracture healing by employing clinical, estrogen level, and histopathological assessments.

Materials and Methods

Ethical approval
The ethical approval granted by University of Mosul, College of Veterinary Medicine, Institutional Animal Care and Use Committee in 15/10/2022, the issue number is UM.VET.2022.058

Experimental animals
Thirty adult female canines weighing 15-20 kg and 2-3 years old were chosen for the study. The animals were housed in controlled conditions throughout the experiment for inspection and adaptation.

Experimental design
Animals were randomly assigned to three groups, each one with ten dogs; the first group animals only have tibia holes (17,18). The second group; animals in this group had tibia holes 21 days after they underwent a unilateral ovariecetomy (19). The third group; The tibia holes in the animals of this group were made 21 days after the ovariohysterectomy operation (20).

Clinical assessment
During the first week following surgery, the animals were checked clinically and physically for temperature, heart rate, respiration rate, lameness, and walking.

Estrogen examination
Blood samples were collected to analyze the amount of estrogen before and during surgical procedures (ovariecetomy and ovariohysterectomy) for all animals and continued every week until the fourth week after the tibial hole was made to evaluate hormone levels.

Histopathological evaluation
Bone samples were carried out 15 and 30 days after surgery for evaluation under a light microscope; samples were sectioned off paraffin blocks after being fixed for a minimum of 72 hours in 10% formalin and stained with Hematoxylin and Eosin.

Surgical technique
All animal surgeries occurred during the same session period, outside the menstrual cycle. Before surgery, the animals went without food for 12 hours and drink for 6 hours. Females received atropine sulfate intramuscularly at a dosage of 0.04 mg/kg B.W. (as a premedication); after 15 minutes, they received a combination of xylazine hydrochloride 2 mg/kg B.W and ketamine hydrochloride 5% (10 mg/kg B.W) parenterally and if necessary, repeated as a half dosage (21,22). After positioning the bitches in a dorsal recumbent position, a mid-ventral incision was used to perform a unilateral ovariecetomy in the second group. At the site of the planned procedure, the abdominal wall was shaved, cleaned, and covered with an aperture of the perforated drape. After catching the right ovary with such a uterine hook and ligation the oviduct and blood supplies, the right ovary was removed through a lengthy 10 cm mid-ventral skin and abdominal wall incision. In the third group, the same as in the second group, the right ovary was removed; beyond that, the left ovary was excised via a similar approach. In addition to the uterus, every ovary was dissected to a cranial level to the cervix. Digital pressure was used to tear the suspensory ligament when the ovary was isolated and externalized. Both broad ligaments were separated and sealed, and the ovarian and uterine vasculature obtained hemostasis. The surgical incision was expanded to remove the uterus, then 3.0 Polyglactin 910 and 2.0 silk sutures were used to suture the abdominal wall. The same anesthesia and preoperative protocols mentioned above were used to perform tibial holes on all animal groups 21 days after ovariecetomy or ovariohysterectomy. The operative area, which included the distal portion of the tibia, was prepped using a sterile technique. Drapes were placed around the operative site, and the animal was placed on lateral recumbence. An incision was created in the skin over the distal portion of the tibia to expose the bone, and a ten-millimeter-diameter drill bit was then used.
to punch a hole in the bone. The skin was closed over using 2.0 silk material (23).

Statistical analysis
Results were presented as means plus standard error, and parametric information was evaluated in two methods. Least Significant Difference (LSD) was used to continue the analysis of variance (ANOVA), and P≤0.05 was regarded as significant. SPSS (Statistical Package for Social Sciences) was employed (24).

Results
Clinical examination
The physical exam findings showed that the inflammatory symptoms, such as pain and edema at the surgical site, started subsiding six days after the surgery in the first group on day two. While the inflammatory symptoms in the second group are similar to the severity in the first group. However, in the third group, all of the symptoms above were lower than those mentioned in the first group, and they vanished by the fourth post operations day.

Serum estrogen levels
Estrogen levels have been determined using blood samples and transversely to serum. Table 1 displays the hormone levels for all samples collected before and during surgical procedures (ovariectomy and ovariohysterectomy) and continues weekly until the fourth week after the tibial hole was performed. Table 1 showed no significant difference in the mean values of the estrogen levels between groups one and two in all periods after overcomes because removing one the main source of estrogen production will decrease estrogen levels. Still, this lack is not very effective for bone healing. At the same time, group three showed a significant decrease at P≤0.05 compared to groups one and two on different days postoperatively. This significant drop in estrogen levels was mainly in the fourth week following surgery.

Table 1: Clarify Estrogen level (Pg/ml) mean ± SE during the studying period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before operation</th>
<th>At hole making</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.65+0.54</td>
<td>22.45+0.22</td>
<td>23.31+0.28</td>
<td>22.85+0.37</td>
<td>23.00+0.32</td>
<td>23.27+0.40</td>
</tr>
<tr>
<td>T1</td>
<td>23.58+0.52</td>
<td>20.88+0.12</td>
<td>20.56+0.12</td>
<td>20.25+0.11</td>
<td>14.10+0.26</td>
<td>14.02+0.21</td>
</tr>
<tr>
<td>T2</td>
<td>22.45+0.53</td>
<td>11.15+0.30</td>
<td>9.97+0.13</td>
<td>1.58+0.18</td>
<td>0.19+0.005</td>
<td>0.19+0.003</td>
</tr>
</tbody>
</table>

Different Capital letters in columns mean a significant difference at P≤0.05. Different small letters in rows mean there is a significant difference at P≤0.05.

Histopathology
The first group's histopathological analysis on the 15th day after surgery showed blood vessel congestion around the fracture site and connective tissue fiber forming due to the proliferation of fibroblast that occupied the fracture position (Figure 1). The findings revealed fibrous connective tissue with macrophage and plasma cell infiltration. Compared to other groups, this time revealed osteoblast proliferation that created osteoid tissue and the bone trabeculi being thicker. It is more regularly spaced apart (15th day) (Figure 2). While the histopathology examination on 30th day post-operation marked the production of lamellar bone rather than compact bone, particularly increased in the haversian canals (Figure 3), multiplying osteoblasts, thickening and regularizing bone trabeculae, and thickening and regularizing the interior callus of the bone plate (Figure 4).

The second group's histological analysis on the 15th day after surgery revealed the existence of a network of connective tissue fibers as well as the inflammatory cells infiltration of like fibroblasts, macrophages, and neutrophils and the existence of network of connective tissue fibers with the proliferation of osteoblast and osteocyte cells (Figure 5 and 6). While some external calluses formed by day 30 after surgery, most cartilages had changed to bone tissue (Figures 7 and 8).

![Figure 1: Histopathological section in the tibial bone of the first group on 15th day post-holing shows the presence of congested blood vessels (yellow arrow) with the proliferation of fibroblast (red arrow) and formation of new blood vessels (black arrow) (H&E 100X).](image-url)
Figure 2: Histopathological section in the tibial bone of the first group on 15th day post-holing shows external callus formation (yellow arrow) with infiltration with macrophages and plasma cells (red arrow) and proliferation of osteoblast (blue arrow) and osteocyte (black arrow) (H&E 100X).

Figure 3: Histopathological section in the tibial bone of the first group on 30th day post-holing shows the lamellar bone formation (red arrow) with wide haversian canal (yellow arrow) and presence of osteocyte (H&E 100X).

Figure 4: Histopathological section in the tibial bone of the first group on 30th day post-holing shows internal callus marked by extra bone plate regularity and thickness (black arrow) and proliferation of osteoblast (yellow arrow) and presence of osteoclast (red arrow) (H&E 100X).

Figure 5: Histopathological section in the tibial bone of the second group on 15th day post-holing shows inflammatory cell infiltration (black arrow) and the existence of fibrous tissue network (red arrow) (H&E 200X).

Figure 6: Histopathological section in the tibial bone of the second group on 15th day post-holing shows proliferation of osteoblast (red arrow) and osteocyte cells (yellow arrow) with granulation tissue formation (black arrow) (H&E 200X).

Figure 7: Histopathological section in the tibial bone of the second group on 30th day post-holing shows external callus formation (red arrow) with the presence of osteocyte (black arrow) and osteoblast lining the haversian canal (yellow arrow) (H&E 200X).
The third group's histological evaluation on the 15th day after surgery showed fibrous tissue development of necrotic osteocytes free of nuclei in cavities (Figures 9 and 10). While at the 30th day after surgery, there was an expansion in the thickness of the bone trabeculi that made the internal callus, as well as a rise in osteoblasts that lined the bone marrow; this time was characterized by a rise in the thickness and consistency of bone trabeculi contrasted to the 15th day results and a decrease in the size of the gap among bone trabeculi (Figure 11 and 12).

Figure 8: Histopathological section in the tibial bone of the second group at 30th day post-holing shows the presence of osteocyte (red arrow) and osteoclast (yellow arrow) with callus formation (black arrow) (H&E 200X).

Figure 9: Histopathological section in the tibial bone of the third group on 15th day post-holing shows the presence of osteocyte (red arrow) and proliferation of osteoblast lining the haversian canal (black arrow) with the formation of fibrous tissue (yellow arrow) (H&E 200X).

Figure 10: Histopathological section in the tibial bone of the third group on 15th day post-holing shows the presence of the necrotic osteocyte that is free of the nuclei (black arrow) in the haversian lamellae (yellow arrow) that surround the haversian canal (blue arrow) (H&E 400X).

Figure 11: Histopathological section in the tibial bone of the third group on the 30th day post-holing shows the proliferation of osteoblasts that line the haversian canal (red arrow) and proliferation of osteocyte (yellow arrow) increased in thickness of internal callus (H&E 200X).

Figure 12: Histopathological section in the tibial bone of the third group at 30th day post-holing shows bone osteon increasing in regularity and thickness (yellow arrow) presence of osteocytes (red arrow) (H&E 200X).
Discussion

The development of swelling, reddening, pain, and elevated heat at the surgical site during the second postoperative day may be due to elevated blood flow to the region, as well as increased capillary permeability and blood vessel distention, which cause inflammatory cells and white blood cells to migrate out of the surgical site and cause edema, these were agreement with Chiu and Chien (25) and Margraf et al. (26). Edema surrounding the fracture region that increases the pressure on nerve termination may cause pain. Moreover, the degree of inflammation stimulates cells to create prostaglandin, which causes vasodilation, an increase in blood vessel infiltration, and an accumulation of exudate in the fractured area (27,28).

In contrast to the other groups, the third group's clinical signs were less severe compared to the first and second groups at four to five days following surgery. This could be caused by the vasculature effect of the estrogen hormone, which produces large numbers of phagocyte cells at the surgical site and increases the severity of the tissue inflammatory process. Also, the estrogen hormone has a highly vascular action authorized by a rich source of angiogenesis factors that encourage the creation of blood vessels in any tissues it is situated close to. These findings agreed with the findings that estrogen is a potent vascular hormone Sina et al. (29) and Vazgiourakis et al. (30). The delamination of the circulatory system to the bone and its surrounding tissues, as well as the exudation of white blood cells and plasma, in addition to the two groups of factors mentioned above, result in reduced oxygen levels and increased acidity in the area, which lead to centralized swelling and inflammation and the incapability to extract necrotic tissues (25).

Due to the removal of the significant source of estrogen production in the second group, the estrogen level was modestly reduced; nonetheless, the effects on bone healing were ineffective; these results concurred with Salih and Al-Khashab (31), Khired et al. (32) and McMillan et al. (33), whom declared that ovariectomy can cause bone loss due to lack of estrogen, which results in osteoporosis in females. By encouraging osteoblast activity and restraining osteoclast activity, estrogen improves bone mineralization. Moreover, it lowers the levels of reactive oxygen species and bone demineralization. By regulating the period of osteoclast apoptosis, estradiol decreases bone resorption (34). Conversely, estrogen levels declined dramatically in the third group in the fourth week following surgery. This drop in estrogen levels was a key threshold that had an effect on bone healing, and this is in agreement with Beil et al. (35) and Salih and Al-Khashab (36), whom says that after hysterectomy, estrogen insufficiency increases bone resorption and decreases renal phosphate output, which affects calcium absorption and increases bone demineralization. An organic matrix controls the deposition of inorganic minerals during every step of bone formation. Calcium and phosphate make up the mineral phase. Phosphate, a component of hydroxyapatite crystal, is essential for the normal mineralization of bone. Phosphorous levels in the urine and serum are indirectly impacted by reduced estradiol at different levels (34).

Because fibroblasts and inflammatory cells infiltrate the bone under prostaglandin mediation, the important signs on the 15th day after surgery were more severe in the first and second groups than in the third group. As a result, granulation tissue forms, vascular tissue grows, and mesenchymal cells migrate. The exposing cancellous bone and muscles, which occurred as a consequence of decreasing estrogen hormone, agreed with Loi et al. (37) and Seko et al. (38), whom said that estrogen is the principal source of nutrients and oxygen for this early process. At 15th and 30th days after surgery, the periosteal response and osteoblast and osteoclast proliferation were more pronounced in the first and second groups than in the third group. Additionally, osteocytes occurred more frequently in the first group than in the second and third groups, which may have altered the inflammatory response and inhibited the healing process due to estrogen levels. Fibroblasts begin to set down the stroma that supports vascular angiogenesis through the healing stage (39,40).

A collagen matrix is developed as vascular ingrowth advances. Osteoid is released and subsequently calcified, resulting in the creation of a soft callus around the healing site. During the first four weeks of the healing process, this callus has little movement resistance. Thus, it needs proper protection, such as a brace or fixation (41). The callus solidifies Between the fracture fragments, and the woven bone formation is bridging. Alternately, if adequate immobilization is not employed, and instead of ossifying as intended, calluses can create an instability fibrous union (42).

Because of the typical estrogen level, recognized as killing bacteria and is highly oxygen dependent, the remodeling stage was more mature in the first and second groups than in the third group. Estrogen can also have a significant pharmacological antimicrobial property via neutrophils, directly inhibiting anaerobes and enhancing the effects of antibiotics like aminoglycosides, which are inhibited in a hypoxic environment. Estrogen also increases fibroblast and angiogenesis activity to speed up the healing of problematic wounds. These actions are desirable when orthopedic and soft tissue trauma is present (39). When the healing of fractures is concluded, the bone heals and enters the remodeling phase, where it regains its architecture, mechanical properties, and initial shape. Physical stress that is applied to the bone speeds up the slow remodeling of the bone that takes place over months to years, which is in accordance with Eliaz and Metoki (43) and Liu et al. (44).
Conclusions

The current investigation found that estrogen is an essential component for the healing of fractures; the decline in estrogen levels had an influence that altered bone healing. Periosteal reaction close to the fracture site was shown earlier in the first and second groups, but it took longer in the third group; thus, hysterectomies and ovariectomized females showed given estrogen in case of bone fracture.

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Conflict of Interest

The researchers affirm that nothing exists of potential conflict of interest.

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