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## Association of calcium level and bone tissue in ovariectomized female adult rabbit (An experimental study).

### ABSTRACT:

**Background** Ovariectomy induced estrogen depletion, which affected bone healing and remodeling. Calcium closely tied to magnesium and phosphorus regulation.

**The aim** of the study was to find the effect of bilateral ovariectomy on femur bone tissue in sexually mature female rabbit and on calcium magnesium and phosphorus level.

**Subjects and Methods:** Forty- eight sexually mature female rabbits were used and divided in to 6 groups; control, groups A, B, C, D, E. Bilateral overectomy was made on the experimental groups. The overictomized groups were scarified at the interval of zero, 7, 14, 30, 60 and 90 days. Blood samples were taken from the experimental animals twice, one was before the overectomy operation and the sconed was at the end of experimental time and before scarifying the animals. Blood samples were centrifuged and serum was obtained and then tested for calcium, phosphorus and magnesium levels. Femur bone was also taken and proessed for H&E staining method. Highly statically difference was found between the control and groups D&E and no significant difference with group A&B in calcium level .phosphorus levels showed difference between the control and the study groups magnesium showed no difference between the control and group A, but statically difference with group B and highly significant deference with groups C,D and E.

**The result** with the femur bone showed no major changes between the control and groups A&B,while in group C resorption cavities appeared clearly. In group D multiple wide vacuoles or resorption cavities was clear .Group E showed osteoporotic changes with wide resorption area were obvious. It is clear from the present result, that OVX had osteoporotic changes on femur bone increased with time in association with calcium level decrease. The effect was minor with phosphorus and less pronounced with magnesium.

**In conclusion** of this study found that OVX is associated with calcium serum level decrease but not fast more than one month after OVX, the phosphorus level declined in rapidly after OVX then gradually rises up again in other groups, for magnesium showed no statistically significant difference.

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## **Introduction:**

Oestrogen has an important role in the development and growth of bones and later in the maintenance of the bone mass. It is believed that the major action of oestrogen on the skeleton in vivo is through the inhibition of bone resorption (1). Oestrogen deficiency triggers an increase in bone remodelling, which is characterized by increased osteoclastic activity (bone resorption), and it is increased but relatively deficit osteoplastic activity (bone formation). This causes rapid loss of bone mass and micro-architecture, resulting in osteoporosis(2) Menopause leads to rapid bone loss in women because oestrogen deficiency reduced calcium absorption and increases excretion; as a result, bone loss far outpaces bone deposition , Although serum calcium levels are usually normal in individuals with osteoporosis, total body calcium stores are greatly diminished (3). Calcium is closely tied to magnesium and phosphorus regulation (4) The aim of the study was to find the effect of bilateral ovariectomy on femur bone tissue in sexually mature female rabbit and on calcium magnesium and phosphorus level.

## **Martial and methods:**

Forty eight sexually matured adult female rabbits of the local breed were

selected. They were divided randomly in to 6 group control, group A ,B,C,D and each group contained 8 rabbits and have OVX then were scarified at the time intervals zero, 7days, 14days, 30days, 60days, 90days respectively. Blood samples were collected from the female rabbits twice one before then before sacrificing .The blood was collected from ear vein and from lateral saphenous vein in the leg. Blood samples were placed in non-heparin tubes for half an hour at room temperature for the purpose of clotting. Then, tubes were placed in the centrifuge for 15 minutes and 3,000 rpm then serum was obtained, which was withdrawn and placed in a new tubes, they were placed in a freezer until use. And then were tested for Calcium, Phosphorus, and Magnesium level. Also femur bone was collected and processed through Fixation , Decalcification, Dehydration, Clearing, Infiltration , embedding , Tissue sectioning with Rotary microtome , Tissue attachment , De-wax and hydration , Staining with Haematoxylin and Eosin and lastly Mounting . Microscopic investigation of tissue sections involved descriptive histology. A light microscope (Motic microscope) was used to perform the microscopic investigations of this study. Microscopic photographs were taken using (Optica\ SN 212973\Italy)

then Data were analysed statistically using a statistical Minitab program under SPSS and Microsoft Excel XP system. Data were presented in simple measure of mean  $\pm$  SD (standard deviation), minimum and maximum values

**Results:**

results of biochemical tests showed highly statistically significant difference between the studied groups regarding calcium level, but when compared to control by Post hoc analysis using LSD test each group, they show that there was highly statistically significant difference between control group and E , F groups only .while , statistically significant difference in group C and no statistically significant difference in both group A and B when compared to control . When we compare the control group with 90 day OVX group (11.94  $\pm$  0.54) vs (10.21

$\pm$  0.39) mg/dl it shows great difference. Phosphorus level also showed difference between control group, (6.13  $\pm$  0.08) and the other studied groups (Table1&figure1). The level was declined in group A rapidly (5.10  $\pm$  0.08) then gradually rises up again in other groups till reach ( 5.55  $\pm$  0.11 ) in group E but still all were below the control it seems that OVX made rapid decline in phosphorus (Table2 and figure2) , The results of magnesium showed no statistically significant difference when compare group A and control , Also the study found that statistically significance on group B and highly statistically significance with the rest of the groups when compared to control .although, all result still in the normal range for serum magnesium in rabbits(Table3,and figure 3)

**Table-1-Mean and SD of calcium values for the control and experimental groups.**

Groups	Calcium (mg/dl)		One-Way ANOVA	
	Mean $\pm$ SD	Range	F	P-value
Control (n=8)	11.94 $\pm$ 0.54	11.09 – 12.9	40.011	<0.001
Group A (OVX 7 days) (n = 8)	11.77 $\pm$ 0.11	11.65 – 11.9		
Group B (OVX 15 days) (n = 8)	11.74 $\pm$ 0.10	11.56 – 11.87		
Group C (OVX 30 days) (n=8)	11.44 $\pm$ 0.03	11.4 – 11.49		
Group D (OVX 60 days) (n=8)	11.01 $\pm$ 0.18	10.7 – 11.2		
Group E (OVX 90 days) (n=8)	10.21 $\pm$ 0.39	9.79 – 10.8		
<b>Post hoc analysis using LSD test</b>				
Control Vs Group A	Control Vs Group B	Control Vs Group C	Control Vs Group D	Control Vs Group E
0.250	0.166	0.001	<0.001	<0.001

Figure (1); Comparison between the studied groups regarding Calcium (mg/dl)

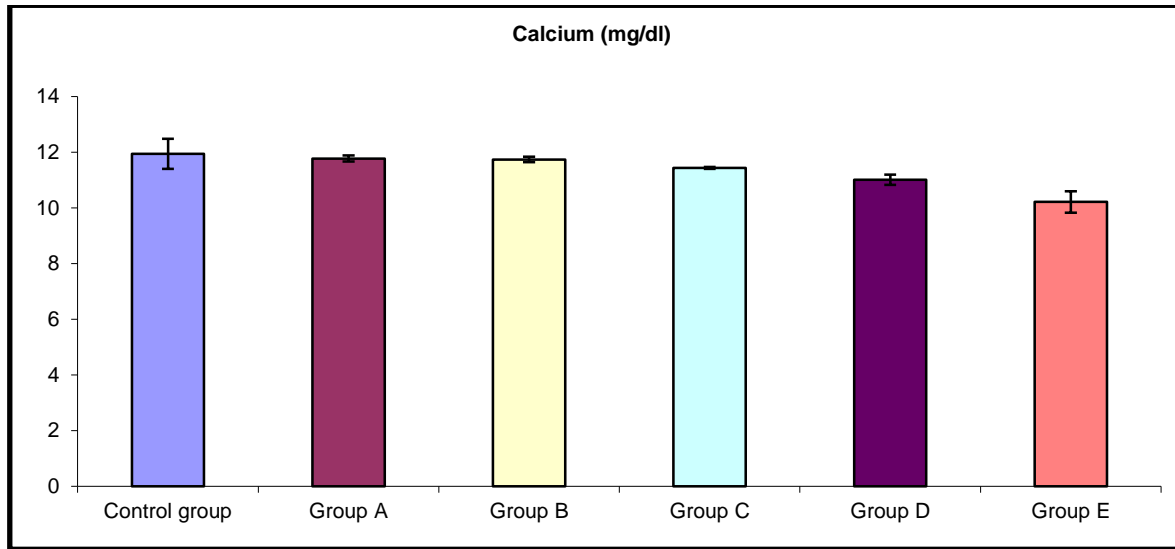


Table-2-Mean and SD of phosphorus values for the control and experimental groups

Groups	phosphorus (mg/dl)		One-Way ANOVA	
	Mean ± SD	Range	F	P-value
Control (n=8)	6.13 ± 0.08	6.01 – 6.22	74.078	<0.001
Group A (OVX 7 days) (n = 8)	5.10 ± 0.08	5.01 – 5.21		
Group B (OVX 15 days) (n = 8)	5.34 ± 0.06	5.24 – 5.44		
Group C (OVX 30 days) (n=8)	5.46 ± 0.21	5.23 – 5.9		
Group D (OVX 60 days) (n=8)	5.53 ± 0.07	5.45 – 5.65		
Group E (OVX 90 days) (n=8)	5.55 ± 0.11	5.34 – 5.67		
Post hoc analysis using LSD test				
Control Vs Group A	Control Vs Group B	Control Vs Group C	Control Vs Group D	Control Vs Group E
<0.001	<0.001	<0.001	<0.001	<0.001

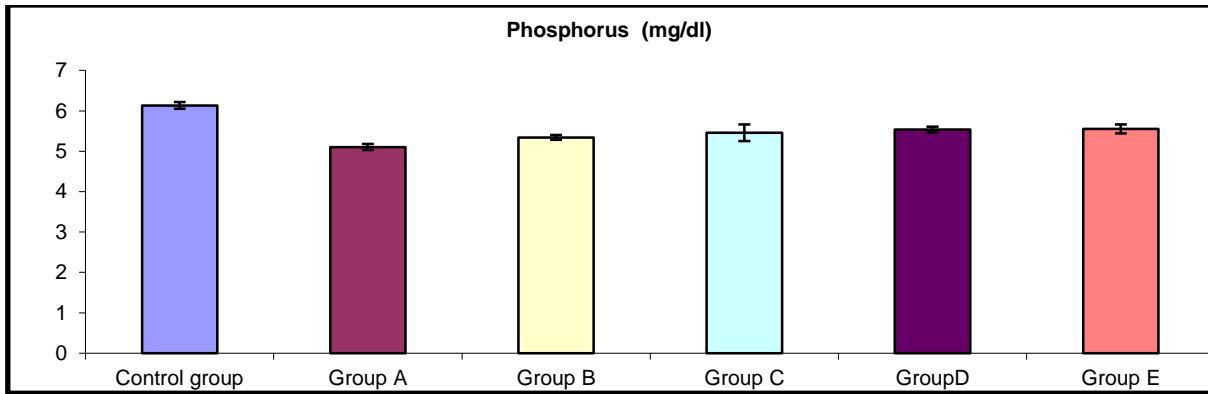


Figure 2 : comparison between the studied groups regarding phosphorus (mg/dl)

Table-3-Mean and SD of magnesium values for the control and experimental groups

Groups	Magnesium (mg/dl)		One-Way ANOVA	
	Mean ± SD	Range	F	P-value
Control (n=8)	2.75 ± 0.11	2.57 – 2.9	6.778	<0.001
Group A (OVX 7 days) (n = 8)	2.80 ± 0.02	2.77 – 2.83		
Group B (OVX 15 days) (n = 8)	2.82 ± 0.01	2.8 – 2.84		
Group C (OVX 30 days) (n=8)	2.86 ± 0.02	2.83 – 2.89		
Group E (OVX 60 days) (n=8)	2.87 ± 0.02	2.83 – 2.9		
Group F (OVX 90 days) (n=8)	2.89 ± 0.07	2.78 – 2.98		
Post hoc analysis using LSD test				
Control Vs Group A	Control Vs Group B	Control Vs Group C	Control Vs Group D	Control Vs Group E
0.076	0.012	<0.001	<0.001	<0.001

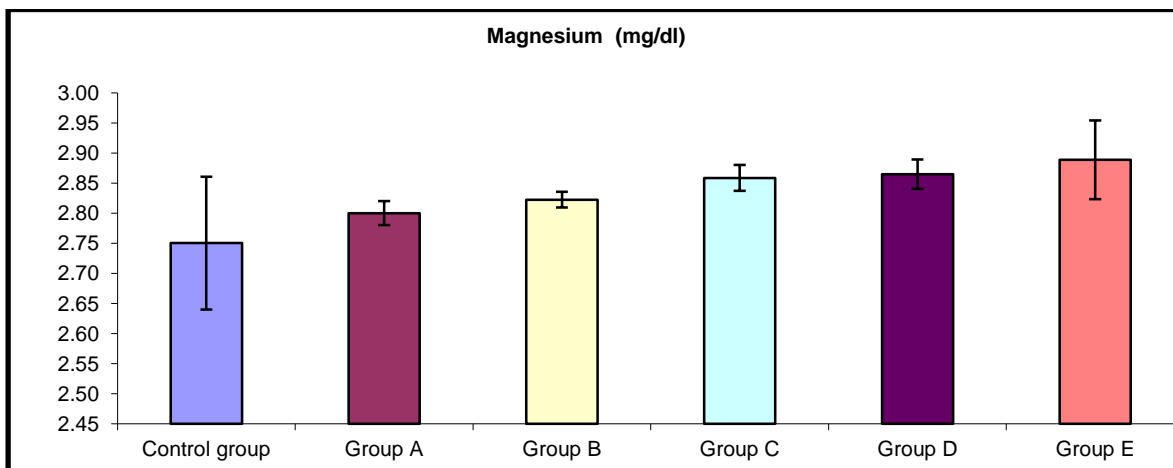
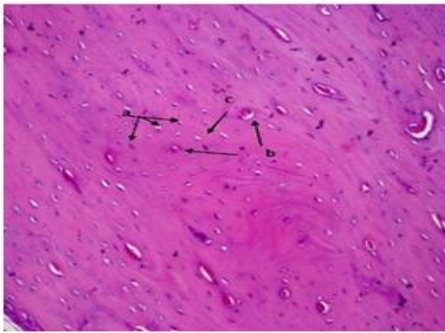


Figure 3; Comparison between the studied groups regarding phosphorus (mg/dl)

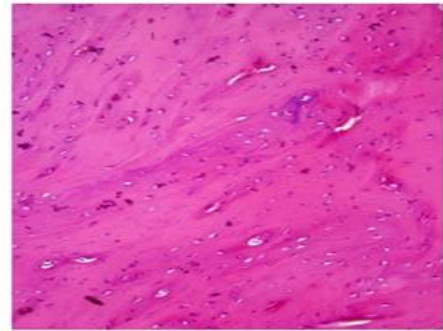
## Histological changes

The results of the present study showed the control rabbit femur bone with normal architecture of lamellar bone in form of concentric lamellae of collagen and oval osteocytes imprisoned in lacunae, surrounding Haversian canal (Figure 4). There was no major changes when it was compared to group A OVX 7 days more or less normal appearance of lamellar bone structure as the effect of OVX on the bone (Figure 5) while group B (OVX 14D), osteocytes lacunar spaces increases and become

more irregularly arranged with alternating of acidophilia with basophilic osteoid matrix (Figure 6). In group C OVX 30D resorption cavities appeared evident and more irregularly osteocyte distribution (Figure 7), when compared it with group D OVX 60D multiple wide vacuoles within bone matrix resembling wide resorption cavities with hyperactivity of osteoclast were obvious (Figure 8). Group E OVX 90D osteoporotic changes were very clear with multiple wide irregular resorption areas and more multiple active osteoclasts (Figure 9 a, b).



**Figure (4):** section of control rabbit femur bone shows, normal architecture of lamellar bone in the form of concentric lamellae of collagen and oval osteocytes imprisoned in lacunae a, surrounding Haversian canal b. Notice the distinct cement line demarcation c and acidophilic osteoid matrix (H&E, Mic. Mag. X 200)



**Figure (5):** Group (A) (OVX. 7 days) section of overectomy rabbit femur bone after one week showing more or less normal appearance of lamellar bone structure (H&E Mic.Mag X200).



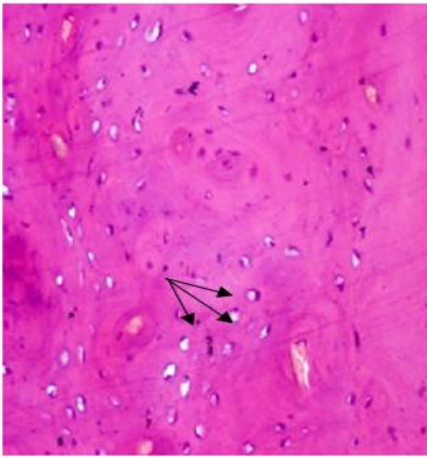


Figure (6) Group (B) OVX. 14 day, the osteocytes lacunar spaces increases and become more irregularly arranged. (H&E.Mic.Mag X200).

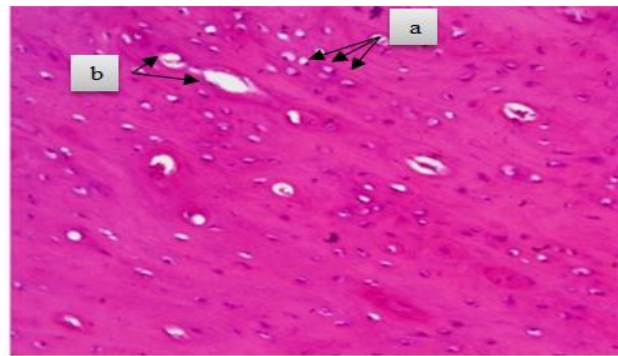


Figure (7) Group (C) OVX. 30 days, resorption cavities become more evident (a) and irregularly arranged osteocytes(b). (H&E Mic Mag X 200).

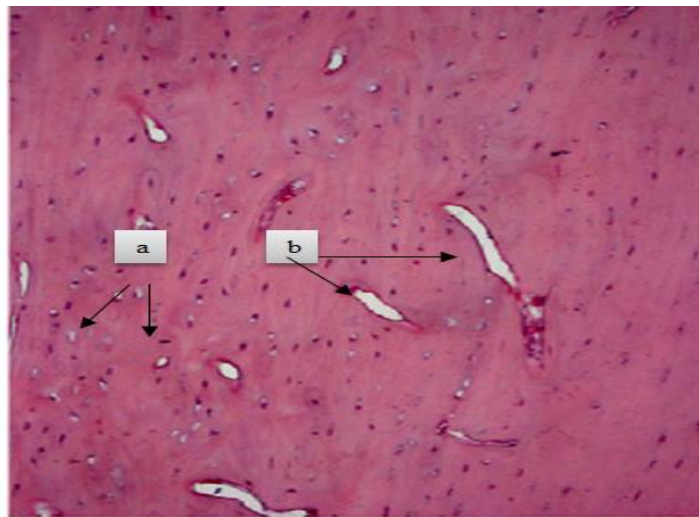


Figure (8) Group (D) OVX. 60 days. Pale acidophilic cytoplasm, irregular small-sized osteocytes (a) and multiple wide vacuoles within bone matrix (b). (H&E Mic Mag X200)

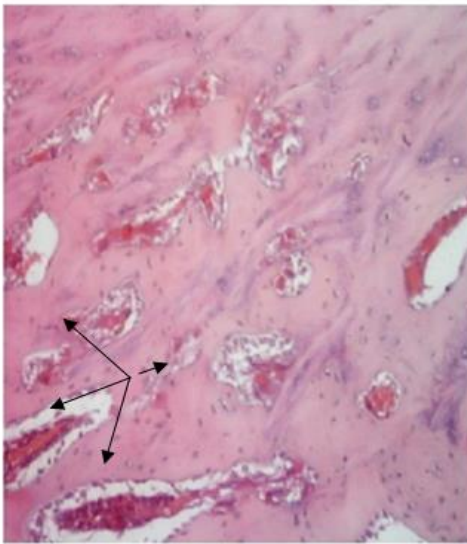


Figure 9a X200



Figure 9b (N) x1000 oil emersion

Figure (9a, b): Group (E) OVX. 90 days illustrating signs of osteoporotic changes with multiple wide irregular resorption areas. With more multiple active osteoclasts (H&E Mic Mag X200)

## Discussion

Highly statistically significant difference between the studied groups regarding calcium level but show that there was highly statistically significant difference between control group and D , E groups only. While, statistically significant difference in group C and no statistically significant difference in both group A and B when compared to control by Post hoc analysis using LSD test. The level was declining by time after bilateral ovariectomy. When the control group was compared with 90 day OVX group ( $11.94 \pm 0.54$ ) vs ( $10.21 \pm 0.39$ ) mg/dl it shows great difference it seems that decrease the level of calcium and it is associated with oestrogen level. Most of the studies shows that calcium level decreases after OVX which is contestant with the present study (4). The

results indicate a significant reduction in the serum calcium in OVX rats when compared with sham ( $2.35 \pm 0.036$ ) mmol/l vs ( $2.58 \pm 0.014$ ) in order they used the mmol/l which equal (9.4) mg/dL and (10.32) after 16 week. Group 2: Ovariectomized control and received vehicle: Group 1: Sham control and received vehicle. Also on other study by Funsun Sumar 2009 (5) ( $10.43 \pm 0.39$ ) mg/d in control group and it was much lower in OVX normal diet ( $8.12 \pm 0.42$ ) mg/d, A highly statistically significant difference between the studied groups regarding phosphorus level. Also the difference between control group ( $6.13 \pm 0.08$ ) and the other studied groups regarding phosphorus level. The level declined in group A rapidly ( $5.10 \pm 0.08$ ) then gradually rises up again in other groups till reach ( $5.55 \pm 0.11$ ) in



group E but still all below the control it seems that OVX made rapid decline in phosphorus and that body compensate the deficiency by time by somehow . In Funsun Sumar 2009 (5) study the control group level was  $(4.74 \pm 0.55)$  which decline to  $(3.47 \pm 0.66)$  in OVX group which is consistent with results.

The results of magnesium showed no statistically significant difference when compare the control  $(2.80 \pm 0.02)$  and group A  $(2.75 \pm 0.11)$  for A, that was similar to multiple study results obtained by Funsun Sumar 2009 (5) were not significantly different in any group  $(2.36 \pm 0.11)$  vs  $(2.34 \pm 0.15)$  mg/dl Serum magnesium levels. Also in the study found that, statistically significance in group B  $(2.82 \pm 0.01)$  and highly statistically significance with the rest of the groups when compared to control although, all result still in the normal range for serum magnesium in rabbits  $(0.8 - 1.2 \text{ mmol/l}) = (1.95 - 2.9 \text{ mg/dl})$ . (7) But another study by Muneyyirci-Delale (6) and his colleagues concluded that Serum levels of  $\text{Mg}^{2+}$  and total Mg were inversely correlated with the oestrogen concentration in menopausal women.

The histological results of the present study showed that control rabbit femur bone, with normal architecture of lamellar bone in the form of concentric lamellae of collagen and oval osteocytes imprisoned in lacunae, surrounding Haversian canal. Also cement line

demarcation and acidophilic osteoid matrix there was no major changes when compare to group A more or less normal appearance while group B osteocytes lacunar spaces increases and become more irregularly arranged with alternating of acidophilia with basophilic osteoid , in group C resorption cavities appeared evident and more irregularly osteocyte when compare it with the group D there are added multiple wide vacuoles within bone matrix resembling wide resorption cavities with hyperactivity of osteoclast At last, group E OVX 90D osteoporotic changes is very clear with multiple wide irregular resorption areas with more multiple active osteoclasts , Study done by Weitzman, and Pacifica (8) showed that acute effects of menopause are modelled by ovariectomy which, like natural menopause stimulates bone resorption by increasing osteoclast formation). Studies done by Cheng et al (9).and Persson et al (10). Showed reduction in the osteoblasts number, osteoporosis induces bone resorption, from mild decreases in cortical bone to severe destruction, this is in agreement with the present result while other studies showed the importance of oestrogen on osteoclast, bone resorption . This is in agreement with the present result. Where oestrogen is a well-known regulator of bone metabolism and is assumed to act by inhibiting osteoclast formation and by reducing the bone-resorbing activity of

terminally differentiated osteoclasts in study done by Lerner (11) in 2006.

## CONCLUSION

In conclusion of this study found that OVX is associated with calcium serum level decrease but not fast more than one month after OVX to show statistically significant difference that may be due to calcium resorption from the bone take time. the phosphorus level declined in rapidly after OVX then gradually rises up again in other groups but didn't reach the control group level may be due to effect of OVX on GIT absorption or kidney excretion of calcium, for magnesium showed no statistically significant difference. As for Histological changes on femur bone found that osteoporotic changes started to appear after 14 day which was mild osteocytes lacunar spaces increases and become more irregularly arranged and increased by time where more evident in last group after 90days with multiple wide irregular resorption and hyper active osteoclast appeared which is consistent with lack of calcium deficiency

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