RESEARCH ARTICLE

# Potential Role of Local Estrogen in Enhancement of Fracture Healing: Preclinical Study in Rabbits

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## Abstract

**Background:** Effects of estrogen on bone metabolism and its protective role on prevention of osteoporosis are well documented. However, the efficacy of estrogen treatment on bone healing is not well investigated. The drug can be delivered both systemically or locally to the bone with differences in concentrations and side effects. The aim of this study was to investigate the effect of local and systemic administration of estrogen on the fracture healing process.

**Methods:** Standardized tibial fractures with 4 millimeter gaps were created in twenty four adult male Dutch rabbits. Fractures were fixed using intramedullary wires and long leg casts. Rabbits were randomly divided into three groups. Group A was treated with twice a week administration of long acting systemic estrogen; group B was treated with a similar regimen given locally at the fracture gap; and group C received sham normal saline injections (control). Fracture healing was assessed at six weeks post fracture by gross examination, radiographic and histomorphometric analysis.

**Results:** Group B had significantly higher gross stability, radiographic union and gap reduction than the two other groups. Histomorphometric analysis showed higher cartilaginous proportion of periosteal callus area in the control group.

**Conclusions:** Our results showed that estrogen may enhance fracture healing of long bone in rabbits. Furthermore, local estrogen treatment might have better effect than systemic treatment.

**Keywords:** Local estrogen, Fracture healing, Preclinical study, Rabbit

## Introduction

The healing process in a fracture is influenced by a variety of biomechanical and biological (cellular, hormonal) factors. Bone metabolism is a complex process that involves a balance between bone resorption by osteoclasts and bone formation by osteoblasts (1-3). These processes are regulated by a series of growth factors including bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), transforming growth factors (TGFs), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), osteonectin, fibronectin, and osteocalcin (4-7).

Female sex hormones appear to be mandatory, not only for the acquisition of peak bone mass in both females and males, but also for the maintenance of bone mass in adults (8, 9).

Effects of estrogen on bone metabolism and its protective effect on bone mineral density (osteoporosis prevention) are well documented in the literature, but its effect on bone healing is still under investigation (10-17). A former study using micro-CT-based angiography on the role of estrogen receptor beta (ER-β) on femoral fracture healing in ER knockout (KO) mice has reported larger total vessel volume at the fracture site in the KO group at 1 and 2 weeks post-fracture. They concluded that ER-β blockade can be considered as a treatment strategy for osteoporotic or non-union fractures (18). Investigation of estrogen effects on mice fracture healing showed that estrogen is effective in all stages of fracture healing (19). Also, increased estradiol binding sites especially on the early and middle stages of fracture healing have been reported in a study on the presence and effect of estrogen receptors in fracture healing in New Zealand rabbits. However, no estrogen receptors were found in late

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fracture callus, growth plate, or periosteum. They have suggested a role for estrogen in the early inductive phase of endochondral ossification in fracture healing (21).

To the best of our knowledge there is no study comparing the effect of local and systemic estrogen administration on fracture healing. We sought to examine the effect of local estrogen on fracture healing in comparison with systemic therapy.

## Materials and Methods

### *Experimental Animals*

The Animal Experimentation Ethics Committee of Shiraz University of Medical Sciences had approved the care and experimental protocol of this study (project number 89-01-01-1957). The study sample consisted of 24 adult male Dutch rabbits weighing 1.8 to 2.2 kilograms who were selected and diagnosed healthy by a veterinarian. Male gender was selected to eliminate the effects of endogenous estrogen. Right tibial bone was selected due to ease of operation as well as post-operative splinting and casting.

### *Fracture Model and Surgical Procedure*

General anesthesia was induced using 8mg/kg of Xylazine and 44mg/kg of Ketamine for all animals. A 10mm longitudinal incision was made over the anterolateral crest of mid-shaft of tibia under sterile condition and after usual fashion shaving, prep and drape of the surgical site as for each animal. The fascia and periosteum were incised and handled with minimal trauma in all cases. A 4 mm bone defect was created using an oscillating saw at the middle of incision, making this middle point as the fracture zone marker. Another skin incision was made over the anterior aspect of the knee. The patellar tendon was identified and split longitudinally. An appropriate size K-wire was inserted into the medullary cavity from an entry point 5mm posterior to tibial tuberosity to fix the fracture according to intramedullary bone diameter. The periosteum and fascia were repaired over the fracture site with running-locked non-absorbable sutures and the skin was closed by nylon. Local 3% Tetracycline spray and sterile dressing were applied. This method of fixation produced more angular stability than rotational stability. A cylindrical cast was applied to provide more rotational stability and a window was made over the wound at the fracture site. Intramural Penicillin (2200mg/kg/day) was given pre-operatively and continued for 3 days post-operatively. Intramural Flunixin (1.1 mg/kg/Bid) was used for analgesia exactly after operation and continued for 5 days. Animals were postoperatively kept in a standard cage (12 hours in sunlight and 12 hours in dark) and allowed to bear weight on their operated extremity as their pain subsided with standard food and water for 6 weeks. All rabbits, their casts and surgical wounds were inspected daily and wound care and cast trimming was performed as needed.

### *Estrogen Treatment and Experimental Design*

Animals were divided into three equal groups. Group A (systemic estradiol) were treated with twice a week long-acting estradiol injections (200µg/kg) given in the subcutaneous area of back. A sham injection of normal saline was also given at fracture site at the middle part of incision under the periosteum twice a week to mimic the stress induced by injection in other groups (24, 25). Group B (local estradiol=LE) were treated with twice a week injection of long-acting estradiol (200µg/kg) at fracture site under the periosteum and daily sham injection of normal saline in subcutaneous area of back. Group C (control) were treated with sham injections twice a week both at the fracture site and at the subcutaneous area of back. Animals were sacrificed six weeks postoperatively with an overdose of diethyl ether inhalation.

### *Assessment of Fracture Healing*

*Gross Examination and Mechanical Assessment*

Right legs were separated from the bodies and sent to a blinded observer. The intramedullary K-wires were removed and the fracture sites were grossly evaluated for presence of motion at the fracture site. After fixation of both bone ends, standardized 20N bending force was applied to the specimens very slowly in the anteroposterior and lateral directions and the amount of movement was assessed visually. The specimens were divided into 4 groups based on the amount of movement at the fracture site to resist the applied force ; no motion, mild movement(1-3 mm), moderate movement (3-6 mm) and excessive movement (>6 mm).

*Radiographic Assessment*

AP and lateral radiographs of the specimens were obtained using standard technique before sending the specimens for gross examination. Radiographs were examined to measure the fracture site gap using Agfa® software. With regards to callus formation, the specimens were divided into three groups: complete union (3 or 4 cortices), partial bridging (one or two cortices) and nonunion (no cortices).

*Histomorphometric Analysis*

The specimens were dissected free of soft tissue, fixed in buffered 10% formalin and decalcified for 2-5 days depending on the density of bone. Five micrometer sagittal sections were prepared. Hematoxylin and eosin (HE) as well as toluidine blue stains were used to evaluate the cellularity and cartilaginous portions of callus area respectively.

Histomorphometric analysis was performed by a blinded pathologist to calculate the tibial diameter (TD), cortical width (CW), periosteal callus area (PCA), the cartilaginous proportion of the PCA (CPCA), the mineralized proportion of the PCA (MPPCA), and the mineralized to cellular area ratio of cortex (M/C ratio). The PCA was also examined to determine the percentage of osteoblasts, osteocytes, and chondrocytes separately in oil immersion fields per section.

### *Statistical Analysis*

Statistical analysis was carried out using SPSS, version 15 (Chicago, IL). All variables were examined for normality by Shapiro-Wilk test. Non parametric data analysis was performed using Kruskal-Wallis, followed

by Mann Whitney U test. The level of significance was considered at $P<0.05$.

## Results

Two rabbits were excluded from the study groups (in groups A and C) and sacrificed due to uncontrollable infection at the fracture site.

### *Gross Evaluation*

Figure 1 shows a comparison of gross evaluation between the three groups. Complete union was seen in 57% (4 of 7) in group A, 75% (6 of 8) of cases in group B and 14% (1 of 7) in group C .These differences were statistically significant ($P=0.03$).

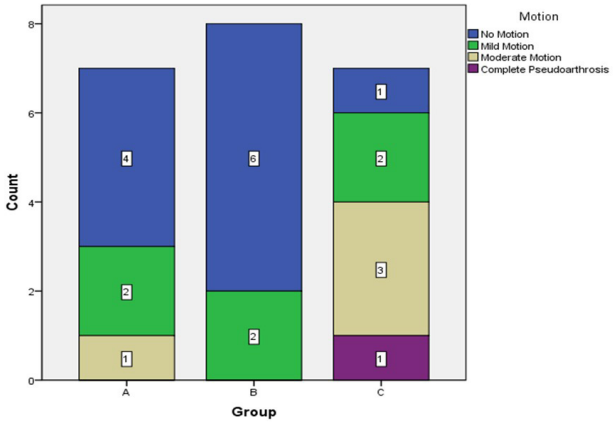


**Figure 1. Bar graph showing the numbers of cases with amount of motion in each group.**

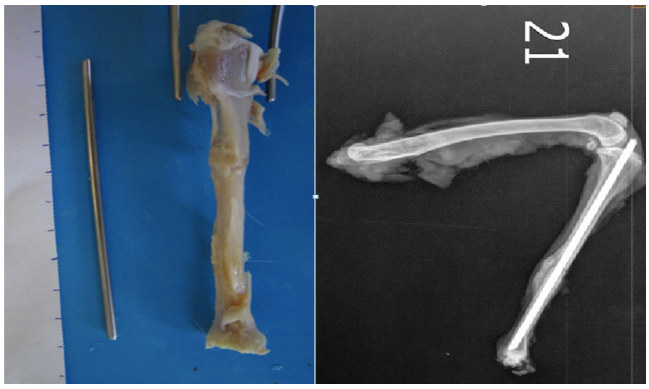

**Figure 2. Specimen (left) and its radiographic appearance (right) in a case of complete healing in group A.**

### *Radiographic Assessment*

Fracture gap was reduced in all cases expect two cases in group C which developed pseudarthrosis. A significant difference was found in the rate of complete closure of the fracture gap [Figure 2] between group A, 57% (4 of 7); group B, 75% (6 of 8); and group C, 14% (1 of 7) ($P=0.03$). Full data of radiographic evaluation is

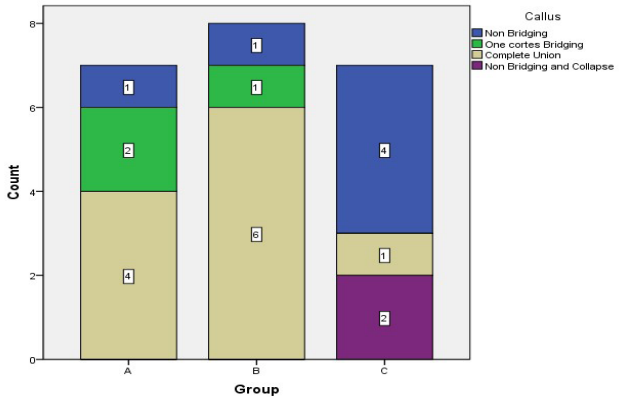


**Figure 3. Bar graphs illustrating the types of callus formed in each of the three groups.**

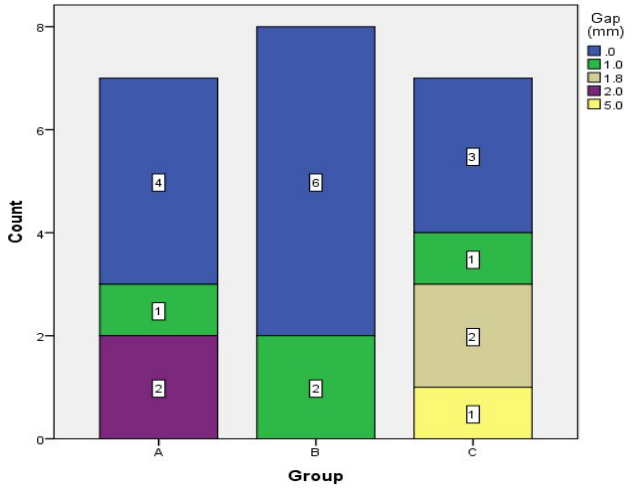


**Figure 4. Bar Graph showing size of gap in millimeters in three groups.**

**Table 1. Results of gross clinical evaluation for each case**

| Groups | A | A | A | A | A | A | C | C | C | C | C | B | B | B | B | B | B | B | B | C | A | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Specimen no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
| Motion | 2 | 2 | 1 | 1 | 3 | 1 | 1 | 3 | 4 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 3 |
| Bridging callus | + - | + - | + + | + + | - - | + + | + + | - - | - - | - - | - - | + + | + + | + + | + + | + + | + + | - - | + _ | - - | + + | - - |
| Gap (mm) | 2 | 1 | 0 | 0 | 2 | 0 | 0 | C* | 1 | 5 | 1.8 | C* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1.8 |

No motion: 1, Mild: 2, Moderate: 3, Severe (pseudo arthrosis): 4, (- , -): Non-bridging callus, (+, -): One or two cortices bridging callus (partial union), (+, +): Three or four cortices bridging callus (complete union), C*: nonunion with collapse

**Table 2. Results of tibial diameter (TD),cortical width (CW), periosteal callus area (PCA) the cartilaginous proportion of the PCA (CPCA), mineralized proportion of the periosteal callus area (MPPCA), and the ratio of mineralized to cellular area of cortex (M/C ratio)**

| | A | B | C | P |
|---|---|---|---|---|
| **Number** | 7 | 8 | 7 | |
| **TD In mm (mean)** | 6.78 | 7.00 | 6.60 | 0.929 |
| **CW in mm (mean)** | 0.971 | 1.087 | 1.442 | 0.189 |
| **PCA in mm²(Mean, Max)** [µ] | 17.31, 28 | 23.03, 44 | 13.6, 20 | 0.423 |
| **CPCA % (Mean)** | 34 | 22 | 54 | **0.02** |
| **MPPCA% (Mean ,range)** [µ] | 30 (10-50) | 50 (30-80) | 42 (10-50) | 0.481 |
| **M/C ratio % (Mean, range)** [µ] | 34 (5-60) | 46 (10-90) | 42 (30-60) | 0.623 |
| **Osteoblast%** | 60 | 43 | 41 | 0.085 |
| **Osteocyte%** | 51 | 48 | 55 | 0.102 |
| **Chondrocyte%** | 63 | 63 | 74 | 0.468 |

**Table 3. Percentages of CPCA for each case**

| **Groups** | A | A | A | A | A | A | C | C | C | C | C | B | B | B | B | B | B | B | B | C | A | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Specimen no.** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
| **CPCA %** | 50 | 50 | 10 | 20 | 80 | 10 | 40 | 70 | - | 40 | 80 | 20 | 20 | 5 | 30 | 5 | 20 | 40 | 35 | 30 | 20 | 65 |

summarized in Table 1. Figures 3 and 4 show bar graphs for callus and fracture gap in the three groups.

### *Histomorphometric Analysis*

The results of histomorphometric analysis are summarized in Table 2. No significant differences were observed in tibial diameter (TD) and cortical width (CW) between the three groups. Although group B had the highest percentage of PCA and MPPCA as well as the M/C ratio, the differences were not statistically significant. The CPCA was significantly lower in group B ($P$=0.02) compared to the other groups [Figure 5], while no significant differences were observed between group A and C ($P$=0.423). No significant differences were found in the percentage of osteoblast, osteocyte and chondrocyte between the three groups.

The percentage of cartilaginous part of PCA (C/PCA) was significantly lower in group B compared to the other groups ($P$=0.02), while no significant difference was observed between group A and C [Table 3].

Figures 6 and 7 describe the histopathologic finding of diversity and differences of osteocartilaginous callus formation between the three groups.

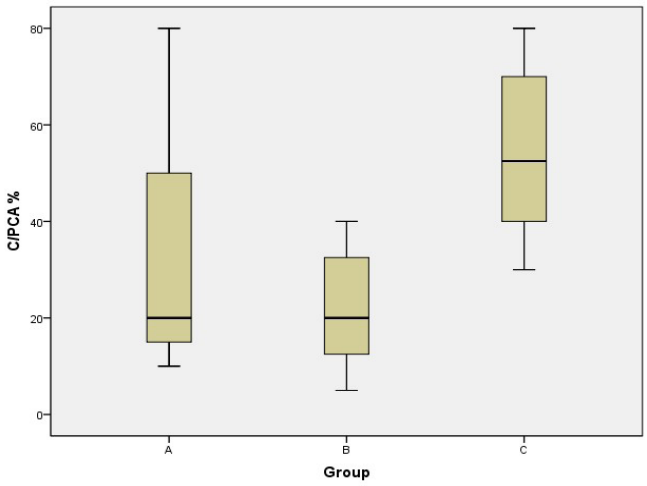


**Figure 5. Box plot graph showing the median and interquartile ranges of CPCA percentage in three groups.**

### *Complications*

Two cases in group C had collapse at fracture site due to underlying nonunion while another case of this group developed pseudo arthrosis [Figure 8]. These findings were not seen in any cases of the other two groups. One case from each of the groups A and B had some degree of malunion due to loss of fixation.

## Discussion

We evaluated the effect of local administration of exogenous estrogen on fracture healing in adult male rabbits. Few studies have evaluated the effect of estrogen treatment on fracture healing by creating ovariectomy or hypophysectomy induced osteoporosis and evaluated the effect of estrogen on bone healing in the absence of endogenous estrogen (19,22,23). All previous studies have used systemic estrogen treatment in fractures without gap; whereas we produced 4 mm fracture gap in order to create more instability and try to induce more enchondral ossification than direct bone healing.

Generally, union assessment in clinical settings is performed by clinical and radiographic evaluation. We

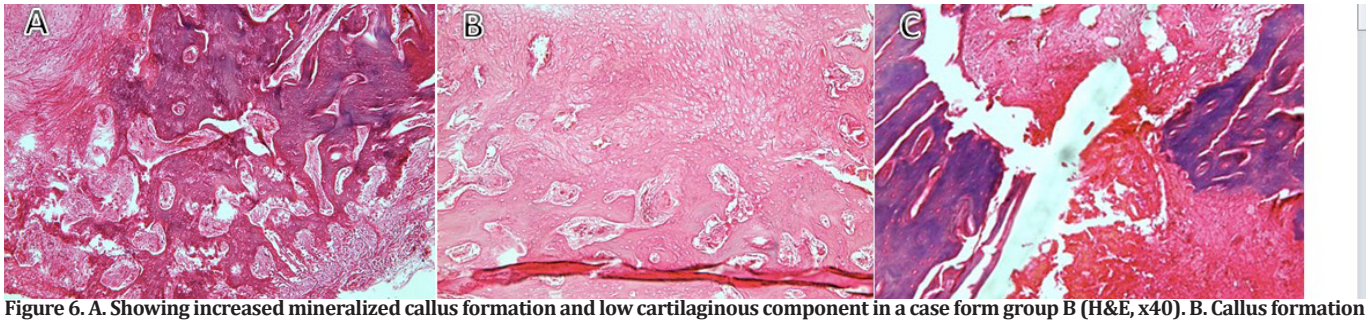


**Figure 6. A. Showing increased mineralized callus formation and low cartilaginous component in a case form group B (H&E, x40). B. Callus formation with small foci's of mineralization. The callus is mostly composed of cartilaginous component (H&E, x40) (Group A). C. mostly blood clot (hematoma) and small focus of callus formation (at left side) between two fracture ends at fracture gap (H&E, x40) (case of control group).**

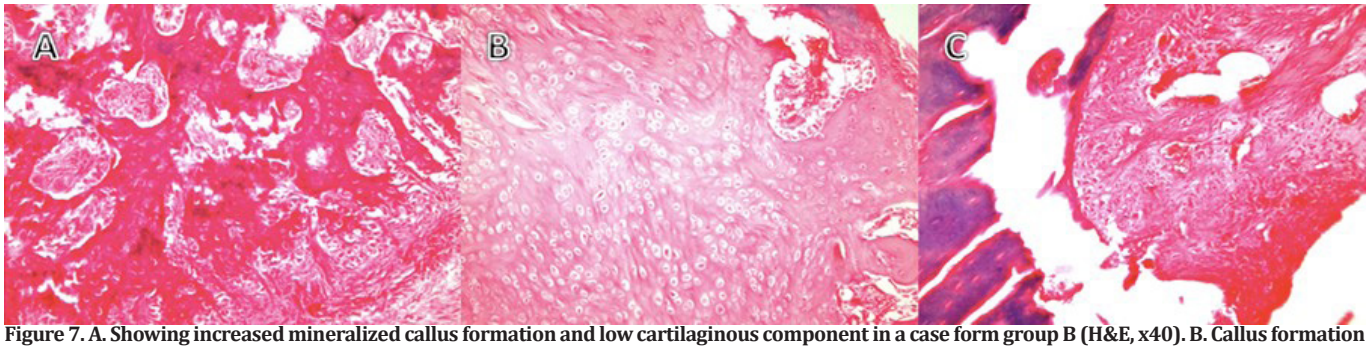


**Figure 7. A. Showing increased mineralized callus formation and low cartilaginous component in a case form group B (H&E, x40). B. Callus formation with small foci of mineralization. The callus is mostly composed of cartilaginous component (H&E, x40) (Group A). C. mostly blood clot (hematoma) and small focus of callus formation (at left side) between two fracture ends at fracture gap (H&E, x40) (case of control group).**

used the same method for evaluation of healing process in late phase (6 week) of fracture healing in rabbits. Radiographic assessment revealed higher rates of union in estrogen treated groups comparing with control. There were also higher rates of union and reduction of fracture gap in locally treated group comparing to systemic administration. These findings are compatible with Beil et al study, although they used micro-computed tomography (19). Considering the results of these gross assessments there seems to be a role for estrogen treatment in fractures with gap as well as a role for local estrogen treatment compared to systemic regimen. The higher rate of healing in the locally treated group suggests that higher local concentrations of estrogen might have an effect on bone healing. Previously, a case

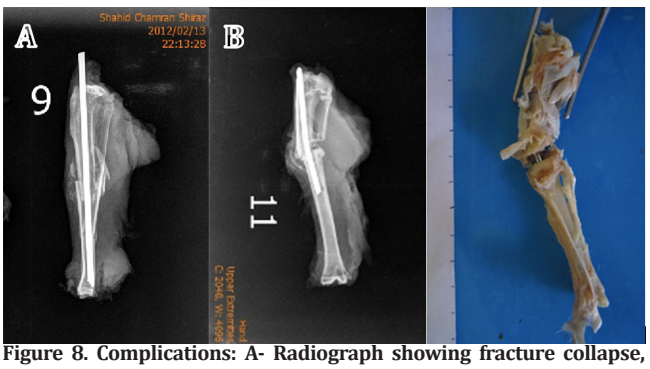


**Figure 8. Complications: A- Radiograph showing fracture collapse, B- Radiograph and picture showing pseudarthrosis both in control cases.**

of accelerated tibial fracture healing in the third month of pregnancy has been reported by Ahmad et al. They suggested that the high serum level of estrogen as well as the hyper-dynamic circulation in pregnancy contribute to accelerated fracture healing (20).

The results of mechanical stability test in all three groups of our study were exactly similar to the radiographic findings, suggesting that an increase in the number of bridging callus cortices will be associated with increased stability. Although Beil et al. used a different fracture healing stability test, however they showed that estrogen treatment can significantly increase the stiffness compared to the control and estrogen deficient groups (19).

Although no statistically significant difference was observed in the PCA between our three groups, the estrogen treated rabbits and particularly the local-estrogen treated group showed higher values of this parameter. Increased PCA values in the early phase of estrogen treated cases (7 and 14 days of healing) have already been reported in Beil et al study. This difference can be attributed to the presence of gap at the fracture site in our study which makes the healing process much more prolonged. We found a higher percentage of cortex mineralization in locally treated group compared to the other two groups. Beil et al also showed that callus mineralization occurs significantly faster in estrogen treated cases in 14 and 42 days of fracture healing (19).

CPCA ratio is an indicator of maturity of bone healing process where lower ratios mean higher maturity rate of healing bone. Lower CPCA ratio in locally treated

rabbits compared to the other groups emphasizes the effect of estrogen in enhancement of osteoid maturation in late phase of healing. Negulesco et al and Beil et al had similar findings and showing that the amount of cartilaginous part of newly formed bone decreased in late phase of healing process (19, 22). Our findings show a reverse relationship between the CPCA ratios [Table 2] and clinical bone strength. In other words, as the rate of healing maturation increases, the bone is clinically more resistant to applied forces.

Comparison of the population of osteoblast, osteocyte and chondrocyte in the three groups did not show any statistically significant differences while a higher number of osteoblast was seen in systemically treated group. Negulesco et al had similar findings in late phase, but they could also show increasing osteoblasts population in the early phase which was not studied in our work (22).

The presence of collapse (two cases) and pseudarthrosis (one case) in the control group, along with absence of these findings in estrogen treated groups suggest a role for estrogen effect on bone healing in presence of gap.

Authors admit several limitations in the current study. This study was not designed to evaluate the different phases of fracture healing process. We were not able to formally evaluate and compare the biomechanical strength of callus due to lack of biomechanical instruments. Instead we have used our proposed visual scale for this purpose with blinded assessors and good inter and intra-observer reliability for this assessment method.

Estrogen may enhance the long bone fracture healing in rabbits, while local estrogen treatment might have better effects compared to systemic treatment. Studies with bigger sizes as well as more confirming tests are advised as suggestions for future studies. Use of a scaffold that contains slow released estrogen near the fracture site, would allow for a more precise release of estrogen which may be more relevant in a clinical setting. It has to be examined if a sustained released would produce similar results to a pulsed treatment or not.

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