



## Histopathological study about the effect of nano magnesium oxide and platelets rich fibrin on the healing of induced radial fracture in dogs

Y.F. Abdulmawjood<sup>1</sup> , M.G. Thanoon<sup>2</sup> , S.M. Ibrahim<sup>2</sup>  and J.H. Alsofy<sup>3</sup> 

<sup>1</sup>Veterinary Hospital, Mosul, Agricultural Nineveh directory, <sup>2</sup>Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Mosul, <sup>3</sup>Private Veterinary Clinic, Mosul, Iraq

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#### Correspondence:

M.G. Thanoon  
[moyaserthanoon@uomosul.edu.iq](mailto:moyaserthanoon@uomosul.edu.iq)

### Abstract

The aim of this study was a histopathological evaluation of the effects of nano magnesium oxide and platelets-rich fibrin on induced radial fracture bone healing in local stray dogs. We used 18 adult animals weighing  $17.5 \pm 0.6$  kg and aged  $2.0 \pm 0.1$  years; these experimental animals were divided into three main equal groups. In the first group (control), the fracture was left without treatment of any bioactive materials; in the second group (nano magnesium oxide), the fracture line was injected with  $20 \mu\text{g}$  of nano magnesium oxide, the third group (platelets rich fibrin) the fracture line was surrounded by platelet-rich fibrin. The bone specimens were taken from all experimental animals 6 and 10 weeks after the surgical operation. A Colorimetric method was used for measuring the alkaline phosphatase enzyme and calcium concentrations in all trial animals. The histopathological results at the 6th and 10th weeks showed that the best response was in the platelets-rich fibrin group, then the nano magnesium oxide group, and lastly, the control group according to the formation of fibrocartilaginous tissue, trabeculae, and woven bone. The concentration rates of alkaline phosphatase enzyme and calcium were increased in the weeks that followed the surgical operation. In conclusion of this study, platelets-rich fibrin and nano-magnesium oxide accelerated the healing of the radial fracture.

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### Introduction

The radius is articulated with the ulna along the caudal border then extended parallel to each other's to form antebrachium (forearm) (1). The radius bone constitutes the largest part of the forearm area, as it bears the greatest weight. Therefore, it is most susceptible to fractures compared to the rest of the fore limb bones (2). Forearm fractures are the most common fracture in dogs and account for approximately 19.33% of all fractures that affect dogs (3). Radial bone fractures in dogs can be successfully treated and immobilized by using external fixation such as a cast (plaster of paris), or external splints as well as using internal surgical fixation (4). Nanoparticles (NPs) are particles containing the smallest size between 1-100 nanometers and have unique physical or chemical properties as they have

emerged in recent years to help restore fractured bone to its normal function (5). These NPs have been shown to enhance the proliferation, adhesion and differentiation of osteoblasts by affecting the function of mesenchymal stem cells (MSCs), which can later differentiate into osteoblasts to form new bone tissue. The NPs have ability to cross physiological barriers on their own or allow the biologically active agent loaded with it to move across those physiological barriers, thus achieving optimal drug action in pathological sites (6). Nano-magnesium oxide is an essential mineral that is non-toxic to living organisms, and its role has emerged in recent years in the field of surgical medicine to repair fractured bone due to its mechanical and biological properties in addition to its rapid decomposition (7,8) as it self-degrades when added to the fractured bone and does not require secondary surgical operations to remove it (9). Platelet-rich

fibrin (PRF) is a biologically active surgical additive that has more than one role not only in stopping hemorrhage, but also in regulating the inflammatory process by accelerating the healing of wounds, fractures and bone regeneration (10,11). The PRF can be obtained by a simple technique that does not require more than a centrifuge of natural blood taken directly from the animal and without any anticoagulants or other chemicals used in transfusion and preservation of blood (12,13). Platelets are highly specialized cells that release growth factors and cytokines to improve the healing process (14). As well as having the ability to release many growth factors, including transforming growth factor (TGF), platelet-derived growth factor (PDGF), insulin like growth factor (ILGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factors (PDGF), especially PDGF, TGF and ILGF, are promote chemotaxis, differentiation, enhancing healing, and promote tissue regeneration, as well as their effect on the activity of osteoblasts themselves (15,16).

The purpose of this experimental study was histopathological evaluation of effects of nano magnesium oxide and platelets rich fibrin on radial fractured bone healing in local breed stray dogs.

## **Materials and methods**

In this experimental study we are used 18 adult local stray breed dogs (males and non-pregnant females), weighing  $17.5 \pm 0.6$  kg and aged  $2.0 \pm 0.1$  years. The animals housed indoor under the same feed and management conditions, in the animals' house of Veterinary Medicine College, University of Mosul, after obtaining an official approval from the ethical committee of the college. All experimental animals were subcutaneously injected by Ivermectin 1% at a dose of 0.3 mg/kg BW then repeated after 14 days before surgical operations. The food withheld for 12 hours before the surgery, and the water was free access until the time of operation. The surgical operations were conducted under general anesthesia; started by premedication of Atropine sulphate 1% in a dose 0.04mg/Kg BW, then after about 10 minutes later a mixture of Xylazine hydrochloride 2% and Ketamine hydrochloride 10% in a dose 5mg/Kg BW, 15mg/Kg BW respectively intramuscularly injection. A transverse fracture was inflicted in the radius bone of all experimental animals, by using wire saw and the best surgical approach is craniomedial aspect (15). The experimental animals were randomly divided into three equal main groups, each group included 6 animals. The first group C (Control) the fracture line didn't treated with any bioactive materials, the second group NMO (nano magnesium oxide) the fracture line was injected by 2 cc suspension of distill water containing 20 $\mu$ g of nano magnesium oxide produced by the US research nanomaterials, Inc., the 99% of average particle size (APS)

20nm with specific surface area (SSA) 60 m<sup>2</sup>/g, while the third group PRF (platelets rich fibrin) the fracture line was surrounded by platelet-rich fibrin which was prepared just before the surgical operation from the same animal by drawing 10 cc of fresh blood from the cephalic vein collected in a test tube without anticoagulant for direct centrifugation 3000 rpm for 15 minutes to obtain on the platelets rich fibrin in the middle layer of the test tube (16). The surgical wound closed by routine manner, then the fractured bone was immobilized by external fixation cast of gypsum. Post-operative care, intramuscular injection of penicillin-streptomycin at a dose of 10000 IU, 20 mg/kg BW, respectively; for five successive days post-surgical operation; the surgical thread stitches were removed at 12-14 days after operation. Daily wound dressing with daily full clinical examination for the first week, then weekly till the end of the experimental study (follow up 10 weeks).

The bone specimen was taken from all trial's animals in two periods at 6 and 10 weeks after induced osteotomy of radius bone. The bone biopsy was immediately preserved in a solution of neutral formalin for 72 hours, and then decalcified by passing it in a formic acid with sodium citrate solution. After that these bone specimens entered a series of passes with alcohol, xylol and paraffin wax until they were ready for histopathological sections. The tissue slices were cutting within a thickness 5  $\mu$ m, and they were stained with two dyes, the first hematoxylin and eosin (H&E), and the second specific to connective tissue, which is Masson trichrome (17). Histopathological slides were examined under a light microscope to observe the differences in their responses to inflammatory reactions for repairing the fractured bone and return it to normal position.

A Colorimetric method used for measuring the alkaline phosphatase enzyme and calcium concentrations, by drawn blood samples from all experimental animals, starting at zero time (before the surgery) and 1, 2, 3, 4, 5, 6, 7 and 8 weeks post-surgical operation. For this purpose, we are used Cobas® kit from the German Roche Company to measure the concentration of alkaline phosphatase, while the calcium 3L79 kit from Abbott GmbH & Co. KGa used for measuring calcium concentration. Sigma Stat (Jandel scientific software V3.1), was used for statistical analysis of our obtained results data, at  $P < 0.05$  (18,19).

## **Ethical approve**

The study was performed after obtaining of approval of ethical Committee of Medical Researchers at College of veterinary Medicine/University of Mosul; UM.VET.2021.062.

## **Results**

All animals suffered from partial loss of appetite at the first- and second-day post operation, and the normal appetite returned for all experimental animals after 3-5 days of

operation. Gross examination at the surgical site revealed slight swelling, redness and pain around the wound without inflammatory exudate; these inflammatory signs subsided within 4-5 days after operation. Skin sutures stitches were removed from all trial animals at the 10-12<sup>th</sup> day after operation and the wound was well healed. Limp was evident in all experimental animals; the animals of the PRF group showed its ability to use the broken limb at the third week after operation, while the animals of the NMO group showed their ability to walk by using the broken limb at the fourth week after operation, and the animals of the C group came later than the second and third groups.

The microscopic examination of the histopathological slices at the sixth week showed a variation in the

inflammatory response between the three main experimental groups. In the first group there is proliferation of fibrous tissue with very little fibrocartilaginous tissue and without presence new osseous tissue. The space between the two edges of the fractured bone was filled with fibrous tissue with few amounts of fibrocartilaginous tissue (Figure 1: A), and this confirmed by using Masson Trichrome dye (Figure 1: B).

In the second group (NMO) there is large quantities with density of fibrous tissue as well as fibrocartilaginous tissue observed fill the space between the ends of fractured bone. (Figure 2: A and B).

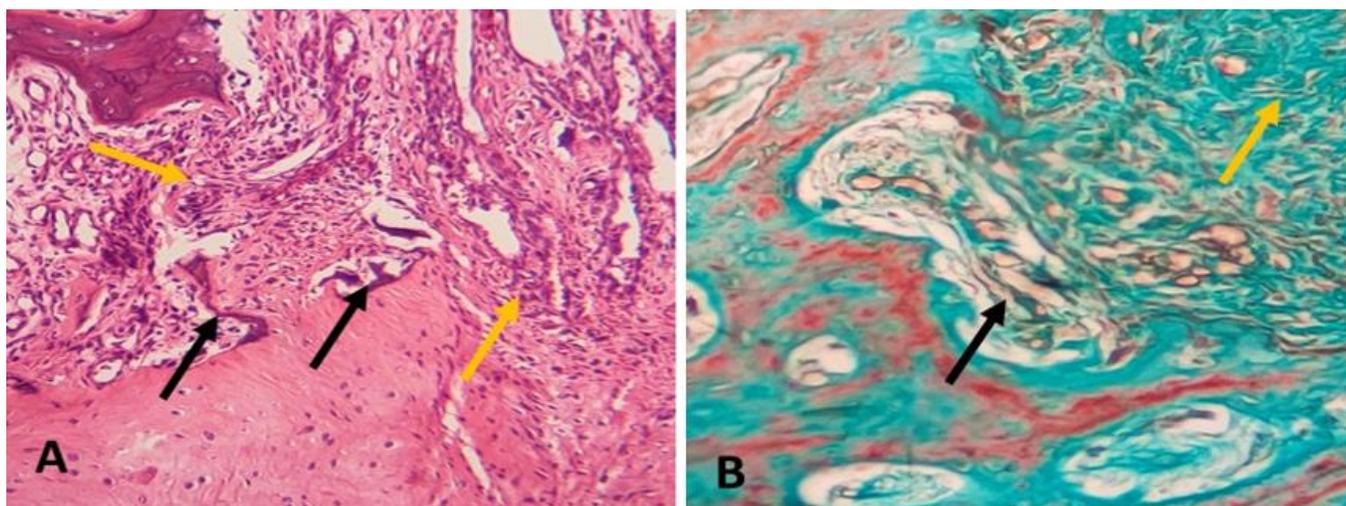


Figure 1: Histopathological section of radial bone after inducing radial fracture, in the first group at the sixth week, shown fibrous tissue (yellow arrow) in the fracture gap, with fibrocartilaginous tissue (black arrow). A: H&E  $10 \times 4.4 X$ , B: Masson Trichrome  $10 \times 3X$ .

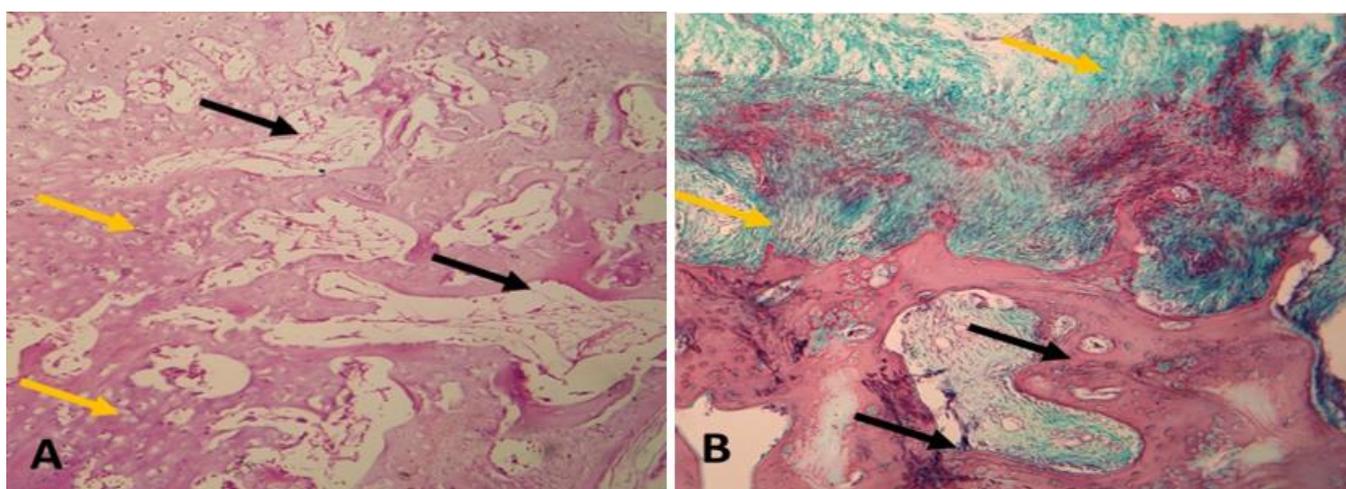


Figure 2: In the second group at the sixth week, shown proliferation and density of fibrous tissue (yellow arrow), as well as fibrocartilaginous tissue in the fractured bone gap (black arrow). A: H&E  $10 \times 2.4 X$ , B: Masson Trichrome  $10 \times 2.8X$ .

While in the third group (PRF) the gap of fractured bone manifested under light microscope by proliferation of fibrous tissue with prevalent of abundant fibrocartilaginous tissue (Figure 3: A and B).

The results of histopathological examination at the tenth week post operation; in the first group (C) showed there was proliferation of fibrocartilaginous tissue as well as fibrous tissue, and the two edges of the fractured bone were attached by fibrocartilaginous tissue with slight ossification of cartilage cells (Figure 4: A and B).

In the second group (NMO), the histopathological examination showed a large proliferation of fibrocartilaginous tissue with presence of cartilaginous

tissue, as well as ossification of some chondrocyte to form woven bone in some areas of the fractured site (Figure 5: A and B).

While in the third group (PRF), the examination of histopathological slices revealed presence of fibrocartilaginous tissue with large amount of cartilaginous tissue as well as woven bone which represented by bone trabeculae at the site of fracture (Figure 6: A and B).

The results of the biochemical markers showed an elevation in the concentrations of alkaline phosphatase and calcium at the weeks that follow the surgical operation. These results with statistical analysis summarized in table 1 for alkaline phosphatase and table 2 for calcium.

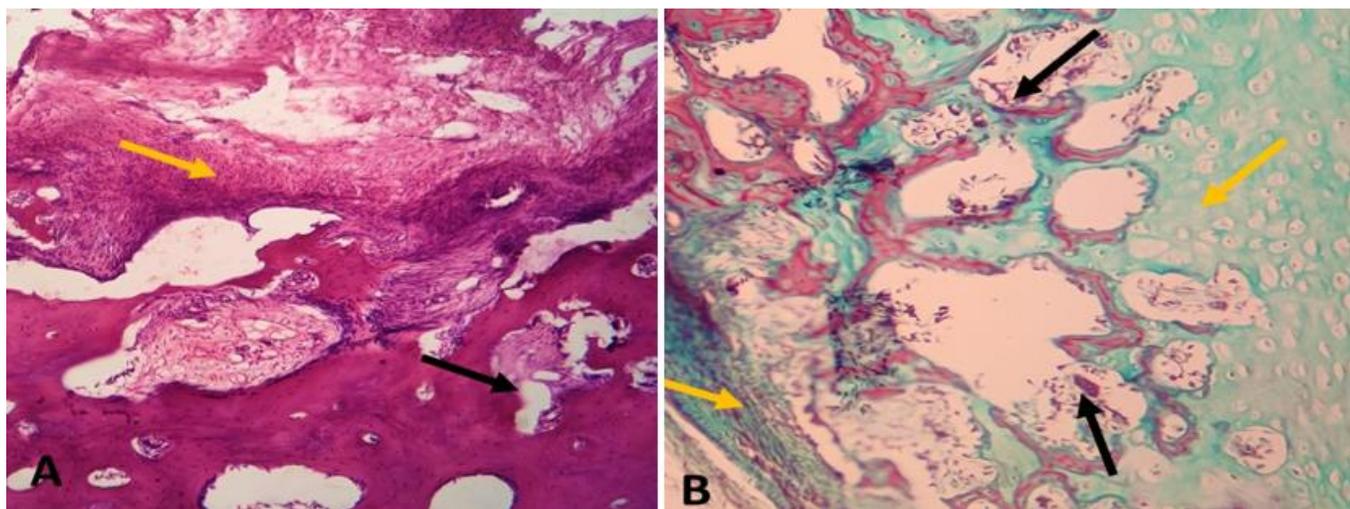


Figure 3: In the third group at the sixth week, shown presence fibrous tissue (yellow arrow) with abundant fibrocartilaginous tissue (black arrow) in the fractured bone gap. A: H&E  $10 \times 1.8X$ , B: Masson trichrome  $10 \times 3.9X$ .

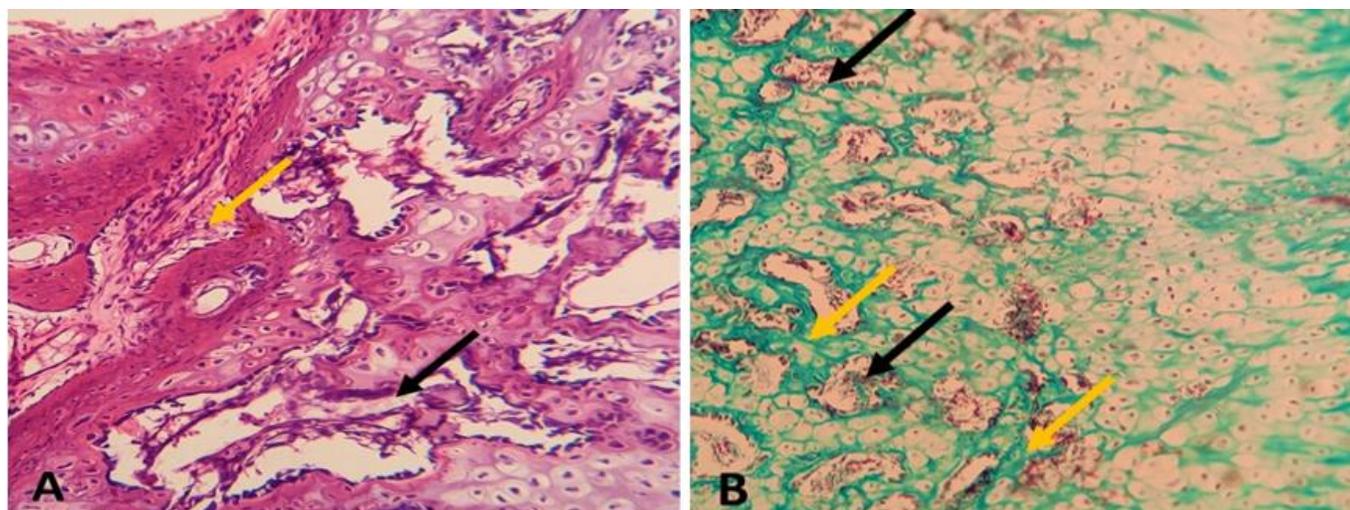


Figure 4: In the first group at the tenth week, shown proliferation of fibrocartilaginous tissue as well as fibrous tissue (yellow arrow), and the two edges of the fractured bone were attached by fibrocartilaginous tissue with slight ossification of cartilage cells (black arrow). A: H&E  $10 \times 4X$ , B: Masson trichrome  $10 \times 3X$ .

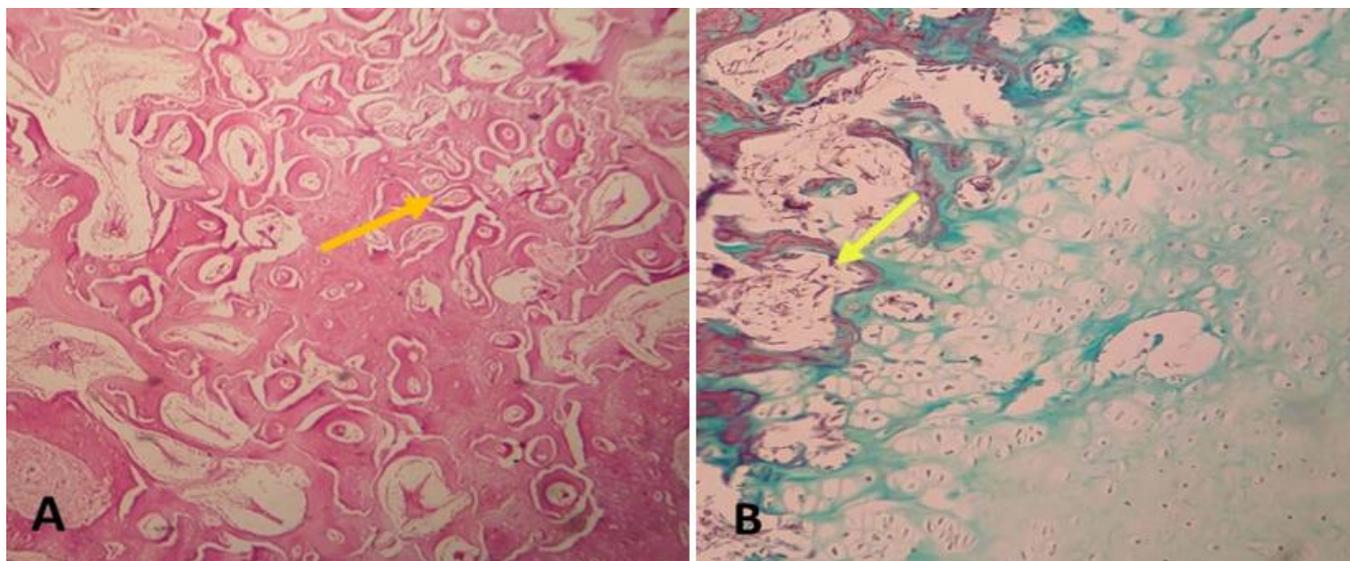


Figure 5: In the second group at the tenth week, shown a large proliferation of fibrocartilaginous tissue with presence of cartilaginous tissue, as well as ossification of some chondrocyte to form woven bone in some areas of the fractured site (yellow arrow). A: H&E 10 × 2.8X, B: Masson trichrome 10 × 3.5X.

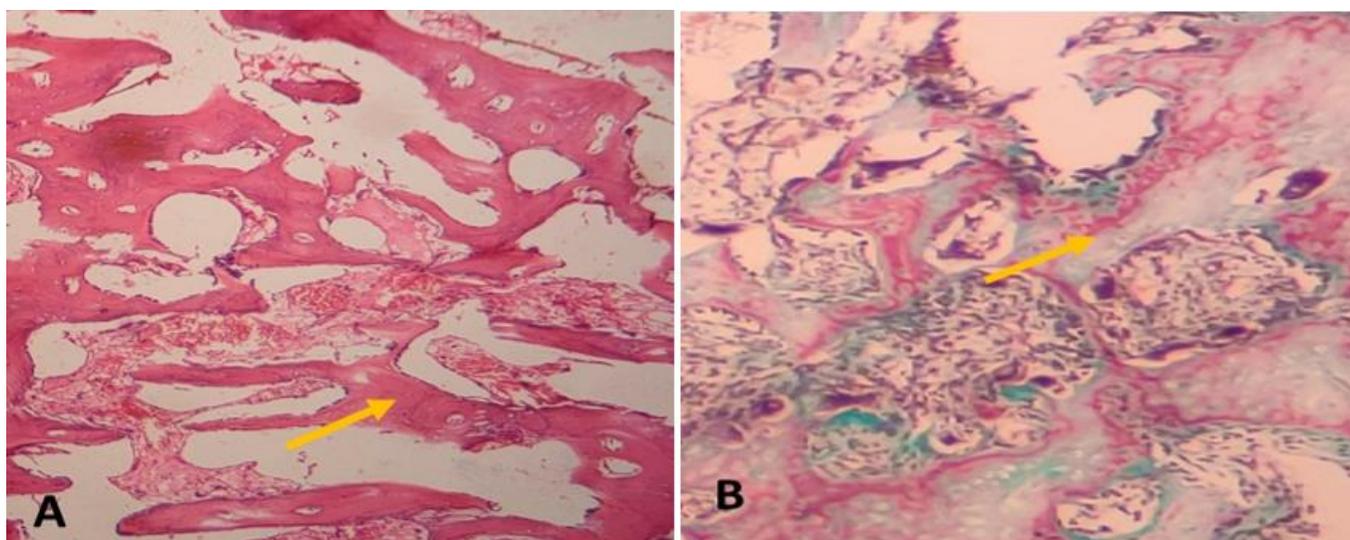


Figure 6: In the third group at the tenth week, shown fibrocartilaginous tissue with large amount of cartilaginous tissue as well as woven bone which represented by bone trabeculae at the site of fracture (yellow arrow). A: H&E 10×4.2 X, B: Masson trichrome 10 × 10X.

Table 1: Alkaline phosphatase concentration levels (U/dL) with its statistical analysis at the three main experimental groups

Time (weeks)	C group	NMO group	PRF group
0	19.37±0.57 a	20.123±0.69 a	18.608±0.64 a
2	19.382±0.39 a	20.618±0.859 ab	23.143±1.18 b
4	20.472±0.592 a	21.99±1.089 ab	26.54±0.616 c
6	23.823±0.627 a	27.76±0.909 b	30.538±0.948 c
8	21.942±0.961 a	24.457±1.537 ab	28.448±0.947 b

The different small letters show the significant difference between the experimental groups at P<0.05.

Table 2: Mean of calcium concentration levels (mg/dL) with its statistical analysis in the three experimental main groups

Time (weeks)	C group	NMO group	PRF group
0	10.633±0.292a	10.75±0.253a	10.833±0.167a
2	11.205±0.324 a	11.867±0.361 ab	12.583±0.318 b
4	11.7±0.225 a	12.633±0.223 bc	12.733±0.255 c
6	11.4±0.237 a	11.9±0.200 a	12.00±0.266 a
8	11.5±0.256a	11.183±0.13a	11.667±0.143a

The different small letters show the significant difference between the experimental groups at  $P < 0.05$ .

## Discussion

All experimental animals suffered from limping which consider a major and clear clinical sign of radial bone fracture, and it was evident in the first and second week and less evident in the third and fourth week then disappeared in the sixth week, and this was confirmed by Watrous (4) and Moens and Manchi *et al.* (20), they said that the lameness is clinically evident when the radius bone fracture coincides with ulnar bone fracture and is less obvious when fracture of the radius bone only.

The histopathological examination at the sixth week showed; in the third group, the space between the two edges of the fractured bone has been filled with fibrocartilaginous tissue more than fibrous tissue. The fracture line in this group was surrounded by platelets rich fibrin, and because the platelets having a characteristics feature; such as stimulating stem cells to migrate, multiply and differentiated into cells that needed by the medium in which they are, especially chondroblast and osteoblast cells, and this was confirmed by Oryan *et al.* (21) and El-shafey *et al.* (22) whom said that the platelets containing many biologically active substances, including various growth factors. Which are attributed to a great regulatory role in migration, proliferation, differentiation, and maturation of various cells that have a major role in inflammatory and healing processes, In addition to its role in the production and remodeling of extracellular matrices. In the second group, the fractured ends gap was filled by fibrocartilaginous tissue as well as fibrous tissue. This meaning there is a quantity of fibrocartilaginous tissue equal or slightly more than fibrous tissue and this may due to presence of nano magnesium oxide which added to the fracture line. Because the nano magnesium oxide act as accelerating factor for bone healing, and this agree with Hickey *et al.* (23), who said that the adding of nano magnesium oxide to the fractured bone showed a significantly enhance adhesion and proliferation of osteoblasts, also is suitable for bone applications with no cytotoxicity. While in the first group, the fractured edges gap was filled by fibrous tissue with little amounts of fibrocartilaginous tissue. This is because the fracture line in first group treated without any bioactive materials. We observed the presence of fibrous tissue in abundance, which will turn later into fibrocartilaginous tissue, and this is agree with Loi *et al.* (24), who confirmed that the function of

macrophages is removing the fibrin network and dead cells by the process of phagocytosis, while osteoclasts work on the removing of bone fragments and dead ends of the broken bone, as the macrophages secrete their repertoire of inflammatory and chemical mediators, which begins to recruit fibrogenic cells, mesenchymal stem cells, and osteoblastic progenitor cells from their local niches, These are the bone marrow, periosteum, and endothelium of the capillary blood vessels.

The results of histopathological examination for the tenth week after operation; in the third group, showed there is the proliferation of fibrocartilaginous tissue with large amount of cartilaginous tissue as well as woven bone which represented by new bone trabeculae at the fracture site. The positive and effective role of platelets rich fibrin is in reducing exaggerated inflammation as well as acting as a scaffold to bridge the fracture gap for the rapid and beneficial transfer of progenitor cells, mesenchymal stem cells and multiple growth factors in the rapid access to the fracture healing stage. This may be attributed to the presence of chemotaxis and cytokines, with multiple growth factors which secreted by local platelets and from those that we were added to the fracture line, as these factors activate the migration and differentiation of progenitor cells, and regeneration of tissue by stimulating angiogenesis, this is agreement with Joo *et al.* (25) and Uthappa *et al.* (26), as they confirmed that the platelets are the natural source of growth factors, and factors that attract or stimulate cells, which they have a primary role in all stages of the healing process. The histopathological results of the second group, at the tenth week showed a proliferation of large amount of fibrocartilaginous tissue with presence of cartilaginous tissue, as well as ossification of some chondrocyte to form woven bone in some fractured areas. This may be due to addition of nano magnesium oxide to the fracture line, and this is coinciding with Li *et al.* (27) and Wu *et al.* (28), who stated that the presence of magnesium ions plays a role in promoting osteogenic differentiation from mesenchymal stem cells; than Stimulation of adhesion and proliferation of osteoblasts. In addition to that the biodegradable of nano magnesium oxide, it makes the surrounding environment weakly alkaline, and this has a great role in stimulating mineralization (calcium, phosphorous and phosphate) to form new calculus during the bone healing process. As for the first group, the histopathological examination of 10<sup>th</sup>

week post operation showed a proliferation of fibrous tissue as well as a proliferation of fibrocartilaginous tissue in an amount equal to or greater than the fibrous tissue, and that the two edges of the fracture were bridged by fibrocartilaginous tissue with a slight deposition of calcium salts for some cartilage cells. This slowdown in fracture healing and the failure to reach the formation of woven bone in the first group than what's found in the second and third groups may be due to the absence of any bioactive material that has ability to accelerate fracture healing, such as nano magnesium oxide or platelets rich fibrin. Therefore, what reached first group at tenth week after operation is considered normal and clinically acceptable which confirmed by Ben Ali (29), who mentioned that the complete fracture healing of radius or long bone takes a longer time may reach to 16<sup>th</sup> weeks due to the lack of bloody supply, as well as the lack of soft tissue.

The concentration rates of alkaline phosphatase in table 1 showed higher concentration in the third group than the rest (first and second groups) at the sixth week after surgical operation. this elevation in the rates of alkaline phosphatase indicates to high activity of osteoblast, which produce new callus formation and this is coincides with Singh *et al.* (30), who said that the increasing in the rates of alkaline phosphatase leads to new bone formation by bone matrix formation and mineralization. The rates of total serum calcium concentrations showed significantly statistical differences between the first and the rest groups (second and third) for the second- and fourth-weeks post operation, with no significant differences between the second and third groups at the same periods. Also, there was no significant difference among the main three trial groups for the sixth and eighth weeks after operation. These results indicated that the all-experimental animals didn't suffering from delayed bone union or osteoporosis, confirmed by histopathological pictures which show a new callus with woven bone formation (ossification). This is agreeing with Fischer *et al.* (31), who said that the bones act as a reservoir for calcium to maintain constant blood calcium levels. Also, the calcium gives bones strength and rigidity.

## Conclusion

The conclusion of this experimental study indicated that the using of nano magnesium oxide and platelets rich fibrin on the fractured line was service for enhancing and improving the healing of radial bone fracture in dogs.

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## Conflict of interest

The authors declare that no conflict of interest exists.

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## دراسة نسيجية مرضية عن تأثير نانو أوكسيد المغنيسيوم والليفين الغني بالصفائح الدموية على التئام كسر الكعبرة المستحث في الكلاب

ياسر فارس عبد الموجود<sup>١</sup>، ميسر غانم ذنون<sup>٢</sup>، سحر محمد ابراهيم<sup>٣</sup> و جنان حمودي الصوفي<sup>٣</sup>

<sup>١</sup>المستشفى البيطري، مديرية زراعة نينوى، فرع الجراحة وعلم تناسل الحيوان، كلية الطب البيطري، جامعة الموصل، <sup>٢</sup>عيادة بيطرية خاصة، الموصل، العراق

### الخلاصة

ان الهدف من هذه الدراسة هو التقييم النسيجي المرضي لتأثير نانو أوكسيد المغنيسيوم والليفين الغني بالصفائح الدموية على التئام كسر عظم الكعبرة المستحث في الكلاب المحلية الضالة. تم استخدام ١٨ حيواناً بالغاً تزن ١٧,٥±٠,٦ كجم وأعمارهم ٢,٠±٠,١ سنة، وقسمت حيوانات التجربة إلى ثلاث مجاميع رئيسية متساوية. المجموعة الأولى (السيطرة) لم يتم معالجة خط الكسر بأي مواد نشطة بيولوجيا، المجموعة الثانية (نانو أوكسيد المغنيسيوم) تم حقن خط الكسر بـ ٢٠ ميكروغرام من نانو أوكسيد المغنيسيوم، المجموعة الثالثة (الليفين الغني بالصفائح الدموية) تم إحاطة خط الكسر عن طريق الليفين الغني بالصفائح الدموية. أخذت العينات العظمية من جميع حيوانات التجربة على فترتين في الأسبوع السادس والعاشر بعد إجراء العملية الجراحية. تم استخدام طريقة الطيف اللوني لقياس تراكيز إنزيم الفوسفاتيز القلوي والكالسيوم في جميع حيوانات التجربة. أظهرت النتائج النسيجية المرضية في الأسبوعين السادس والعاشر أن أفضل استجابة كانت في مجموعة الليفين الغنية بالصفائح الدموية ثم مجموعة نانو أوكسيد المغنيسيوم وأخيراً مجموعة السيطرة وذلك وفقاً لتكوين الأنسجة الليفية الغضروفية والحويجات العظمية والعظم المنسوج. لوحظ زيادة في معدلات تراكيز إنزيم الفوسفاتيز القلوي والكالسيوم في الأسابيع التي أعقبت العملية الجراحية. في ختام هذه الدراسة، فإن استخدام الليفين الغني بالصفائح الدموية ونانو أوكسيد المغنيسيوم يسرع من التئام كسر عظم الكعبرة.