

Treating of the Articular Cartilage in Rabbits Using Different Doses of Platelet-Rich Plasma**Mustafa Abdullah khaleefah and Hassanain A. Emran***

Department of Surgery and Obstetrics/ College of Veterinary Medicine/ University of Baghdad

*Corresponding author; Dr. Hassanain A. Emran hassanain.j@covm.uobaghdad.edu.iq

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<http://creativecommons.org/licenses/by/4.0/>.**Abstract**

The current study was designed to evaluate the effects of autologous platelets rich plasma (PRP) on the articular cartilage repair. The present study was conducted on twenty-four adult male rabbits. Surgically (24) animals have received the same defect (5mm in diameter) which is made on the lateral condyle of the distal end of the femoral bone. The animals were divided randomly into three equal groups. Group (I) remain without treatment as a positive control group, group (II) which treated with a single dose of prepared PRP injected into the intraarticular space immediately after the creation of the defect, group (III) treated with two doses of PRP (the first dose administered immediately after the surgery and second dose after (15) days of the first dose. Results were evaluated through the Biochemical analysis of the alkaline phosphatase (ALP) and Ca^{2+} at 15, 30, and 60 days after treatment. The biochemical findings of the current study showed that the total ALP was not significantly differed in animals of a group (II, III), whereas animals of a group (I) showed an elevation of ALP in the last period which indicates the osseous reaction especially in late stages of cartilage repair; however, the other two groups (II, III) didn't show a great change in ALP levels in all periods of blood sample collection. The Ca^{+} levels also showed no significant differences among groups, only in the last period in the control positive group, so we concluded that the increase in ALP, and Ca^{+} levels in control group is an evidence of continues inflammatory process, and increase ability of ossification of the new tissue formed in the site of the lesion.

Keywords: Platelets rich plasma (PRP), ALP, Articular Cartilage, Rabbits

علاج اصابات الغضروف التمثلي باستخدام جرعة مختلفة من البلازما الغنية بالصفائح الدموية في الارانب

الخلاصة

استخدم في هذه التجربة 24 ارنباً بالغاً، حيث تم اطلاق اللقمة الوحشية للنهاية السفلى لعظم الفخذ للطرف الايسر لجميع الحيوانات، وقسمت هذه الارانب بصورة عشوائية الى ثلاثة مجاميع متساوية، المجموعة الاولى اعتبرت مجموعة سيطرة ايجابية، والمجموعة الثانية اعتبرت مجموعة معالجة، حيث تم حقنها بالبلازما الغنية بالصفائح الدموية لمرة واحدة مباشرة بعد اجراء احداث الاصابة، والمجموعة الثالثة عولجت بالحقن بنفس المادة المحقونة في حيوانات المجموعة الثانية بجرعتين الاولى مباشرة بعد اجراء احداث الاصابة، والثانية بعد (15) يوماً من موعد الجرعة الاولى. المعايير التي تم حسابها خلال فترة التجربة هي: اخذ عينات دم من جميع الحيوانات بعد 15, 30, و60 يوماً لغرض قياس مستوى الكالسيوم ومستوى انزيم ALP. اظهرت النتائج في هذه الدراسة عدم وجود فروقات معنوية في مستوى الكالسيوم وانزيم ALP في جميع الحيوانات للمجموعتين الثانية والثالثة، في حين اظهرت نتائج حيوانات المجموعة الثالثة ارتفاعاً في مستوى الانزيم في الفترة الثالثة مما يشكل فرقاً معنوياً مقارنة مع المجموعتين الاولى والثانية اعتباراً من اليوم الثلاثون وحتى اليوم الستون. اما فيما يخص ايون الكالسيوم، لم تظهر النتائج فروقات معنوية بين المجاميع، الا المجموعة الاولى في الفترة الاخيرة حيث اظهرت فرقاً معنوياً بارتفاع مستوى ايون الكالسيوم ونسنتج من ذلك ان ارتفاع تركيز انزيم ALP وايون الكالسيوم في المجموعة الاولى دليل على استمرار العملية الالتهابية وزيادة قابلية التعظم للنسيج الجديد المتكون في مكان الاصابة.

Introduction

Arthritis is a complex family of musculoskeletal disorders consisting of more than 100 different diseases or conditions that destroy joints, bones, muscles, cartilage and other connective tissues, hampering or halting physical movement. With a progressively ageing population, there exists an increasing need for therapies to replace or regenerate compromised musculoskeletal tissue (1). The main treatment of OA is the intraarticular injection of hyaluronic acid in addition to the oral administration of anti-inflammatory non-steroidal drugs. These treatments are partially effective in reducing the symptoms and pain because they do not help to stop the cartilage degeneration (2). Tissue engineering has emerged a new method involving the combining of cells, scaffold, and bioactive factors to fabricate functional new tissue to replace the damaged tissue (3).

This study is focusing on the changes of calcium ions, and alkaline phosphatase levels in blood serum during two months of treatment of mechanically damaged articular cartilage using platelet-rich plasma in rabbits.

Materials and Methods

Twenty-eight healthy male rabbits, aged between (1-1.5) years and weighing approximately (2.5±0.15) kg, were employed for this study. Animals were housed in individual cages under the same circumstances and provided

with food and water *ad libitum*. The experimental animals were performed in compliance with the principles guide of ethical used in laboratory animals approved by the University of Baghdad, College of Veterinary Medicine's Animal Care and Use Committee.

Experimental design:

24 animals received the same surgical operation to create a mechanical defect in the lateral condyle of the distal end of the femoral bone (hind limb). All animals divided into three symmetrical groups, according to the keeping duration of two months after the operation and labelled as a group I, II, and group III.

1-Group (I) remained without treatment as a positive control.

2-Group (II) treated with a single dose of prepared (PRP) directly after surgery.

3-Group (III) treated with two doses of (PRP), the first dose was given directly after surgery, while the second dose was given (15) days post-surgery.

Blood samples collected after 15, 30, and 60 days after treatment of each group.

Surgical Procedure:

The animals have withheld the food and water for (8) hrs. Intramuscular prophylactic antibiotic: streptomycin penicillin (Penoksal®, Vilsan-turkey) was also administered before the operation and followed by a daily dose of

1ml/20kg B.W. for five days. The animals were administered aesthetic drug by intramuscular injection of a dual content syringe of 5 mg/kg Xylazine hydrochloride (TROY, Australia) and 35 mg/kg BW. of Ketamine hydrochloride (TROY, Australia) (4). The animals were positioned in lateral recumbence and the left hind limb of each rabbit was prepared surgically. An approximately three cm incision was made in the skin at craniolateral surface over the knee joint. The subcutaneous fascia was incised, the extensor tendon deviated medially, and then the synovial sac was opened. A 5mm-diameter articular cartilage defect was created in the stifle joint (including all layers) using a manual proceeding using the same instrument (5 mm auger) to destroy the cartilage. The defects were made on the most teared portion of the lateral femoral distal aspect. The wound was surgically closed in three layers, the synovial sac and subcutaneous fascia with (3-0 vicryl) absorbable sutures. The last layer closed is skin using silk (No.3\0). Streptomycin penicillin (Penoksal®) at the dose of 1mg/20kg body weight was administered by intramuscular injection for five consecutive days in all animals.

Eight animals left without any treatment as a positive control (group I). The administration of prepared (PRP) was made by intraarticular injection of (0.5 ml ($650,073 \pm 62,739/\mu\text{l}$) of the re-suspended (PRP) from the anterolateral aspect of the affected knee joint, a single dose is given to eight animals directly after the skin suturing (group II), and double dose in the rest eight

animals (the first is injected directly after the skin suturing, and the second injected after 15 days of the operation) (group III).

Preparation of platelet rich plasma (PRP)

In the current study the PRP prepared by double centrifugation method for blood samples collected via heart puncture, initial centrifugation was done to separate different constituents presented in blood, this centrifugation is followed by another spin to condensate platelets who diffuse on the thinner part (lower part) of the tube. Blood with all its constituents initially aspired from the heart in (5) ml tubes with anticoagulants. The first spinning is done at a fixed speed to separate different blood cells at (1800) rpm extends (10) minutes. Then the sample forms three obvious levels: which are (from the top), 1st level consists mostly from platelets and leukocytes, 2nd level which is thinner is rich with leukocytes (also known as buffy coat), and 3rd level which is contains erythrocytes. To form enriched plasma with platelets the 1st layer is carried out to another sterile tube for second spin (4000) rpm for (15) minutes must be enough for production of mushy small tabs (erythrocyte-platelet) on the most lower portion. The higher part of the tube consists from PPP (platelet-poor plasma) must withdraw, and Pellets (most lower part of tube) is re suspended in plasma to form PRP (Platelet-Rich Plasma, (5) after re suspend platelets, the PRP injected in the articular space of the affected joints as designed. One sample of each group sent to the lab for haematological

analysis to determine the number of platelets presented in the concentrated plasma using blood sample analysis device (Vet. Scan 5 HM) produced by ABAXIS, serial no. (CA 94587) USA.

Biochemical analysis (ALP and Calcium)

Alkaline phosphatase and calcium levels were measured in the serum after making the defect as a negative control, and at days 15, 30, and 60 after treatment. ALP activity and Ca^{2+} were measured using automated clinical chemistry analysers 902 Hitachi.

Statistical data analysis

Results were expressed as mean \pm standard deviation for each parameter examined. The data were analysed using the non-parametric test (Mann-Whitney U). Correlations between ALP and Ca^{2+} of treated groups and control at days 15, 30, and 60 after treatment; the level of significance chosen was $p < 0.05$. All descriptive and inferential statistical analysis was conducted using SPSS version 17.0.

Results and Discussion

The platelets counting in the current study shows increased number about three times of the normal counting of the whole blood; this is due to the method of preparation of PRP (double centrifugation method). This method must have followed to condensate platelets in blood samples; however, a low platelet concentration can be obtained from a onetime centrifugation, which have to form a mixture of PRP and PPP

which is not requested PRP. The finding of ALP levels in this study showed that there are no significant differences between periods of treatment in the same group, and different groups except elevation of ALP level in animals of a group (I) which was significantly higher ($p < 0.05$) after 30, and 60 days than treated groups (II, III) (Table 1). The value of the plasma calcium significantly decreased ($p < 0.05$) and remained low after 30-60 days in (II and III groups), and then still in the same level; decreased at the end of the experiment. However, the range of calcium value increased significantly in a group (I) as compared to the other groups; whereas, the range of calcium value was not significantly different (non-parametric test, $P < 0.05$) for groups (II and III) (Table 2).

Table (1) shows the ALP values in the columns (Mean \pm SD), the capital letters indicate significant difference at $P \leq 0.05$; NS = Non-significant.

Time Group	15 day	30 day	60 day
Control (I)	54.46 \pm 2.1 a	100.42 \pm 1.7 B	103.578 \pm 9.988 B
Single (II)	61.165 \pm 8 a	86.8775 \pm 1 a	63.598 \pm 6.22 a
Double (III)	58.4225 \pm 5 a	85.825 \pm 1.4 a	31.038 \pm 8.84 a
LSD	NS	NS	30.25

Table (2) shows the Ca values in the columns (Mean \pm SD), the capital letters indicate significant difference at $P \leq 0.05$; NS = Non-significant.

Time Group	15 day	30 day	60 day
Control (I)	14.14 \pm 0.4 a	14.06 \pm 0.3 a	14.58 \pm 0.1 B
Single (II)	12.21 \pm 0.4 a	11.78 \pm 0.2 a	10.54 \pm 0.2 B
Double (III)	12.43 \pm 0.2 a	10.85 \pm 0.5 a	10.34 \pm 0.5 a
LSD	NS	NS	4.40

Two time spinning method technique have to made to actual condensed cells of autologous samples; however, a low platelet concentration can be obtained from a one-time centrifugation, that it would results in a mixture of plasma enriched with platelets, and Poor plasma to these cells which will not be useful in clinical trials. These findings are the same as (6). Many basic agents affects the type, and the enrichment of the plasma such as gravity effect (G), the type of the anticoagulant, and the results of blood counting of the animal, and outcome of actual use of the plasma (7). Also the number of spinning times affect the gravity force (G) used in the separation method is of high value factors. The rise of gravity can be the cause of enriching the plasma in (8). The significant increase of ALP levels of the control group after 30m and 60 days is due to the activity of osteoclasts and

osteoblasts, which is activated in a group (I) due to osseous reaction in the site of the defect which is also confirmed by the histopathological findings. Similar results were noticed by Tablin, (2010) (9). It seems that ALP activity measurements as an indicator of the subchondral bone remained energetic during the inflammatory process. Therefore, the situation of this process of osteochondral tissue was evident after 30, and 60 days of the repair, this is, for example, reflected by the fact that collagen type II which is the component of cartilage expressed in osteochondral tissue was lower during the ingrowth of 15 days than it was in native articular cartilage. The results of tested serum enzyme may be caused by rapid proliferation of subchondral bone as it is formed by cells responsible for osseous structures formation called osteoblasts. The ALP measures in this study all over the 60 days of experiment was continued high in a group (I) in comparison to the enzyme levels in other groups (10).

The increased calcium ions levels influence the osteoblasts to form ALP, who is responsible of the rising of surrounding PO_4^- ions. The increase in calcium ions and PO_4^- values influence more increase in calcium ions, whereas ossification is initiated. In this time of increase Ca^{2+} and PO_4^- levels in fluids of interstitial tissue, the bone forming cells forms tiny vacuoles filled with ALP and pyrophosphatase that degrade PO_4^- ions of the ground substance (11). The ALP analysis also demonstrated a high osteogenic capacity from 60 days of a group (I). The Ca^{2+}

levels as presented (Table 2) also may be fixed in position to go through surrounding capillaries, it may also be absorbed by blood stream to precipitate on another subchondral bone. Calcium levels aggregation by interstitial tissue capillaries occurs due to osteoblast function which increased already, in association with the degree of tissue repair. The ground substance capillaries which condensate Ca^{2+} and degrade PO_4^- produce a condition of neutral electric stage, which cause formation of crystals of CaPO_4 in ground substance capillaries, and nearby tissues. So, the CaPO_4 molecules integrate precipitation of minerals in the matrix by synthesis and precipitation of $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ which is also known as (hydroxyapatite) in the surrounding tissue of the osteoblast were shown on the time of bone synthesis on time of Ca^{2+} rising (10; 11).

Conclusions

The results of this study showed significant increase of both ALP, and Ca^{+} levels in group (I) in 60 day period of experiment, while groups (II, and III) revealed normal level, and no significant changes in all periods, this is an evidence of continuous inflammatory process in control group with increase ability of replacing the damaged cartilage with new bony tissue, which represents a big problem of joints causing osteoarthritis.

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