Research Article

# The Histological and Biochemical Changes Occurred by Adding Platelet-Rich Plasma on Intra-bony Defect in Glucocorticoids -Induced Osteoporosis in Rabbits in Iraq

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**ABSTRACT**

Background Platelets are small discoid blood cells made in bone marrow with a lifespan of 7–10 days. Inside the platelets are many intracellular structures containing glycogen, lysosomes, and two types of granules. The alpha granules contain the clotting and growth factors that are eventually released in the healing process. Alpha granules are storage units within platelets, which contain pre-packaged growth factors in an inactive form). The main growth factors contained in these granules are transforming growth factor beta (TGFB), vascular endothelial growth factor (VEGF) platelet-derived growth factor (PDGF), and epithelial growth factor (EGF) the granules contain vitronectin, a cell adhesion molecule that helps with osseointegration and osseoconduction. Normally at the resting state, platelets require a trigger to activate and become a participant in wound healing and hemostasis. Autologous platelet rich plasma could be used in many clinical fields of oral and maxillofacial bone, implant reconstructive surgery, conservative and periodontology. Osteoporosis has an impact on bone healing process, platelets rich plasma (PRP) ameliorated the deleterious effect of osteoporosis on bone healing process. This study was carried out to evaluate histologically the regeneration capacity of autologous (PRP) on defect in the maxillary bone of osteoporotic rabbits.

**Keywords:** Platelet rich plasma, osteoporosis, glucocorticoids.

## INTRODUCTION

Bone is a hard connective tissue consisting of metabolically active cells that are integrated into a rigid framework and are imbedded into a matrix of mineralized substance and collagen fibers, composed of approximately 75% inorganic and 25% organic material [1] The ability of this tissue to store minerals and especially calcium mostly hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$], is the principal characteristics that distinguish bone from all other connective tissues [2]

Glucocorticoids (GC) are a class of steroid hormones that bind to the glucocorticoids receptor (GR), which is present in almost every vertebrate animal cell. The name glucocorticoids (glucose + cortex + steroid) derive from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal cortex, and their steroidal structure[3].

*Osteoporosis* (op) literally means 'porous bones'. The two Greek words, which make up the term osteoporosis, are "osteon" which means bone and "poros" which means pore.[4] In osteoporosis the bone mineral density (BMD) is reduced, bone microarchitecture is deteriorating, and the amount and variety of proteins in bone is altered. Osteoporosis is defined as a bone mineral density that is 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual x-ray absorbtiometry DXA. Osteoporosis itself has no specific symptoms; its main consequence is the increased risk of bone fractures. Osteoporotic fractures are those that occur in situations where healthy people would not normally break a bone; they are therefore regarded as *fragility fractures*.

OP-like conditions often manifested themselves in the geriatric female population, for which bone fracture has become common.[5]

The healing of bone tissue is a complex process, because it involved a number of cellular functions and mineralization of the defect followed by an eventual remodeling of the defect site to attain the original structure [6].

OP has received attention in all clinical field, as it is characterized by decreasing of bone structure, mass, and function. OP is thought to be an outcome of altered bone remodeling capacity, i.e., bone formation diminished while restorative capacity stays relatively stable.[7]

Several studies have shown us that bone repair procedures in normal defect or osteoporotic defect may be improved by the addendum of specific growth factors[5]. Growth factor is a protein molecules naturally occurring substance capable of stimulating cellular growth, proliferation and cellular differentiation. Usually it is a steroid hormone [8].

Platelet-rich plasma (PRP), also termed autologous platelet gel, plasma rich in growth factor (PRGF), platelet concentrate (PC) is essentially an increased concentration autologous platelet suspended in a small amount of plasma after centrifugation. PRP contains different growth factors and other cytokines that stimulate healing of bone and soft tissue.

Based on this principle PRP are used to accelerate the healing of injured bone, tendons, ligaments, muscles and joints. In this way, PRP injections use each individual patient's own healing system to improve musculoskeletal problems.

All of the known clinical applications of PRP highlight an accelerated tissue cicatrization because of the new formation of effective neovascularization, accelerated bone healing with fast tissue regeneration, and nearly total absence of side effect.[9]

## MATERIALS AND METHODS

Twenty four local breed adult female healthy rabbits of average weight 1.5-2 Kg ± 100 gm aged (8-12 months) were used in this study. These rabbits are divided into 4 groups (6 rabbits for each), Intra bony defect were made for each rabbits in the alveolar bone near the first molar in the maxilla these groups are:

Group A: (6 rabbits) control group with normal healing

Group B: (6 rabbits) control group received platelet rich plasma.

Group C: (6 rabbits) experimental group - osteoporotic group - with normal healing.

Group D:(6 rabbits) experimental group - osteoporotic group- received platelet-rich plasma.

### Animal Preparation

All rabbits in this study were received Ivermectine injection subcutaneously at a dose of 0.8ml/kg B.W. to prevent external and some internal parasite infection 2 days before surgery and the dose repeated after 21 days. Then the animals were fasted prior to operation for about 6-8 hours. One hour before operation, each rabbit had systemic administration of antibiotics using Procaine Penicillin and Streptomycin at a dose of 10,000 IU, 0.5 ml/Kg.

B.W I.M. respectively. These were injected into rear limb-thigh muscle of the rabbits, repeated after one week postoperative. The surgical procedures were done under general anesthetic drugs by using atropine sulfate at dose (0.4 ml/`Kg body weight) I.M. as a premedication to reduce salivary and mucous secretion, followed 10 minutes later by a mixture of ketamin hydrochloride 10% at a dose (50 mg/kg B.W) and xylazin 2% (5 mg/kg body weight) at a dose were injection I.M. respectively I.M. Application of eye ointment to prevent dryness of the cornea. Lidocaine hydrochloride 2% with adrenaline.

### Stimulation of osteoporotic-like syndrome

Twelve rabbits were received (10 mg/kg body weight) of hydrocortisone daily by i.m injection for 8 weeks to induced osteoporotic –like syndrome.[10]

### Production of Platelet Rich Plasma (PRP)

Five milliliters autologous blood taken from each rabbit prior to surgical operation from marginal ear vein[11]. integrated with1 ml of anticoagulant citrate dextrose phosphate (ACD-A) in sterile glass test tube to prevent coagulation.

The blood was centrifuged at 1200 rpm for 20 minutes to separate plasma-containing platelets from red blood cells.

The plasma was drawn off the top centrifuged. An additional 15 minutes centrifugation at 2000 rpm was done to separate the platelets. The platelets poor plasma was separated from platelets rich plasma along with puffy coat [12] which is about 0.5 ml. At the time of application, the P.R.P. was integrated with equal volume of sterile solution of 10% CaCl2, a citrate inhibitor that allow the plasma to coagulate which causes the platelets to be activated and released the growth factor and cytokines 13. After activation, PRP turned into gel – like solution with adhesive properties (within 30 min). In addition, freshly used by inserting it inside a syringe for application [14]

### Histological Preparation of Bone

The rabbits of all groups were sacrificed by Islamic staggering at 2, 4 and 6 weeks interval (6 rabbits for each interval). The maxilla of each rabbit was dissected, removing all soft tissue. The extracted maxillary bone processed as follow: [15]

Fixation: The specimen fixed in 10% buffered formalin solution immediately after resection for 72 hours. After fixation the specimens were decalcified in 10% formic acid for 10-15 days solution was changed every 48hours for best result, small needle must be penetrate the specimens to make insure from bone decalcification. After decalcification, the specimens were washed for 8-10 hours in running water.

Dehydration: Dehydrated it through graded series of alcohol 60%, 70%, 80% up to absolute alcohol then by xylene cleared.

Embedding: Each specimen was placed in a dish of melted paraffin wax and the dish was kept into a constant temperature oven regulated to about 60 c , during the course of several hours the specimen was changed to two or three successive dish of paraffin so that all of the xylene in the tissue was replaced by paraffin. Pour the wax on oriented specimens in metal blocks to get block of wax with the studied specimens.

Sectioning of the blocks as serial sections of 5µm buccolingually.

### *H&E staining procedure* [16]

1. Deparaffinized and hydrated to distilled water.
2. Staining with Harri's haematoxylin for 15 min.
3. Washing in tab water.
4. Differentiate with 1% acid alcohol for 10-20 min.
5. Washing in tap water for at least 5 min.
6. Dip in ammonia water 3-5 sec.
7. Wash in running water 10-20 sec.
8. Counter stain with 1% aqueous yellow eosin for 15 sec-2 min.

9. Dehydrate in 95% alcohol for few sec. until excess eosin removed.

10. Dehydrating with absolute alcohol for 3 min. tow changes.

11. Clearing with xylene and mounting in D.P.X. 12. Slides were examined on light microscope.

**Biochemical serum analysis**

Serum calcium* and phosphorus**, tests were investigated for both experimental and control groups on the sacrifice day. The anesthetized animal was placed in right recumbence, the area over the heart was swabbed with alcohol, and blood specimen was sampled by heart puncture. The heart beat was palpated by fingers and the needle introduced carefully over the point of strongest beat, beneath the xyphisternum into the chest, the needle was advanced until blood is aspirated. Five ml of blood was collected from the heart using 5cc disposable syringe. The blood was immediately poured into clean and sterile test tube (10cm× 1.5cm).

***Calcium**: Direct measurement by Ion Selective Electrode analyzer (ISE).

****Phosphorus**: Activity determined colorimetrically (Randox Kit, Germany). **

The sampled blood was left for coagulation in water bath at 37˚C for 15 min. Then the test tube was centrifuged (1500 rpm) for 5 min. Using Pasteur pipette, 2 ml of supernatant serum was collected. After that, the sample were stored frozen at (-20˚C) in polyethylene tubes for laboratory analysis. Using automated laboratory techniques.

**Statistical Analysis**

The mean of all fields within each group calculated, after that we find the standard error of them, by using one- way ANOVA test for testing the equality of means and two – way ANOVA was design in factorial experimental. We find the significant differences between groups and between healing periods. The data obtained were analyzed by using (SPSS) statistical software package, to determine the group's differences. All multiple comparisons significant p-value was at ($p< 0.05$).

## RESULTS

***Histomorphometric analysis for bone microarchitecture*:**

**Trabecular number**

Control group with PRP (Group B) was showed highest mean values of Tb.No, while the lowest values were determined in experimental group without PRP (Group C), these differences were found at 2ndand 4th week ($P\leq0.01$), but there was non-significant differences between groups at 6th week postoperatively. Furthermore, all groups had significant differences in the values of Tb.No with time progression ($P\leq0.05$) except group C showed no significant differences with increasing time. Table 1.

**Osteoblast number**

The highest mean values of osteoblast number were shown in group B than other three groups in almost 3 healing periods. However, the values tended to increased significantly with time progression from 2nd week to 4th week. While at 6th week duration, values of osteoblast number decreased significantly ($P\leq0.05$) in groups A & D, but showed no significant change for group C (experimental without PRP) while group B (experimental with PRP) show significant increase in osteoblast value ($P\leq0.05$). Table 2.

The highest mean values of osteoclast number were seen in group C, while the least mean values seen in group A. Almost all groups had significant reduction in OCL. No. with time progression ($P\leq0.05$), except group D (experimental with PRP) which showed non-significant differences in OCL no. with time. Table 3.

The results of this study denoted non- significant differences of calcium level in all groups with time progression. On the other hand, the highest mean values of calcium level were seen in osteoporotic group (C & D) than control groups (A&B) at 2nd and 4th week interval, but there were non- significant differences between groups at 6th week duration Table 4.

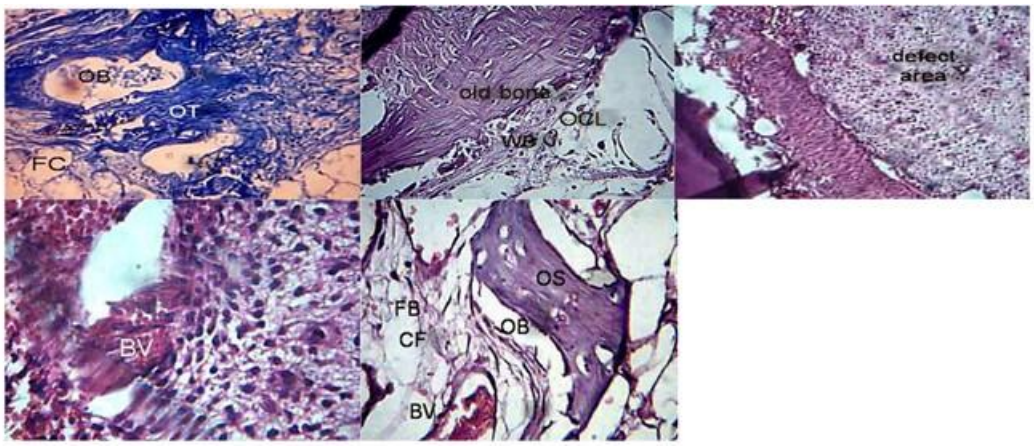


**Figure 1:** View of group A at the end of 2nd week showing newly osteoid tissue (OT) formation surrounded by osteoblast (OB), fat cells (FC) and haemopoeitic cells. (Gomori Blue Trichrome stain X100) while group B at the end of 2nd week showing presence of osteoclast

(OCL) on the periphery of woven bone (WB). (H&E stain X100 group C at the end of 2nd week showing that defect area (H&E stain X40) Other view of group C at the end of 2nd week showing (BV) and new bone matrix formation by active osteoblast (arrows) . (H&E stain group D (experimental with PRP) at the end of 2nd week showing newly bone trabeculae with osteoblast (OB), osteocyte (OS), fibroblast (FB), collagen fiber (CF) and blood vessel (BV). (H&E stain X200).

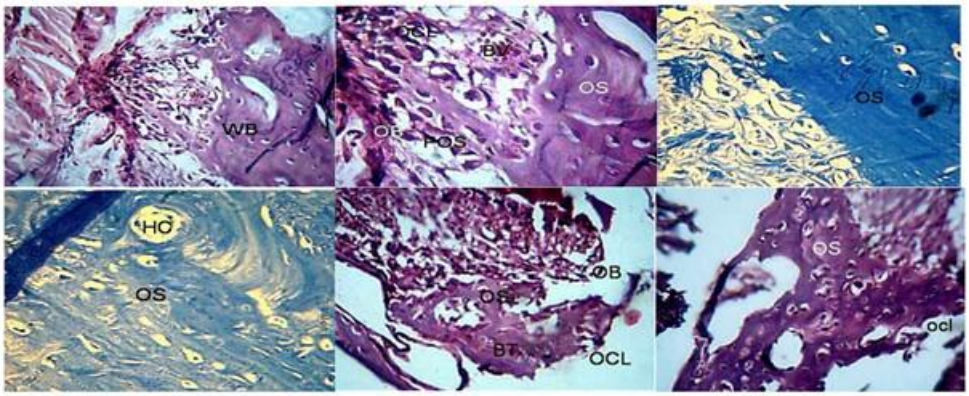


**Figure 2**: View of group A at 4th weeks duration showing new woven bone(WB) formation in the defect area (H&E stain X100) view of previous figure showing blood vessel (BV), preosteocyte (POS), osteocytes (OS), osteoblast (OB) and osteoclast (OCL).

(H&E stain X200) The histochemical picture of control group not treated with PRP at the end of 4th week duration revealed immature bone formation in the defect area surrounded by blood vessels and fatty tissue View of group A at the end of 4th week showing immature new bone formation filled by irregular arranged osteocytes (OS) (Gomori Blue Trichrome stain X200) Other view of group B at the end of 4th weeks showing osteocytes(OS) around Haversian canal (HC) (Gomori Blue Trichrome stain X100 View of group C at 4thweeks duration showing new bone trabeculae (BT) formation with osteocyte (OS), osteoblast (OB) , osteoclast(OCL) and degeneration necrosis of bone (arrow) (H&E stain X200) View of group D at 4th weeks duration showing new bone with osteocyte (OS) inside it and osteoclast (OCL) on periphery (H&E stain X200)

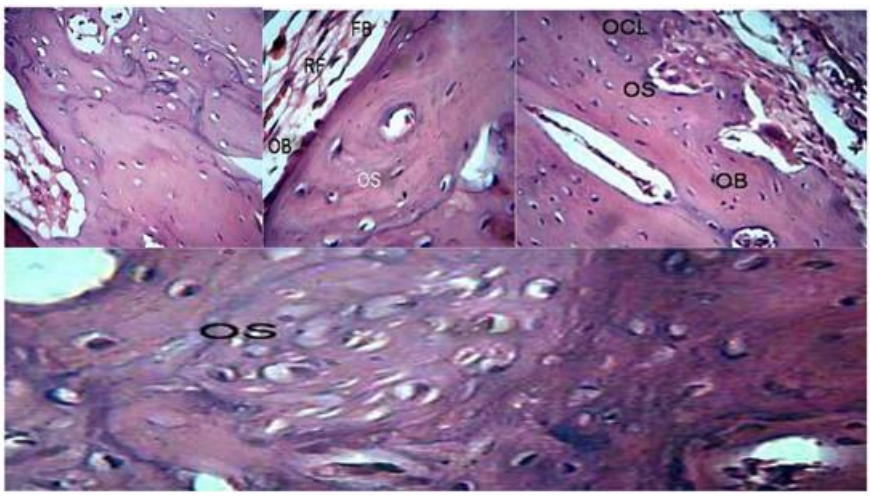


**Figure 3:** View of group A at the end of 6th week showing reversal line within new bone (arrows) .(H&E stain X200) View of group B at the end of 6th week showing circular arrangement of osteocytes (OS) around Haversian canal , osteoblasts (OB) reticular fiber (RF) and fibroblast(FB) (H&E stain X400) View of group C at the end of 6th week showing osteoblast(OB), osteocyte(OS), and osteoclast (OCL) (H&E stain X100) View of group D at the end of 6th week showing immature bone with irregular arrangement of osteocytes (OS), and white arrow showing the line between old and new bone(H&E stain X400)

**Table 1:** Trabecular number (mm-1 /mm$^2$) in different healing

| ***Studied Groups*** | ***Healing Period*** | | | |
|---|---|---|---|---|
| | **2 weeks** | **4 weeks** | **6 weeks** | **P value** |
| **Group A: Control without PRP** | 1 ± 0.4 | 2 ± 0 | 0.75 ± 0.25 | P ≤0.05 |
| **Group B: Control with PRP** | 3.5 ± 0.3 | 2.7 ± 0.2 | 1.2 ± I.03 | P ≤0.05 |
| **Group C: experimental without PRP** | 0.5 ± 0.25 | 0.5 ± 0.3 | 0.75 ± 0.25 | P ≤0.05 |
| **Group D: experimental with PRP** | 1.7 ± 0.28 | 1.5 ± 0.3 | 1.2 ± 0.5 | P ≤0.05 |
| **P Value** | P ≤0.01 | P ≤0.01 | P≥0.05 | P ≤0.05 |

Data represent means ± SE

P≤0.01:- highly significant differences in the mean value of trabecular number.
P≤0.05:- significant differences in the mean value of trabecular number.
P≥0.05:- non significant differences in the mean value of trabecular number.

**Table 2:** Osteoblast number (no /mm$^2$) in different healing period of studied group

| *Studied Groups* | *Healing Period* | | | |
|---|---|---|---|---|
| | **2 Week** | **4 Week** | **6 Week** | **P Value** |
| **Group A: Control without PRP** | 6.50 ± 0.50 | 7.75 ± 0.25 | 5.00 ± 0.41 | P≤0.05 |
| **Group B: Control with PRP** | 7.00 ± 0.71 | 9.00 ± 0.58 | 9.4 ± 0.58 | P≤0.05 |
| **Group C: experimental without PRP** | 2.50 ± 0.29 | 2.60 ± 0.29 | 2.75 ± 0.25 | P≥0.05 |
| **Group D: experimental with PRP** | 5.25 ± 0.25 | 6.50 ± 0.29 | 4.75 ± 0.25 | P≤0.05 |
| **P Value** | P≤0.01 | P≤0.01 | P≤0.01 | |

- Data represent means ± SE
- P≤0.01: - highly significant differences in the mean value of trabecular number.
- P≤0.05: - significant differences in the mean value of trabecular number.
- P≥0.05: - non significant differences in the mean value of trabecular number.

**Table 3:** Osteoclast number (no. /mm$^2$) in different healing period of studied group:

| *Studied Groups* | *Healing Period* | | | |
|---|---|---|---|---|
| | **2 Week** | **4 Week** | **6 Week** | **P Value** |
| **Group A Control without PRP** | 0.5 ± 0.28 | 1.2 ± 0.47 | 0.25 ± 0.25 | P≤0.05 |
| **Group B Control with PRP** | 1.5 ± 0.3 | 0.5 ± 0.28 | 0.25 ± 0.25 | P≤0.05 |
| **Group C Experimental without PRP** | 4.5 ± 0.28 | 3 ± 0.4 | 2 ± 0.48 | P≤0.05 |
| **Group D Experimental with PRP** | 1 ± 0.4 | 1 ± 0.4 | 0.75 ± 0.25 | P≥0.05 |
| **P Value** | P≤0.01 | P≤0.01 | P≤0.01 | |

- Data represent means ± SE
- ≤0.01: - highly significant differences in the mean value of osteoclast number.
- P≤0.05: - significant differences in the mean value of osteoclast number.
- P≥0.05: - non significant differences in the mean value of osteoclast number.

**Table 4:** Calcium level (mg/dL) at day of sacrifice in different healing period of studied group

| *Studied Groups* | *Healing Period* | | | |
|---|---|---|---|---|
| | **2 Week** | **4 Week** | **6 Week** | **P Value** |
| ***Group A Control without PRP*** | 10.1 ± 0.45 | 11 ± 0.39 | 10.6 ± 0.4 | P≥0,05 |
| ***Group B Control with PRP*** | 10.2 ± 0.3 | 11 ± 0.39 | 10.7 ± 0.37 | P≥0,05 |
| ***Group C Experimental without PRP*** | 11.5 ± 0.21 | 11.9 ± 0.4 | 10.9 ± 0.36 | P≥0,05 |
| ***Group D Experimental with PRP*** | 11.4 ± 0.16 | 11.8 ± 0.4 | 10.9 ± 0.36 | P≥0,05 |
| ***P Value*** | P≤0.05 | P≤0.05 | P≥0,05 | |

- Data represent means ± SE.
- P≤0.05: - significant differences in the mean value of Calcium level.
- P≥0.05: - non significant differences in the mean value of Calcium level.

**Table 5:** Phosphorous level (mg/dL) at day of sacrifice in different healing period of studied group

| *Studied Groups* | *Healing Period* | | | |
|---|---|---|---|---|
| | **2 Week** | **4 Week** | **6 Week** | **P Value** |
| **Group A Control without PRP** | 5.6 ± 0.035 | 4.8 ± 0.06 | 4.9 ± 0.05 | P≤0.05 |
| **Group B Control with PRP** | 5.5 ± 0.037 | 4.8 ± 0.04 | 4.9 ± 0.08 | P≤0.05 |
| **Group C Experimental without PRP** | 4.5 ± 0.1 | 4.1 ± 0.04 | 4.7 ± 0.075 | P≤0.05 |
| **Group D Experimental with PRP** | 4.4 ± 0.012 | 4.01 ± 0.03 | 4.7 ± 0.07 | P≤0.05 |
| **P Value** | P≤0.05 | P≤0.05 | P≥0.05 | |

- Data represent means ± SE.
- P≤0.05: - significant differences in the mean value of Phosphorous level.
- P≥0.05: - non significant differences in the mean value of Phosphorous level.

The highest mean values of phosphorous level were seen in group A and B (control groups), while the lowest were seen in group C and D (experimental groups). These results mean that there was negative relation phosphorous and both calcium and alkaline phosphatase. In addition, there were significant differences in all groups with time interval (P≤0.05). Table 5.

## DISCUSSION

The usage of autologous blood products such as platelet-rich plasma to facilitate healing in a variety of applications has been increased recently specifically about growth factors, which play a crucial role in the healing process. The platelets are responsible for activity extruding growth factors, which initiate soft tissue healing, bone formation, and stem cell recruitment. Growth factors contained in PRP signal local mesenchymal and epithelial cells to migrate, divide and increase synthesis of collagen and bone matrix, thus providing a scaffold that encourages migration of osteoblasts, also they stimulate chemo taxis, metabolism and proliferation in osteoblasts and in bone marrow osteoprogenitor cells.[17]

With that knowledge there is abundant enthusiasm in the application of concentrated platelets, which release a supra-maximal quantity of these growth factors to stimulate recovery in non-healing injuries especially in patients who suffering from systemic diseases. One of these diseases are osteoporosis, the mechanism has been proposed to explain the great incidence of delayed healing of fractures in osteoporosis is an imbalance between bone desorption by osteoclasts and bone deposition by osteoblasts[18]**.** In osteoporotic bone injury, the application of (PRP) restored early cell proliferation during healing levels comparable to non-osteoporotic controls [19].

The rabbit is one of the most commonly used animals for medical research being used in approximately 35% of musculoskeletal research studies [20]. This is in part due to ease of handling and size. The rabbit is also convenient in that it reaches skeletal maturity at around 6 months of age [21] The histological structures of teeth and their supporting tissues in the rabbits resemble largely those of a human [22]

In this study, we evaluated the bone healing process following the application of PRP in experimentally created intra-bony defects in normal and osteoporotic rabbits.

### Histological and Histomorphometrical analysis

The histological features of control groups (with and without PRP) revealed the maturity of bone formation, in which, the osteocytes and osteoblast arranged in circular manner around Haversian canal. In addition, there was fewer amounts of spaces between cortical bones in the control groups (group A and B) than that in the experimental groups (group C and D). This might be attributed to the effect of hydrocortisone in delay bone formation. This result correlated with previous study done by [23]**,** who found that when the defect in the bone of oral cavity and OP-like conditions were introduced simultaneously in rabbits, cortical porosity was noted.

Results of histomorphometric analysis for bone architectures parameters showed that using of autologous PRP in the normal bone defect or osteoporotic one has benefits for organizing the formative cell (specially osteoblast), formation of neovascularization and more rapid and faster apposition of bone matrix with its mineralization process. These results were supported by increase in number of trabecular bone and osteoblast, in comparison to defect not treated with PRP as differences values shows to be highly significant this could explained that PRP stimulates proliferation of fibroblasts and promote collagen and extracellular matrix synthesis and act as a chemoatractant for fibroblasts, monocytes and neutrophils.[24]

### Biochemical serum analysis

Serum Calcium and phosphorous ratio may provide a sensitive measures of bone mineral changes and may add to our understanding of the changes that take place in bone disease [25]**.** Also this study revealed a significant increase in the calcium serum level in the animals of group C&D than their controls at 2nd & 4th weeks duration postoperatively, while no significant differences between control and osteoporotic animals at 6th week. These changes in serum

calcium levels may indicate suppression of bone formation in osteoporotic rabbits. The result suggest that GC inhibits bone growth mainly by decreasing bone formation.[26]

Regarding the serum phosphorous level in the present study, there were reduction in its level in control groups compared with their experimental at the end of 2nd & 4th weeks duration postoperatively and like calcium there were no significant differences in its level at 6th weeks duration. These results showed disagreement with[10] who suppose that there were no significant differences in the phosphorous level between GC-induced osteoporotic rabbits and normal rabbits in all healing periods. However, other study[27] concluded that the balance of calcium and phosphorous level results from an inverse relationship.

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