Research Article

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The Effects of Autologous Platelet-Rich Plasma on the Bone Fracture Healing in Rabbits

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Abstract

The purpose of this study was to evaluate the effects of an autologous platelet rich-plasma on femoral fracture healing in experimentally induced rabbits' model. In this study, 34 local breed rabbits, around 2-2.5 kg body weight, were divided into two equal groups randomly; Group A: platelet-rich plasma (PRP) and Groups B: Control group. Rabbits in both groups underwent a complete mid-shaft transverse osteotomy of the femur by a fine electrical saw. After fracture induction, the rabbits in group A (PRP group) were treated by application of 0.5 ml of autologous PRP at the site of the fractured bone, while in group B (control), rabbits were injected with distilled water. Samples from the fractured femur were collected at 10^{th}, 20^{th}, 30^{th} days post operation for radiological evaluation and at 10^{th}, 14^{th}, 20^{th}, and 30^{th} days for histopathological evaluation. In radiological study, it was found that the rate of callus formation in rabbits treated with PRP (Group A) were faster than the control group (Group B) at different periods. Similarly, in histological finding it found that the stages of healing were faster in Group A when compared with Group B. It was concluded that using autologous PRP has beneficial effect to enhance the process of bone healing in the rabbit's model.

Keywards: Fracture, Platelet rich-plasma, Rabbit

تأثير استخدام الخلايا الأقراص الدموية الذاتي على التئام كسور العظام في الارانب

الخلاصة

هدفت الدراسة تقييم استخدام الخلايا الأقراص الدموية في الارانب. تم استخدام 34 ارنباً ناضجاً تراوحت اوزانها (1.5 – 2) كيلو غرام واعمارها تتراوح بين(5-7) أشهر، قسمت الى مجموعتين متساوية عشوائياً . المجموعة الأولى هي حيوانات مجاميع التجربة ، والمجموعة الثانية هي مجاميع السيطرة. لقد تم اجراء عملية الكسر لعظم الفخد في الارانب لجميع المجموعات جراحياً باستخدام منشار كهربائي دوار رقيق. تم حقن المجموعة الأولى – مجاميع التجربة بـ 0.5 ملم من الخلايا الأقراص الدموية وحقن المجموعة الثانية محلول ملحي عادي. واظهرت لنا الفحوصات بأن عظام المجموعة الأولى قد تم فيها الالتئام بدرجة عالية و اعادة تشكيله ابتدأ من الاسبوع الثانى بعد العملية لقد تم احتواء العظم الى شكله الطبيعي و تشريحى كاملامع درجة العالية. مقارنة بمجموعة الثانية.لقد تم ذلك بدراسة سريرية و شعائية نسيجيةوالصِّفَةُ التَّشْرِيحِيَّة

Introduction

A bone fracture is a medical condition in which there is a break in the continuity of the bone (1). It can be the result of high force impact or stress, or trivial injury as a result of certain medical conditions that weaken the bones, such as osteoporosis, osteopetrosis, bone cancer, or osteogenesis imperfecta, osteomalacia, where the fracture called a pathologic fracture (2). With the increased incidence of road traffic accidents and high velocity trauma these days, femoral fractures are very frequently encountered in pet animals. Intramedullary nailing has proven to be a gold standard treatment modality for diaphyseal femur fractures (3). Femoral nailing gives predictable realignment of bone, rapid healing and early functional use of the limb (2). Although, treatment of fractures or a large bone defect represent a major clinical challenge for orthopedic surgeon. In order to overcome this problem several bone substitutive materials developed and applied clinically, to further improve success rate co-adjuvant have been produced, which enhance osteointergration potential and restore normal bone structure (4, 5).

PRP is a product derived from the blood, it is obtained in order to produce high concentration of platelet in a small amount of blood, it plays important role in healing and regeneration process because it contains large number of growth factors (6, 7). It has been reported that activated platelet can release more than 300 molecules, which are responsible for coordination of cell-cell and cell-extracellular matrix integration (8, 9), such as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), insulin like growth factor-1 (ILGF-1) and epidermal growth factor (EGF) (10, 11). PRP have been used since the beginning of 1990s and their clinical benefits were initially reported in oral and maxillofacial surgeries, nevertheless, its commercial incentives of pharmaceutical industries is increasing up to date especially in sport medicine (12). There are many methods for obtaining PRP, each one has a scientific properties that depends on the capacity of platelets concentration and release of certain growth factors (13). One of these methods is by centrifugation, the result of this centrifugation is the production of a large concentration of platelets in small volume of plasma (13). Moreover, when it used in combination with bone grafts it shown positive results in acceleration of bone regeneration in animal models (14-16). Finally, on the basis of the above-mentioned information we aimed to show the effect of PRP on bone healing in experimentally induced femoral fracture in rabbit model.

Materials and Methods

Animals

In this study, 34 clinically healthy local breed rabbits of female sexes, 5-7 months old, weighing 2-2.5 kilograms were used. The animals were divided into 2 equal groups. In each group (n=17), 9 rabbits were randomly selected for x-ray and 8 rabbits for histopathological study. The animals were caged in the same hygienic environment and fed with same ration and water through the period of experiment. The food and water were changed daily with the litter of the cages. The study was approved by the ethic and scientific committee councils of the college of veterinary medicine/ University of Sulaimani. The study was carried out in the Veterinary Teaching Hospital belonging to college of Veterinary Medicine/ University of Sulaimani.

Preparation of the PRP

PRP was prepared from rabbit autologous blood, which were taken from the ear vein under general anesthesia. From each rabbit, 1 ml of the ear dropped blood were collected into a tube contained anti-coagulant (ACD-A). The collected blood directly transferred to the laboratory for PRP preparation. PRP was prepared by two round method (13), at the first spin, the blood

centrifuged at 6000 rpm for 10 minutes, then the buffy coat with the plasma were aspirated into a separate tube which then re-centrifuged at 6000 rpm for 10 minutes. Finally, the plasma separated from the buffy coat layer and stored in a separate tube in a refrigerator for about 24 hours and then applied to the fractured bone during the operation.

Anesthetic technique and preoperative medication

Anesthesia was induced by an intramuscular injection of a mixture of Xylazine and Ketamine at a dose of 5 mg/kg and 35 mg/kg b. w. respectively. The preoperative antibiotic medication (Enrofloxacin) 0.15 mg/ kg b. w. was injected one day before the operation.

Surgical procedure

The animals were placed on the left lateral recumbency in order to localize the right hind limb proximally. The lateral aspect of the right femur was prepared for surgical operation by clipping, shaving and surgical scrubbing with povidone iodine (Dermosept company). The leg was carefully draped with a sterilized drape and leaving enough space for the operation. An incision of an approximately 2-3 cm was made in the skin over the femur bone. Subcutaneous tissue and the fascia latae were dissected down to the muscles (Figure 1 A). Using a blunt dissection, Semimembranosus muscle and Vastus lateralis muscle were separated from each other in order to expose shaft of the femur. Then the periosteum was incised, and the cortical bone of the femur was explored. A complete transverse osteotomy of the mid-shaft of the femur was performed using fine electrical saw (Figure 2) with pattern blade of 10 mm wide and 0.5 mm thick (Figure 1 B). Then the fractured femoral segments were fixed by an intramedullary pin. The pin was introduced into medullary cavity of the proximal segment, continued until it was protruded from the intertrochantric fossa (Figure 1 C). Then the protruded part of the pin was fixed into the mouth of the pin chuck and the pin was redirected to the distal fragment. Then the autologous PRP were applied to the fractured site by a syringe in group A (PRP group) (Figure 1 D), while in the control group only the distilled water was applied. Finally, surgical incision of skin and the fascia were sutured using simple interrupted suture, size (0), non-absorbable silk. The post-operative care was undertaken by injecting enrofloxacin for 5 days with a dose of 5 mg\ kg body weight.

Treatment of the rabbits

1. Group A (PRP group) (n=17); 0.5 ml of an autologous PRP was injected into the site of the experimentally induced transverse fracture, which then followed by closure of the fascia and the skin with (0) non absorbable silk.
2. Group B (Control group) (n=17); in this group the fractured site of experimentally induced transverse fracture was injected with distilled water. The skin and fascia were sutured with (0) non absorbable silk.

Clinical and postmortem examination

Clinical assessment of the lameness in rabbits of each group was evaluated by observation for weight bearing and pain by palpation of fracture limbs. The early ambulation and proper limb movement suggesting a faster process of healing in all animals whether they were treated or not. Moreover, the other clinical features such as feeding, drinking and defecation were also suggestive of good quality healing.

By the end of 10^{th}, 20^{th}, and 30^{th} post-operative days, three rabbits in all groups were sacrificed. The entire femur of the operated limps was detached. The skin is removed and soft tissues such as muscle, fascia, tendons and ligaments were carefully removed by scalpel blade. Postmortem evaluation of these specimens taken from control and experimental groups was assessed by gross observation and palpation of the osteotomized bones particularly development

of the calluses around the fracture site suggesting faster process of fracture healing.

Radiographic imaging

Radiographs of osteotomies were taken by digital X-rays machine at the 10th, 20th and 30th post-operation days. This imaging was performed to evaluate the healing process in both groups, as well as to compare the size of callus formation in each group.

Histopathological examination

The femur bone of all animals was removed, dissected from surrounding soft tissue including skin, muscles and the fascia. Two samples were collected at the fractured site at 10th, 14th, 20th, 30th days post operation and fixed in 10% neutral buffered formalin for microscopic evaluation. The specimens were decalcified in a mixture of hydrochloric acid and formic acid solution for about 24 hours. After decalcification the specimens were rinsed in water and then transferred to ammonia solution for 30 minutes in order to neutralize acids that left in the specimens. The specimens were washed in running tap water thoroughly for up to 24 hours. Then they were dehydrated overnight and embedded in paraffin wax, then routinely processed for staining with hematoxylin and eosin according to the Luna (17).

Results and Discussion

Clinical examination of the rabbits included daily observation of the physical activity such as movement, weight bearing of osteotomized legs. Feeding, drinking and defecation were found normal in all groups from the first day after the operation. The early ambulation of the operated limbs in both groups is also suggestive of proper healing, as they were observed in all animals in group A and B, they were having proper locomotion after the first day of the operation. However, lameness was observed for about one week post the operation, was due to a painful swelling of the operated limbs which were improved in course of time. Although, the other clinical observations such as feeding, drinking, defecation and the physical activity were not observed in both groups after the operation.

Postmortem examination

Four rabbits in each group were executed at 10th, 20th and 30th day post-operation and the femur bones were detached. The skins were removed and the soft tissue such as muscle, tendons and ligaments were carefully removed by a scalpel blade. At 10th day post-operation there was not any significant change at the size of the soft callus between group A and B, that could be detected by naked eyes. At the 20th day of post-operation, hard calluses were developed and observed by the naked eye. At the site of the fractured bone, the calluses were developed and surrounded the ends of the fractured segments. Although, the amount of these calluses was varied between group A and the control group (Figure 3). Similarly, at 30th day post-operation there were differences in the size of the callus formation between the two groups, in which the amount of the callus in group A was greater than the control group. It was apparently more compact and uniform with the bone contour in group A in contrast to group B (Figure 3).

Radiological examination

Radiographic images were taken at 10th, 20th and 30th post-operation days. The X-ray photograph was taken in veterinary teaching hospital using Digital x-ray device. For each group and at different periods after the operation the status of the bone healing and the amounts of callus formation were estimated. In these radiographic images it was found that the process of bone healing was not similar between the groups and there was a considerable variation in the amount of callus bone union and their healing. The radiographic finding of the fractured femur after 10th day in all groups were showed that the

calluses at the fractured lines were not apparent. Although, small area of radiolucent tissues was noticed around the fractured lines in the group A. Then the fractured femurs were radiographed after 20 days of the operation and it was observed that the calluses were developed at the fractured area in both groups, where the fractured lines were completely disappeared in group A (PRP) rather than the control group. It appeared that the amount of callus was greater in group A when compared to the group B (control). Similarly, the degree of radio-opacity of the calluses were varied between the groups, it was more evident and uniform in group A when compared to the group B. Finally, at 30th day post the operation, there was a complete formation of the hard callus around the fractured sites. Where the calluses were completely radio-opaquer and uniform in group A than the group B. In the group B, the callus was also developed but the amount and the radio-opacity were lesser than the group A. Although, the fractured lines were still present in the group B but they were completely disappeared in the group A (Figure 4).

Histopathological study

The histopathological study showed that the process of fracture healing was varied between group A and group B. Initially, during the 10th day of the operation in group A, the tissue showed marked diffuse dissemination of granulation tissue, which is actively proliferated from the periosteum, indicating the inflammatory phase of healing. Meanwhile, a number of apparently non-cellular trabecular bones were present at the site of fracture (Figure 5). While, in group B, there was proliferation of the osteoblasts and the granulation tissue in combination with the obvious fibrous connective tissue at the fracture site, which indicates an early inflammatory phase of fracture healing (Figure 6).

The photomicrographic of the fractured bone at 14th day post operation, showed advanced progress of the bone healing in group A when compared to the group B. During this stage, the bone trabeculae were increased and became thicker when compared to the control group. Moreover, there was an enormous proliferation of thicker pinkish granulation tissue that extended considerably among the bone trabeculae, and numbers of large blood vessels were found that engorged with a blood within the granulation tissue (Figure 7). The proliferation of these substances at the fracture site indicates the early development of soft calluses that bridge the fracture gaps. In the group B, these alterations were completely different, where it showed only diffuse proliferation of granulation tissue that extended considerably within the bone trabeculae (Figure 8).

During 20th day post operation, group A were showed complete formation of hyaline cartilage at the fracture site, which then extended deeply to form a woven or trabecular bone. This alteration of soft tissue into the cartilaginous tissue indicates complete formation of the hard callus around the fracture site, which assumed to be developed from the perichondrium tissue, where it acts as a bridge between the bone fragments in order to immobilize the fractured segments. Moreover, the developed bones at the fractured site were showed development of excessive amount of the regenerated hematopoietic tissues (Figure 9). However, in the control group, there was formation of the hyaline cartilage at the fracture sites, which extended deeply to form a woven or trabecular bone, but it was less dense and a smaller number of chondrocytes were present when compared to group A (Figure 10).

The histological features of the fractured bones at 30th day post operation in group A, showed development of condensed hyaline cartilage at the fracture site, which indicate a continuous hard callus formation at the fractured site. Moreover, there were clear establishment of the woven bone (Figure 11). In group B, there

was development of small amount of less condensed hyaline cartilage and a clear woven bone with their hematopoietic tissue, as well as the bone matrices were appeared lesser than the group A (Figure 12).

Discussion

In this study, an autologous PRP were used on the experimentally induced bone fracture in order to enhance the process of fracture healing in the female rabbit. Currently, different materials were applied to the fractured bones in different animal model that underwent experimentally induced fractured. PRP is among the most applicable materials because of its safety, minimal invasive during application and enhance the process of wound healing (18). It has been recorded by different studies that PRP enhances bone healing through proliferation of granulation tissue due to the presence of a high level of growth factors. In this study, a single dose of autologous PRP were applied to the site of the fractured bone at the end of the operation. The results were showed the proper locomotion in animals earlier in the group A. The anatomical configuration of the fractured bones showed production of more tough and uniform callus in the group A when compared to the group B. In a study on 40 New Zealand rabbits that underwent tibial reconstruction using bone autograft alone, bone allograft alone and in combination with PRP for 24 weeks. It was found that when the PRP when combined with the bone grafts it was significantly increase the process of bone resorption and acceleration of bone union (19). In this study, all the animals were observed for about 30 days post operation and were radiographed at 10^{th}, 20^{th} and 30^{th} days post operation in both groups, where they showed evidence of a faster fractured healing in all stages in the treatment group when compared to the control group.

The radiological study showed a significant difference in the callus formation between the group A and the group B. Nevertheless, the callus formation was appeared more radiopaque in the group A with no evidence of the fractured line during 30^{th} day in the group A. Similarly, in a study on 20 New Zealand rabbits, it was found that PRP was significantly enhance the processes of fracture healing in the duration of only one month, however, this enhancement were not continued during the second month (20). However, in other studies, they were recorded that the beneficial effect of PRP on bone regeneration could not be demonstrated in combination with every bone substitution materials and every animal models; in goats, using an autogenous cancellous bone with addition of PRP were not improved bone healing of critical-size on the forehead-bone defect (21). Similarly, the addition of PRP to β-tricalcium phosphate used in pigs model were not have an effect on bone healing on the anterior spinal fusion and the forehead-bone defect (22). As well as, adding of PRP to bovine cancellous blocks, were recorded no positive effect in a rat mandibular defect (23) and when applied around titanium dental implants in the dogs model (24). Although, in the current study, we found that application of PRP were enhance the process of bone healing and faster callus formation in contrast to the control group were healed normally.

The histopathological findings further confirmed the significant difference between group A and group B, in which application of PRP were enhanced the process of bone healing. Similarly, other studies were also confirmed similar finding (25), for instances in a study on twenty adult male New Zealand rabbits, were underwent partial resection of the tibia, which then followed by application of PRP in combination with β-tricalcium in the first group and the aspirated bone marrow in combination with β-tricalcium in the second group. After 4 weeks the rabbits were euthanized and the tibia

were evaluated using digital radiography and histomorphometry, the result showed that the PRP provided a greater amount of bone consolidation than bone marrow at the fractured site (26). However, different studies, they were recorded that neither calcium phosphate alone or with PRP was produces any effect on bone formation in the dogs' models (27, 28). Further, in another study on 20 lambs, aged 4 months, were undergoing osteotomy for femur bone, which the followed by application of PRP to the fractured site at the 1st, 10th, and 20th days post operation, the results showed no beneficial effect of PRP on fracture healing. Therefore, different animal species showed different results, which might be contributed to the species of the animals, sources of PRP and the optimized dose of PRP for instances studies on ovine species were not showed a beneficial effects of PRP on bone healing (29). However, in our study the results were interestingly differ, it was found that the stages of healing were faster in Group A when compared with Group B, as they were confirmed on histological study section from the fractured bones.

Conclusion

In conclusion of this study it was appeared that application of autologous PRP enhanced the process of callus formation at the site of fractured bone and the fractured lines were disappeared faster than the control group.

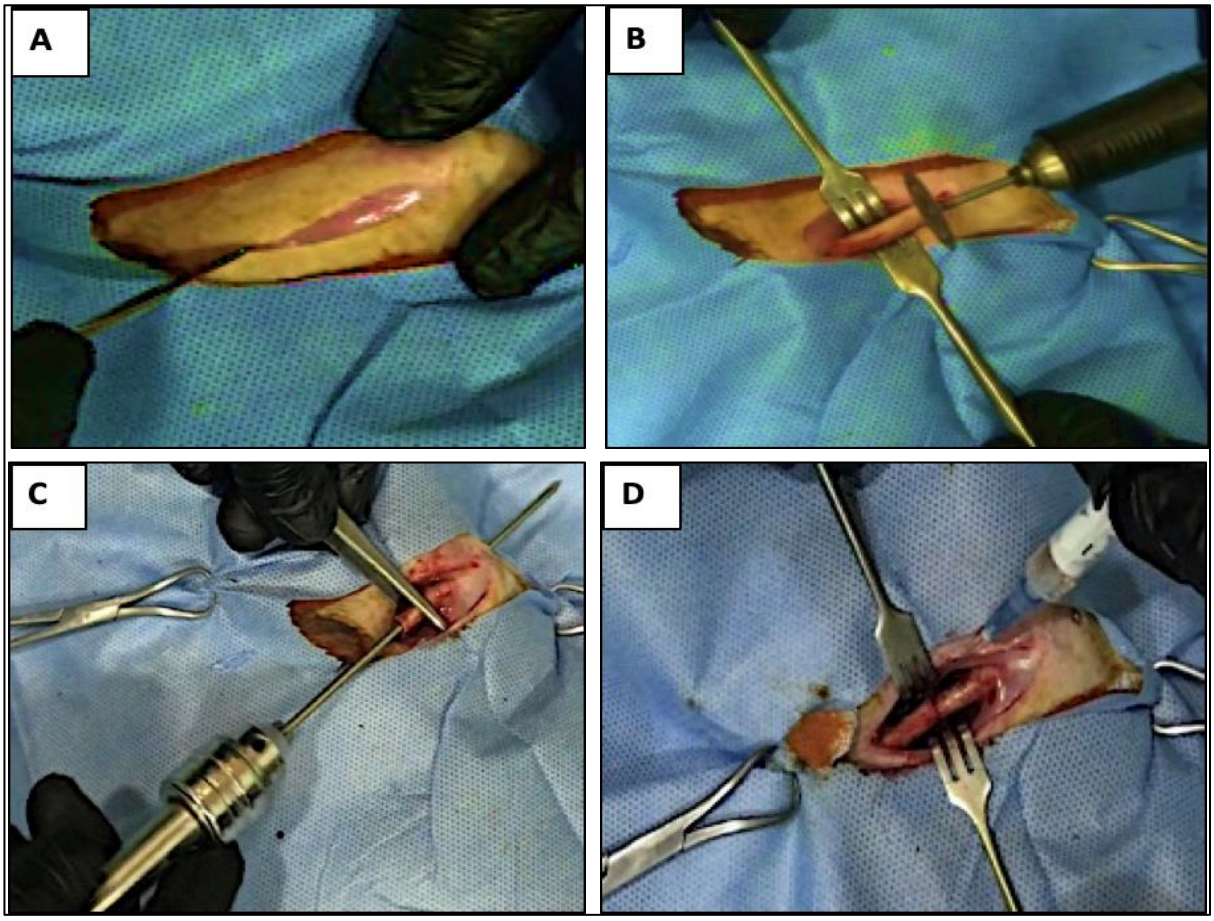

Figure 1: A; show the surgical incision of the skin and the subcutaneous tissue over the femoral bone, B; The femur is exposed and transversely fractured using electrical bone saw, C; introducing the intramedullary pine by the pine chuck, D; application of the PRP into the fracture site.

Figure 2: Electrical rotating saw with 10 mm width and 0.5 mm thickness blade used for osteotomy.

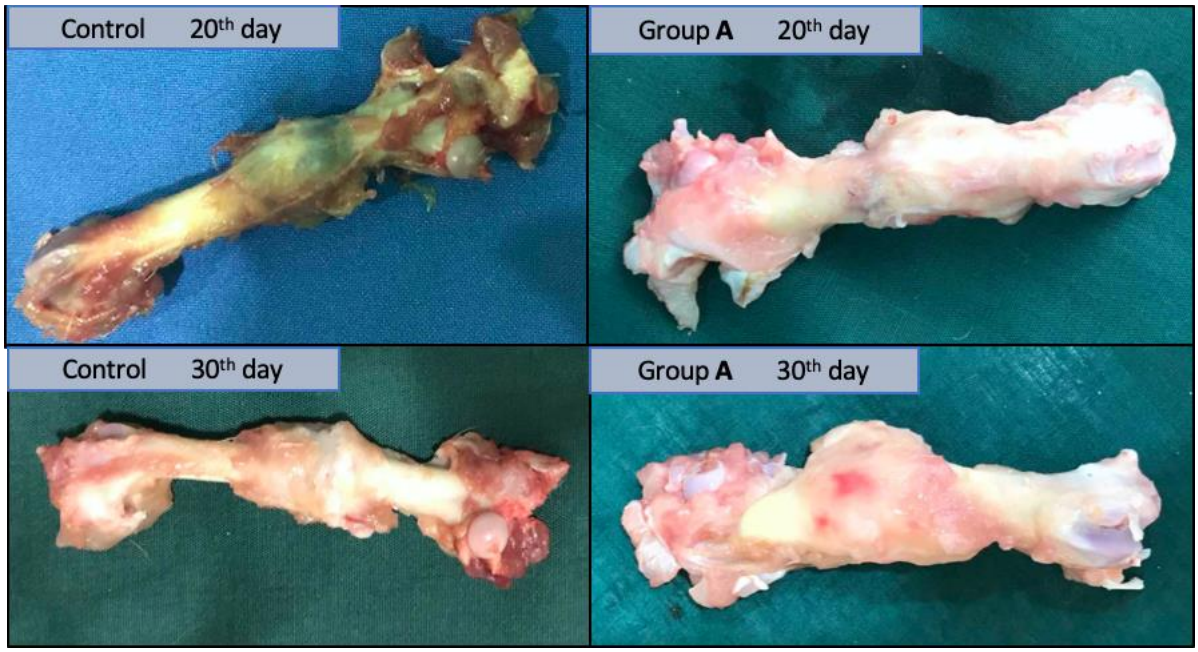

Figure 3: Show postmortem finding of the fractured femur bone in groups A and B at 20th and 30th day post operation. There was formation of the callus at the fractured site in both groups, but the amount of callus was greater in group A than the group B.

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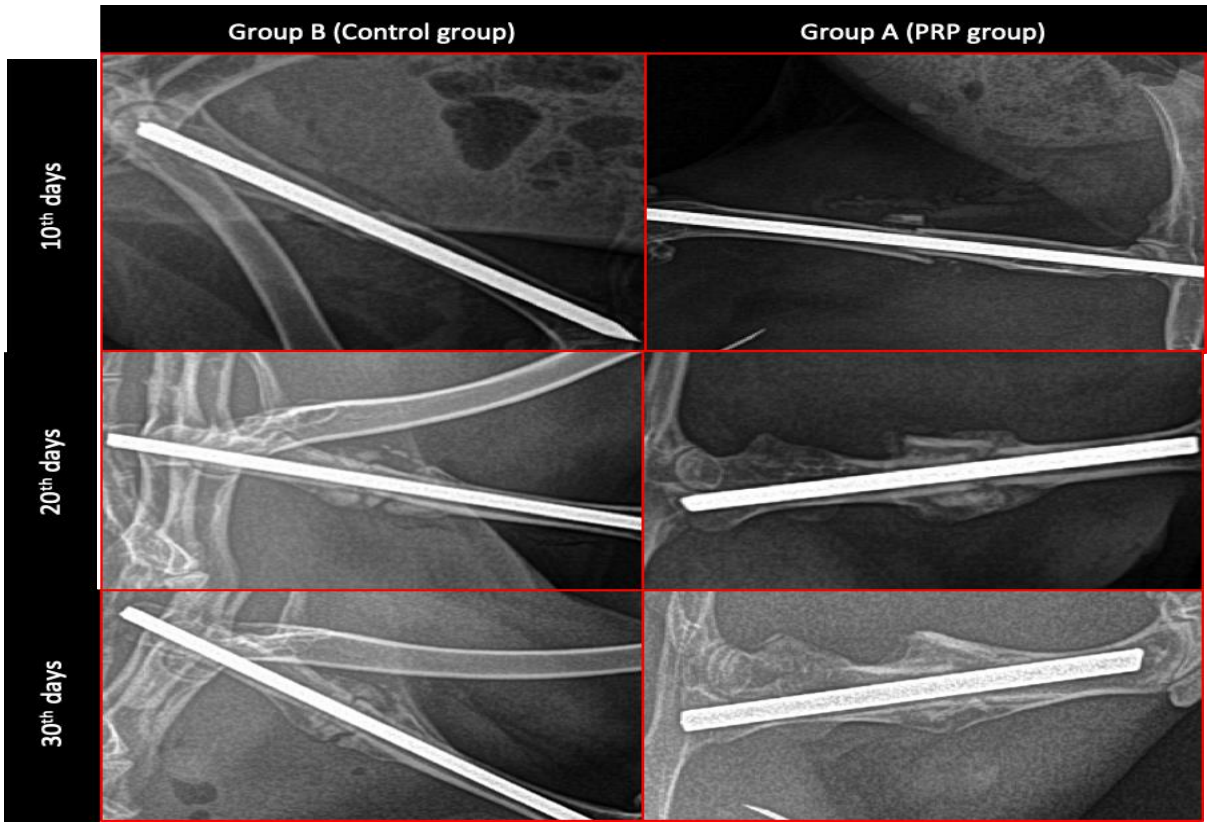

Figure 4: Radiographic finding of the fractured bones at 10th, 20th and 30th day of post-operation in group A and B. At 10th day there is no evidence of hard callus formation in both groups. During 20th and 30th day there were evidence of callus formation in both groups, and the amount with their radio-opacity were greater in group A than the group B.

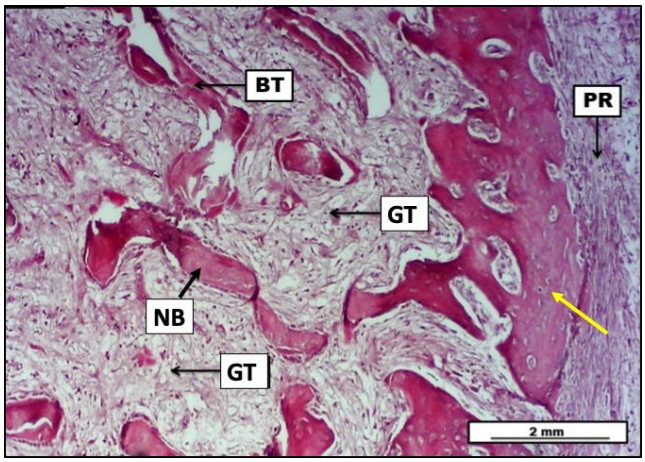

Figure 5: Photomicrograph of fractured bone, group A at 10th day post operation, showed diffuse dissemination of lose granulation tissue (GT), which is actively proliferated from the periosteum (PR) that contained active osteoblast (Yellow arrow). The section shows many remaining of dead bone trabeculae (BT) with necrotic bones (NB) at the fractured site. H&E stained. X100.

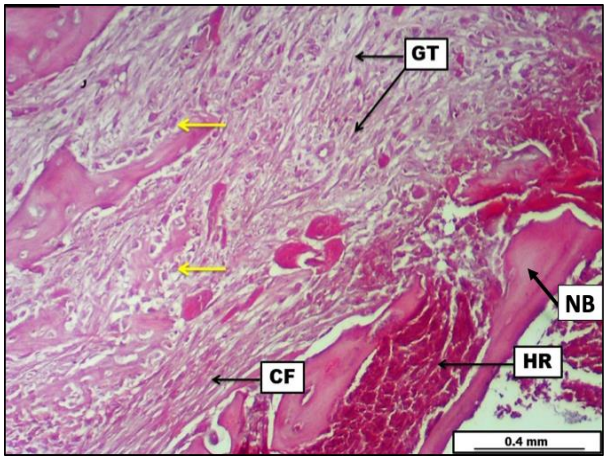

Figure 6: Photomicrograph of fractured bone, group B at 10th day post operation, revealed obvious fibrous connective tissue proliferation and granulation tissue (GT) with bundles of proliferated collagen fibers (CF). Presence of hemorrhage (HR) at the fracture site and necrotic bones (NB). The section shows proliferated osteoblast parallel to the bone trabeculae (yellow arrows). H&E stained. X100.

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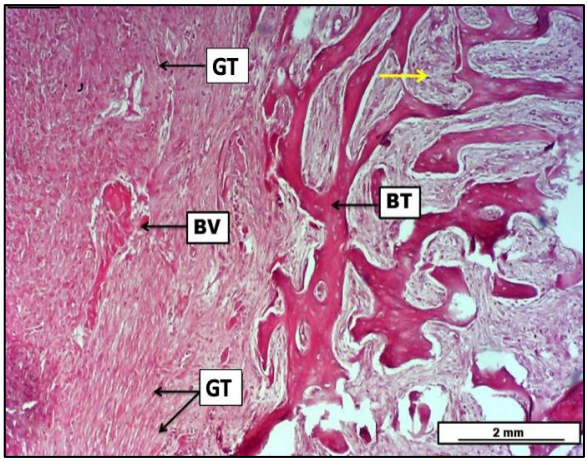

Figure 7: Photomicrograph of fractured bone, group A at 14th day post operation, displayed enormous proliferation of thicker pinkish granulation tissue (GT), in which extended considerably among the bone trabeculae (BT) (yellow arrow). Additionally, large section of engorged blood vessel (BV). H&E stained. X100

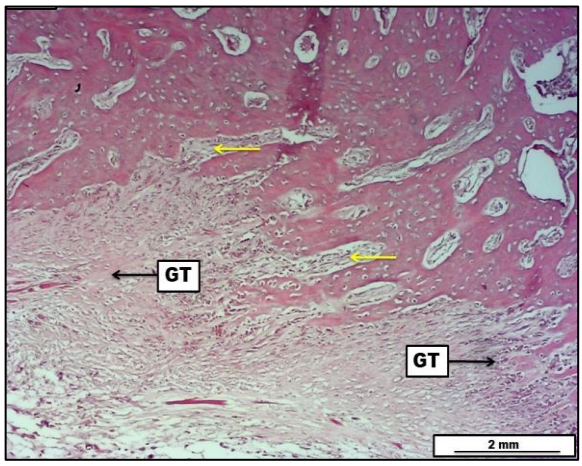

Figure 8: Photomicrograph of the fractured bone, group B at 14th day post operation, displayed diffuse proliferation of granulation tissue (GT), in which extended and interlaced considerably within the bone trabeculae (yellow arrow). H&E stained. X100.

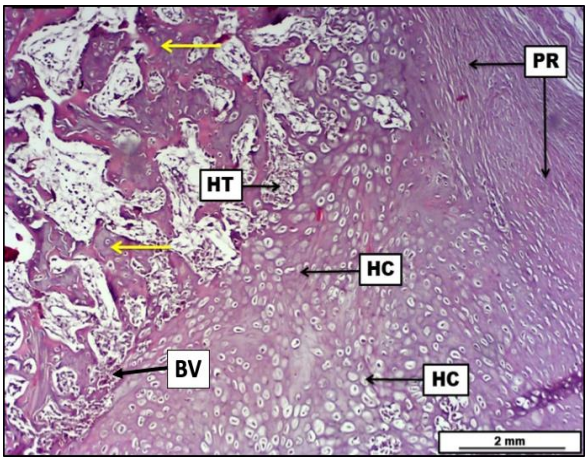

Figure 9: Photomicrograph of fractured bone, group A at 20th day post operation, showed formation of hyaline cartilage (HC) at the fracture site, which extended deeply to form a woven or trabecular bone or osteoid tissue (yellow arrows) and a newly formed blood vessels (BV). The hyaline cartilage assumed to proliferate from the perichondrium tissue (PR). The section shows considerable amount of regeneration of hematopoietic tissue (HT). H&E stained. X100

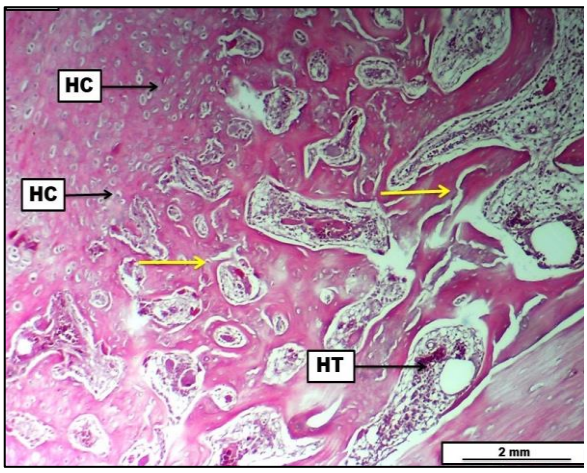

Figure 10: Photomicrograph of fractured bone, group B at 20th day post operation, showed clear formation of hyaline cartilage (HC) at the fracture site, which extended deeply to form a woven or trabecular bone (yellow arrows). Moreover, the section shows excessive amount of regeneration of hematopoietic tissue (HT). H&E stained. X100.

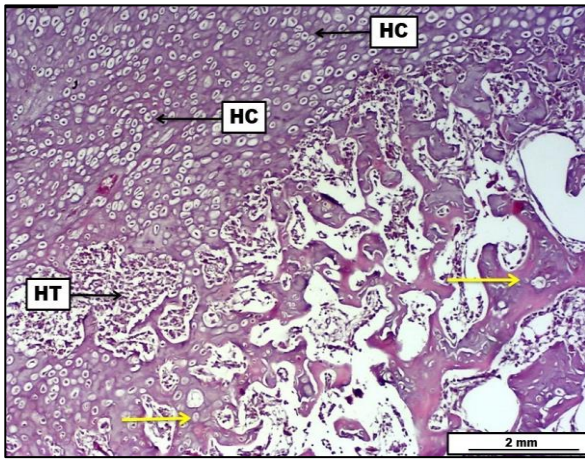

Figure 11: Photomicrograph of fractured bone, group A at 30th day post operation, showed remarkable hyaline cartilage (HC) together with clear establishment of woven bone (yellow arrows). In addition, the section revealed hematopoietic tissue regeneration (HT). H&E stained. X100.

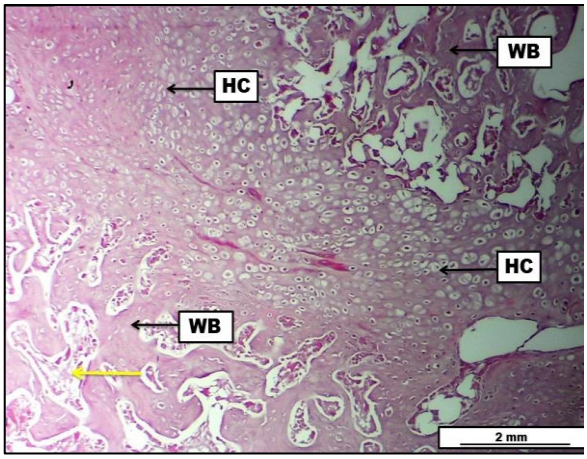

Figure 12: Photomicrograph of fractured bone, group B at 30th day post operation, showed notable amount of hyaline cartilage (HC), clear woven bone formation (WB) and a hematopoietic tissue regeneration (yellow arrows). H&E stained. X100.

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