Iraqi Journal of Veterinary Sciences, Vol. 36, Supplement I, 2022 (203-210)
Proceedings of the 7th (1st International) Scientific Conference, College of Veterinary Medicine, University of Mosul

Iraqi Journal of Veterinary Sciences

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Histopathological study of the effect of using repeated doses of platelets-rich plasma on articular cartilage repair in Rabbits

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Article information

Article history:
Received September 14, 2022
Accepted December 09, 2022
Available online December 09, 2022

Keywords:
Osteoarthritis
Articular cartilage
PRP

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Abstract

The present study was performed on platelet-rich plasma compound from animals engaged in this work, to be directly injected in the joint space of the affected stifle joint 5mm diameter full-thickness articular cartilage removal in the knee joint manually using the same instrument 5 mm drill to remove the cartilage, by different doses to evaluate its influence on articular cartilage repair. In this experiment, 28 adult rabbits were used. The same defect is created on the lateral condyle of the distal end of the femur. Three identical groups of animals were formed randomly. Group I was untreated. Group II was treated with a single dose of PRP (which was administered immediately after the creation of the defect), and group III was treated with two doses of PRP (the first dose was given immediately after the creation of the defect, and the second doses after 15 days of the first one. The histological examinations were recorded of four rabbits of each group, which were euthanized one and two months after treatment, and the assessment of cartilage tissue repair was checked. The control group showed destruction of articular cartilage at the site of the lesion with involvement of subchondral bone in both periods 30 and 60 days of examination, while specimens taken from groups II and III after 30 days showed a new tissue formation which is characterized as the homogenous extracellular matrix, with the proliferation of active chondrocytes. At the same time, slides on day 60 of treatment showed an increased number of newly formed chondrocytes and a clear line of demarcation between articular cartilage and subchondral bone, representing the tissue filling the gap had been created at the beginning of the experiment.

DOI: 10.33899/ijvs.2022.135928.2543,

Introduction

Osteoarthritis (OA) is a degenerative disorder of the articular cartilage. One of the most prevalent joint illnesses is OA, which is characterized by pain, stiffness, and inability to move the affected joint (1,2). There are two types of OA primary which affects the articular cartilage directly such as in aged people, obesity, and joints injuries, and secondary which may occur due to inflammatory arthritis, vascular necrosis, and infectious arthritis (3), which is mostly treated with intraarticular hyaluronic acid injections and oral nonsteroidal anti-inflammatory medication. Although some symptoms and pain are reduced by this treatment (4). As a new therapeutic agent of OA, so-called disease-modifying OA drugs (DMOADs), such as chondroitin sulphat and glucosamine, attracted attention (5,6). There is therefore a great need for an efficient, simple, and inexpensive therapeutic method that prevents cartilage degeneration in the initial stages of OA. Recent studies have explored the regeneration of hyaline cartilage of injured articular cartilage using different growth factors that inhibit cartilage degeneration and accelerate cartilage metabolism (7). Results from these studies show that basic fibroblast growth factor (bFGF), insulin-like growth factor-1(IGF-1),

transforming growth factor- β1 (TGF- β1) and platelet-derived growth factor (PDGF) are powerful chondrocyte mitogens as well as matrix metabolism stimulators (8,9). Platelet - rich plasma (PRP) which prepared by several methods such as PRP method (10), buffy coat method (11), contains a high concentration of platelets in a small volume of plasma and high concentrations of several autologous growth factors, including PDGF, TGF-β1 and IGF-1 (12). In the field of oral surgery and plastic surgery, PRP has been clinically used to improve tissue regeneration and wound healing (13). A previous study reported increased bone regeneration by the administration of PRP - containing gelatine hydrogel microspheres, which provided continuous release of growth factors (14). A similar treatment has also been reported to promote the regeneration of intervertebral disks (15). We aimed in current study to investigate the effect of repeated dose of PRP on articular cartilage regeneration.

Materials and methods

Experimental design

The study used 28 healthy male rabbits that ranged in age from 1 to 1.5 years and weighed about 2.5 kg. Animals were kept in separate cages in the same conditions. Animals were given unlimited access of food and water. 24 Animals submitted to same surgical procedure to make a mechanical defect in the lateral condyle of the femoral bone (distal end). They were labelled as group 1, group 2, and group 3 after being randomly divided into three equal groups and kept for two months following surgery.

Group I served as the positive control and received no treatment. Group II was given a single dose of prepared PRP soon after surgery. Group III was given two doses of PRP, the 1st immediately after surgery, and the 2nd 15 post surgery PRP. The rest four animals were euthanized, and the distal end of femoral bone (same site of defect) examined histologically as negative control.

Ethical approve

The study was reviewed and approved by the scientific committee of the Department of surgery and obstetrics, and the college council session of the College of Veterinary Medicine, University of Baghdad (No. 2164/ P. G. in 26-11-2018).

Surgical procedure

The animals were fasted for six hours prior to operation. The operation is made under general anaesthesia which is achieved by administration of 20 mg/kg of body weight of ketamine hydrochloride, and 5 mg/kg body weight of xylazine (Troy, Australia) (16). The site of the operation is prepared surgically, animal was secured to the table lying down on its right side. Layers covering the stifle joint were incised including skin, sub cutaneous fascia, extensor tendon deviated medially, then synovial sac to expose the lateral condyle of the distal end of femur (weight bearing part). A 5 mm auger is used in all animals of the experiment to remove the full thickness of the articular cartilage reaching the subchondral bone (Figure 1). The site was closed then as follow: synovial sac is closed by simple continuous suture technique, sub cutaneous fascia is closed by simple continuous suture technique, both are closed by absorbable suture material 3/0 vicryl. Post operatively 1 mg/kg body of penicillin-streptomycin is administered to each animal. The prepared PRP is administered by intra-articular space injection 1 ml of resuspended plasma.

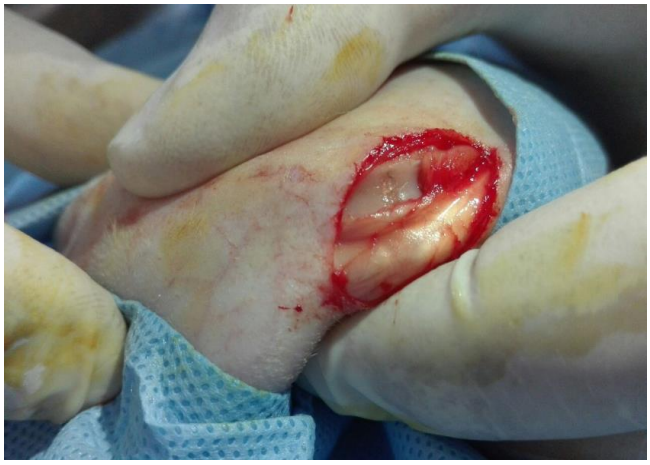

Figure 1: Photographs show the full-thickness articular cartilage defect created.

Preparation of PRP

To prepare PRP, many procedures can be followed, the double centrifugation methos is used in this research, in which an initial centrifugation was done to separate red blood cells as a first spin, then followed by a second spin to condense platelets, which are suspended in the smallest final plasma volume. A 5 ml of whole blood is collected in a 5 ml glass tube that contain anticoagulants. The first run is made at constant acceleration to separate RBCs from the WB 1800 rpm for 10 minutes, the sample is obviously separated three layers: the superficial layer that contains mainly platelets and WBC, and the middle thin layer that is known as the buffy coat and that is rich in WBCs, and a bottom layer that consists mostly from RBCs. The upper layer and superficial buffy coat were transferred into a new sterile tube. The second spin step was then performed, for the second spin 4000 rpm for 15 minutes should be just effective to help in forming soft pellets at the lower part of the glass tube. The superficial part of the plasma is mainly composing PPP (platelet-poor plasma) which should be removed, and concentrated platelets then homogenized (resuspended) in lower 1/3rd of plasma to create the PRP (17,18).

The counting of platelets is estimated by sending one sample from all group to the laboratory analysis to determine

the number of platelets condensed in the remaining plasma using a device for blood sample detection (Vet. Scan 5 HM) serial no. (CA 94587) USA.

Results

Platelets counting

The platelets counting of prepared PRP showed an increase in the platelet's concentrations compared to the whole blood counting. Furthermore, in all samples, a small number of RBCs, WBCs were noticed in the plasma. The average whole blood platelet count was 650,073±62,739 µl, which were higher than the whole blood counting.

Histopathological examination

Histopathological examination of group I after 30 days showed cartilage surrounding site of the lesion was collapsed and gradual absence of demarcation line from normal tissue toward site of defect, with exposing of subchondral bone into articular surface, and complete destruction of cartilage was extended to involve the bone trabeculi (Figure 2). In addition, marked fibrillation of mineralized tissue that extended along surface of the defect was observed (Figure 3) in compare to normal medial condyle tissue (Figure 4). Histopathological sections of animals of group I after 60 days of creation of defect, revealed marked extensive damaged cartilage, with complete absence of chondrocytes as well as loss of extracellular matrix, in addition of losing the smooth articular surface (Figures 5 and 6).

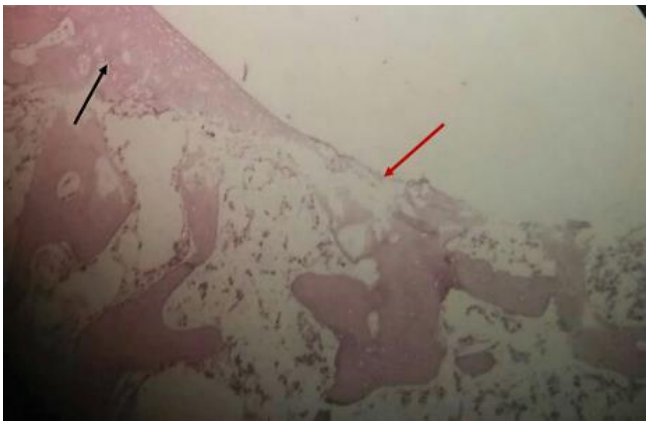

Figure 2: Histopathological section in the joint of positive control group I at one-month post-injury, shows complete destruction of cartilage (red arrow) with absence of demarcation line (black arrow) (x 40 H&E stain).

After 30 days of induced injury, the histopathological finding of specimens of group II showed a proliferation of active chondrocytes with a heterogeneous extra cellular matrix with complete absence of demarcation line between newly formed cartilage and subchondral bone with spots of degenerative changes. The repair processes have formed a mixed tissue at the site of the defect where the lesion undergoes differentiation into cartilaginous precursor cells and bony tissues. The site of healed condyle tissue revealed a mixture of fibrocartilage and variable amounts of hyaline cartilage (Figure 7). After 60 days of induced injury, group II showed advanced stages of cartilage repair represented by semicomplete filling of the gap with a new tissue, which has an increased number of chondrocytes with copious homogenous extra cellular matrix, also normal subchondral bone and clear line of demarcation was seen (Figure 8). The repair processes at the site of defect of the group II revealed a cartilaginous precursor cells undergo differentiation into hyaline cartilage with normal appearance of isogenic groups of mature chondrocytes surrounded by translucent extracellular matrix (Figure 9).

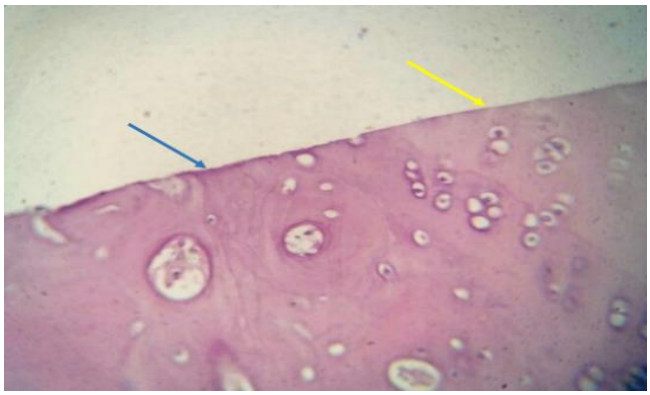

Figure 3: Histopathological section in the joint of positive control group I at one-month post-injury, shows the exposed subchondral bone (blue arrow) compared to normal cartilage (yellow arrow) (x 400 H&E stain).

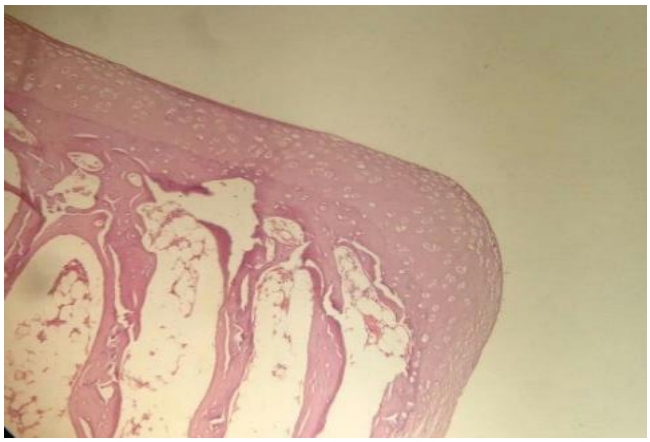

Figure 4: Histopathological section in the joint of normal animal of (medial condyle) shows healthy tissue including subchondral bone, chondrocytes occupying its lacune, extracellular matrix (x10 H&E stain).

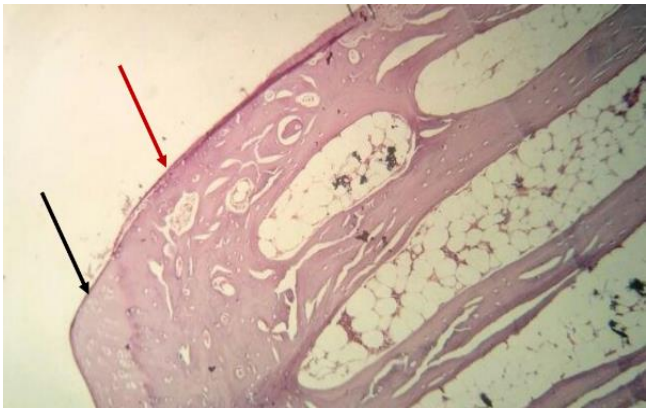

Figure 5: Histopathological section in the joint of the positive control group I at two months post-injury, shows irregular or absent of chondrocytes, and extra cellular matrix (red arrow), and congestion of bone marrow of subchondral bone, (black arrow) represents the normal cartilage (x10 H&E stain).

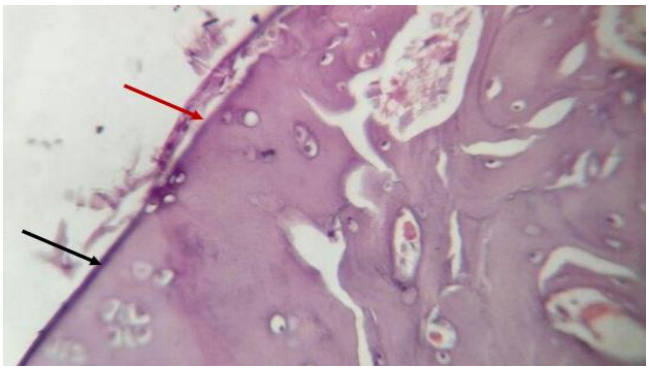

Figure 6: Histopathological section in the joint of positive control group I at two months post-injury, shows subchondral fibrous tissue (red arrow), and degeneration of chondrocytes, (black arrow) represents the normal cartilage (x 40 H&E stain).

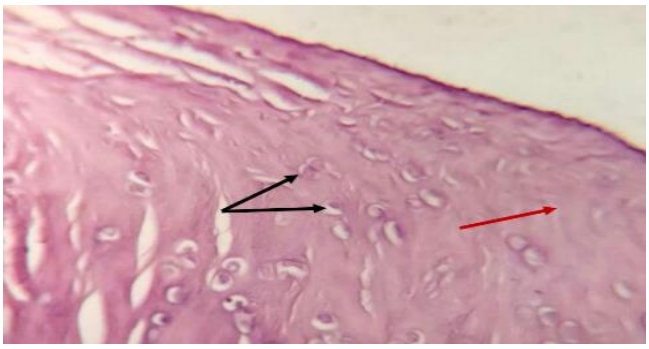

Figure 7: Histopathological section in the bone of animal of group II after one month of injury, shows the repair tissues are a mixture of fibrocartilage (red arrow) and active chondrocytes (black arrow) (x 40 H&E stain).

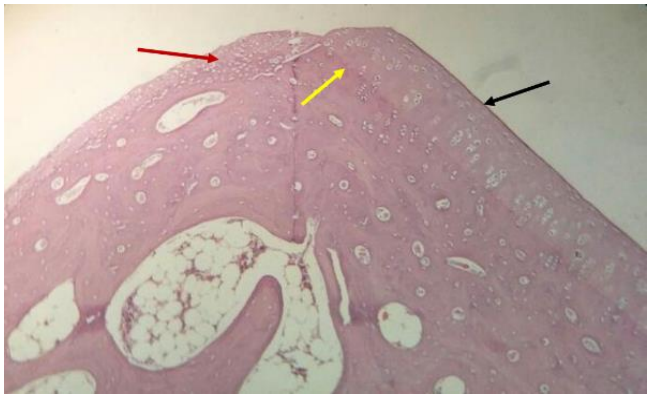

Figure 8: Histopathological section in the joint of group II at 2 months post-injury, showed new tissue with proliferative feature by chondrocytes (red arrow), (black arrow) represents the normal cartilage with clear line of demarcation (yellow arrow). (x 100 H&E stain).

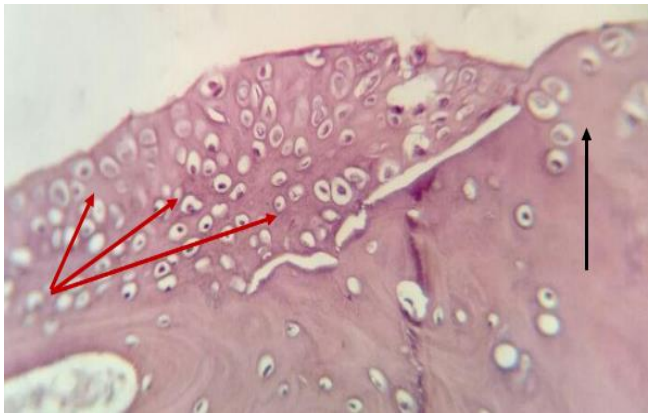

Figure 9: Histopathological section in the joint of group II at 2 months post injury shows invasion of the new tissue with clusters of chondrocytes (red arrow) and normal subchondral bone, (black arrow) represents the normal cartilage. (x 40 H&E stain).

In group III after 30 days of induced injury, the defect site of the articular cartilage revealed marked focal degeneration of extracellular matrix, with the presence of active chondrocytes, and spots of subchondral bone destruction periosteum associated with the proliferation of progenitor cells to form a migration line of chondroblast with forming chondroblast and immature chondrocyte (Figures 10 and 11). After 60 days of induced injury, slides of group III showed complete filling of the gap (site of the defect) by the tissue formed newly. This new tissue takes the same appearance of the medial femoral condyle with a numerous cluster of new chondrocytes, and seminormal extracellular matrix, with clear tidemark between cartilage and the subchondral bone which appeared normal (Figures 12 and 13).

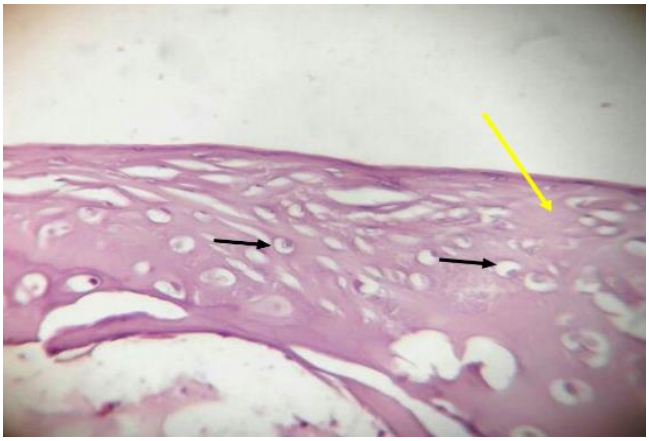

Figure 10: Histopathological section in the joint of animal of group III one month after injury shows degenerative changes in the site of lesion with presence of numerous chondrocytes (black arrow), with proliferation of fibrous connective tissues (yellow arrow) (x 40 H&E stain).

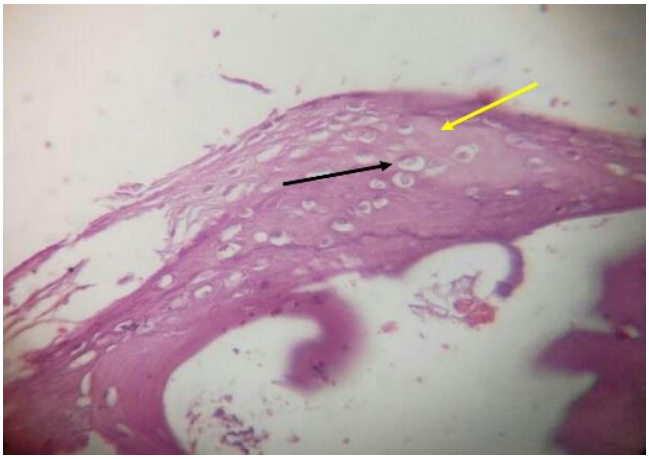

Figure 11: Histopathological section in the bone and cartilage of group III animals after one month of injury, showed substantial thickening of the cartilage tissue invaded by chondrocytes (black arrow) and fibrous connective tissue (yellow arrow) (x 40 H&E stain).

Discussion

The number of platelets obtained by double centrifugation method shows a markable elevation about 3 times of the normal number in the blood, this is due to method of preparation of PRP (double centrifugation method). This technique should be applied to truly concentrate platelets from autologous blood. These findings are the same as Marx (19).

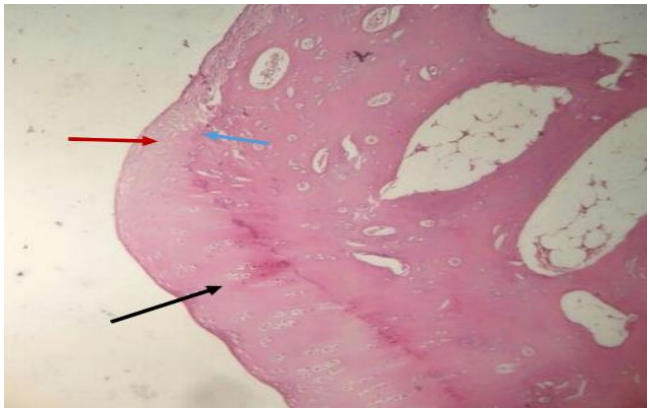

Figure 12: Histopathological section in the lateral part of femur of group III animals after two months of injury showed tissue filling the lesion site with a numerous chondrocyte (red arrow) and fibrous connective tissue (black arrow) with presence of line of demarcation separates cartilage from subchondral bone (blue arrow) (x 10 H&E stain).

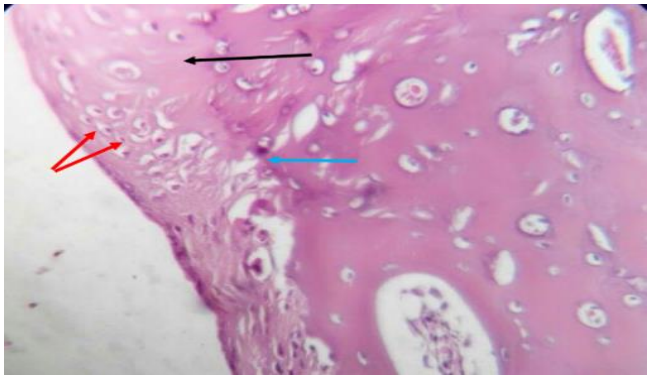

Figure 13: Histopathological section in the distal part of the femur group III after two months of injury, showed tissue filling the lesion site with numerous chondrocytes (red arrow) and fibrous connective tissue (black arrow) with presence of line of demarcation separates cartilage from subchondral bone (blue arrow) (x 40 H&E stain).

Many factors may interfere with the results of both quantity and quality of PRP such as the effect of gravity (G), the type and method used to prevent coagulation of blood, and the normal value of platelets present in the whole blood before preparation (19). In addition to all these factors, also the times of spinning applied in the centrifugation steps are considered very important factors (20-22).

The histopathological findings of group I samples examination after 30 days of defect induction showed collapse of the cartilage surrounding site of the lesion, and gradual absence of demarcation line from normal tissue toward site of defect, with exposure of subchondral bone into articular surface, and complete destruction of cartilage, also

the damaged was extended to involve the bone trabeculae. In contrast, groups II, and III there were several histological changes such as proliferation of chondrocytes, extracellular matrix synthesis, and normal structures of subchondral bone, these findings are agreed with Nebras *et al.* (23) who declared that the triggering of angiogenesis, activation, multiplication and chemotaxis of mesenchymal cells is occur due to presence of thrombocyte derived growth factor secreted from the platelets during fracture healing process.

Schmidt *et al.* (9) studied the correlation between platelet counting and the amount of growth factor, suggesting that the elevation of platelets to eight times more than normal level, the amount of PDFF-BB, TGF-β1, VEGF and endothelial growth factor were increased as well, and this elevation is differed from individual to another (9).

PRP is considered an autologous of a biological origin treatment of choice in which the individual has growth factors secretion is generally influenced by platelets are very important during the process of to enhance many steps during healing times, for instance, the formation of newly blood vessels (angiogenesis), cell division and multiplication, and cell movement due to presence of chemotactic factors. Many researches have stated that PRP interferes with joint formation and enhance restoration of joint homeostasis. Schmidt *et al.* (24) designed a study to investigate the response of adult porc. Chondrocytes cultured in the existence of 10% PRP, 10% PPP, or 10% fetal bovine serum, and stated that PRP therapy was responsible for the significantly higher DNA and proteoglycan content, as well as improved the formation of new collagen (24).

Additionally, Akeda *et al.* (25) mentioned that, compared to human serum, PRP had a very important role in cell multiplication, and chondrocyte physiology and activity, and also stated that this preparation method may be effective for the safe and easy engineering method of cartilaginous structures, contended that injection of PRP inside the synovial space of the joints rich in growth factors may be effective in restoring the hyaluronic acid level and balancing formation of new blood vessels (25,26).

There are many studies applied to estimate the role of PRP to minimise the sings and the suffering caused by osteoarthritis, Spreafico *et al.* (26). Mediators that are responsible for activation of platelets are growth factors and cytokines. Other studies, suggested that the PRP may enhance chondrocyte differentiation, multiplication, and maturation (27-29). The PRP also have anti inflammatory effects, and this activity may exert this effect by inhibiting the NF-KB pathway (27). In another study on pigs, Lippross stated that the administration of PRP inside the articulate space may significantly reduces joint inflammation (30).

The presence of GF-b considered the most important actor presented in PRP, which is responsible for the process of cartilage repair and suggesting that the functions include increase of chondrocyte phenotype expression, these results exist in treated groups of our study, which agreed with findings of Pujol *et al.* (31). The differentiation chondrocyte of mesenchymal stem cells, extracellular matrix precipitation, and respond with most of the suppressive effects of inflammatory mediators IL1 on cartilage-specific macromolecules synthesis (32), also PDGF has a very important influence of the maintenance of hyaline-like chondrogenic phenotype, which lead to increases in number of chondrocytes, the formation of upregulates proteoglycan, and is a potent chemotactic factor for all cells of mesenchymal origin (33).

The intra-articular injection of any lubricated media such as HA, Autologous condition serum, and PRP plays an important role in protection of the articulated bone surfaces, which is mainly decreases pain, and decreases damage progression in the affected joints. This agreed with the findings of Anitua *et al.* (27) Increased hyaluronic acid production and secretion were detected in synoviocytes from the patients with OA cultured in PRP. This suggests that intra-articular PRP injection could potentially serve as the endogenous source of chondroprotection and joint lubrication (27).

More than all these descriptions of the role of PRP, there are many other descriptions for the effect of PRP on the natural healing process especially in knees suffering from OA. As a beginning numerous growth factors are usually aggregates in alpha-granules inside platelets as the latent form. During activation process of platelets, these factors are gradually freed and involved with other factors in the regeneration process (17). The normal healthy cartilage surrounding the lesion site also releases Chondrocyte, which then moved into the affected part of the cartilage. Also, the activation and migration of mesenchymal stem cells to reach the site of defect (34-37).

One of the most important marks in our study, is the number of chondrocytes, which is appeared in group II slightly more than samples of group III, this is may be due to adverse action of PRP which is decrease the proliferative activity of chondrocytes, and cartilage surrounding the site of the lesion, this notice is explained by Forogh *et al.* (38) The results showed that intra-articular injection of PRP could provide a therapy for knee osteoarthritis with the potential to relieve symptoms even for 12 months. However, the frequent use of PRP injection increases the risk of adverse reactions (39-41).

Conclusion

Based on the results obtained from this study, we concluded that the injection of PRP in the intra articular space increases the ability of the cartilage surrounding the lesion to regenerate due to the effect of growth factors which influence the proliferation, maturation of chondrocytes, also extracellular matrix synthesis. The use of single dose or repeated doses of PRP make no significant difference in articular cartilage regeneration.

Acknowledgments

I would like to express thanks to College of veterinary medicine, University of Baghdad for supporting the present work, and the laboratory facilities.

Conflict of interest

The authors declare that no conflict of interest exists.

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دراسة نسجية مرضية لتأثير استخدام جرعة مكررة من البلازما الغنية بالصفيحات الدموية على شفاء الغضروف التمفصلي في الأرانب

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الخلاصة

استهدفت هذه الدراسة تحضير البلازما الغنية بالصفيحات الدموية من الأرانب المستخدمة في هذه التجربة، ومن ثم حقنها في تجويف مفصل الركبة لهذه الحيوانات والذي سبق وتم إحداث إصابة في الغضروف التمفصلي للنهاية القاصية لعظم الفخذ ميكانيكيا لدراسة تأثير البلازما على شفاء الغضروف التمفصلي في الأرانب. استخدم في هذه التجربة 28 أرنبا بالغا، حيث تم إتلاف اللقمة الوحشية للنهاية السفلى لعظم الفخذ للطرف الأيسر لجميع الحيوانات، وقسمت هذه الأرانب بصورة عشوائية الى ثلاثة مجاميع متساوية، المجموعة الأولى اعتبرت مجموعة سيطرة إيجابية، والمجموعة الثانية اعتبرت مجموعة معالجة، حيث تم حقنها بالبلازما الغنية بالصفيحات الدموية لمرة واحدة مباشرة بعد إجراء إحداث الإصابة، والمجموعة الثالثة عولجت بالحقن بنفس المادة المحقونة في حيوانات المجموعة الثانية بجرعتين الأولى مباشرة بعد إجراء إحداث الإصابة، والثانية بعد ١٥ يوما من موعد الجرعة الأولى .أظهرت نتائج الفحص النسجي للعينات المأخوذة من حيوانات المجموعة الأولى وللفترات ثلاثون وستون يوما تحطما كاملا في نسيج الغضروف التمفصلي في المنطقة المصابة، في حين أظهرت العينات المأخوذة بعد ٣٠ يوما من إحداث الإصابة من حيوانات المجموعتين الثانية والثالثة نمو نسيج جديد يملا منطقة الإصابة، والذي امتاز بنمو النسيج خارج الخلايا إضافة الى تكاثر الخلايا الغضروفية. في حين أظهرت المقاطع النسجية للعينات المأخوذة بعد ٦٠ يوم من إحداث الإصابة بظهور خلايا غضروفية جديدة ووضوح الخط الفاصل بين الغضروف التمفصلي والعظم تحت الغضروف.