Autologous Matrix-Induced Chondrogenesis *vs* Microfracture with PRP for Chondral Lesions of the Knee in a Rabbit Model

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Currently, microfracturing is the most commonly used cartilage repair procedure in cartilage defects. Our aim was to study the mechanism of in vivo cartilage repair in case of full-thickness articular cartilage damage of the knee using a three-dimensional matrix implanted without any preseeded cells in the defect. We also investigated whether platelet-rich plasma application after microfracture procedure of the knee is associated with improved outcome compared with traditional microfracture treatment alone in a rabbit model. Histological examination of the chondral defects, revealed the largest amount of new tissue with hyaline-like cartilage features in Hyalofast group. At 12 weeks from implantation of the Hyalofast scaffold demonstrated complete filling of the defect with hyaline cartilage in admixture with the scaffold and bone metaplasia in the deepest areas. In the PRP group, complete filling of the defect with an admixture of fibrous and hyaline-like cartilage tissue appeared with a discreet tendency of endochondral ossification. We confirmed the superiority of the autologous matrix-induced chondrogenesis compared to microfracture and PRP or microfracture alone in case of full-thickness articular cartilage damage of the knee.

Key words: autologous matrix-induced chondrogenesis, hyaline cartilage, microfracture, platelet-rich plasma

Primary (idiopathic) osteoarthritis, the most common disease of the joints, is partly a result of natural aging of the joint with a multifactorial pathogenesis involving the articular cartilage, but also synovium, subchondral bone and bone marrow, menisci, ligaments and supporting musculature [1].

Hyaline cartilage protects the underlying bone from excessive load and trauma by dissipating the forces produced during movement [2]. Articular cartilage lesions may be associated with pain, loss of function and long-term complications such as osteoarthritis [3]. Due to its poor blood supply and self-renewal capacity, the normal structure and function of cartilage are difficult to restore when it is injured or degenerated [4].

In recent years, a completely new approach has been developed to treat cartilage lesions, namely biological strategies, which are increasingly being used on the scale. The new therapeutic methods are based on the revolutionary idea of *regeneration*, unlike the traditional approach, based on the concept of *repair* [5-7]. A variety of surgical techniques that aim to resurface the damaged articular cartilage have evolved [8].

These include bone marrow stimulation methods, such as Pridie drilling, abrasion and microfracture (MF) [9]; implantation techniques, such as autologous chondrocyte implantation (ACI), matrix-induced autologous chondrocyte implantation (MACI) [10], mosaicplasty [11, 12]; and a more recently tissue engineering techniques using scaffolds. MF is recommended as the treatment of choice for smaller areas of full-thickness articular cartilage damage of the knee (<2.5 cm^2), while ACI or MACI are frequently used for larger defects [13].

Currently, microfracturing is the most commonly used cartilage repair procedure in cartilage defects [8]. In microfracturing, the subchondral bone plate below the cartilage lesion is perforated to initiate bleeding and induce a reparative response [14]. The blood clot formed is thought to develop a favorable microenvironment capable of stimulating attraction, proliferation, and differentiation towards various cell types, including chondrocytes and bone cells [15]. However, the fibrin clot is not mechanically stable to withstand the tangential forces [8]. The perforation of the subchondral plate may also accelerate the development of intralesional osteophytes [16]. In long term, the initial benefit tends to decrease between 18 and 36 months after the procedure [14].

Some authors have modified the traditional marrow stimulating techniques to enhance its efficacy by combining it with an intraarticular injection of platelet-rich plasma (PRP) or in vitro manipulated mesenchymal stem cells [17].

Platelet-rich plasma with higher platelet concentrations than the mean blood measures represent a rich source of autologous growth factors. After activation, the alpha granules of concentrated platelets in PRP release growth factors at concentrations significantly higher than the baseline blood levels, such as: platelet derived growth factor (PDGF), transforming growth factor (TGF-β), basic fibroblast growth factor (bFGF), insulin growth factor (IGF), vascular endothelial growth factor (VEGF), and epithelial cell growth factor (ECGF) [18]. It has been reported that local concentration of these factors might stimulate the articular chondrocyte proliferation and matrix biosynthesis [19]. Also, PRP could effectively promote the maturation as well as the calcium depositing of the subchondral bone [20]. PRP can easily be obtained on the day of surgery by two centrifugation steps from autogenous whole blood [18].

The ACI procedure, first introduced by Brittberg and coworkers in 1994, involves the use of a periosteal flap or

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a collagen sheet, which is fixed to the surrounding cartilage to create a reservoir for injection of a suspension of culture-expanded autologous chondrocytes [21]. Over the last 20 years, the procedure has become more widespread and it is currently probably the most developed articular cartilage repair technique. It has a few well-known drawbacks such as hypertrophy of the repair tissue in 25% of cases and a difficult surgical technique [22].

Autologous matrix-induced chondrogenesis (AMIC) technique is a novel biological regenerative method of articular cartilage developed by Behrens. It is a one-step procedure that combines microfracture with the fixation of a biological scaffold [17]. An implanted exogenous scaffold may improve the mechanical stability and durability for endogenous cells and may provide a proper stimulus for chondrogenic differentiation and cartilage regeneration [8]. There are different types of scaffolds available: natural protein–based or carbohydrate-based scaffolds, and synthetic scaffolds [17].

Our aim was to study the mechanism of in vivo cartilage repair in case of full-thickness articular cartilage damage of the knee using tissue engineering technique. The cell source was mesenchymal stem cells migrating from subchondral bone and invading a three-dimensional matrix that was implanted without any preseeded cells in the defect. We also investigated whether platelet-rich plasma application after microfracture procedure of the knee is associated with improved outcome compared with traditional microfracture treatment alone in a rabbit model.

Experimental part

Materials and methods

Animal care- as in [23].

Our study included 18 six-month-old male rabbits, weighing 1.5 kg to 3.0 kg. The 18 rabbits were randomly divided into 3 experimental (n=6 rabbits/group).

All experimental procedures with animals followed the international recommendations for the use and care of animals and the study protocols were approved by Institutional Review Board of University of Medicine and Pharmacy Targu Mures no. 35 from 21st of March 2016. The animals were acclimatized to the usual laboratory conditions 7 days before the experiment and were housed with appropriate bedding and provided water ad libitum and free access to standard laboratory rodent feed. Rabbits were kept in standard single cages under controlled temperature and light conditions.

Experimental design

After 4 h of stopping nutrition, all studied animals were sedated by intramuscular injection of ketamine hydrochloride 60 mg/kg and xylazine 6 mg/kg. This drug combination ensures post procedural analgesia also. In sterile conditions, a medial para-patellar arthrotomy was made in both knees. A full-thickness cylindrical osteochondral defect of 5 mm in diameter and 2 mm in depth was created in the patellar groove through use of a standard size drill head (fig. 1A). In all the studied animals, microfractures were performed as recommended in the literature [24]. Multiple holes were made in the exposed bone with a 1 mm K-wire. By picking holes in the subchondral bone, blood and fat droplets are given a pathway to flow into the defect or lesion. This develops in to a mesenchymal clot.

In the first experimental group (Hyalofast group), the defect area knee was than treated by implanting a of a biomimetic biodegradable, hyaluronan based scaffold. The stability of the implanted scaffold was tested by cyclic flexion-extension of the knee while the graft was visualized (fig. 1B).

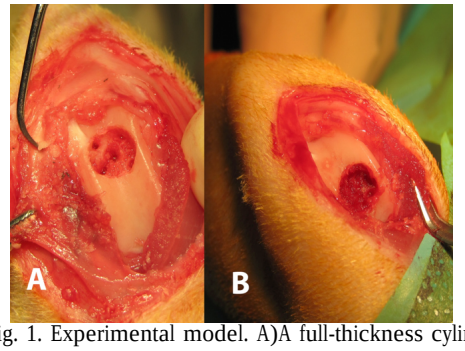

Fig. 1. Experimental model. A)A full-thickness cylindrical osteochondral defect of 5 mm in diameter and 2 mm in depth was created in the patellar groove through use of a standard size drill head. In all the studied animals, microfractures were performed in the exposed bone with a 1 mm K-wire. B) In the Hyalofast group, the defect area knee was than treated by implanting a of a biomimetic biodegradable, hyaluronan based scaffold press fit into the area

In the second experimental group (PRP group), 1 mL of the PRP solution was injected into the joint following tight closure of the capsule to prevent leakage of the PRP solution from the joint. The PRP development was done using the centrifuge ORMA CN45. For preparing 1 mL of PRP, 10 mL of whole blood was first collected from the central auricular artery with a 20-mL syringe pre-filled with 1 mL 2.5% sodium citrate as anticoagulant. An aliquot of each blood sample was drawn out for cell counting. The sample was centrifuged for 10 min at 3600 revolutions per minute into three layers, the plasma, the platelets and the red blood cells. Then the plasma and the platelets are extracted to undergo another centrifugation at 3600 revolutions per minute for 10 min and most of the supernatant plasma is discarded. Subsequently, we obtained 1 mL of PRP. Platelet counts were also performed for samples of PRP.

In the third experimental group (microfracture group) the defect area of knee was left treated with microfracture alone.

After the surgery, the knee joint capsule and skin were closed using resorbable surgical sutures. The rabbits could move freely postoperatively. To avoid the postoperative infections, antibiotic injections were given three days postoperative.

At twelve weeks after the surgery, the rabbits in each group were sacrificed by injecting pentobarbital sodium (>100 mg/kg intraperitoneal), according to Annex IV from Directive 2010/63/UE- L 276/72. The knees were excised, photographed and graded for cartilage repair, according to the international cartilage repair society score (ICRS) macroscopic assessment scores. Afterwards, the joints were CT scanned and further processed for the histological analysis.

Histopathological examination -as in [23]

For the histopathological examination, the femoral condyles were fixed for 7 days by immersion in 10% neutral-buffered formalin (NBF). Following complete aldehyde fixation, the femoral condyles were cleaned of muscle and connective tissue and decalcified for two weeks in a 1:1 mixture of formic and hydrochloric acid (8%). When decalcification was completed, the tissues were transversely trimmed, briefly washed in tap water and dehydrated in ethylic alcohol in ascending concentration (70, 80, 90, 95, and 100%), clarified in xylene and embedded in high temperature melting paraffin wax. Tissue sections were cut from each paraffin block at 4 µm thickness with

a rotary microtome and routinely stained with hematoxylin and eosin (H&E). The histological slides were examined using an Olympus BX41. Images and morphometric evaluation were obtained with an Olympus UC30 digital camera.

The re-created tissue was scored blindly according to the ICRS scale.

Statistical analysis

For statistical analysis we used SPSS version 20 (USA, CA). To characterize groups, we employed descriptive statistics (mean, standard deviation, minimum, maximum). The means between groups were compared for statistical significance using the t-test for Equality of Means. The confidence intervals were set at a 95%. A value of $p<0.05$ was considered statistically significant.

Results and discussions

Macroscopic observation

All rabbits in the experimental groups, survived the follow-up period of twelve weeks without wound infection, or synovitis in the operated knees. The platelet count in PRP was about five times higher compared with platelet count in whole blood (fig. 2).

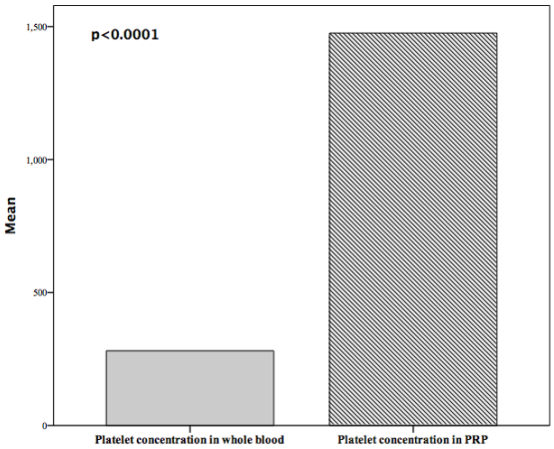

Fig. 2. Platelet count in whole blood and in PRP

The macroscopic evaluation of the femoral condyles in Hyalofast group revealed a white, smooth and uniform in texture, regenerated tissue which was well integrated into the surrounding cartilage as compared with other two groups. After microfracture repair, tissue appeared with an irregular texture and often depressed topology. The defects treated with PRP were filled with regenerated glossy white tissue, although the boundary was still notable between the defective tissue and the normal tissue (fig. 3).

According to the ICRS scores from macroscopic observations of the femoral condyles, the average score in the Hyalofast group was higher than those in the PRP and microfracture group (fig. 4).

Joints CT scanned

Joints CT scanned evaluation showed superior outcome in the Hyalofast group, especially with regard to the surface of the regenerate and degree of integration (fig. 5).

Histological examination

Histological assessment confirmed the macroscopic results. The mean results of the histological assessment are presented in figure 6-11. The value of the histological ICRS scale in Hyalofast group was higher compared to microfracture group in all criteria, but with a statistically significant value in case of mean surface, mean matrix and mean cell distribution.

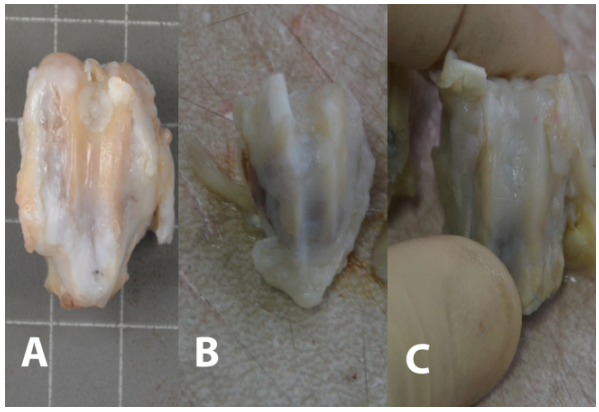

Fig. 3. Gross appearance of defects in the trochlear groove. A)Microfracture group. The defect is partially vacant and clearly noticeable from the surrounding cartilage. B) PRP group. Repaired tissue covers the defect completely, but is distinguishable from the surrounding cartilage. C) Hyalofast group. Repaired tissue covers the defect completely and no obvious margin is notable

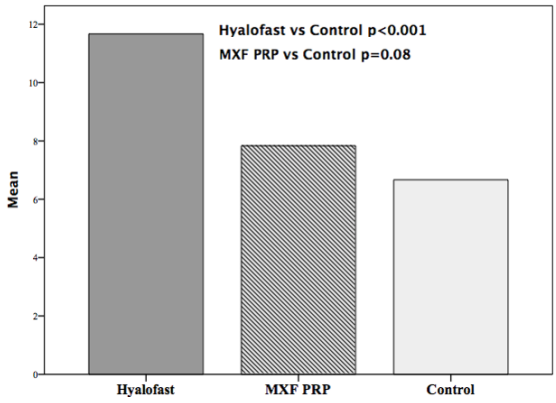

Fig. 4. ICRS macroscopic evaluation of cartilage repair

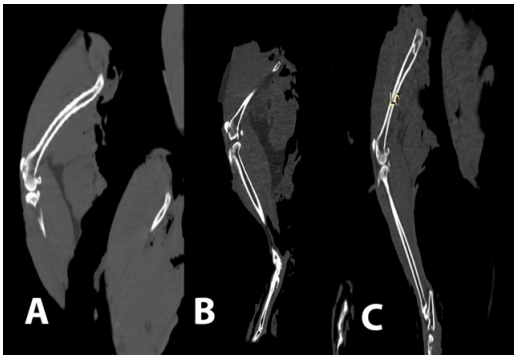

Fig. 5. Joints CT scanned images
A) Microfracture group. Sclerotic bone formation in the defect area with microcysts in the subchondral bone. B) PRP group. The defect is partially filled with newly formed bone. C) Hyalofast group. Complete filling of the defect with newly formed bone

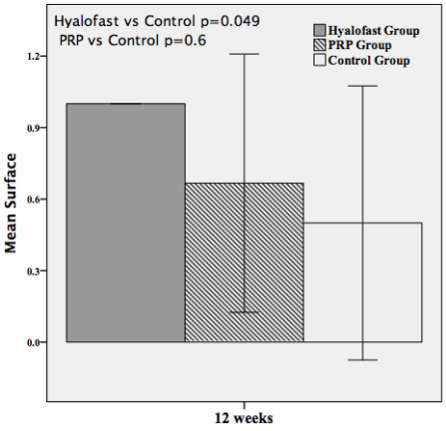

Fig. 6. ICRS visual histological assessment scale - Mean Surface

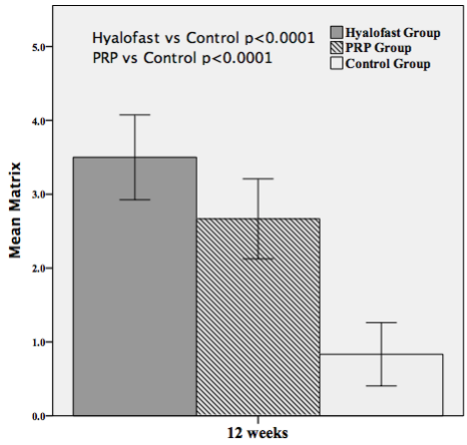

Fig. 7. ICRS visual histological assessment scale - Mean Matrix

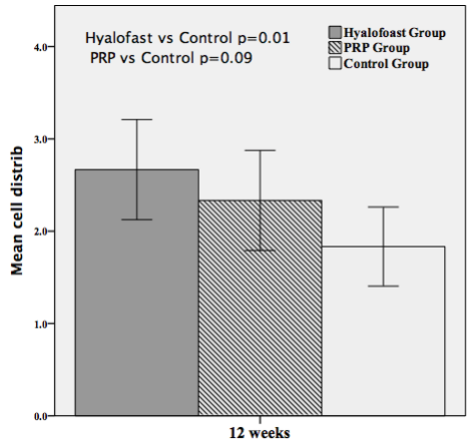

Fig. 8. ICRS visual histological assessment scale - Mean Cell Distribution

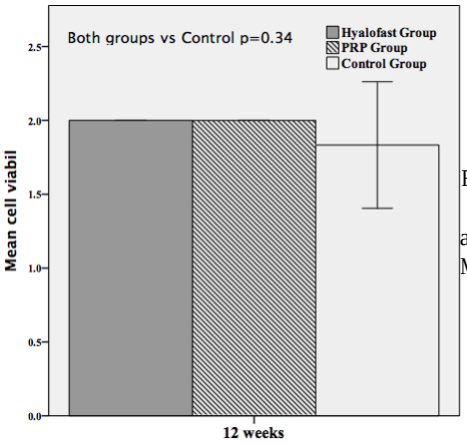

Fig. 9. ICRS visual histological assessment scale - Mean Cell Viability

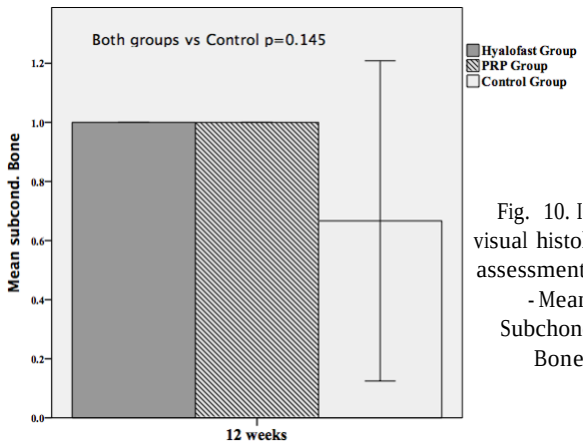

Fig. 10. ICRS visual histological assessment scale - Mean Subchondral Bone

The value of the histological ICRS scale in PRP group was higher compared to microfracture group in all criteria, but with a statistically significant value only in case of mean matrix.

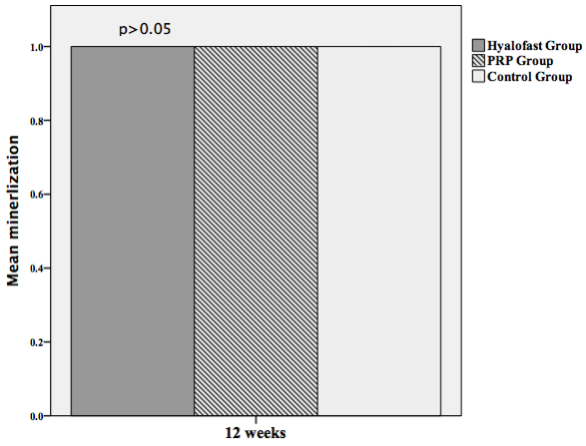

Fig. 11. ICRS visual histological assessment scale- Mean Mineralization

Histological examination of the chondral defects, revealed the largest amount of new tissue with hyaline-like cartilage features in Hyalofast group (fig. 12).

Histological features of biopsy specimens taken at 12 weeks from implantation of the Hyalofast scaffold demonstrated complete filling of the defect with hyaline cartilage in admixture with the scaffold and bone metaplasia in the deepest areas suggesting that the repair tissue was still undergoing remodeling. The neotissue was fully integrated into the host cartilage (fig. 12).

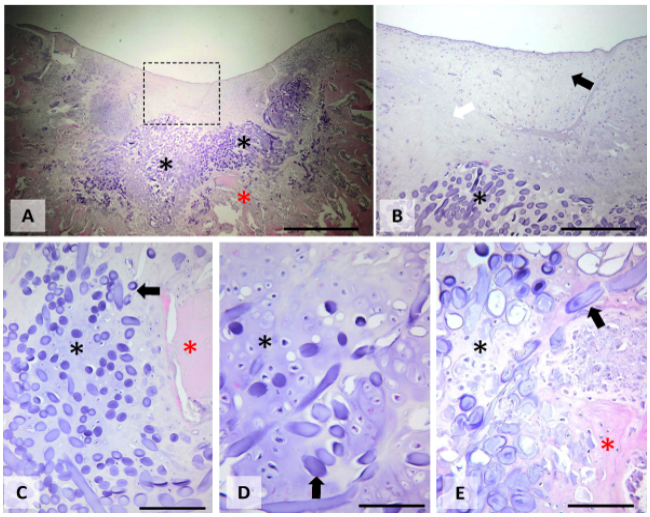

Fig. 12. Histopathological examination of the femoral trochlea from the Hyalofast group at 12 weeks;
Picture A captures the cross-sectional appearance of the femoral trochlea and the induced defect. We observe the total coverage of the defect with cartilaginous tissue in admixture with the scaffold (reticular, regular, basophilic aspect - black asterisk). The red asterisk indicates the underlying bone tissue;
Picture B represents the histological detail of the area marked by a rectangle in the image A. Compete filling of the defect with cartilage tissue (hyaline-like cartilage - arrow) is observed. The presence of implant material (reticular, basophilic) fully integrated into the cartilage mass (asterisk) is noticed.
Picture C represents the deep defect area characterized by the presence of cartilaginous tissue (black asterisk) with areas of bone metaplasia (red asterisk) and implant material (reticular, basophilic - arrow) fully integrated into the cartilage mass;
Picture D and E illustrate the deep defect area where the focal presence of the implant material is observed (basophilic, cylindrical - arrow) in admixture with cartilaginous tissue (hyaline-like - black asterisk) with a tendency of bone metaplasia (red asterisk). H & E, ob x 4 for image A (scale = 1000 μm), obx20 for image B (scale = 200 μm), obx40 for C, D and E images (scale = 100 μm

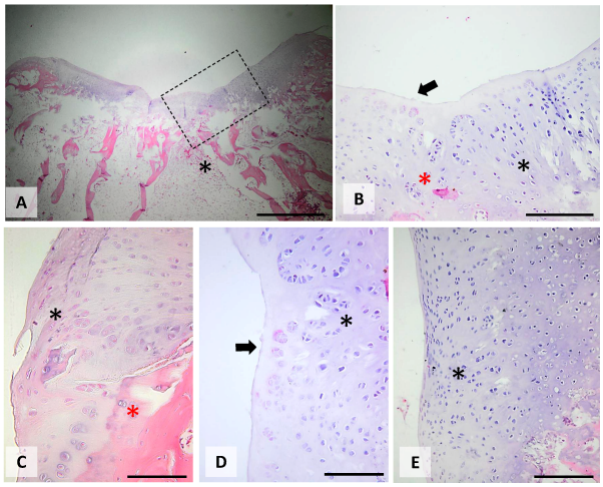

Fig. 13. Histopathological examination of the femoral trochlea from the PRP group at 12 weeks. Picture A captures the cross-sectional appearance of the femoral trochlea and the induced defect (marked by asterisk). The total defect coverage with cartilaginous tissue is noticed. Picture B represents the histological detail of the area demarcated by a rectangle in image A. Complete filling of the defect with cartilage tissue is observed (admixture of fibrous (red asterisk) and hyaline-like cartilage (black asterisk)). The differentiation of articular cartilage with the presence of flattened chondrocyte is noticed in the superficial area. We can observe the tendency of the chondrocytes to order in cords corresponding to the area of proliferation in endochondral ossification.
Picture C, D and E represents the superficial area of the defect with the presence of an admixture of fibrous (red asterisk) and hyaline-like cartilage (black asterisk). In Picture E an orderly layout of chondrocytes is observed with specific flattened arrangement in the superficial area. In the deeper layer, the chondrocytes disposition appears in the shape of cords (chondrocyte proliferation). The superficial layer of the cartilage is almost smooth (arrow). H & E, ob x 4 for image A (scale = 1000 μm), obx20 for image B (scale = 200 μm), obx40 for C, D and E images (scale = 100 μm).

In the PRP group, complete filling of the defect with an admixture of fibrous and hyaline-like cartilage tissue is observed with a discreet tendency of endochondral ossification (fig. 13). The superficial layer of the cartilage is almost smooth.

By contrast in the microfracture group, the neotissue fills incompletely the defect and is characterized by the presence of fibrotic scar tissue with dense appearance in the deep area, a lax appearance in the superficial layer with only a discreet marginal cartilaginous tissue (fig. 14).

One of the main features of modern medicine is the multifactorial approach of pathology resulting in interdisciplinary research, not only from medicine, but from basic research, too [25].

Marrow stimulation techniques such as microfractures are relatively simple, minimally invasive and inexpensive. The principle behind this regenerative strategy is the migration of non-differentiated bone marrow-derived multipotent stem cells from the subchondral bone into the defect site leading to the formation of new cartilage tissue [26]. The limitation for marrow stimulation techniques is that the bone marrow stem cells and growth factors are released into the joint rather than being contained at the site of the defect [17]. In good agreement with the literature, in the present study, the microfracture technique alone lead to predominantly fibrous type cartilage tissue at the repair site with limited filling of the defect and poor integration with the surrounding tissue.

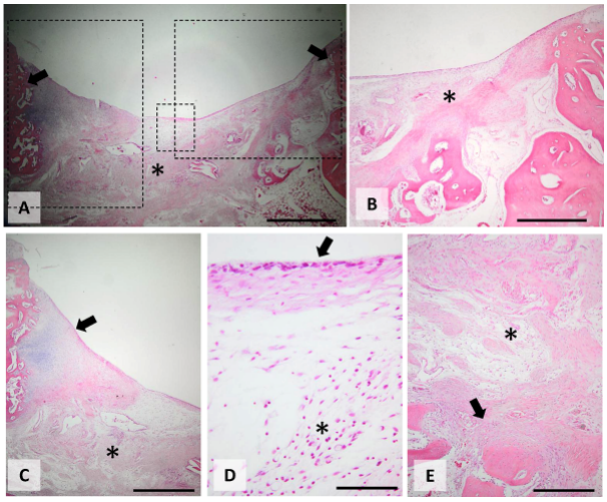

Fig. 14. The histopathological examination of the femoral trochlea from the Microfracture group at 12 weeks. Picture A captures the cross-sectional appearance of the femoral trochlea and the induced defect (marked by black asterisk). The arrows indicate the marginal area of the defect. The neotissue fills incompletely the chondral defect and is characterized by the presence of fibrotic scar tissue in various stages of maturity (the dominant structure) and a discreet marginal cartilaginous tissue (minimal, in shape of a border). Picture B. represents the histological detail of the area demarcated by a rectangle on the right side of image A. The defect is filled with fibrous scar tissue, with dense appearance in the deep area and a lax appearence in the superficial layer.
Picture C represents the histological detail of the area demarcated by the rectangle on the left side of image A. The defect is filled with fibrous scar tissue (red asterisk), with dense appearance in the deep area, a lax appearence in the superficial layer and a discreet marginal cartilaginous tissue (indicated by arrow).
Picture D represents the histological detail of the area demarcated by the rectangle on the centre of image A, showing the superficial appearance of the defect. It is characterized by the appearance of young cicatricial connective tissue (inflammatory granulation tissue - asterisk). Picture E represents the histological detail of the deep area of the defect. The defect is filled with fibrous lax (asterisk) and dense (arrow) scar tissue. Areas of bone metaplasia do not appear.H & E, ob x 4 for image A (scale = 1000 ìm), obx10 for image B and C (scale = 400 μm), obx20 for image E (scale = 200 μm), obx40 for image D (scale = 100 μm).

PRP has been extensively investigated for bone regeneration and soft tissue healing and many reports claim a positive effect of PRP [27]. Most published studies describing the effect of PRP on the histologic appearance of the articular cartilage repair concluded that PRP improved the histology of the cartilage repair [18, 27, 28], and a few reports stated that it worsened the histological scores [29, 30]. In another recent review, the authors suggested that there is limited evidence to support the use of PRP in the management of chondral or osteochondral defects, whether alone or as an adjunct to surgical treatment [31].

Our study showed that intra-articular use of PRP during microfracturing led to better cartilage integration and improved quality of the repaired tissue than did microfracturing alone twelve weeks after the experiments.

These divergent findings might be due to several reasons. Despite the increasing use of PRP, there is no standardized protocol for PRP preparation in clinical practice. and different protocols may result in PRP formulations that differ in componential composition [32]. Also, the amount of PRP solution is controversial, and might not be enough for an adequate response to impact the long term. A lack of repeated PRP injections might be another reason. Milano

et al. suggested administering repeated platelet concentrate injections after application of microfractures to full thickness cartilage injuries and reported superior and more durable reparative responses than isolated microfractures in an animal model [33].

In the treatment of cartilage injuries, tissue engineering techniques using scaffolds have led to promising results. Scaffolds rapidly fill cartilage defects and provide a temporary substrate onto which invading cells can adhere [17]. The 3 scaffolds that have been reported in the literature for AMIC are ChondroGide (Geistlich Biomaterials, Wolhausen, Switzerland), Hyalofast (Fidia Advanced Biopolymers, Padua, Italy), and Chondrotissue (BioTissue, Zurich, Switzerland) with comparable clinical results [17].

In this study, within the pale of Autologous Matrix-Induced Chondrogenesis(AMIC) we combined microfracturing with a hyaluronan based matrix. Hyalofast (Fidia Advanced Biopolymers, Italy) is a 3-dimensional structure nonwoven graft made from semisynthetic derivative of hyaluronic acid. The use of hyaluronan in cartilage tissue engineering scaffolds may establish an embryonic-like microenvironment to support the reparative events [34]. Hyaluronic acid has been shown to induce mesenchymal stem cells from the bone marrow to differentiate along the chondrogenic lineage [35, 36]. It is resorbed following the breakdown pathway of endogenous hyaluronic acid [37].

The AMIC procedure provides two major advantages; on one hand, it is a one-step procedure with no need of cartilage harvesting potentially leading to donor site morbidity and on the other, it is cost effective with no need of in vitro cell expansion [8]. As AMIC was aimed to extent the applicability of MF from small- to medium-size cartilage defects the results might be expected to be equivalent to MF.

In the present rabbit animal model, the Hyalofast group had the largest amount of hyaline cartilage repair tissue compared with the microfracture group, or microfracture and PRP group. In the microfracture and PRP group, hyaline cartilage was also noted, but it was in admixture with fibrocartilage. In the microfracture group we only noticed a discreet marginal cartilaginous tissue. Fibrocartilage contains more collagen and less proteoglycans than normal intra-articular hyaline cartilage, and a much greater concentration of type I compared with type II collagen [38]. The fibrocartilage, lacks the biomechanical characteristics of hyaline cartilage that are necessary to withstand the compressive forces distributed across the knee, facilitating the occurrence of osteoarthritis [26].

So, the repaired tissue formed after AMIC procedure is of a higher quality and durability than the regenerate formed following bone marrow stimulating techniques, such as MF alone or combined with PRP. The present data demonstrate that the addition of PRP had a positive effect on osteochondral formation as shown on histology and on CT images compared to microfracturing alone.

Also, areas of bone metaplasia appeared only in the Hyalofast group, suggesting that the scaffold favors the regeneration of articular tissue with an ordered histoarchitecture.

But, when we quantified the histological findings with histologic ICRS score we obtained a statistically significant higher value in favor of Hyalofast group only in few domains and in even fewer domains in favor of microfracture and PRP group compared to microfracture alone.

Conclusions

We confirmed the superiority of the AMIC technique compared to microfracture and PRP or microfracture alone in case of full-thickness articular cartilage damage of the knee. The injection of PRP combined with microfracture might also enhance chondrogenesis and improve cartilage healing, leading to the formation of an admixture of fibrous and hyaline-like cartilage tissue. However, the fibrous cartilage might be responsible for the rapid deterioration in cartilage quality with time.

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