Encapsulation of Rat Bone Marrow Derived Mesenchymal Stem Cells (rBMMSCs) in Collagen Type I Containing Platelet-Rich Plasma for Osteoarthritis Treatment in Rat Model

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Abstract

Osteoarthritis (OA) is the most common form of degenerative joint disease, affecting more than 25% of the adult though prevalent in the elderly population. Most of the current therapeutic modalities aim at symptomatic treatment and lingering the disease progression. In recent years, regenerative medicine such as stem cell transplantation and tissue engineering has been suggested as a potential curative intervention for OA. The objective of current study was to assess the safety and efficacy of an injectable tissue-engineered construct composed of BMMSCs, PRP, and Collagen type I in rat model of OA. To produce collagen type I, PRP and BMMSCs, male Wistar rats were ethically euthanized. After expansion and characterization of rat BMMSCs (rBMMSCs), tissue-engineered construct was formed by combination of appropriate amount of collagen type I, PRP and rBMMSCs. *In vitro* studies were conducted to evaluate the effect of PRP on chondrogenic differentiation capacity of encapsulated cells. Then tissue-engineered construct was injected in knee joint of rat model of OA (24 rat in 4 groups: OA, OA+MSC, OA+Collagen+MSC+PRP, OA+MSC+Collagen). After 6 weeks, the animals were euthanized and knee joint histopathology examinations were performed to evaluate the effect of each treatment on OA. Tissue-engineered construct was successfully manufactured and *in vitro* assays demonstrated that relevant chondrogenic genes and proteins expression were higher in PRP group than the others. Histopathological findings of the knee joint samples showed favorable regenerative effect of rBMMSCs+PRP+Collagen group comparing to others. In this study, we introduced an injectable tissue-engineered product composed of rBMMSCs+PRP+Collagen with potential regenerative effect on cartilage damage caused by OA.

Introduction

In the last decades, advancement in health care system improved the prevention, diagnosis and treatment of great number of disorders which restrict human life expectancy [1, 2]. These advancements led to population aging and high prevalence of degenerative disorders same as osteoarthritis (OA). OA considered as the fourth leading cause of physical disability in both the young and the old people in the world. Conventional medical treatment approaches for OA are focused mainly on pain relief and symptom management including nonsteroidal anti-inflammatory drugs (NSAIDs), physical exercises and braces. Besides, some invasive curative strategies consist of steroids, hyaluronic acids (HA), platelet-rich plasma (PRP) injection. When Conventional treatments strategies fail, final curative option may be the surgical approaches [3]. Unfortunately, there are no effective therapeutics, hence, scientists attempt to find novel treatment to restore the function and structure of damaged articular cartilage. Regenerative medicine has been exploiting tissue engineering approaches to treat OA for decades. Numerous pre-clinical and clinical studies have been conducted using SVF, PRP and Stem cells of various kinds (especially mesenchymal in origin) either single or in combination [1]. A few studies revealed that the transplanted stem cells die within a few days after transplantation due to a harsh environmental conditions (e.g., nutrient and oxygen deprivation, oxidative stress, mechanical stress) coupled with death signals, inflammation and anoikis, a programmed cell death due to loss of anchorage to the ECM. [2, 3]. Tissue engineering is an attractive

field to regenerate damaged tissues and restore their functions with three components including cells, 3D scaffold and growth factors. Recently, the autologous chondrocyte implantation (ACI) has appeared as a promising cell-based therapy aim to provide functional repair of injured cartilage to manage the disease symptoms [4]. In fact, ACI approach is restricted by some hindrances including insufficient donor tissue supply, donor site injury and the rapid dedifferentiation of transplanted chondrocytes during two-dimensional expansion [5]. To overcome such obstacles, tissue engineering recommends scaffolds in combination with stem cells as poly-therapy to provide three dimensional environment [6]. Multipotent mesenchymal stem cells (MSCs) with chondrocyte differentiation capacity proposed as an attractive source to OA injuries regeneration (direct intra-articular injection) [7–9]. Also, the homing ability of MSCs to injured sites has been shown [10] and after transplantation exerted its effectiveness to cartilage repair [11, 12]. One limitation of utilizing MSCs alone is insufficient ratio of implanted MSCs and chondrogenic differentiation difficulty. Leijset al demonstrated the low homing of injected MSCs into cartilage while the majority of MSCs detected in joint space or adhered to synovial membrane [13]. Unlike MSCs transplantation, tissue engineering strategies using stem cells seeded or encapsulated in appropriate scaffold such as hydrogels which is impregnated with growth factors revealed more acceptable results for cartilage repair [10, 14, 15]. In cartilage tissue, chondrocytes are encapsulated in a thin rim named pericellular matrix (PCM) that play a critical role in chondrocyte anchorage to the extracellular matrix and preserving chondrogenic differentiation capability [16, 17]. The PCM and the entrapped chondrocyte make functional cartilage unit termed 'chondron'. However, the injected stem cells are encapsulated with a biomimetic scaffold can facilitate cell migration to a targeted niche and promote chondrogenic potential capability [17]. For clinical application, the cells should be encapsulated in an injectable biocompatible scaffold with low immunogenic reaction which could be easily prepared in large scale. Biomaterials like hydrogel mimic ECM that provides support to vulnerable stem cells through cell-to-cell adhesion, proliferation and adherence to host cells. Collagen, one of the different hydrogels is a major component of ECM. Study shows that collagen successfully improved the MSCs viability and enhanced chondrogenic differentiation [4]. Our study precisely opted bone marrow derived mesenchymal stem cells encapsulated with collagen type I in combination with PRP in order to reduce inflammation, promote chondrogenic differentiation to repair OA in rat model successfully. After extensive literature review, we found this combinatorial approach is quite unique, novel and scientifically sound. Because of the great importance of this factor, collagen type I appears to be a hopeful candidate with PCM mimicry to form an injectable chondron. MSCs encapsulated in collagen hydrogel reside in the specific niche and differentiate to chondrocyte properly [18, 19]. Incorporation of collagen hydrogel in various tissue-engineered-based scaffolds has gained priority in recent studies in order to increase the biocompatibility, biodegradability, and regenerative potential [6]. Collagen type I has a high connection affinity to fibronectin (FN) that is expressed at the damaged sites so collagen hydrogel provide a biocompatible injectable scaffold to find cartilage damaged and aim to chondrocyte differentiation promotion of encapsulated MSCs [20, 21]. Besides, the injected cells need adequate growth factors to accelerate their differentiation and conduct them to their determined fate. Recent studies detected the regenerative therapeutic effects of PRP because of the great number of growth factors presence that increase healing rate of damaged tissues by growth promotion in the site of injection [22–24]. In this study, we prepared an

injectable collagen hydrogel that contains MSCs and PRP to evaluate the effect of this tissue-engineered construct transplantation in rat model of osteoarthritis.

Materials And Methods

Experimental animals

Male Wistar rats (2 months old, 200–220g) were purchased from the animal house of Pasteur Institute (Tehran, Iran). The study was performed according to instructions approved by the Animal Care and Use Committees of Tehran University of Medical Sciences (ethical code: IR.TUMS.

VCR.1398.034). The animals were kept in a room with controlled temperature (21–25 °C) on a 12 hours light/12 hours dark cycle.

Rat bone marrow mesenchymal stem cells (rBMMSCs) isolation and culture

Adult rats were euthanized under proper anesthesia with ethically approved protocol. Femora and tibiae were harvested aseptically. Connective tissues were removed and both ends of each bone were cut. Bone shafts were thoroughly washed with sterile PBS and the bone marrow was flushed-out with Dulbecco's Modified Eagle's Medium (DMEM). The cell suspension was collected in a 90 mm culture dish, then filtered by strainer (70µm) and centrifuged at 1200 rpm for 5 min and re-suspended in growth medium containing low glucose DMEM supplemented with 10% FBS, penicillin (100U/mL), L-glutamine (2mM), streptomycin (100µg/mL), and amphotericin-B (0.25µg/mL) (all from Thermo Fisher Scientific, USA). Finally, cells were plated at a density of 10^5 cells/cm² in a T25 tissue culture flask (Nunc, USA) and maintained at 37°C in a humidified atmosphere of 5% CO₂. The medium was refreshed after 24 hours and then every 3 days. Cells at 70-80% confluency were serially passaged. We used cells at passage 3 for subsequent experiments.

rBMMSCs characterization

To characterize the cell population as multipotent MSCs, cells at 3rd passage were characterized to meet the minimal criteria such as morphology, plastic-adherent property, expression of MSC-specific surface markers by flow cytometry and the ability for multi-lineage differentiation *in vitro*.

Surface marker expression

Flow cytometry (FACS Calibur, BD Bioscience, USA) was performed to determine the phenotypic expression of rBMMSCs. At 3rd passages, 1×10^6 cells were stained with anti-CD-90, anti-CD-105, anti-CD-45, and anti-CD-31 (All from Abcam, USA). We used the following antibodies: primary antibodies anti-CD-105 mouse monoclonal (ab2529), anti-CD-90 mouse monoclonal (ab222781), anti-CD-34 mouse monoclonal (ab81289), and FITC-conjugated anti-CD-31 mouse monoclonal (ab33858). Unstained cells were used for iso type control. Data were analyzed by recording 10,000 events using FlowJo Version 7 software.

In vitro differentiation of rBMSCs

After three passages, cells were re-plated in growth medium at 2×10^5 cells/well in 24-well tissue culture plates. After 24 h incubation, the growth medium was replaced with osteogenic differentiation medium containing dexamethasone (10nmol), β -glycerophosphate (10mmol), L-ascorbic acid (0.3mM) (all from Sigma-Aldrich, USA). For adipogenic differentiation, 2×10^5 rBMSCs/well were cultured in 24-well tissue culture plates and after 24h incubation, adipogenic differentiation media containing insulin (10 μ L/mL), dexamethasone (1 μ M), indomethacin (0.5mM), and 3-isobutyl-1-methylxanine (60 μ M) (all from Sigma-Aldrich, USA) was added. The humidified atmosphere with 5% CO₂ was maintained all through the incubation. The differentiation media was replaced every three days. After 21 days of differentiation period, the media was removed and cells were fixed and stained by alizarin red for osteoblast cells and Oil Red O staining for adipocyte cells. Calcium deposition and lipid droplets in the cells were observed using microscopy.

PRP preparation and activation

Approximately 8ml blood was collected from each euthanized rat by cardiac puncture and transferred to a centrifuge tube pre-loaded with 2ml acid citrate dextrose solution. The tubes were then centrifuged at 2000 rpm for 10 min at 20°C and plasma collected carefully then further centrifuged at 4000 rpm for 10 min at same temperature. The supernatant alone is platelet-poor-plasma (PPP) and the precipitate at the bottom of the centrifuge tube with supernatant is the PRP. When PRP volume was more than 2mL, amount 1ml of PRP was used for blood count. We activated PRP immediate prior to application with CaCl₂ 10% (0.2ml CaCl₂ to 0.8ml PRP).

Collagen type I gel preparation and cell encapsulation

Collagen type I was extracted following the protocol described by NavneetaRajan and colleagues (25) from rat tails and processed using acetone, 70% (vol/vol) isopropanol, and 0.02N cold acetic acid. The pure acetone, isopropyl alcohol, acetic acid were purchased from Merck (Germany). The collagen solution was sterilized using 1% chloroform (Merck (Germany)) and the resultant solution was mixed with 10X DMEM-F12 (Gibco, USA) and triple-buffer system-HSS at a volume ratio of 8:1:1 vol/vol respectively. The medium and HSS buffer were filtered through 0.22 μ m strainer prior to mixing. The HSS buffer composed of HEPES 4.77g and 2.2g sodium bicarbonate in 100 mL of 0.5M NaOH solution. All the components of HSS were purchased from Sigma Aldrich (USA). The final concentration of collagen type I hydrogel was 2mg/ml.

rBMSCs encapsulation procedure is shown in graphical abstract. Briefly, the activated PRP and collagen type I solution were mixed properly (1:1 vol/vol) in a falcontube and kept half an hour in room temperature. MSCs were harvested by using trypsin/EDTA and 2×10^6 cells suspended in 100 μ L of complete culture media. The cell suspension was added to PRP-Collagen solution and mixed by gentle pipetting. The composition transferred and cultured in 24-well plate.

Cell morphology and attachment by SEM

The attachment of rBMMSCs in collagen was investigated by scanning electron microscope (SEM) after 48 h post cell seeding. For SEM analysis the samples were fixed by an aqueous solution of Karnovsky's fixative (consisting of 2.5% glutaraldehyde and 2% paraformaldehyde) for 90 min at room temperature. Thereafter, the prepared specimens were washed two times with PBS and dehydrated using a graded ethanol series of 30, 50, 70, 90, 95, and 100%. Finally, the specimens were dried under vacuum and after coating with gold, examined via SEM (SEM, AIS2300C SEI, Korea) at 20 kV.

MTT assay

MTT assay was performed to measure the viability of encapsulated cells alone and with PRP at different incubation period (day 1, 3, and 5) in a 96-well plate. This method is based on the reduction of the yellow MTT-salt [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide] to blue-purple formazan crystals by mitochondrial enzyme succinate dehydrogenase. For this purpose, 1×10^4 cells (encapsulated) were plated on each well with PRP (controls without PRP). Then 200 μ L MTT solution (5 mg/mL) was added in each well and the plate was incubated at 37°C for 3–4 hours. Then the supernatant was replaced with 100 μ L DMSO in order to dissolve formazan crystal producing purple-blue colour. Optical density measured by using microplate reader at 570 nm.

In vitro chondrogenic differentiation of encapsulated rBMMSCs

After passage 3, the cells, at a concentration of 2×10^6 cells/mL, encapsulated in collagen solution then cultured in the 24-well tissue culture plate. After 4 h incubation, differentiation groups were divided to three groups. 1. TGF- β 1 group: treated with chondrogenic differentiation media that contains DMEM/F12 supplemented with 10% ITS + 10^{-7} M dexamethasone (Sigma), 1 μ M ascorbate-2-phosphate, and 10 ng/mL transforming growth factor-beta 1 (TGF- β 1, SIGMA, USA). 2. PRP- TGF- β 1 group: treated with chondrogenic differentiation media + PRP with a ratio of 1:1, and 3. Control group without differentiation media and PRP. The cell culture maintained for 21 days with media change in every 3 days. Finally, the cells were collected for the real time PCR and were fixed for immunocytochemistry for expression of related genes to chondrocyte.

Real time PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used for the mRNA expression patterns of chondrogenic specific genes in control, TGF- β 1 and PRP-TGF- β 1 groups. Total RNA was prepared by using RNeasy Plus Mini kit (Qiagen, USA, 74134) as described by the manufacturer, and complementary DNA (cDNA) synthesis from 1 μ g of extracted RNA was performed by Revert Aid First Strand cDNA Synthesis kit (Takara, USA, K1632). qRT-PCR reactions were carried out in the 48-well optical reaction plates on StepOne™ Real-Time PCR machine. For each PCR reaction, 30 ng synthesized cDNA was used for PCR by mixing with 10 μ L of Power SYBER Green master mix (2 $\times$, Applied Biosystems) primed with 0.5 μ M of each primer (Table 1) in a total volume of 20 μ L at the annealing temperature. The

comparative Ct method ($\Delta\Delta$ CT Method) was used for relative gene expression analysis. All Ct values calculated from the normalization of target genes to GAPDH as an internal control and calibrated using calculation from the undifferentiated BM-MSCs. The relative gene expression values presented as mean of three independent experiments.

Immunocytochemistry

After 21 days' post-induction to chondrogenic differentiation, encapsulated cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 20 minutes. Subsequently permeabilized with 0.1 % Triton X-100 in PBS. The cells were blocked for 30 min at room temperature with 5 % bovine serum albumin (BSA), then incubated with primary antibodies (diluted in 5 % BSA in PBS) against aggrecan (mouse monoclonal antibody; Abcam, USA, 1:200), collagen type I (mouse monoclonal antibody; Abcam, 1:200), and collagen type II (mouse monoclonal antibody; Abcam, 1:200) overnight at 4°C. Secondary antibodies included Alexa fluor 488 donkey anti-mouse (1:500; Gibco) and the nuclei were counter-stained with DAPI (Sigma-Aldrich, D8417). For negative controls, only the secondary antibody was used.

In vivo experiment

The 8 weeks male wistar rats (200-220g) were randomized into four groups each of 6 rats: (i) OA group (OA induction without treatment), (ii) OA+MSC group, (iii) OA+Collagen+MSC+PRP group, and (iv) OA+MSC+Collagen group. OA of the knee joints was induced as previously described by Tang et al (2017). In brief, under sterile conditions, the animals were anesthetized and a medial parapatellar approach was used to release the joint capsule and to perform a medial collateral ligament transection (MCLT) of the right knee joints. The wounds were closed and covered with a local antibiotic ointment. All rats were returned to their cages after the operation and were allowed to move freely, and 0.2 mg/kg/day of intramuscular Meloxicam was administered for 5 days for pain relief and administrated by penicillin once a day for the first 3 days. On fourth weeks post-induction, OA was confirmed by radiography. Then the knee joints were injected by MSC, Collagen+MSC, and Collagen+MSC+PRP. The recipient rats were injected with 4×10^6 collagen-encapsulated MSCs (passage 3). All rats were euthanized 6 weeks post-treatment, and knee joint samples were collected for histopathological evaluations.

Statistical analysis

Tukey's HSD test and repeated measures analysis of variance test was used for multigroup comparisons according to the GraphPad Prism software, Version 6.00 (GraphPad Prism, Inc., San Diego, CA). $P < 0.05$ was considered significant.

Results

Isolation and characterization of rBMSCs

The isolated rBMSCs from the rat femur and tibia bones exhibited typical fibroblastic morphology (Fig. 1A). At passages 3, flow cytometry analysis showed that the cells were negative for CD31 and CD34 and positive for CD90 and CD105 (Fig. 1B). The adipogenic and osteogenic differentiation were approved by oil red O and Alizarin red staining, respectively (Fig.1Ab, 1Ac).

PRP analysis and classification

Platelet rich plasma contained 1298900 ± 248000 platelets/ μ l compared to platelet level (978000 ± 164000 platelets/ μ l). White blood cell count (WBC) was $10000 \pm 2100/\mu$ L in plasma and $6800 \pm 3580/\mu$ l in PRP. Neutrophils/ μ L in plasma was 395 ± 220 and in PRP 388 ± 256 . This PRP is classified as P4-x-B β according to the PAW classification system (26).

MTT assay

The proportion of live cells in the Collagen+Cell+PRP group was higher than those of in the Cell alone and Collagen+Cell groups (Fig. 2). This result confirmed that PRP increases the survival rate of MSCs. SEM image of the rBMSCs seeded on the collagen is shown in Fig. 2A. This micrograph shows cell attachment and interaction with collagen at 48 h post- encapsulation.

rBMSCsencapsulation and chondrogenic differentiation

The collagen scaffolds were successfully produced and micro-encapsulated with MSCs. All scaffolds were bead-like and remained in its original gel state during the 21 days of chondrogenic differentiation induction. All scaffolds were able to maintain and induce the most basic morphological feature of a chondrocyte, its round shape. The cell distribution was homogeneous, occupying all the scaffold area. Two hours post-seeding, the cells were in close contact with the scaffold, however, lacunae were observed housing the cells after one week, due to ECM production confirmed at histological sections. Some chondrocyte-like features were observed on the phase-contrast microscopy such as cells inside lacunae and cells grouped together resembling isogenous groups commonly found in hyaline cartilage.

Cartilage genes expression

At 21 day of differentiation, the expression of chondrogenic of genes collagen types I type II, and aggrecan, in various groups were analyzed with qRT-PCR. The chondrogenic genes expressions were up-regulated and these levels in group of TGF- β 1 and PRP-TGF- β 1 were obviously higher than that of in the control group. Furthermore, aggrecan expression in the PRP-TGF- β 1 significantly higher than that of the TGF- β group (Fig.3).

Chondrogenic differentiation

The effect of PRP on cartilage-specific proteins is shown in Fig.4. Collagen types I, II and aggrecan are the main structural proteins of extracellular matrix (ECM) in cartilage. In the PRP-TGF- β 1 group, the mean amount of collagen types I, II and aggrecan proteins was significantly more than that of TGF- β 1 group. In

comparison, when the cells were treated with PRP along with TGF- β 1, they synthesized more collagen types I, II and aggrecan than that of the TGF- β 1 group (Fig.4).

***In vivo* experiment**

Osteoarthritis was confirmed by radiographic evaluation indicating the severe loss of joint space in medial compartment using (Fig. 5, 6). Histopathological features of the knee joint in various groups are shown in the Figs 7 and 8. Histologically, the control group showed severe abrasion or superficial fibrillation characterized by microscopic cracks into the superficial, mid and deep zones of articular cartilage (AC) (Fig. 7A). In the control group, cartilage matrix loss led to delamination of superficial, mid, and deep layers and cyst formation associated with chondrocyte death, regeneration (clusters), replication, hypertrophy, and proliferation. The deformative change also caused articular bone plate micro-fracture. The organization of the trabecular bone and subchondral was disrupted. Severe subchondral sclerosis and extensively increasing bone volume and bone marrow (BM) distance from cartilage was also identified. Finally, osteonecrosis associated with the infiltration of osteoclastic-like cells associated with severe infiltration of multinucleated giant cells indicating granulomatous inflammation and area of bone resorption was detected in the control group.

In the OA+MSC group, moderate abrasion or superficial fibrillation characterized by microscopic cracks into the mid and deep zones of AC was seen (Fig. 7B). Mild fibrillation characterized by microscopic cracks into the deep zone of AC was detected in the OA+MSC+Collagen group (Fig. 7C). A mild subchondral sclerosis as well as micro-fractures in the trabecular bone was seen (Fig. 7C). All histopathological features including articular bone plate micro-fracture, chondrocytes proliferation, clusters and death, and osteonecrosis without infiltration of multinucleated giant was also detected in the OA+MSC group with the lesser severity than that of the OA+MSC+Collagen group. In the OA+MSC+Collagen, the disrupted organization of the subchondral bone was improved compared to both control and OA+MSC groups. The OA+MSC group also showed the better organization of the subchondral bone than that of the control group. No microscopic cracks into all three zones of AC were seen in the OA+MSC+Collagen+PRP group. In the most part of knee joint the cartilage was intact and the cartilage matrix swelling led to cartilage hypertrophy in this group. No evident subchondral sclerosis was detected. The trabecular bone and subchondral plate were approximately intact. The induced osteoarthritis was improved in the MSC+PRP+Collagen group compared to other treatment groups as well as the control group (Fig. 7D). High magnifications of histopathological features are shown in Fig.8.

Discussion

Knee OA (KOA) is one of the most prevalent degenerative joint diseases with persistent pain and loss of function. It was published that among elders, the risk of lower limb movement disability caused by KOA is at least 40% (27). Natural polymers such as collagen has been studied the most owing to their biocompatibility, making them suitable for cell therapy (28). Tissue engineering approaches using MSCs and scaffolds is new strategy for cartilage regeneration(29). Therapies with encapsulated MSCs have

been new approach for knee OA. Encapsulated MSCs show prolonged survival subsequent injection that could be beneficial for long-term efficacy (30). However, the absence of functional inducing chondrogenic differentiation is drawback. In this study, the effect of cartilage regenerative capacity of a tissue-engineered composite of PRP + MSCs + collagen hydrogel was evaluated *in vitro* and *in vivo*.

The growth factors, such as TGF- β , indicate that has important capacity as a tool for stimulating chondrocyte proliferation and repair of cartilage defects (31). PRP is obtained from peripheral blood, which has pain-relief and anti-inflammatory effects, exhibited good efficacy in knee-osteoarthritis and other musculoskeletal morbidity (32). In our study, we investigated that collagen hydrogel that contain MSCs and PRP might improve the efficiency of injured articular cartilage treatment in animal model. We theorized that PRP affects MSC proliferation and differentiation, especially chondrogenic differentiation.

Drengk et al. reported that PRP activated proliferation of mesenchymal stem cells in 3D culture (33). PRP as a source of important growth factors could efficiently facilitate differentiation and proliferation of MSCs (34). In this study, the viability and proliferation of MSCs was evaluated by MTT assay. The proliferation rate of MSCs-collagen-PRP group significantly higher than other groups. PRP contains a natural cocktail of growth factors such as TGF- β demonstrated upregulated cartilage gene expression and differentiation (33). The capacity of chondrogenic effects of PRP are attributed to growth factors such as TGF- β , vascular endothelial growth factor, basic fibroblast growth factor produced in the alpha granules of platelets (35). In this study, Immune-staining of differentiated cells in TGF- β -PRP group shown that, aggrecan, collagen type I and collagen type II protein expression are higher than TGF- β -PRP group and confirmed that enhance effect of TGF- β transcription factor in chondrogenic differentiation. Next, we investigated the gene expression of chondrogenic pathway. Zhu et al. reported that TGF- β is reported to initiate the chondrogenic differentiation process by induction of collagen and aggrecan genes (36). Collagen type I, collagen type II and aggrecan are the main genes of cartilage extracellular matrix and play an essential role in maintenance of cartilage (28, 37). The gene expression of aggrecan in PRP-TGF- β 1 group was higher than that of PRP and control groups. In this study, RNA levels of cartilage-specific markers, including collagen type I, collagen type II and aggrecan were similarly higher in the PRP-TGF- β 1 and TGF- β 1 group than control group. Liou et al. found that MSCs encapsulated in a PRP-containing hydrogel, resulting in optimal MSC chondrogenic differentiation (38). Intra-articular injection of MSCs has limitations being considered to cell death after injection and leakage from the injection site. Encapsulation of MSCs by biomaterial has therefore been developed to overcome these drawbacks and to deliver therapeutic factors in intra-articular knee (39). Rogan et al. reported that MSCs encapsulation obviously resulted in more cartilage formation in KOA models. Luca et al, founded that encapsulated cells show long-standing survival following injection that could be useful for long-term efficacy of MSCs in cell-based therapy of OA. In current study, histological assessment of articular cartilage was conducted 6-week post injection. Cartilage repair exhibited better healing result in MSC-PRP-Collagen group compared to other groups. Although the chondrogenic differentiation effect of PRP was poorer than TGF- β . The result is consistent with the research of Chellini et al. (40), and the anti-fibrotic potential of PRP underlies the formation of high-quality cartilage.

Conclusions

Our study revealed that MSCs encapsulated in a PRP-containing collagen hydrogel can be used as a potent chondrogenic inducer substitute on cartilage tissue engineering. *In vivo* experiment demonstrated that these composite have a beneficial effect on OA treatment via the stimulation of ECM synthesis and chondrocyte proliferation and provides a new insight into cartilage tissue engineering strategies.

Abbreviations

ACI: Autologous Chondrocyte Implantation, BM: Bone Marrow, BMMSCs: Bone marrow derived Mesenchymal Stem Cells, cDNA: Complementary DNA, DMEM: Dulbecco's Modified Eagle's Medium, ECM: Extracellular Matrix, FBS: Fetal Bovine Serum, FACS: Flow Cytometry FN: Fibronectin, HA: Hyaluronic Acids, MCLT: Medial Collateral Ligament Transection, KOA: Knee Osteoarthritis, MSCs: Multipotent mesenchymal stem cells, NSAIDs: Non-Steroidal Anti-Inflammatory Drugs, OA: Osteoarthritis, PBS: Phosphate Buffered Saline, PCM: Pericellular Matrix, PPP: Platelet-Poor-Plasma, PRP: Platelet-Rich Plasma, qRT-PCR: Quantitative Reverse Transcriptase-Polymerase Chain Reaction, rBMMSCs: Rat Bone marrow derived Mesenchymal Stem Cells

Declarations

Ethics approval and consent to participate:

Animal experiments were approved by the ethics committee of the Tehran university of medical sciences (ethical code: IR.TUMS. VCR.1398.034) and were carried out in accordance with the university's guidelines.

ETHICAL STATEMENT:

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Conflicts of interest/Competing interests:

The authors have no conflict of interest

Availability of data and material

All data are available based on request via contacting with corresponding authors by email.

Consent to participate

NA

Consent for publication

NA

Authors' contributions:

Authors' Contributions

Md Shahidul Islam contributed to all experimental work and drafted the manuscript. Mamun Al Mahtab revised the manuscript and contributed to the interpretation of data. Sadegh Shirian participated to the establishing animal model and surgical study. Hamid Reza Aghayan and Babak Arjmand contributed to the study design and stem cell isolation. Vajiheh Taghdiri Nooshabad participated in evaluation and characterization of PRP. Amin Bigham Sadegh participated in histological and radiographical study. Amir Allahverdi contributed in molecular analysis. Faezeh Esmaeili Ranjbar contributed in stem cell characterization. Jafar Ai and Somayeh Ebrahimi-Barough were responsible for the overall supervision, contributed to design research, data interpretation and manuscript revision.

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Tables

Table 1. Primers sequences for real time-PCR

primer	forward	reverse	Accessation number
Aggrecan	TCCACATCAGAAGAGCCATAC	AGTCAAGGTCGCCAGAGG	NM_022190.1
Collagen I	GCTGTGGAAGTGGATGAAGA	TGAGGAACTGTGGAGAGACG	NM_053304
Collagen II	ACCTGGTACCCCTGGAAATC	CACCAGGATTGCCTTGAAAT	NM_012929.1
GAPDH	TGAGGACCAGGTTGTCTCCT	ATGTAGGCCATGAGGTCCAC	NM_017008.4

Figures

Figure 1

Bone Marrow derived Mesenchymal stem isolation and characterization. (A) Cell isolation and differentiation. (a) Isolated MSCs showed spindle and fibroblast-like shapes. (b) Osteogenic and adipogenic differentiation were visualized by Alizarin Red and Oil Red O staining respectively. (B) CD marker characterization by flow cytometry (FACS). In this representative data set, the cells were positive for CD90 and CD105, negative for CD31 and CD34.

Figure 2

Cell attachment and viability in collagen scaffold. A) SEM micrograph from encapsulated cells in collagen after 48h. B) Cells viability of BMMSCs with basal culture medium as a control group, collagen encapsulation with basal culture medium, collagen encapsulation with basal culture medium supplemented with PRP. a (cell vs collagen+cell) *** $P < 0.001$. b (cell vs collagen+cell+PRP) *** $P < 0.001$. c (collagen+cell vs collagen+cell+PRP) *** $P < 0.001$

Figure 3

Expression of cartilage-specific genes. Relative expression of aggrecan, collagen type I, collagen type II in control, TGF- β 1 and PRP- TGF- β 1 groups during chondrogenic differentiation. The expressions of collagen type I, collagen type II and aggrecan genes were significantly upregulated in TGF- β compared to that in control group. Results are presented as mean $\pm$ SD. Significant levels are a $p < .001$.

Figure 4

The effect of PRP and TGF- β 1 on the biosynthesis of the extracellular matrix, as detected by immunofluorescence. (A) Cells were stained for Aggrecan (green), collagen type I (green), collagen type II (green), and nuclei were stained with DAPI (blue) after 21 days in culture.

Figure 5

The X-ray assay shows the loss of joint space in medial compartment indicating Osteoarthritis (black arrow).

Figure 6

Radiographical images the severe osteoarthritis indicated by loss of joint space in the control group (A, arrow), moderate osteoarthritis in the MSC group (B, arrow), mild osteoarthritis in the MSC+Collagen group (C, arrow), and without osteoarthritis in the MSC+Collagen+PRP group (D, arrow)

Figure 7

Histopathological findings of the knee joint in various groups, A: Cartilage matrix loss resulted in delamination of superficial, mid, and deep layers and cyst formation (black arrow) and vascular invasion in subchondral bone (yellow arrow). The organization of the subchondral and trabecular bone was disrupted (green arrow). Severe subchondral sclerosis and extensively increased in bone volume (star) and bone marrow (BM) distance from cartilage was seen. Osteonecrosis associated with the area of trabecular bone fracture (green arrow) was identified in the control or osteoarthritis (OA) group. B: in the OA+MSC group, mild cartilage matrix loss resulted in delamination of mid and deep layers and cyst formation (black arrow). The organization of the subchondral and trabecular bone was disrupted (green arrow). A distinct subchondral sclerosis (star) was seen. Osteonecrosis associated with the area of trabecular bone fracture (green arrow) was identified. C: mild cartilage matrix loss resulted in delamination of mid and deep layers and cyst formation (black arrow). The organization of the subchondral and trabecular bone was disrupted (green arrow). A mild subchondral sclerosis (star) was seen. Osteonecrosis associated with the area of trabecular bone fracture (green arrow) was identified in the OA+MSC+Collagen groups. D: There were no microscopic cracks into all three zones of articular

cartilage in the OA+MSC+Collagen+PRP group. The cartilage was intact in the most part of knee joint and cartilage matrix swelling leads to cartilage hypertrophy in this group (brown arrow). There was no evident subchondral sclerosis. The subchondral plate and trabecular bone was approximately intact.

Figure 8

High magnification of histological findings: A, cartilage matrix loss leading to cyst formation (stars); B; chondrocyte death (yellow arrow), regeneration (clusters), and hypertrophy; C: severe infiltration of multinucleated giant cells (black arrow) indicating granulomatous inflammation; D: osteonecrosis

Supplementary Files

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