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Evaluation of low Level Laser and Autogenous Platelet-Rich –Plasma (PRP) in Repair of experimental Stifle articular Cartilage defect in Rabbits

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Abstract

The objective of this study was to evaluate the direct effects of low level laser following intra articular PRP injection for repair of distal femoral cartilage defect. The experiment was conducted on 25 male adult New Zealand white rabbits. Under general anesthesia, using a dental drill, a whole 4 milimeter in diameter and 1.5 milimeter in depth was made in the inner aspect of the medial condyle of femoral bone in each rabbit. The rabbits were admeasured into 5 groups of 5 rabbits each. The first group, considered a normal group, whereas the second group, distilled water injected. In third group, the created hole was filled with autologous PRP (1 ml intra-articular) for three times with one week interval. In the fourth group, the defect area was subjected to low level laser therapeutic regimen of low level laser irradiation with (P= 100 mW, WL= 650 nm, A = 1 J cm^2, T= 1 minute) for 15 days. Whereas in the fifth group, the area was immediately subjected to therapeutic regimen of low level Laser irradiation for 15 days with 1 ml intra-articular administration of PRP extracted from ear vein blood triple times with one week interval duration. The sample from cartilage defected and treated area was evaluated histopathologically at the end of the two months and was assessed histomorphometrically too. Histopathology evaluation of defects was performed with H&E and Trichrome staining. The findings demonstrated that intra-articular injection of PRP and even laser alone provides suitable ground for lying ground substance at the cartilage defect area. The defects were filled with smooth, shiny white tissue macroscopically at two months after triple PRP administration along with laser irradiation. Despite much connective tissue formed in defect area, in control group there was no evidence of chondrocytes in this group, whereas there was trace of chondrocytes in defects area in group III, IV and group V were almost completely filled with hyaline cartilage; but it seems to need more time to fill the defect perfectly in other three groups. The results indicated there is positive possibility for partial resurfacing of cartilage defect using PRP along with laser.

Keywords: PRP, rabbits, low level laser, joint cartilage, defect.

Introduction

Articular cartilage is a major avascular tissue combined of chondrocytes embedded in a matrix consisting mainly of type II collagen and glycosaminoglycans such as chondroitin sulfate and hyaluronic acid (HA). Its main function is to allow smooth articulation of weight-bearing joints and to cushion the underlying bone from forces involved in joint movement. Because of its avascular nature, articular cartilage has very finite capacity for repair and partial-thickness defects do not heal spontaneously. Full-thickness lesions that penetrate the subchondral bone can undergo repair PRP and even laser treatment which can be effective to stimulate cells to penetrate at the site of injury. Joint cartilage allows gliding action of synovial joints. It is recipient of most blows and jolts to the skeleton. Its resilience buffers these blows, preventing erosion of the subchondral bone with subsequent shortening[1]. Injury to articular cartilage can affect joint role as adult articular cartilage has a finite capacity for repair[2-4]. Articular cartilage is a avascular tissue, it is not innervated and normal mechanisms of tissue healing perform poorly to form only fibrocartilagenous tissue. The existing therapies for repairing of cartilage are limited and physicians are often compelled to wait until the cartilage degeneration gains the point where a partial or total joint replacement can be applied as a treatment[5]. Various treatments also have been attempted for those with focal osteochondral lesions. Surgical approaches for this include a variety of forms of abrasion chondroplasty, microfracture, and transplantation of osteochondral plugs or the use of cultured autologous chondrocytes[6]. Over the past decade, The growing concern in the use of platelet-rich plasma (PRP) to optimize the healing process of tissues has sparked the development and marketing of a plethora of commercial procedures that are designed to concentrate platelets and suspend them in plasma or a fibrin construct of varying densities[1,3]. Although these techniques and their resulting products have been summarily grouped under the generic term “platelet-rich plasma,” their precise make up and, so their potential efficacy, can vary widely. For instance, some PRP products include white blood cells, whereas others do not. In some methods, calcium chloride or exogenous thrombin is added to activate platelets or to initiate the clotting cascade. Eventually, variations in the initial

volume of whole blood used as well as the efficiency of platelet improvement varies markedly among PRP techniques and has resulted in a high variation (3- to 27- fold) of growth factor concentration and availability[4]. Accordingly, because all PRP products are not the same, the success or failure of a special PRP or PRP-related product for a specific pathologic indication cannot be universally applied to all PRP products[7]. The goal of the present study was to evaluate the biological compatibility of PRP alone with low level laser speeding up cartilage regeneration in minimum possible time.

Material and Methods

All rabbit procedures were carried out according to the guidelines of the animal ethics committee of Islamic Azad University. Twenty 40-week- old New Zealand male rabbits weighing 3.250 to 3.750 kg were used. They divided into five groups (I, II, III, IV and V) of 5 rabbits each, according to the procedure performed. Rabbits in each treatment were kept in individual cage and were clinically observed for two consecutive months under strict hygienic conditions. They were fed with standard ration for rabbits and water ad labium during the experimental period.

In vitro study : Ten blood samples from ten adult male rabbits were collected. A 10 ml ear vein blood sample was aspirated under strict aseptic conditions. The general anesthesia induced with premedication of diazepam (1mg/kg, IM) and intravenous Ketamin hydrochloride (30 mg/kg, Alfasan) and Xylazine (4 mg/kg, Alfasan). And rabbits were connected to anesthetic machine with 1-2 % isoflurane (Minrad INC) throughout the experiment.

The blood was aspirated with a 21 G needle. A 10 ml syringe preloaded with 1.3 ml of anticoagulant citrate dextrose (ACD) solution was used to avoid coagulation. One millimeter was set apart for cell counting. Each blood sample was centrifuged for 15 minutes at 72 gat 4°C resulting in the three following layers: the inferior layer composed of red cells, the intermediate layer composed of white cells and the superior layer made up of plasma. The 6 ml plasma layer was centrifuged for another 5 minutes at 1006 g in order to obtain a two-part plasma, the upper part consisting of 5.5 ml of poor- platelet plasma (PPP) and the lower part consisting of 0.5 ml of platelet-rich plasma (PRP). The PPP was first aspirated to avoid its mixing up with the PRP.

The PRP was then gently aspirated with another pipette and placed in a sterile tube. The PRP was thus prepared for activation by calcium chloride ($CaCl_2$), which inhibits the blood-thinning effect of ACD. After activation, PRP turned into a gel-like solution with adhesive properties and ready for use. The coagulation time of PRP after CaCl instillation was evaluated in 250 µl samples placed in Eppendorf tubes with different $CaCl_2$ concentrations. Coagulation was determined by the visualization of the clot at 20°C and carried out always by one person[8,9].

Surgical procedure: The general anesthesia induced with premedication of diazepam (1 mg/kg, IM) and ketamine hydrochloride (30 mg/kg, IV, Alfasan), xylazine (4 mg/kg, IV, Alfasan) and rabbits were connected to anesthetic machine with 1-2 % isoflurane (Minrad INC) throughout the experiment.

Post operative care: Antibiotics (penicillin G procaine 40000 IU/kg IM, bid), gentamicin sulfate (5 mg/kg, IM), vitamin B. complex (0.2 ml/case, IM) and analgesic such as Tramadol hydrochloride (4mg/kg, SC, bid) were administered for 4 post-operative days.

Operation procedure : Under the same general anesthesia described above, the right knee joint was opened with a Medial parapatellar approach. The patella was dislocated medially and the surface of the femoropatellar groove was exposed. A full-thickness rectangular cartilage defect of 4 mm diameter with 1.5 mm depth was created in the patellar groove of the right knees using a stainless-steel dental bit (figure 1). After irrigating the joint with sterile isotonic saline, suturing of the joint capsule and the skin was done in all rabbits. The rabbits were returned to their cages and allowed to move freely without joint immobilization.

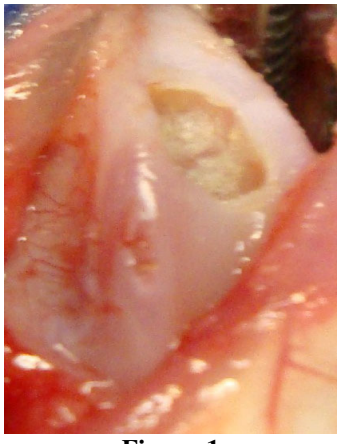

Figure-1
A full-thickness rectangular cartilage defect of 4 mm diameter with 1.5 mm depth was created in the patellar groove of the right knees using a stainless-steel dental bit

Histological examination of repair tissue (Quantitative assessment of cartilage quality): The rabbits were sacrificed at two months after defects. Each cartilage defect area was evaluated both macroscopically and histologically with H&E and Trichrome staining. All the animals were operated on according to the guidelines for animal experiments of the Iran society for prevention of cruelty to animals (ISPCA). The right stifle joint of rabbit was resected and stripped of soft tissue leaving no layer of connective tissue over the experimental sites. These specimens were stored in a 10% buffered formaldehyde solution for fixation. After fixation, the extra tissues of the distal condyle of right femoral bone were separated to have only defected or experimental site. The parts were dehydrated in graded series of alcohol and embedded in methylmethacrylate.

Light-microscopically analysis consisted of a description of the tissue response within the denuded area and was carried out by two investigators using an optical microscope.

Histomorphometry: Apart from a subjective description, each experimental site was quantitatively assessed using a grading scale for cartilage and bone organization. Specimens were evaluated for bone and cartilage organization at the edges and center of the lesion and the amount of cartilage and bony tissue bridging of the defect (table-1). Three sections for each specimen were evaluated by two blinded reviewers. The reviewers reached a consensus on the score of each section and then assigned a final score to each specimen. The scores of the specimens in each material group were averaged to determine the overall score for the material group. In all groups, histomorphometry was also carried out on the sections to quantify the bone fill percentage of newly formed cartilage and bone covered the area.

Statistical design and analysis: Kruskal Wallis one-way ANOVA was used to evaluate differences between the experimental groups. Differences between the various groups were calculated using Wilcoxon paired-rank test. $P<0.05$ was defined statistically significant.

Results and Discussion

Operation and postoperative course, no preoperative complications occurred and all rabbits recovered well from anesthesia. All rabbits underwent uneventful ealing, tolerated the active distraction procedure well and gained weight. Healing and recovery were uneventful in all rabbits with no signs of wound dehiscence or infection.

Descriptive histology: Light-microscopic examination of the sections revealed not much difference between the PRP and the laser group. At 8 weeks, there was a limited inflammatory reaction with sporadic giant cells. Fibrous tissue was seen in control group with trace of minute inflammatory cells or reactions. Newly formed bone with fibrocatilage tissues with resorption of the PRP particle could be observed (figure- 3) At 8 weeks, there was a decrease of inflammatory cells in almost in all samples in group II. There were complete signs of resorption of the PRP even in groups III, IV and V (figures- 4, 5, 6, 7). Extensive bone formation was seen at the site of defect (figures- 4, 5). It was difficult to judge the amount of resorption of the autogenousparticle of PRP because of the newly formed bone (figures- 4, 6, 7). There were no signs of inflammatory reaction in group IV and V. More newly formed bone was observed in greater quantities of the autogenous PRP bone particles compared group II. Fibrous tissue was extremely seen at the end of 8 weeks in group V. After 8 weeks, the transplanted autogenous PRP gel was surrounded by a fibrous tissue layer. At weeks 8 weeks the amount of fibrous tissue remained the same in control group but replaced with that of hyaline cartilage in group V.

Histomorphometry comparing bone formation at the edge of the defect and the amount of bone bridging the defect, significant differences were seen between the sites with laser and with without PRP for the different section in 8 weeks times in all cases P> 0.05 (table-1).

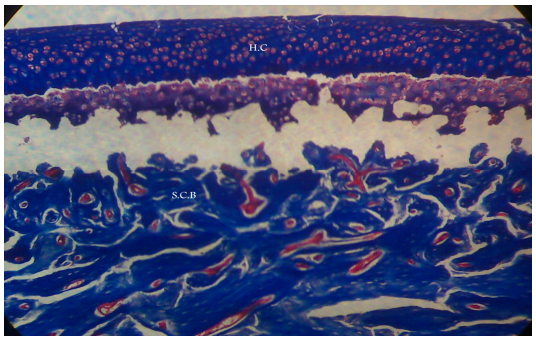

Figure-2
Normal articular cartilage (Trichrome × 64; HC: hyaline cartilage, SCB: subchondral bone)

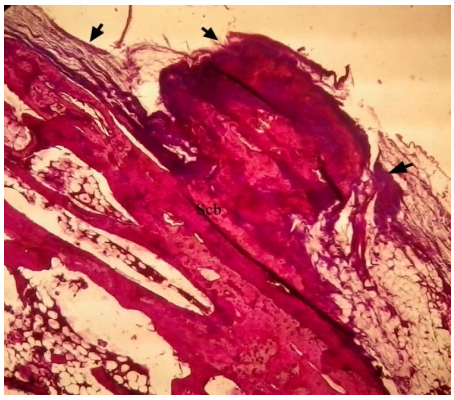

Figure-3
(control group) connective tissue has filled the defect area, but there is no evidence of chondrocytes. A part of hyaline cartilage (HC) and subchondral bone (SCB) are seen (Trichrome*64)

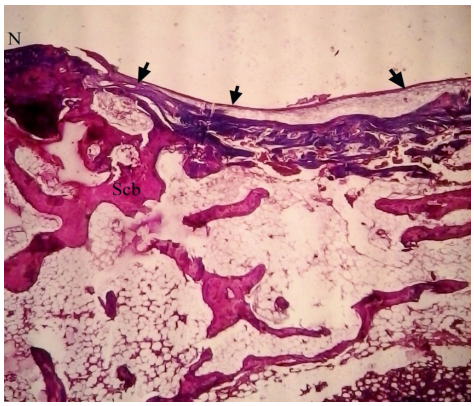

Figure-4
A small amount of connective tissue on defect area of two months Laser group has been formed. A part of damaged articular surface has been covered with connective tissue and there is trace of chondrocytes (FT). (HC: hyaline cartilage, SCB: sub chondral bone) (Trichrome*6)

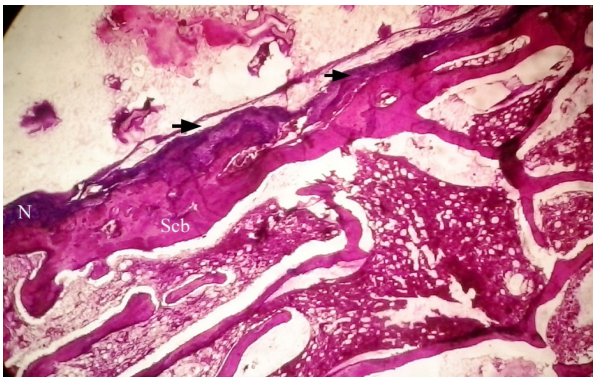

Figure-5
There is trace of connective tissues with chondrocytes cells filling the gap in two months. A part of damaged articular surface has been covered with connective tissue and there is trace of chondrocytes (FT). (HC: hyaline cartilage, SCB: sub chondral bone) (Trichrome*64)

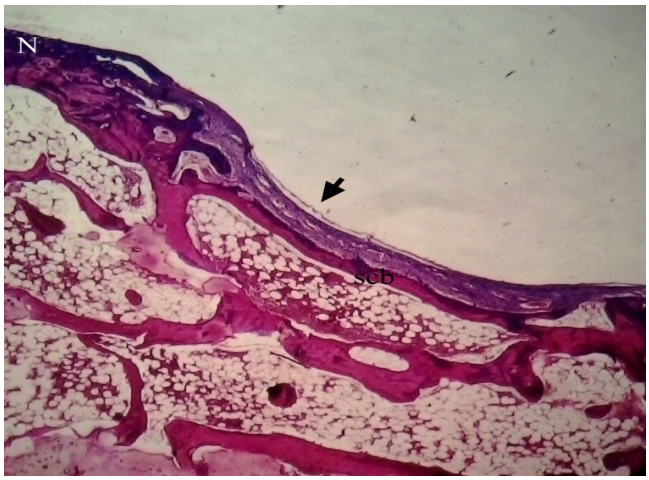

Figure-6
Investigation of samples in group four, the gap was completely filled with normally organized hyaline cartilage after two months (Trichrome*64)

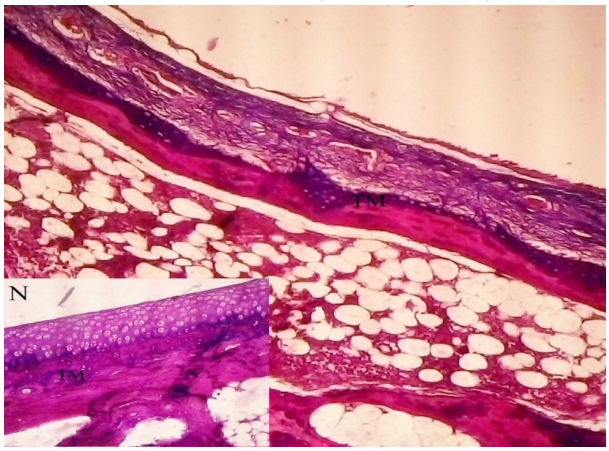

Figure-7
Investigation of samples in group four, the gap was completely filled with normally organized hyaline cartilage after two months (Trichrome*64)

Discussion: PRP is being used during healing, reconstruction, or implantation procedures, whether open or arthroscopic. PRP can be applied intraoperatively or at the end of an open surgical procedure. Alternatively, injections may be administered after the initial surgical hematoma has resolved or arthroscopic irrigation fluid has absorbed as an adjunct to surgery[8]. In this experiment, additional injections given at one week interval for 3 times to speed up the initial healing process. Several articles were published on human subjects, reporting on the outcome after surgery with the addition of PRP [9]. Precise intra-articular application can be performed for meniscal treatment and glenoid labrum restabilization by leaving the needle at the tear site just before performing the repair. In open or mini-open surgery, PRP can be applied as a gel just before closure or by infiltrating the concentrate over the desired area. PRP gel matrices can also be sutured or glued to the surgical site, because all PRP products are not the same, the success or failure of a specific PRP or PRP-related product for a specific pathologic indication cannot be universally applied to all PRP products[4]. The various PRP preparations on the market has led to considerable confusion, not only in trying to evaluate the potential clinical benefits of PRP in different applications but also in trying to resolve the variety of marketing claims that accompany each product. Unlike prescription drugs, whose standards for quality, strength, purity and consistency are regulated by the United State Pharmacopeia. The natural response of articular cartilage to damage is variable and, at best unsatisfactory [10,11,12] . It is well known that partial thickness defects of a certain size in the articular cartilage do not heal spontaneously[12]. If the cartilage lesion does not penetrate the subchondral bone, it will not heal and can progress to the degeneration of the articular layer[13]. Injuries that do penetrate the subchondral bone undergo a repair process characterized by the formation of a transient fibrous cartilaginous tissue with material properties lower than those of native nature, which degrades within 1 year of injury[13]. In this research, the development has explored. Our findings for both cell seeding densities examined provide supporting evidence that integration of a bony base to chondrocyte-seeded into such polymer or constructs, this scaffold continues to support tissue elaboration (maintenance of the chondrocyte phenotype, biochemical content and material properties). In this study we used PRP gel with low level laser. Some chondrocyte appeared to migrate from the surrounding tissues in defect area to the fill it uniformly of the trabeculae bone spreading and assuming spindle like appearance[14-16]. In this case of our study other material like PRP with laser even alone explored for bone tissue underlying boy substrate and the substrate porosity will influence diffusion into the underlying bony tissues and even cartilage tissues too. This study demonstrates the ability to cultivate anatomically shaped tissue constructs aimed at the eventual replacement of the entire articular surface of diarthrodial joint Such as approach for cartilage repair will require the constructs to have functional properties similar to the native tissue. This functional tissue needs promoter like PRP which can be potentiated with laser therapeutic regimen for two weeks after surgery PRP with laser were mainly concern to

mature cartilage healing and repair which permit further design of local and bulk tissue properties. Besides physiologic loading with different cell populations with applied physical environmental conditions within growing tissues. In our finding using PRP with laser on macroscopic examination, the transplanted tissue was compliant and difficult to distinguished from native tissue and histomorpholgic studies demonstrated total resorption of the PRP with laser at 8 weeks.

Table-1
Articular cartilage regeneration with autologous marrow aspirate and hyaluronic acid: An experimental study in rabbit model

Parameters Evaluated	Source	Group Normal	Group Control	Group PRP	Group Laser	group PRP & Laser
% Filling defect relative to surface of original cartilage						
91-100%	**0**	**0**				
71-90	**1**					**1**
51-70	**2**				**2**	
26-50	**3**			**3**		
Less than25	**4**		**4**			
Integration of repair tissue with surrounding articular cartilage						
Normal	**0**	**0**				**0**
Decreased cellularity	**1**			**1**		
Gap 1 side	**2**				**2**	
Gap 2 side	**3**		**3**			
% of bony tissue formation under main trademark (indicate repair of bony tissue under cartilage	**90-100**	**0**				
	75-89					**1**
	25-74			**2**	**2**	
	< 25 %		**3**			
%of bony tissue formation above main trademark (indicate thickness of repaired cartilage	**99-100**	**0**				
	75-89					**1**
	25-74			**2**	**2**	
	<25%		**3**			
% of Trademark formation	**100**	**0**				**0**
	75-99			**1**	**1**	
	50-74					
	25-49					
	<25		**4**			
Cell morphology						
Normal	**0**	**0**				
75% normal cells with morphology of chondrocytes	**1**					**1**
normal cells with morphology of chondrocytes of 50 - 75%	**2**				**2**	
normal cells with morphology of chondrocytes 25-50%	**3**			**3**		
normal cells with morphology of chondrocytes<25%	**4**		**4**			
Intradefect architecture						
Normal	**0**	**0**				
1-3 small voids	**1**					**1**
1-3 large voids	**2**				**2**	
>3 large voids	**3**			**3**		
Cleft with defects	**4**		**4**			
Surface architecture						
Normal	**0**	**0**				**0**
Slight fibrillation	**1**					
Moderate fibrillatio0n	**2**			**2**	**2**	
Marked fibrillation	**3**		**3**			

A final concept that has come to bear is he PRP with laser constructs may play a role in enhancing mature cells of chondrocytes at the site of injury, that may be useful in the design of a different type of inflammatory and non inflammatory cartilage injuries specially in clinical model[17-20]. The preliminary results indicate that the treatment with PRP injections is safe and has the potential to reduce pain and improve knee function and quality of live in younger patients with low degree of articular degeneration [21]. Our preliminary findings support the application of autologous PRP as an effective and safe method in the treatment of the initial stages of cartilage and subchondral bone defects[22]. Platelets supply an autologous source of growth factors for tissue regeneration and healing. In addition, use of a platelet gel can be a positive efficacy in clinical conditions that require rapid healing. Studies of the use of PRP for cartilage degeneration have demonstrated more consistently favorable results. In this study found PRP injections effective at improving pain, function and quality of living in rabbits with chondral injuries and early or advanced osteoarthritis, and a comparison of PRP injections alone or with laser will have preventive measurement for osteoarthritis of the synovial joint found improved grades with the use of PRP.

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