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Infected bone defect healing could be complicated due to more delayed responses to antibiotic therapy in ovariohysterectomized rats

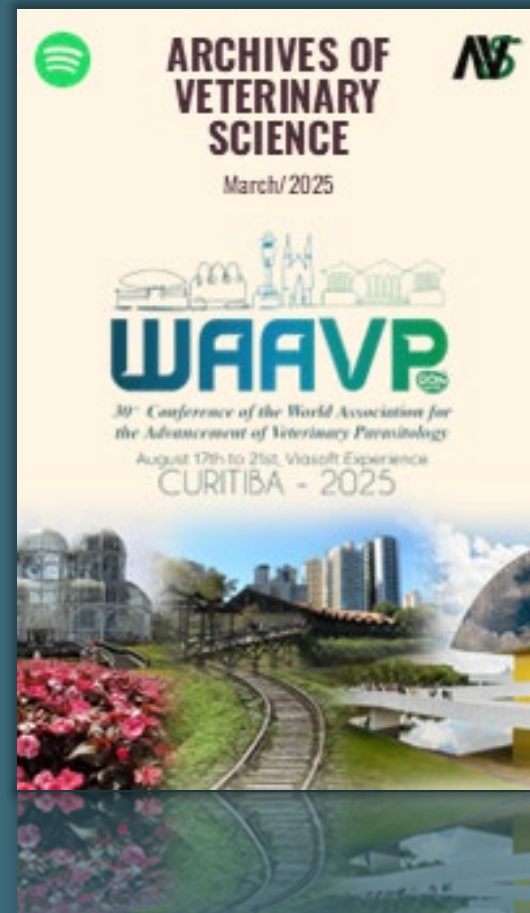
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Infected bone defect healing could be complicated due to more delayed responses to antibiotic therapy in ovariectomized rats

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Abstract: As women age and enter menopause, bone fracture threatens their lives. This study examined the lower steroid hormones that have worse effects on the healing of the infected bone. A total of 36 six-month-old Sprague-Dawley (SD) rats were divided into six groups: sham, group I (infection), group IA (infection + antibiotic), group O (ovariectomy), the OI group (ovariectomy + infection), and the OIA group (ovariectomy + infection + antibiotic). A 2 mm defect in the femur's diaphysis was used to perform the fracture. An inoculation of 4×10^4 CFU/ml of *Staphylococcus aureus* was implemented. On the 30th and 60th days, blood (TNF- α and IL-6) and tissue (histopathology and culturing agar) samples were collected. Agar culture analysis showed that the OI group had a higher bacterial load in the bone compared to the I group on both days. The OI group showed a wider level of callus and inflammation than even the I group, but in terms of bone formation, it was significantly ($p = 0.02$) weaker than the I group. Serum findings showed a significant increase in TNF- α levels in the OI group compared to the O group on day 60. We observed a significant decrease in bone bacterial load and increased healing in IA compared to I, but not in OI compared to OIA. The animal that had an ovariectomy had a more severe infection in its broken bone, resulting in increased bone lysis and a greater bacterial load. Additionally, the healing of the fracture was linked to a longer delay. Systemic antibiotics, but not those given to animal bone that had undergone ovariectomy, greatly decreased the bacterial load and improved the quality and strength of the callus in normal bone that had been infected by a fracture.

Keywords: Bone healing, ovariectomy, Fracture related infection.

1. Introduction

Fracture-related infection (FRI) is one of the most severe complications that occur in trauma surgery. The current consensus regarding the diagnosis of FRI is to perform tests such as histopathological examination to confirm the presence of microorganisms in the depth of bone tissue samples (Metsemakers et al., 2018). As the population increases, fractures caused by bone weakness are often infected by microorganisms, 30% of which are symptomatic. It has become one of the most common problems faced by doctors (Yano et al., 2014). FRI can lead to serious clinical consequences, including abscess formation, prolonged antibiotic therapy, multiple debridement surgeries, long-term disability, and even amputation. The cost of treatment is very expensive per patient, resulting in a significant social and economic burden (Iliens et al., 2021). Today, we know that bone fractures, especially in the condition of osteoporosis, lead to a delay in healing. In cases of osteoporosis during menopause and old age, the possibility of infection increases, along with a weak immune system and a reduced ability to eliminate microbes (Lumsden and Sassarini, 2019). In 2012, Gjertsson et al. discovered that ovariectomized mice developed systemic trabecular bone weakness and thinning that was even more severe than in a septic arthritis model (Gjertsson et al., 2012). However, it is still not clear whether the repair of a broken bone in an animal with an ovarian disorder or hormonal defect is associated with a longer delay and whether the place where the antibiotics reach the fracture site is disturbed or not. For FRI, *Staphylococcus aureus* is the most common infection. When the damaged site is infected, the bacteria form a matrix and attach to the bone surface (Seebach and Kubatzky, 2019). Bacteria that enter the fracture space are placed in the form of biofilms and suffer from a decrease in metabolic activity and speed of division. They also become drug-resistant to antibiotics and resist phagocytosis by the host's immune cells (Ul-Haq et al., 2024). If implantable therapeutic measures, especially implants, are used in the wound at the fracture site, the main problem for the treatment of FRI will be to destroy the biofilm formed on the implant as well as the colony-forming units (CFUs) (Rupp et al., 2020).

The newly identified MicroRNA-186 molecules and the identification of bone morphogenetic protein (BMP) pathways have facilitated understanding of the bone healing process (Wang et al., 2019). Steppe et al. (2023) showed that external frequency vibration stimulation applied to the whole body does not interfere with fracture repair in rodents with estrogen. Whereas in estrogen-deficient (ovariectomized) rodents, post-fracture bone formation was impaired (Steppe et al., 2023). The present study's strength lies in demonstrating, through histopathology, the delayed healing of infected fractured bone in ovariectomized female rats.

The goal of this study was to determine whether FRI in people with ovarian failure and its hormones can respond to antibiotic treatment, as well as in individuals with active ovaries and hormones. Homogenous bone repair in ovariectomized people who are probably affected by osteoporosis can be as beneficial as the repair of normal people.

2. Materials e Methods

2.1. Ethical Approval

All methods used in this study, such as handling, scarification, and animal husbandry, followed the directives provided by the Islamic Azad University's Animal Ethics Committee number IR.IAU.SRB.REC.1403.102.

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2.2. Experimental design and ovariectomy

2.2.1. Animals and Housing

The animals were placed in the animal house. Normal conditions were provided for the mouse cage, which consisted of a 12-hour light/dark cycle. Fresh airflow and temperature were maintained between 22 and 25 degrees Celsius, and commercial pellets of feed and water were maintained freely.

2.2.2. Ovariectomy surgery

Under general anesthesia with intraperitoneal injections of ketamine (25 mg/kg) and xylazine (2.5 mg/kg) (Wong et al., 2019), an ovariectomy was performed via a midline abdominal incision (2 cm in length) in the Linea alba. Using a single-clamp technique, the ovarian ligaments and cervix were ligated with 5-0 silk. The ovaries and the uterus were then removed. Four simple, interrupted sutures were placed in the abdominal wall (Bozkurt et al., 2024). A continuous subcuticular or facial layer was placed. The skin was closed with four wound clips and covered with topical antibiotics. The procedure was completed within 15 minutes. To reduce pain, 0.05 mg/kg, of intra-muscular buprenorphine was given 15 minutes before surgery and three consecutive days after the procedure. Before fracture induction, the rats were housed for three months (Wong et al., 2019).

2.2.3. Chemicals and reagents

Chitosan (molecular weight = 190–50 KDa, more than 75% acetylated), alloxan monohydrate, yeast extract, sodium citrate buffer, and peptone were purchased from Sigma-Aldrich. Polyethylene glycol, silver nitrate (AgNO₃), glacial acetic acid, and glutaraldehyde were purchased from Merck.

2.2.4. Animal groups

On the first day of research, the ovariectomy groups removed the ovary and uterus. Sixty-six-month-old female Sprague Dawley rats (n = 10) were randomly divided into six groups: I (without any treatment), I (with infection), IA (infection + antibiotic), O (ovariectomy), OI (ovariectomy + infection), and OIA (ovariectomy + infection + antibiotic). Then, three months after the research began, all 60 rats were surgically created with a femoral diaphysis defect. In infected groups, the injection of a suspension containing bacteria (described in the section on infection) was done at the time of defect creation.

2.2.5. Bone defect surgery

In this study, rats from all six groups were anesthetized by intraperitoneal injections of 25 mg/kg ketamine hydrochloride and 2.5 mg/kg xylazine (Wong et al., 2019). During the aseptic procedure, a 2.5 cm-long incision was made on the mid-diaphyseal surface of each femur's right mid-region. A periosteal elevator separated the periosteum from the diaphyseal surface. Similarly, a femur defect with a diameter of 2 mm was created using a drill hole. Before applying the preserved periosteum and surrounding muscles, the surgical site was washed with normal saline. The operative site was treated by each rat's cure protocol (Sonbolekar et al., 2022). The surgical site was followed up daily for signs of infection and bleeding (Bozkurt et al., 2024).

2.2.6. Infection of Bone defects

On the defect site, the prepared bacterial suspension (*S. aureus* ATCC25923, Baharafshan Company) with 104 CFU/ml was injected into the fracture site under direct vision. The Seattle 1945 strain was a methicillin-sensitive clinical isolate. The wound was sutured in layers. Cefazolin (5 mg/kg) was administered to the rats that were scheduled for antibiotic therapy (Penn-Barwell et al., 2012). Daily antibiotic treatment was performed from 24 hours after surgery until the end of week 4 or week 8.

After euthanasia, a microbiological sample was taken from the area around the defect in a sterile technique with a swap and gently cultured on an agar plate, and CFU was counted (Sittek et al., 2024).

2.2.7. CFU and bone defect

After euthanasia, a microbiological sample will be taken from the area around the defect in a sterile technique with a swap and gently cultured on an agar plate, and CFU will be counted (Sittek et al., 2024). Weighing and grinding every part of the defect was done. Serial dilutions of the suspension were prepared, and each dilution was streaked on agar plates before being cultured for 24 hours at 37°C. The number of CFU in bone samples revealed the number of CFU/g in the defect. The final CFU/g in bone was calculated using the average of the three dilutions.

2.2.8. Sample Preparation and Staining Procedures

On the 30th and 60th days, five rats were randomly selected from each group, and blood samples were taken directly from the heart into an EDTA tube during euthanasia for TNF α (TNF- α Rat Tumor Necrosis Factor-alpha ELISA Kit, Karmania Parsgen) and IL-6 (IL-6 Rat Interleukin 6 ELISA Kit, Karmania Parsgen) serum level evaluation, after euthanasia with a ketamine-xylazine overdose. Samples from the defect area, along with healthy tissue, were prepared for histopathology. The samples were fixed and decalcified for seven days in 10% formalin buffer and 20 days in EDTA. Then, hematoxylin-eosin staining was performed, and according to Table 1, they were examined and compared in terms of the restoration process.

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2.2.9. Histopathology

Histopathology evaluated the inflammatory tissue response, osseous formation, and integration using (Table 1) a modified Allen's scoring system (Sonbolekar et al., 2022).

Phenomena	Description Phenomena	Score
Union	Failure to fill the defect	0
	Only fibrotic tissue	1
	Equal amounts of fibrotic and cartilage tissue	2
	High amounts of cartilage and little fibrosis	3
	Only cartilage	4
	Lots of cartilage with little immature bone	5
	Immature cartilage and bone in equal proportion	6
	Prominent amount of immature bone and little cartilage	7
	Healing with immature bone	8
	Healing with mature bone	9
Inflammation	Absence of inflammatory cells	0
	Presence of inflammatory cells less than 25%	1
	Presence of inflammatory cells 25-50%	2
	Presence of inflammatory cells 50-75%	3
	Presence of inflammatory cells more than 75%	4
Remodeling	No Remodeling	0
	Remodeling less than 25%	1
	Remodeling 25%-50%	2
	Remodeling 50%-75%	3
	More than 75% Remodeling	4

Table 1 – Basis for grading histopathological lesions of broken bones in ovariectomized rats.

2.3. Statistical Analysis

A Kruskal-Wallis non-parametric test with GraphPad Prism 9.0 software was used for histopathological scoring. The inter-individual consequences were designated as significant if the p-values were less than 0.05 (* $p \leq 0.05$), less than 0.01 (** $p \leq 0.01$), and less than 0.001 (***) $p \leq 0.001$). Finally, the data were presented as means and standard deviations.

3. Results

3.1. Clinical findings

All rats were able to tolerate weight bearing after surgery. All infected rats showed obvious redness and swelling in the affected area. Five rats died and were replaced, possibly due to sepsis.

3.2. Bacteria load on bone defect

On day 30, agar culture revealed that the bacterial load in the OI group's bone defect was significantly higher compared to other groups. Interestingly, this difference also existed in the OIA group. On day 60, the OI group's bone bacterial load was only significant with the IA group, not even with the OI group (Figure 1).

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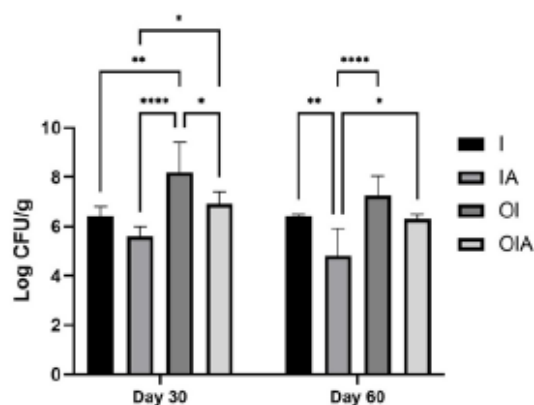


Figure 1 – The Quantification of bacteria load at day 30 and day 60. A, antibiotics; CFU, colony-forming unit; Inf, infection; O, ovariectomized. **p < 0.01; ***p < 0.001.

3.3. Serum Inflammatory cytokines

The ELISA test showed a significant increase in IL-6 levels in the OI group compared to the O group on days 30 and 60. Also, on day 60, a significant increase in IL-6 serum levels was seen in the I, IA, OI, and OIA groups compared to the sham and O groups. Group I, on the other hand, differed significantly from both the sham and O groups. Importantly, there was no significant difference between the OI and OIA groups (Figure 2).

In the case of TNF α , it was also found that a significant increase in its serum level was observed in the OIA group compared to all other groups but not in the OI group on day 30. Additionally, on the 60th day, the serum level of TNF α significantly increased in the OI group compared to the OIA group and other groups. Most importantly, there was a significant difference between the OI and OIA groups (Figure 2).

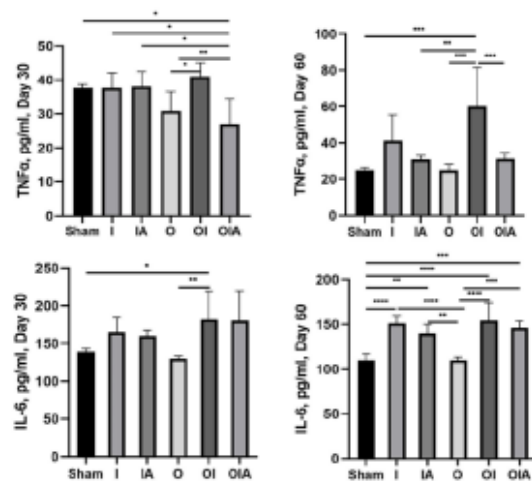


Figure 2 – The serum of serum inflammatory cytokines is presented. A, antibiotics; IL, interleukin; Inf, infection; O, ovariectomized; TNF- α , tumor necrosis factor alpha; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

3.4. Histological findings

Inflammatory cell infiltration, periosteal reaction, and cortical thickening at the fracture site occurred in all four infected groups (Figures 3, 4, and 5). Despite no significant difference, the mean callus union score was 2.4 (SD 0.6) in the I group, showing a trend toward higher callus quality than 3.4 (SD 0.9) in the OI group at day 30. A similar trend was observed at day 60, with more severe inflammatory necrosis identified in OI (Figure 5). Moreover, quantitative analysis showed a significantly enhanced callus quality

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($p < 0.001$), as demonstrated by higher bony callus formation in IA compared to I at day 60. In OIA, the fracture gap was found to be composed of fibroblasts and many inflammatory cells, with no bony callus. In OIA, there was no significant improvement in callus quality compared to OI.

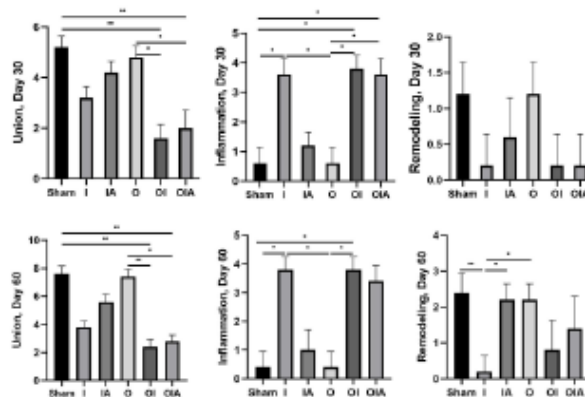


Figure 3 – The quantification of callus quality at day 30 and day 60. A antibiotic; I, infection; O, ovariectomized. * $p < 0.05$; ** $p < 0.01$.

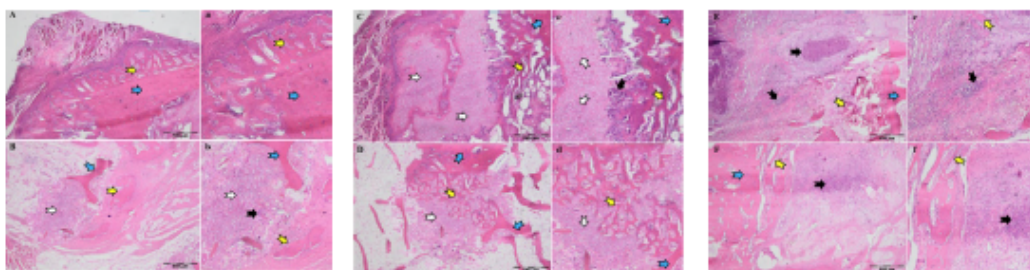


Figure 4 – Histology at the diaphyseal defect site in various groups of rats on the Days 30 post-surgery (H&E ×40). (A, a): Sham, (B, b): I, (C, c): IA, (D, d): O, (E, e): OI, (F, f): OIA. Yellow arrow: New bone trabecula, White arrow: Cartilage, Blue arrow: Old bone trabecula, and Black arrow: Inflammation.

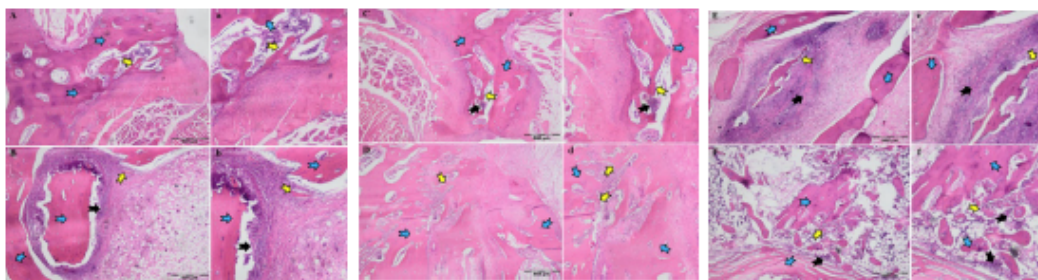


Figure 5 – Histology at the diaphyseal defect site in various groups of rats on the Days 60 post-surgery (H&E ×40). (A, a): Sham, (B, b): I, (C, c): IA, (D, d): O, (E, e): OI, (F, f): OIA. Yellow arrow: new bone trabecula, Blue arrow: Old bone trabecula, and Black arrow: Inflammation.

4. Discussion

The healing of the fracture site is delayed in the bones of ovariectomized or aged menopausal people who may be in the presence of a fraction-related infection (Komrakova et al., 2020). Some research has proved the sex mechanism in bone healing, pain, and

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response to injuries, but the mechanisms remain unclear (Ortona et al., 2023). Gjertsson et al., when studying septic arthritis, found that ovariectomy could induce more bone mass loss, which results from cytokine reduction due to a low level of serum estrogen (Gjertsson et al., 2012). However, it remains unknown if the severity of the infection and bone healing is worse in ovariectomized people with bone infection at the fracture site. One recent animal study showed that the instability of the fracture site without the implant could lead to exaggerated immune responses in an infected fracture model (Sabatè-Brescó et al., 2021). Parenteral antibiotic therapy has a moderate impact on fracture healing and microbe eradication. Systemic antibiotic administration to ovariectomized patients is poorly understood.

In the present study, the healing and repair disorders of infected broken bones were investigated in ovariectomized rats, which probably developed osteoporosis within three months, with severe lysis of cortical bone, increased bacterial load on the bone, inflammation, and necrosis at the fracture site, and decreased bone formation. It was found. More importantly, these changes were partially reversed by systemic antibiotic treatment until day 60 in group I, but not in group OI.

More CFU at the fracture site means more infection of the bone tissues. The bacterial invasion and colonization of the fracture site is another important pathological problem. In 2017, Bentley et al. labeled the bacterial colonies with BrdU and detected *S. aureus* colonization through the haversian system in chronic osteomyelitis, which means bacterial migration. The dense cortical bone may play a major role in the spread of infection because the host systems undergo several remodeling processes in the bone repair (de Mesy Bentley et al., 2017). In this study, a high number of CFU was detected in the bone tissue of ovariectomized mice at the infected fracture site. In addition to the fracture site, the anatomical and tissue holes in the cortical bone can be an escape site for bacterial growth, which prevents the take up of bacteria by the host body's phagocytes (Muthukrishnan et al., 2019). In addition to the physiological barrier, colonies of bacteria that invade the microenvironment undergo various biological changes that lead to increased growth and consumption of various nutrients. In this case, the minimum antibiotic inhibitor concentration will increase to 1000 times (Sharma et al., 2023). Although cefazolin is the first-line antibiotic for treating fracture infection, it cannot effectively reduce the extent of infection at the fracture site (Redfern et al., 2016).

On the contrary, antibiotic therapy in our study was able to significantly reduce the amount of CFU on day 60 but not on day 30 of the study. On the 30th day, the antibiotic reduced the infection in ovariectomized mice, but not on the 60th. A significant increase in serum inflammatory cytokines in bone infection cases has been identified in previous studies (Lühje et al., 2020). The OI and OIA groups (TNF- α only) showed a significant increase in serum levels of IL-6 and TNF- α on the 30th day of the present study, compared to the O group. However, it was not observed between the OI and OIA groups. This means that on day 30, antibiotic therapy in ovariectomized mice (probable osteoporosis) failed to reduce inflammatory cytokines. On the 60th day of the present study, a significant increase in the serum levels of IL-6 and TNF- α was observed in the OI and OIA groups (only IL-6) compared to the O group. Also, between the OI and OIA groups, only the TNF- α level decreased. This means that on the 60th day of antibiotic therapy, ovariectomized mice (probable osteoporosis) were able to reduce TNF- α . In an uninfected fracture, the onset of inflammation and its appropriate reduction cause the formation of calluses, especially the formation of wavy bone blades and their subsequent remodeling. However, excessive and prolonged inflammation will have a disruptive effect on bone healing (Chow et al., 2020). During the prolongation of inflammation in infected bone tissue, pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs) on macrophages and some other inflammatory cells increase in expression, stimulating the release of various inflammatory cytokines, especially TNF, IL-1, and IL-6, leads to excessive activation of bone-eating cells called osteoclasts, resulting in lysis of bone tissue (Chow et al., 2019; Nishitani et al., 2016).

On the 30th day of the present study, in the ovariectomy with infection group, the rate and quality of union formation significantly differed and improved compared to the sham group. Also, there was a significant difference in the ovariectomy with the infection group that had received antibiotics compared to the sham group, which shows that antibiotics in this group cannot have much effect on the bone repair of ovariectomy patients. The rate of union formation in the oophorectomy group with infection was different from the oophorectomy group without infection, and it was higher in the oophorectomy group without infection. The rate of union formation in the oophorectomy group with infection that had received antibiotics differed from both the oophorectomy group and the oophorectomy group without infection. This indicates that the antibiotic did not contribute to bone healing in the ovariectomy group with infection. The infected group's level of inflammation differed from the sham groups, and it was notably lower in the latter. The level of inflammation in the infected group was significantly lower compared to the sham group. This indicates that the antibiotic did not significantly impact the infection rate in the ovariectomy group. In the ovariectomy group with infection, there was a difference compared to the ovariectomy group, and in the ovariectomy group, the inflammation level in the ovariectomy group was different compared to the ovariectomy with infection group that received antibiotics, and it was higher in the group that received antibiotics, and this sign This indicates that the antibiotic did not alleviate the infection in the group of ovariectomy patients with infection. The amount of bone regeneration was higher in the sham and ovariectomy groups compared to the other groups, while it was lower in the infection group, ovariectomy with infection, and ovariectomy with infection that received antibiotics.

On the 60th day of the present study, the rate of union formation in the ovariectomy group with infection was significantly different from the sham group, and it was higher in the sham group. The sham group had a significantly higher rate of union formation than the ovariectomy group with antibiotic infection. The union formation rate significantly differed between the ovariectomy group and the ovariectomy with infection group, as well as between the ovariectomy group and the antibiotic-treated ovariectomy with infection group. In the ovariectomy group, the union formation rate was lower

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than in the infection group, indicating that the antibiotic did not affect the infection. The inflammation rate in the sham group was different compared to the ovariectomy group with infection, and it was lower in the sham group. The inflammation rate was different in the sham group compared to the infected group, and it was higher in the infected group. The amount of inflammation in the ovariectomy group was different compared to the ovariectomy group with infection, and it was lower in the ovariectomy group. The rate of bone regeneration in the infected group compared to the sham group was significantly different, and it was higher in the sham group. The rate of bone regeneration in the infected group was different from the infected group that received antibiotics, and it shows that the antibiotic did not affect the infection. The infectious group showed a lower rate of bone regeneration compared to the ovariectomy group.

Our study did not show a significant increase of TNF- α and IL-6 in the bone defect callus of ovariectomy patients with fracture site infection treated with and without antibiotic treatment at 30 and 60 days. This means that the antibiotic did not affect the severity of the inflammation. These changes, along with a higher bacterial load, lead to a greater tissue response in the periosteum that increases bone loss, especially in the setting of an infected fracture.

Shiels et al. identified that if the treatment time of an open fracture is prolonged, the bacterial load of the wound at the fracture site may increase, reducing the ability of penetration and antibiotic-based therapy (Shiels et al., 2018). In a rat model of infectious fracture, Thompson et al. reported that osteoporosis caused by ovariectomy reduced antibiotic penetration and efficacy (Thompson et al., 2021). We have a similar observation of a significant decrease in bacterial load in group I at day 60 but not in OI. In another study, more or less infectious resistance has been observed in skin and knee joint infections in osteoporosis caused by ovariectomy (Gjertsson et al., 2012; Castleman et al., 2018). Our findings showed that 30 days after infection, systemic injection of cefazolin could not reduce CFU, but on day 60, it could reduce CFU in the non-ovariectomy group. The important point was that on the 60th day, the antibiotic could not reduce the infection in the ovariectomy group (the groups OI and OIA were not significant on the 60th day). Thompson et al. reported that group O reduces the antibiotic effect in a rat model of infected fracture. We have a similar observation of a significant decrease in bacterial load in group I at day 60 but not in OI. In this study, the breakdown of the blades and the disruption of the bone's local immune response, likely caused by ovary removal, made the antibiotic treatment ineffective against the fractured area's bacteria.

The CFUs in the haversian system or cortical cavities contain bacterial colonies, making it difficult for antibiotics to penetrate (Nishitani et al., 2015). We detected a significant increase in bacterial load in the bones of these rats despite only using cefazolin as a treatment. We need to conduct further studies on other systemic antibiotics to explore their impact on fracture healing and bacterial load. Our findings in ovariectomized rats indicated a more severe infection and a long delay in the healing of infected fractured bone, which showed poor clinical outcomes. Since systemic antibiotic treatment can't stop the infection from spreading in the broken bone wound or even on the implants in the OIA group, it may be necessary to look into other treatment options, such as taking out the infected implants from older patients. Progressive bacterial colonization of an infected fractured bone may also fail surgical excision of necrotic bone tissue. To address these issues, new therapies that target these key pathologic changes are necessary to improve outcomes and reduce the rate of recurrent infection associated with fractures in individuals with osteoporotic and incompetent ovaries, as well as the factors and influences on recovery. Aging and menopause also include osteoporosis and hormonal disorders, which are suggested to improve the regulation of bone homeostasis with more appropriate nutrition, such as the use of microbiota (Li et al., 2021).

5. Conclusion

In comparison to normal bone, infected bone fractures displayed increased bacterial load at the fracture site, increased fibrocartilaginous reactivity, worse bone callus healing, and bone lysis. Systemic antibiotics were shown to help lower the bacterial load on bones, enhancing the quality of the callus and increasing mechanical strength in broken bones that were infected in healthy people. However, similar benefits were not seen in rat bones that had been ovariectomized. Parenteral antibiotic administration cannot remove the cortical bone bacterial colonization, which may be the cause of poor healing in infected fractured osteoporotic bone. These results clarified why systemic antibiotic therapy in rats with ovariectomies had a reduced success rate. The major pathological characteristics identified here have a role in the therapeutic strategy for developing the next clinical therapy for infected fracture bone in ovariectomized individuals, given the elevated risk of infected fracture bone in older patients.

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6. References

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