Regenerative therapy of Rabbit ear cartilage using adipose derived stem cells and platelet rich fibrin

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1. Abstract

The objective of the current study is to evaluate using of platelet-rich fibrin (PRF) or adipose-derived stem cells (ADSCs) for the therapy of rabbit ear cartilage defects. Nine adult New Zealand white rabbits were divided into three groups. The rabbit ears developed two defects in the cartilage. Phosphate-buffered saline (PBS) was injected sub-perichondrially into Group I, adipose-derived MSCs (ADMSCs) were injected into Group II, and platelet-rich fibrin (PRF) was injected into Group III in the defective ear. After 4 weeks, the auricular defect was examined morphologically, histologically, and immunohistochemically. In addition, the gene expression of the collagen type II (Col II) and aggrecan. Anatomically, the auricles of all treatments seemed to be fully healed with smooth surfaces. Histopathologically, a small region of immature cartilage was seen in the control positive group's defects. While the group treated with PRF and ADSCs demonstrated the normal characteristics of new cartilage development, including mature chondrocytes within their lacunae and rich extracellular matrix (ECM). The PRF-treated group responded to Masson's trichrome and orcein stains, while produced a faint staining in the case of the control positive and ADMSC groups. Immunohistochemically, there was a strong positive S100 expression in ADSCs and PRF but mild reaction in the control group. The expression of relative col II and aggrecan was substantially highest in PRF (± 0.72 and ± 0.57, respectively). While, Control positive and ADMSC groups recorded (± 0.41 : ± 0.21, ± 0.6 : ± 0.44) respectively**.** The PRF had the highest rate of proliferation and chondrogenic potential when compared to the ADMSCs and control groups.

Keywords: *Auricular cartilage defects; Adipose stem cells; Platelet rich fibrin; Rabbit*

2. Introduction

Cartilage injuries including the auricular cartilage take a long time to repair because the cartilage tissue is avascular and comprises few cells with low mitotic activity [1].

The regenerative therapy was initially based on the use of scaffolds (Natural scaffolds as polylactic acid, poly glycolic acid, Synthetic materials as Silastic sheets, Dacron sheets, Vicryl mesh, or expanded Polytetrafluoroethylene), and multipotent stem cells or combination of both in addition to growth factors [2-6].

An ideal three-dimensional scaffold for cartilage engineering should mimic the construction of the natural extracellular matrix (ECM) to confirm cell adhesion and proliferation, appropriate mechanical strength to preserve the scaffold's shape for

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cartilage regeneration. [7, 8]. The platelet-rich fibrin was an autologous platelet concentrate (Second Generation) and considered as a physiological bio-scaffold that comprised of a three-dimensional fibrin network that rich in integrated platelets, leukocyte, cytokines, as well as the clot they generate which were required for regeneration and healing. [9-13].

Stem cells are unspecialized progenitor cells that used for tissue engineering usually obtained from four basic sources embryonic, fetal, adult types of cells, iPSC. Adult stem cells (ASCs) are also known as mature or somatic (such as fat, bone marrow, and synovium) [14-16]. Adipose tissue was another alternative stem cell source after bone marrow that could be obtained by less invasive methods, less painful with decreased donor site morbidity and in larger quantities than bone marrow [15-21]. ADSCs had the highest proliferation rate, with no significant association between stem cell quality, proliferation capacity and the patient's age as the bone marrow [22].

The purpose of this study was to evaluate the therapeutic effects of ADSCs and PRF in the regeneration of rabbit auricular cartilage defect. This evaluation was done using macroscopic and microscopic analysis. Moreover, biochemical diagnostic studies were performed for collagen type II (col II) and aggrecan expression through a distinctive molecular analysis.

3. Materials and Methods

Ethical approval

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Cairo University (Giza, Egypt; Vet. CU. IACUC reference no. Vet CU 8032022406).

3.1. Animals

Nine New Zealand white rabbits (*Oryctolagus cuniculus*) of both sexes weighing 2 to 4 kg each and between 4 and 6 months of age were housed in a pathogen-free environment (at the Department of Anatomy and Embryology using the Close Battery System) and fed on pellets (that contain 12-14% protein) around 1/5 cup twice a day based on weight. The animals were numbered and divided equally into 3 groups (three rabbits per group). (a) Group I (positive control), (b) Group II (the ADMSC-transplanted group), and (c) Group III (PRF-transplanted group).

3.2. Induction of cartilage defects

The three experimental groups positive control, ADMSC, and PRF involved the nine rabbits' left and right ears. By injecting intravenously into the ear veins of each rabbit, 2% xylazine (Bayer, Leverkusen, Germany) and ketamine (2 mg/kg) (Virbac, Carros, France) were used to anaesthetize them. A 2×2 cm cartilage plate was excised from the middle of each auricle in each rabbit from each group.

3.2.1. Isolation of rabbit ADMSCs

An incision was made in the skin and subcutaneous tissue of the inguinal region of rabbit after general anesthesia by intravenous infusion of 2% xylazine (1-3 mg/kg) and ketamine (2 mg/kg). Bilateral inguinal adipose tissue's subcutaneous fat (10 g) was taken out and cleaned with PBS to get rid of excessive blood. Using blades, adipose tissue was cut into small pieces before being treated with 1 mL of collagenase type II at a concentration of 0.075% (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes at 37°C and 5% CO_2. 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle's Medium (DMEM) was used to neutralize the enzyme activity before the sample was centrifuged at 4000 rpm for 10 minutes to generate a pellet. The pellet was centrifuged for 10 minutes at 3000 rpm to eliminate cellular debris, and the supernatant was carefully aspirated to get cell pellets made up of stromal vascular fraction cells.

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3.2.2. Stem cells characterization

Flow cytometry was used to characterize the phenotypes of the stem cells. Trypsinized stem cells from passage 3 were subjected to flow cytometric analysis for CD73, CD105, and CD45 surface markers using a FACSCalibur flow cytometer (FACSort, Becton Dickinson, Germany) for one-color analysis. The data were analyzed using Cell Quest Pro software (Becton Dickinson, Biosciences).

3.2.3. Tissue culture of the ADMSCs

Cells were cultivated in a 75-cm^2 culture flask that contained 12-mL of DMEM high glucose with 10% FBS and 100 mg/mL of penicillin/streptomycin after the pellet was resuspended in 10-mLPBS. The cells were then incubated at 37°C with 5% humid CO2. Non-adherent cells were removed from the media after 48 hours, and adherent cells were then cultivated until they achieved 80–90% confluence. Cells multiplied up until the third passage while the culture media was changed every three days. Cells were trypsinized with 2 mL of 0.05% trypsin-EDTA when the adhering cell monolayer reached confluence and were then incubated for 2 min. The excess trypsin was then eliminated by adding DMEM, then centrifugation, and subculture again. For the experiments, third or fourth passage of stem cells was used.

3.2.4. platelet Rich fibrin

According to Choukron's methodology, 5 ml of peripheral blood was drawn from a saphenous vein of the rabbit and centrifuged right away (3000 rpm, 10 min) to separate it into three layers: platelet-poor plasma (PPP) in the top layer, PRF in the center (yellow segment), and erythrocytes in the bottom layer.

3.2.5. Subperichondrial injection of stem cells and PRF

On postoperative days (POD) 0, 2, and 4, a 29-gauge needle was used to inject 1 mL of purified adipose stem cells at a concentration of 2×10^7 cells/mL at the edge of the excision site after general anesthesia with 2% xylazine (Bayer, Leverkusen, Germany) and ketamine (2 mg/kg) (Virbac, Carros, France) at ear veins. This was done after the pellet containing the ADSCs had been trypsinized, re-suspended in PBS, and activated using low level laser for 10 minutes. while 1 ml of PRF was injected into the rabbit ear cartilage defect on postoperative days (POD) 0, 2, and 4. However, 1 ml of PBS was injected in the control positive group.

3.3. Morphological evaluation of cartilage regeneration

Inspection of all experimental animals were continued until the auricular defect had fully healed. Digital photos were taken right away on PODs 0, 3, 7, 14, 21, and 28 using a Nikon D40 camera (Nikon, Japan).

3.4. Microscopical evaluation

The rabbits were sacrificed, and the bilateral defective auricles were harvested and fixed in 10% neutral buffered formalin (NBF) for 48 hours, washed, embedded in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax, and sectioned at 3-5 μm thickness for histo-pathological and immune-histochemical examinations.

3.4.1. Histopathological examination

The prepared deparaffinized sections (3–5 μm) were eventually stained with hematoxylin and eosin (H&E), Masson's trichrome, and toluidine blue stains [23], in addition to orcein stain that detects elastic fibers [24]. The stained slides were examined using a light microscope (Leica DM500). The images were captured by a camera (Leica ICC50 HD) attached to the microscope and finally examined by an image analysis software [Leica Microsystems, LAS version 3.8.0 (Build: 878); Leica image analyzer computer system] at the Cytology and

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3.4.2. Immuno-histochemical examination for S100

Deparaffinized sections (thickness, 4 μm) from all groups were prepared for S100 immunohistochemical examination according to Giovannini et al. [25]. S100 immunostaining was quantified as area percentage [using ImageJ software (NIH, Bethesda, MD, USA)] in randomly selected high-power microscopic fields from different sections for each group. Regardless of the severity of staining, areas displaying positive S100 (brown color) were chosen for estimation. Each specimen's mean value and standard error of the mean were calculated and statistically analyzed.

3.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Following the manufacturer's instructions, total RNAs were extracted using the easy-spin Total RNA Extraction Kit (iNtRON Biotechnology DR, Cat. No. 17221) and reverse-transcribed using oligo (dT) and M-MuLV reverse Transcriptase (NEB#M0253). RT-PCR was carried out using HERAPLUS SYBR Green qPCR kit (#: WF10308002) and 10 ng of cDNA. The used qRT-PCR primer sets were col II (sense 5′-CCTGTGCGACGACATAATCTG-3′ and antisense 5′-GGGGTCCTTTAGGTCCTACG-3′) and aggrecan (sense 5′-GCCCTTGGTTTCTTGCAGAC-3′ and antisense 5′-TGTCATTCAGGCCGATCCAC-3′). ACTB (*actin-β*) gene was used as an internal control using the primer (sense 5′-GTGCTTCTAGGCGGACTGTT-3′ and antisense 5′-TCGGCCACATTGCAGAACTT-3′). The program was adjusted as follows: 95°C for 2 min and 40 cycles of 95°C for 10 s and 60°C for 30 s. Each RT-PCR was conducted in triplicate. qRT-PCR data were analyzed using CT, ΔCT, ΔΔCT, and $2^{-\Delta\Delta CT}$ [26].

3.6. Statistical analysis

Data was analyzed using one-way analysis of variance (ANOVA) by SPSS version 17.0 software (IBM, USA) to assess the significance of the mean between the groups, followed by an LSD post hoc test. Statistical significance was described as a P-value less than 0.05.

4. Results

4.1. Stem cell characterization using flow cytometry

Flow cytometric characterization analysis of rabbit adipose stem cells in Figs (1) showed that stem cells were positive for CD73 and CD105, whereas negligible levels for CD45.

4.2. Gross anatomical observations

No auricles showed signs of infection, inflammation, or transplant rejection during the trial. The defect in the control group remained narrow, with no cartilage growth within the damage (Fig. 2C). Gross inspection after 4 weeks of ADMSCs and PRF injection revealed that the cartilaginous defect in the experimental groups had completely repaired with a smooth surface and similar color to the surrounding tissue (Fig. 2A–B).

4.3. Microscopic findings

4.3.1. Histopathological examination:

The histopathological examination of the auricles obtained from all groups revealed some differences. ADMSCs injection resulted in the formation of small immature cartilaginous tissue at the defective edges that appeared light purple in H&E staining, distinguishing it from the dark purple mature original cartilage (Fig. 3A). H&E-stained sections of platelet rich fibrin (PRF) treated group showed fibrous tissue filtration that was organized to fill the

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gap of the defective area (Fig. 3B). Moreover, newly proliferating immature chondrocytes were observed at the boundary of the original cartilage defect (Fig. 3C). The untreated (control) group exhibited the surgically created defect without formation of neocartilage after 4 weeks from the induction of the defect. However, small islet of pale stained cartilaginous tissue was detected near the edge of the original cartilage (Fig. 3D).

In contrast, Masson's trichrome staining revealed a small formed immature cartilage at the defective edges treated with ADMSCs showed a faint reaction to Masson's trichrome (Fig. 4A), and orcein stains (Fig. 4B). The sections obtained from PRF treated group and stained with MT stain showed formation of fibrous tissue that was organized in the form of fibrous plate filling the gap at the defective area (Fig. 4C-E). Orcein staining exhibited light brown elastic fibers of the newly formed tissue, distinguishing it from the dark brown elastic fibers of the original matrix (Fig. 4F). The control group with MT stain showed a pale bluish differentiated tissue with no new cartilage formation at the defective area of the original cartilage (Fig. 4G), however, fibrous tissue formation was observed at the edge of the original cartilage defect (Fig. 4H). This fibrous tissue exhibited dark brown elastic fibers when stained with orcein stain (Fig. 4I).

Moreover, the microscopic structure of the examined groups was investigated using a toluidine blue stain, in which aggrecans exhibit purple color (Fig. 5). The ADMSC-treated tissue revealed a faint bluish tint at the defective edges (Fig. 5A). The newly formed tissue that filled the gap of the original cartilage defect appeared pale when stained by toluidine blue stain in case of PRF group (Fig. 5B). The control group showed dark blue original cartilage with small lighter area at the boundary of the native cartilage (Fig. 5C).

4.3.2. Immunohistochemical evaluation

The control group revealed mild immunoreactivity to S100 with area % 1.75, while both PRF group and ADMSCs treated group revealed moderate expression of S100 with area % 7.4 and 11.37 respectively.

4.4. Gene expression of col II and aggrecan

In ADMSCs, PRF, and the positive control, the relative expression of collagen type II was 0.6, 0.72, and 0.41, respectively. while in aggrecan was 0.44, 0.57 and 0.21 respectively as shown in (Fig. 7). In comparison to the positive control group, the col II and aggrecan gene expression was significantly higher in the ADSCs and PRF groups.

5. Discussion

Regenerative medicine and tissue engineering techniques are the best options for treating and regenerate of the cartilage defects. Stem cells are non-specialized cells that are found in many adults and embryonic tissues. MSCs are source for cartilage regeneration and have Adipogenic, osteogenic, chondrogenic, myogenic, and neurogenic potential [27, 28]. This study was designed to evaluate the regenerative capacity of ADMSCs and PRF.

Based on the fact we agreed on, Arzi et al. [29], who stated that the ear cartilage defects have poor vascularization support. As a result, there would be less potential for the regenerative capacity for self-repairing and regeneration. In this study, it added the use of PRF in comparison with ADMSCs for cartilage regeneration while herewith [27, 28] have used only the ADMSCs for tissue repair and engineering.

This investigation valued what mentioned by [30-32] who clarified the benefits of using of PRF for regeneration as PRF serve as a representative source for growth factors such as platelet-derived

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growth factor, transforming growth factor, vascular endothelial growth factor, insulin-like growth factor 1 and epidermal growth factor that were slowly released by the platelets entrapped over a prolonged period, and didn't need to be activated before use because it was made from venous blood without any anticoagulant in a single step centrifugation procedure making this biomaterial an attractive choice to be used as scaffold for regeneration.

Platelet rich fibrin was the first choice for [33-36] than platelet rich plasma for regeneration. This was similar to our decision for using of PRF for the following reasons; PRF had the capacity to stimulate the creation of long-lasting collagen deposits, viable blood vessels, and a denser fibrin network that made handling and suturing easier. In addition to the simple and effective collecting process, no added substances from the outside to activate the platelets, arrested platelets into the fibrin network, and support for hemostasis, cell migration, and proliferation, the platelets degrade at a slower rate after application, resulting in delayed growth factors and cell release profiles.

Although [19, 37] shown that (ADMSCs derived from adipose tissue) might multiply and mature into chondrocytes, our investigation was able to determine the regeneration effects of ADMSCs, and PRF on an auricular cartilage lesion. In the current investigation, rabbits treated with PRF and ADSCs had their auricular cartilage lesion repaired more quickly. In comparison to normal controls, the regeneration of the auricular cartilage in these rabbits was significantly improved, and the healing improvement was histopathologically characterized by the formation of new cartilage made up of chondrocytes and cartilage-specific ECM at the site of the surgically created defect proved and this in agreement with [1, 37]**.**

Our results of the H&E-stained sections showed minor immature cartilaginous tissue formation only along the damaged borders of the original cartilage after 1 month, in contrast to [37, 38], who noticed significant regenerative effects of ADMSCs after 1 and 2 months. our results of the H&E-stained sections of ADSCs- treated group revealed small immature cartilaginous tissue formation only at the defective edges of the original cartilage after 1 month. The H&E findings were reinforced by a weak response to Masson's trichrome and orcein stains. As opposed to the PRF-treated group, which exhibits a great response to the Masson's trichrome and orcein stains. Furthermore, Bahrani, Razmkhah [19] did not observe the formation of small new immature cartilage islands until 3 months after injection with ADMSCs, but they did not notice the emergence of a mature cartilage plate in the gap until 5 months had passed. Six months following the injection, a mature, well-formed cartilaginous plate that filled the gap was produced.

S100 proteins, on the other hand, are important for numerous intracellular and extracellular biological processes. Cell survival and cell differentiation are examples of intracellular processes [39]. In the experimental group (ADSCs, PRF), immunohistochemistry for S-100 revealed significantly stained chondrocytes and chondroblasts with a substantial increase in area percentages of 11.37 and 7.4, respectively, but not in the control group's 1.75. These results support the claims made by [37], who stated that ADMSCs underwent chondrogenic transformation when S100 protein expression was elevated.

High quantities of aggrecan and link proteins are expressed by chondrocytes, which are retained within the matrix network and capable of surviving in suspension cultures [40]. Aggrecan and Col-II are significant markers of cartilage

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regeneration. In our work, platelet rich fibrin dramatically boosted the expression of the col-II gene and aggrecan compared to adipose derived stem cells and control groups, indicating that growth factors in the PRF can facilitate cartilage repair.

Oh et al. [37] found that in rabbits with greater col II expression, ADMSCs exhibited a regenerative impact on auricular cartilage defect. However, our research showed that PRF had significantly greater relative expression of col II and aggrecan (0.72 for col II and 0.57 for aggrecan) than other groups.

6. Conclusion

PRF in this study had the highest rate of proliferation and chondrogenic potential when compared to the ADMSCs and control groups According to histological analyses. Additionally, PRF produced more col II and aggrecan and had better S-100 protein reactivity, which suggests that it might be useful to combine PRF and ADMSCs for the regeneration of cartilaginous defects.

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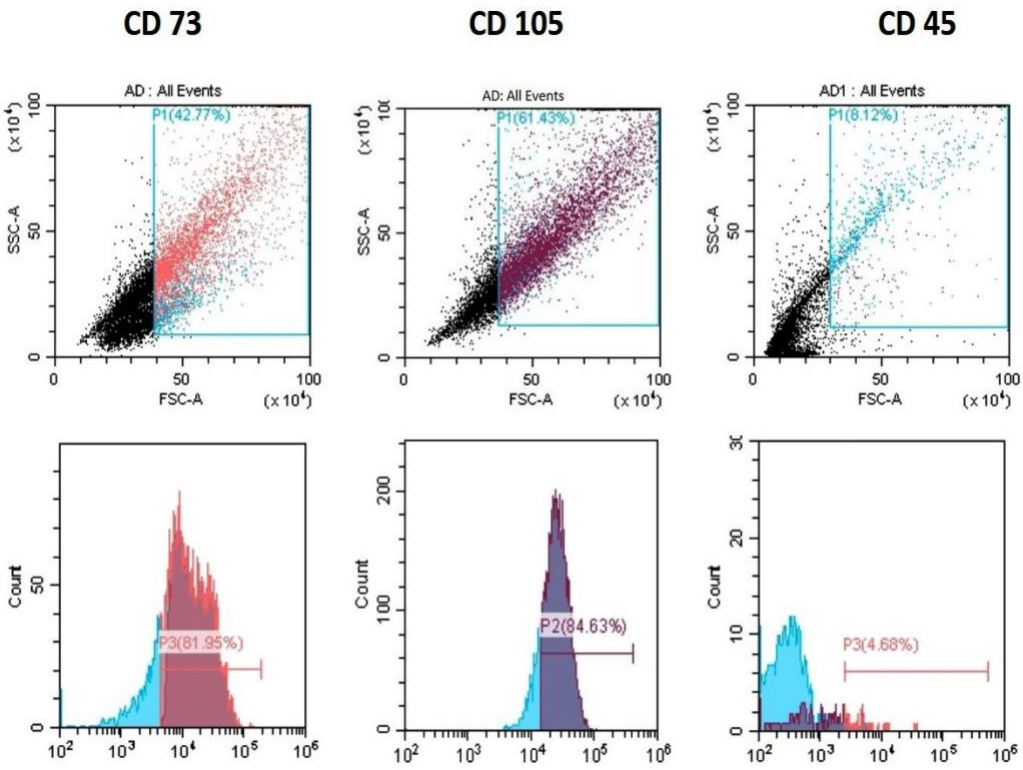

Fig 1. Flow cytometry of ADMSCs at passage 3, typical surface markers were shown in (CD105, CD73) and CD45 as negative marker.

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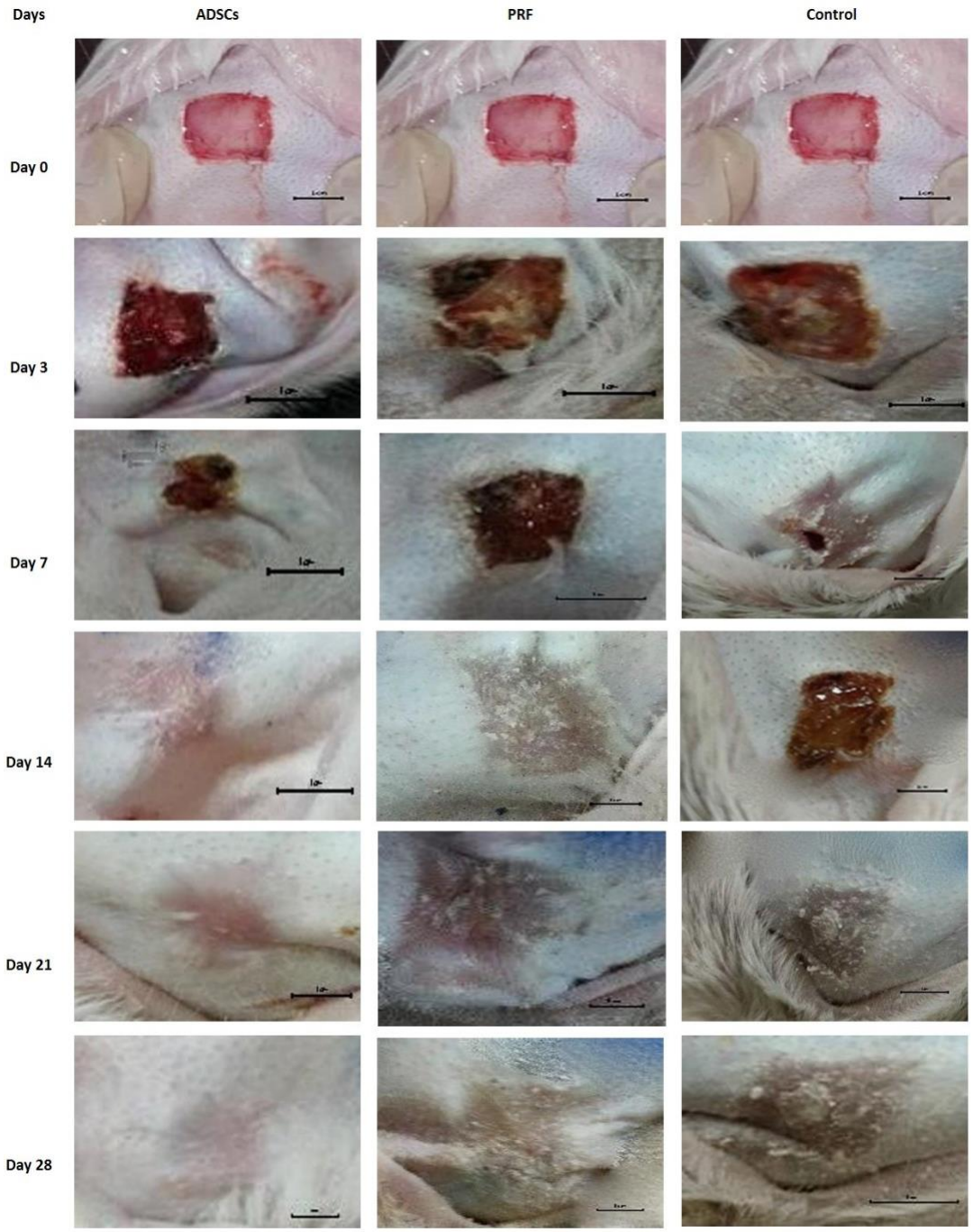

Fig 2. Gross observation of all groups on postoperative days 0, 3, 7, 14, 21 and 28 showed that: 4 weeks after injection of Adipose derived stem cells and Platelet rich fibrin. All the defects were completely healed by chondrocytes with smooth surface and similar color with the surrounding tissue in the experimental groups (ADMSCs and PRF). The defect of the control group (PBS) persisted thin, presenting no chondrocyte propagation around the damage.

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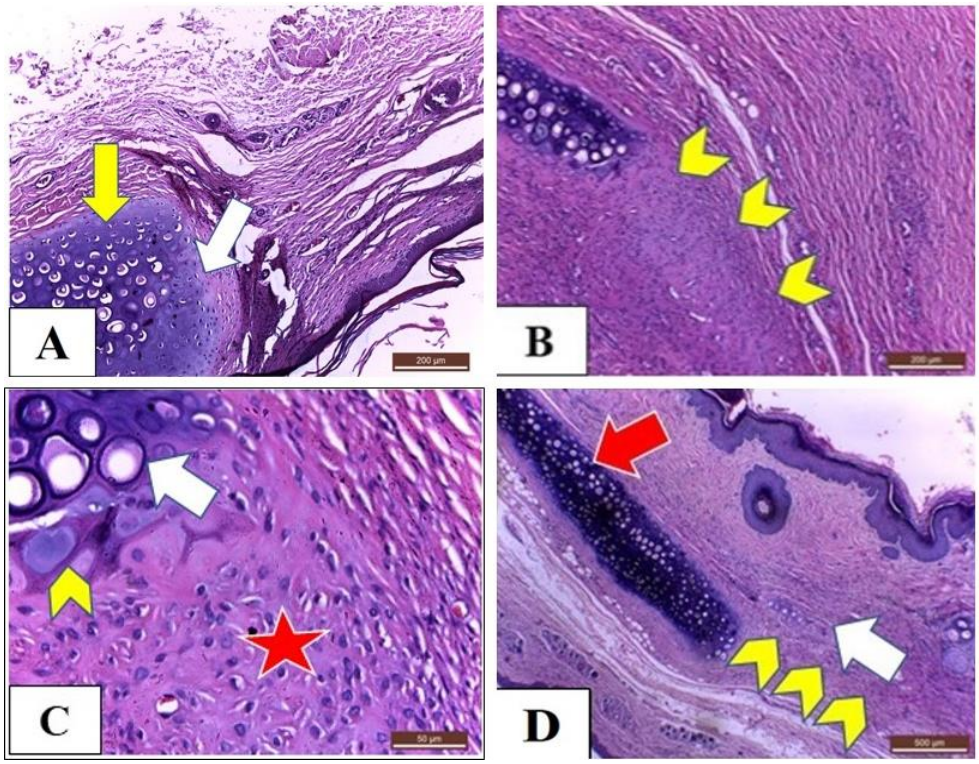

Fig. 3. Photomicrograph of H& E-stained sections of surgically removed auricular cartilage: (A) ADSCs treated group, 100X, shows light purple newly formed cartilage (white arrow) at the defective edges of the deep purple original cartilage (yellow arrow) after treatment with ADSCs. (B) PRF treated group, 100X, showing newly formed tissue (yellow chevron). (C) PRF treated group, 400X, exhibited lacuna of normal cartilage (white arrow) with formation of newly formed cartilaginous tissue (yellow chevron), fibrous tissue was also formed (red star). (D) control group, 40 X, showing native cartilage (red arrow), defective area (yellow chevron) and small islet of cartilage (white arrow).

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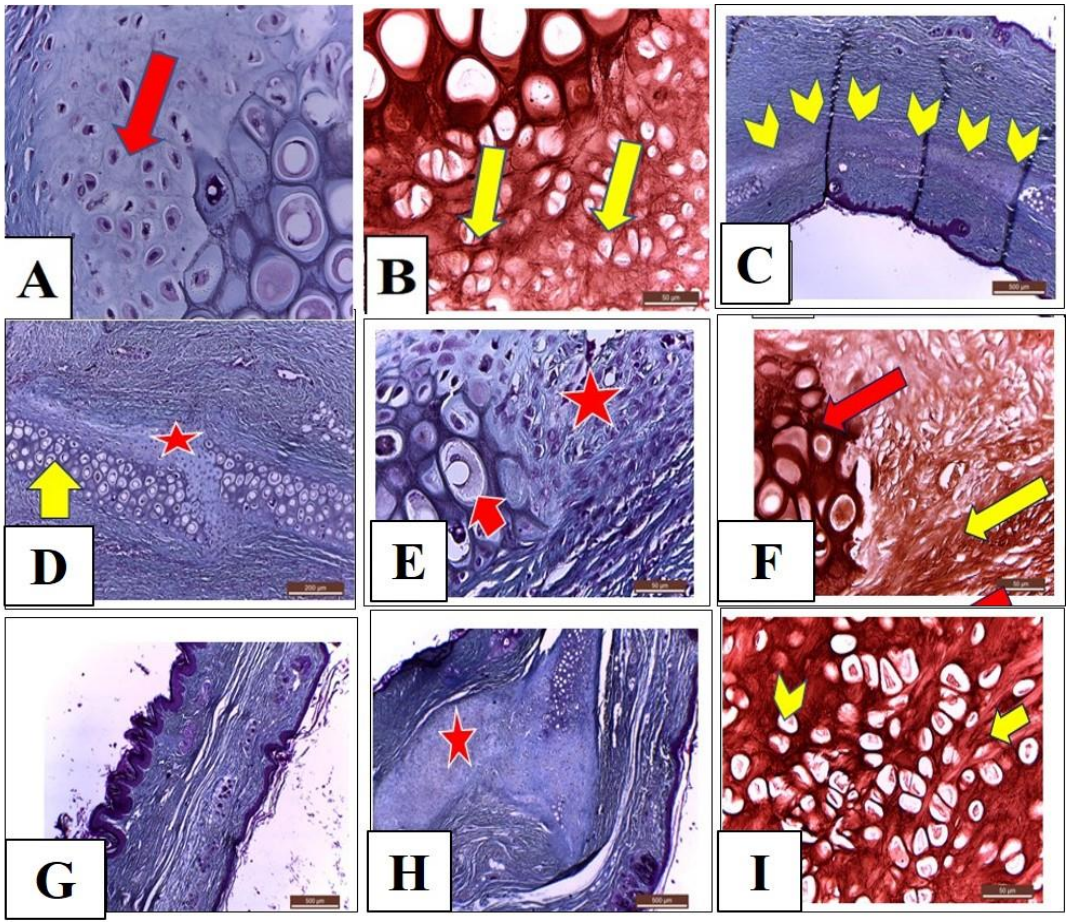

Fig. 4. Photomicrograph of Masson's trichrome and orcein stained sections of surgically removed auricular cartilage. (A) and (B) 400X, show faint reaction of the newly formed tissue to Masson's trichrome stain (red arrow) and orcein stain (yellow arrow), respectively in the ADMSCs. (C,40X, D, 100X and E, 400X) Masson's trichrome stained section of PRF treated group shows new tissue formation (yellow chevron) with fibrous tissue infiltration (red star) at the defect area of the original cartilage (yellow arrow) that exhibits mature chondrocytes (red arrow). (F) orcein stained section of PRF treated group, 400X, reveals light brown elastic fibers of the newly formed tissue (yellow arrow) and dark brown elastic fibers of the native cartilage (red arrow). (G, 40X, and H, 100X) control group stained by Masson's trichrome stain shows newly formed tissue at the edge of the original cartilage (red star). (I) orcein stained section of the control group, 400X, reveals elastic fibers (yellow arrow) around the chondrocytes (yellow chevron).

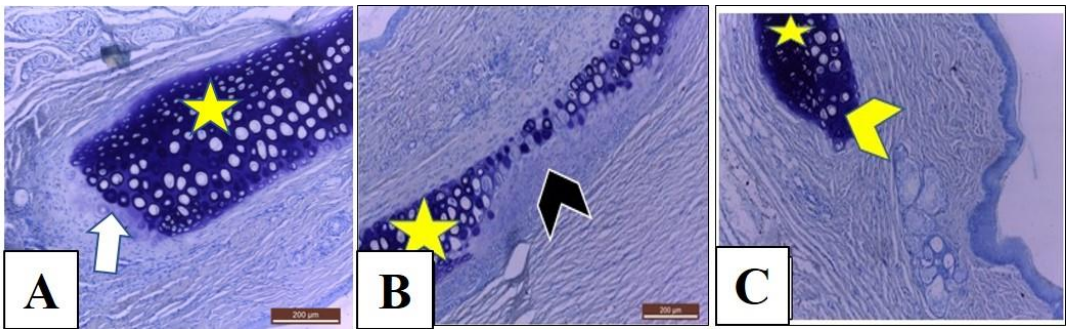

Fig. 5. Photomicrograph of toluidine blue stained sections of surgically removed auricular cartilage. (A). Faint bluish tint (White arrow) of the edges of the original cartilage (yellow star) in ADMSCs treated group is noticed. (B, 100X) newly formed tissue appeared pale stained (black chevron) filling the gaps of the native cartilage (yellow star) that is deeply stained blue by toluidine blue stain. (C, 100X) toluidine blue stained section of the control group reveals new formed tissue (yellow chevron) at the boundary of the original cartilage (yellow star).

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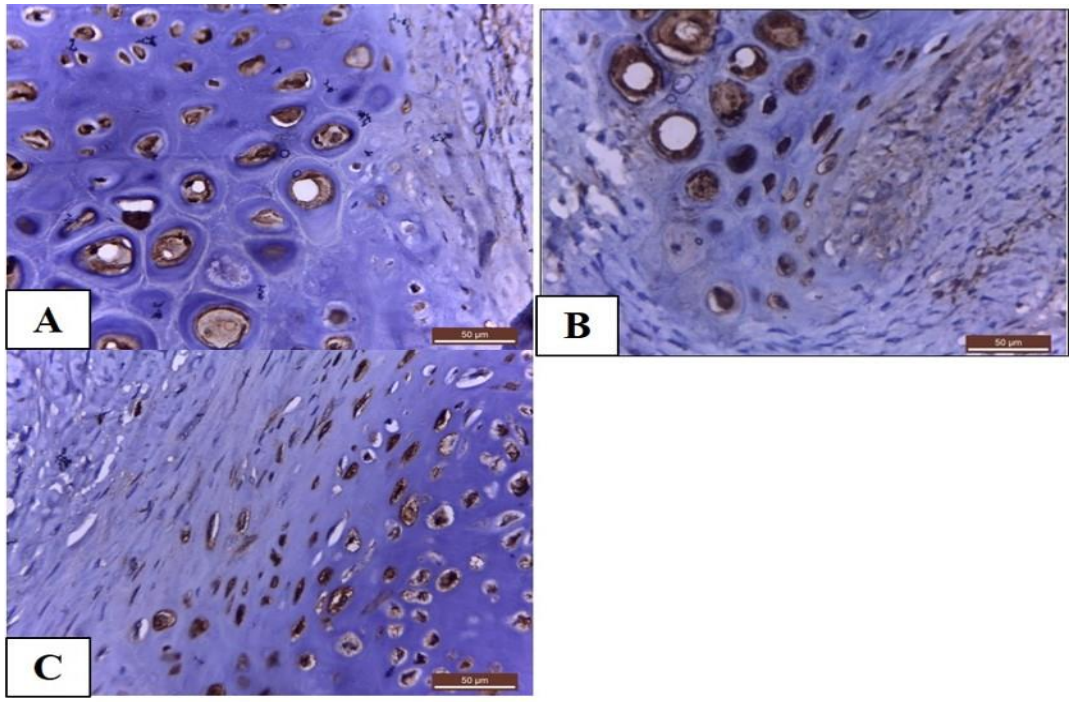

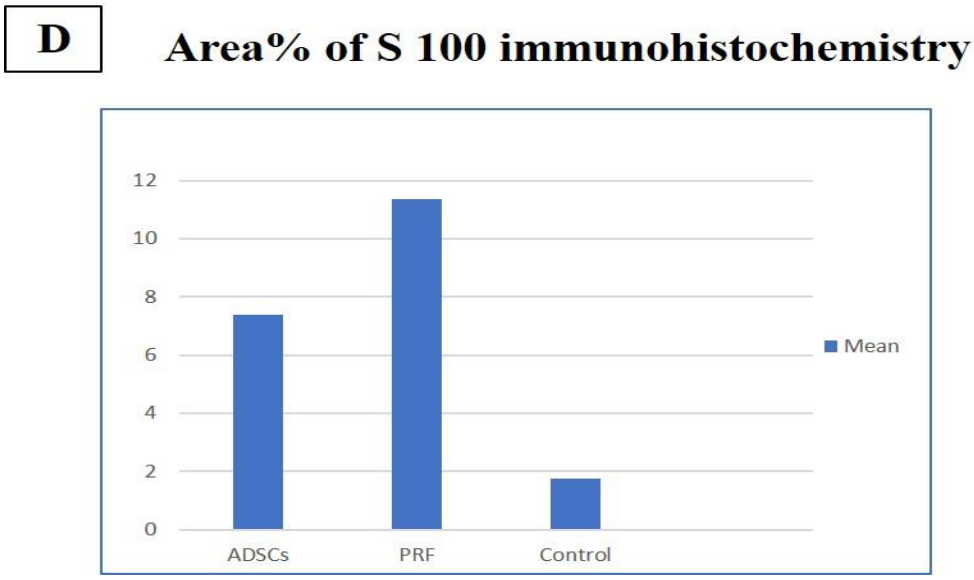

Fig. 6. Photomicrograph of immunohistochemical staining for S100(400X). Both ADMSCs (A) and PRF (B) treated group reveal moderate expression of S100. While the Control group (C) exhibits mild reaction to S100.

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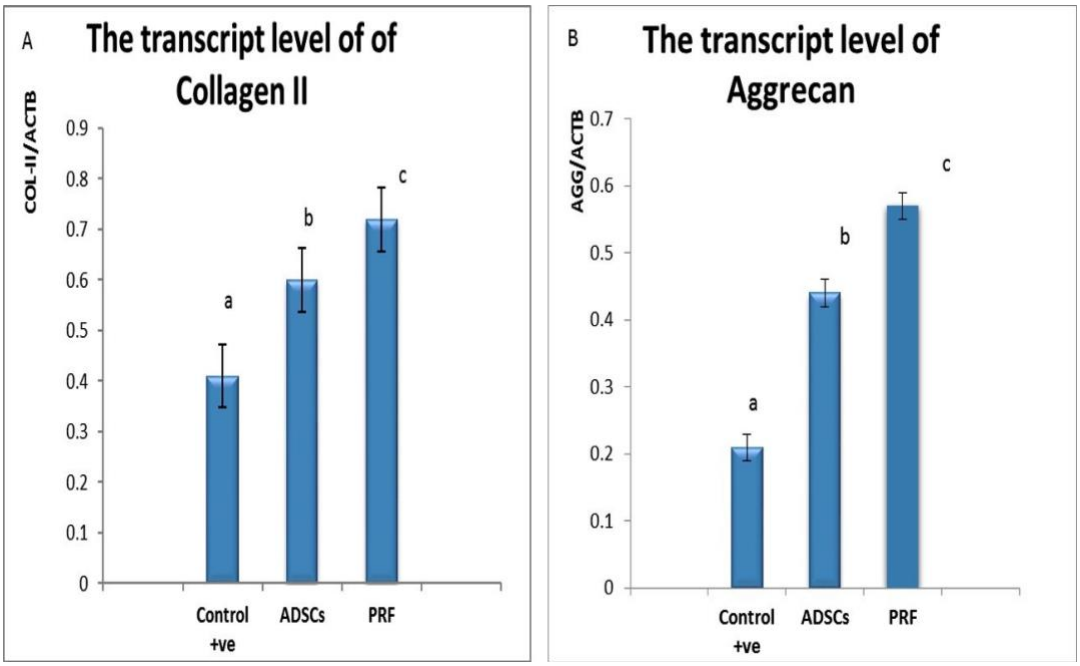

Fig. 7. Gene expression analysis of ADMSCs, PRF and control. (A) The relative gene expressions of Collagen II. (B) The relative gene expressions of Aggrecan. Data were presented as means ± SE. Significant difference was considered at $P<0.05$.

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