The Mechanism of Cartilage Regeneration by Buffy Coat and the Pre-clinical Study

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Research

Keywords: Cartilage Repair, Buffy Coat, Mesenchymal Stem Cells, Osteoarthritis

DOI: https://doi.org/10.21203/rs.3.rs-98538/v1

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Abstract

Background

Autologous bone marrow buffy coat transplantation possesses obvious advantages in the therapy of large cartilage defects and osteoarthritis. However, there is no definite research on the specific effective components of bone marrow buffy coat and its mechanism of cartilage regeneration. Moreover, as the crucial cartilage regenerative cell in bone marrow buffy coat, mesenchymal stem cells (MSCs) are difficult to fix onto the damaged cartilage area without damaging the subchondral bone. We assessed the effect of using hyaluronic acid (HA) as a liquid scaffold mixed with autologous bone marrow buffy coat to fix cartilage defect.

Methods and Materials

We extracted the bone marrow from the anterior superior iliac crest of the white New Zealand rabbit, centrifuged to obtain a buffy coat, and analyzed the components of buffy coat by ELISA. Buffy coat+HA group, MSC+HA group, MSC+TGF-β+HA group were cultured in vitro and observed by staining. In addition, we made damage to the femoral condyle of rabbits, and divided them into groups: HA group, buffy coat group, buffy coat with HA group. Each group was assessed for cartilage regeneration by visual observation, histological and immunohistochemical analysis at 4 weeks and 8 weeks, and biochemical analysis at 8 weeks postoperatively. One-way ANOVA and LSD were used for statistic analysis.

Results

Buffy coat have a variety of growth factors, inflammatory factors and anti-inflammatory factors that stimulate the MSCs’ regeneration. Buffy coat can differentiate into cartilage without TGF-β stimulation in vitro. The cartilage regeneration ability of buffy coat and buffy coat+HA is strong, and the combination of buffy coat and liquid scaffold HA can make cartilage formation ability more stable in vivo.

Conclusion

MSC, multiple growth factors and cytokines in buffy coat synergistically promote cartilage regeneration. Liquid scaffold HA enhances the effect of buffy coat on cartilage attachment and regeneration of cartilage defects.

Introduction

Injuries to the articular cartilage are challenging to repair because cartilage possesses a limited capacity for self-repair [1]. Articular cartilage is avascular, aneural, and alymphatic, leading to difficult to access to stem cells; additionally, low cell mobility due to surrounding matrix and a limited number of progenitor cells could be another contributing factor. Remarkable efforts have been made to repair cartilage defects. Several techniques concerning implantation of different kinds of cells or tissues aiming to instance autologous chondrocyte implantation and mosaicplasty were developed to restore the normal structure
and function of cartilage [2]. These techniques have been demonstrated to be valid in enhancing cartilage repair. However, the regeneration of hyaline cartilage has not been adequately accomplished.

Numerous studies have demonstrated the therapeutic potential of mononuclear cells (MNCs) extracted from bone marrow for tissue regeneration after ischemic femoral head necrosis, myocardial infarction, and cartilage injury [2-5]. In addition, BMSCs constitute 0.001% to 0.01% of mononuclear cells in bone marrow aspirate after undergoing density gradient centrifugation to remove platelets, granulocytes, red blood cells, and immature myeloid precursors [6]. Chondrogenesis of MSC requires stimulation of a variety of growth factors, such as transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) [7,8]. Jin et al. (2011) first observed that the buffy coat containing monocytes, lymphocytes, macrophages, platelets, and various cytokines displayed satisfactory cartilage regeneration [9], as the achieved number of MSCs in the buffy coat are higher than that of the bone marrow stimulation (100 times). The buffy coat of 4 ml bone marrow contained 2400 MSCs and 1 * 10^7 MNC. Buffy coat possesses an acceptable cartilage differentiation capability in vitro [10]. However, the mechanism of cartilage regeneration of the buffy coat is unclear and is difficult to fix onto the damaged cartilage area.

Hyaluronic acid (HA) is a biodegradable, biocompatible, non-toxic, non-immunogenic, and non-inflammatory linear polysaccharide [11]. In cartilage, HA chains are used as aggregated anchors of aggrecan monomers, and play a pivotal role in assuring cartilage hydration, joint surface lubrication, and synovial fluid viscosity [12,13]. In OA, the molecular weight and concentration of HA are reduced, thereby lowering fluid viscosity and elasticity. Protection against articular injury is compromised and OA damage ensues [14,15].

Hence, in the present study, the buffy coat components and chondrogenic ability of the buffy coat in vitro and cartilage regeneration of the mixture of hyaluronic acid (HA) and buffy coat of in vivo was analyzed. This study may provide an important theoretical and experimental basis for the use of buffy coat in regenerating cartilage.

**Materials And Methods**

2.1 Materials

Healthy 16-week-old (range of weight, 3.0-3.5 kg) New Zealand white rabbits were purchased from the Experimental Animal Center of Yanbian University (Yanji, China). All of the rabbits were housed during the period of experiment under the conditions of a 12:12 h light/dark cycle at 27 °C.

2.2 Component analysis of buffy coat

In the present study, 8 rabbits were selected for the experiment. Moreover, 2 ml of bone marrow was extracted from the anterior superior iliac crest of rabbits using a 20 ml syringe containing 2 ml of 0.9% sodium chloride solution. The collected bone marrow was placed in a tube (17-1440-02; Amersham Biosciences, Sweden), and centrifuged at 1000 g for 30 min at 4 °C.
The analysis of PDGF, TGF-β1, vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), insulin like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), tissue necrosis factor-α (TNF-α), interleukin-4 (IL-4), and interleukin-10 (IL-10) was performed using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, Houston, TX, USA), according to the manufacturer's instructions.

2.3 In vitro study

The mixing ratio of fibrinogen (Shanghai RAAS Blood Products Co., Ltd., Shanghai, China) to HA (Hunan Jingfeng Pharmaceutical Co., Ltd., Shanghai, China) was 10:1. Cells with density of $5 \times 10^6$ cells/well were then mixed homogeneously with aprotinin, 60 U/mL thrombin, fibrin stabilizing Factor XIII, and 50 mM CaCl$_2$. Then, 250 µL of the fibrin/HA mixture was dropped into an empty Petri dish to form a gel [30].

Buffy coat was re-suspended in an α-minimum essential medium (α-MEM) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum (FBS). Buffy coat was cultured in an incubator with 5% CO$_2$ at 37 °C. After 6 days, non-adherent cells were removed and adherent cells were further cultured. After 12 days of cultivation, the adherent MSCs were retrieved by trypsin treatment and re-cultivated at density of $3 \times 10^5$ cells/plate (100 mm) for expansion. The second-generation cells were harvested into fibrin/HA composite gel ($5 \times 10^5$ MSCs/scaffold). After 90 min of incubation, they were transferred into a chondrogenic medium. The bone marrow aspirates (2 ml) were obtained aseptically from the iliac crest, and buffy coat was obtained by Ficoll gradient centrifugation method.

After placing the buffy coat into a 1.5 ml tube, it was then centrifuged (1000 g for 5 min); after removing 450 µl of the supernatant, 50 µl of buffy coat was seeded into fibrin/HA composite gel ($5 \times 10^5$ MNCs/scaffold). After 90 min of incubation, they were transferred into the chondrogenic medium.

2.4 In vivo experiment

Herein, xylazine hydrochloride (0.1 ml/kg) was injected intramuscularly, and 2% pentobarbital sodium (2 ml/kg) was injected intraperitoneally into white New Zealand rabbits. After completing anesthetization of these rabbits, the operation was carried out. An arthrotomy was made through a midline longitudinal incision on the lateral parapatellar with the patella dislocated laterally to expose the femoral condyle. A surgical drill (5 mm in diameter) was used for the creation of an osteochondral defect of 2 mm in depth in the patella groove.

For the in vivo study, we used a polyglycolic acid (PGA) (Albany International Inc. Mansfield, MA, USA) scaffold after flattening to cover the cartilage defect. Absorbable sutures were cross-secured simply to prevent the graft from falling into the joint cavity.

The rabbits were randomly divided into three groups: 1. Injecting HA (Hunan Jingfeng Pharmaceutical Co., Ltd., Shanghai, China) after appearance of defect, and the defect was left untreated as a negative control (HA group); 2. Supplemented autologous buffy coat was isolated from the iliac crest (buffy coat
group); 3. Buffy coat and HA composite were injected into the cartilage defect site (buffy coat+HA group). Regeneration of cartilage was evaluated by macroscopic, histological, and immunohistochemical analyses at 4th and 8th weeks post-operation, and additionally by biochemical analysis at 8th week post-operation.

The design of experiment was conducted based on “Animal research: reporting in vivo experiments: the ARRIVE guidelines” [31].

2.5 Macroscopic and histological evaluations

At 4th and 8th weeks post-operation, the rabbits were euthanized by injection of an over-dose of pentobarbital. Femoral condyles were subsequently retrieved. Macroscopic images of the condyles were initially observed; the samples were then embedded into paraffin, sectioned, and processed for performing Safranin-O staining, Sirius Red staining, and immunohistochemically analysis.

2.6 Histological scoring

To minimize the effects of subjective bias, the Safranin-O stained slides were graded using the Bern Score, consisting of seven categories (range of score: 0–9) [32]. The quality of the regenerated articular cartilage of the defects and a modified version of the histological grading scale system were used for the International Cartilage Repair Society (ICRS) score [33]. The ICRS consists of seven categories, and a score ranging from 0 to 18 was accordingly assigned.

2.7 Chemical assay of cartilage tissue engineering

For chemical assays of the repaired tissue, the cartilage specimens were minced by a surgical knife. The harvested tissue was dried and dissolved in papain solution (5 mM L-cysteine, 100 mM Na2HPO4, pH 6.4, 5 mM EDTA, and 125 mg/ml papain type III) at 60 °C for 24 h, and then centrifuged at 12,000 g for 10 min. The GAG contents were measured by subjecting the supernatant to 1,9-Dimethylmethylene Blue (DMB) assay.

2.8 Statistical analysis

Statistical analysis for comparing the experimental data was carried out by using (one-way ANOVA), and specific inter-data differences between mean values were determined by the LSD test. P-values less than 0.05 were considered statistically significant.

Results

3.1 Composition analysis of buffy coat

The results showed that buffy coat contains a great number of growth factors that promote cartilage regeneration, and also contains inflammation and anti-inflammation factors. Buffy coat contains growth factors, such as TGF-β1 (1.349±0.382 ng/ml), PDGF (1.514±0.292 ng/ml), FGF-2 (1.326±0.818 ng/ml),
IGF-1 (0.195±0.056 ng/ml), EGF (0.162±0.110 ng/ml), VEGF (0.031±0.009 ng/ml) (Table 1), in addition to other inflammatory factors, including IL-1β (0.753±0.138 ng/ml), IL-6 (0.219±0.066 ng/ml), TNF-α (1.031±0.151 ng/ml), and other anti-inflammatory factors, involving IL-4 (1.115±0.657 ng/ml) and IL-10 (1.969±0.095 ng/ml) (Table 2). It can be seen that numerous factors promoted cartilage regeneration in buffy coat.

### Table 1. Growth factors' density in buffy coat

| Growth factors (pg/ml) | TGF-β1     | PDGF      | FGF-2      | IGF-1      | EGF        | VEGF      |
|------------------------|------------|-----------|------------|------------|------------|-----------|
|                        | 1349.4±381.8 | 1513.7±292.9 | 1326.3±818.3 | 195.0±56.2 | 162.0±110.1 | 32.5±9.9 |

### Table 2. Inflammation factors and anti-inflammation factors in buffy coat

| Inflammation factor (pg/ml) | anti-inflammation factor (pg/ml) |
|----------------------------|---------------------------------|
| IL-1β                      | IL-6                            |
| 753.3±138.2                | 219.2±66.0                      |
| IL-6                       | TNF-α                           |
| 219.2±66.0                 | 1031.2±151.6                    |
| TNF-α                      | IL-4                            |
| 1031.2±151.6               | 1114.8±657.4                    |
| IL-4                       | IL-10                           |
| 1114.8±657.4               | 196.9±94.8                      |

### 3.2 In vitro evaluation

On examination with naked eyes, it was found that there was no difference in the size of the three groups after inoculation. After 21 days of culture, it was revealed that the MSC group was generally smaller with blur edges. There was no remarkable change in the size of the buffy coat group and the MSC+TGF-β1 group, both with round and clear edges and high cartilage transparency (Figure 1A). Cells on the fibrin/HA composite gel were stained with Safranin-O, and a significant intergroup difference was noted. On microscopic observation, cells in the outer region of the MSC group displayed differentiation into chondrocytes and production of GAG; however, the central cells did not differentiate into chondrocytes, and apoptosis was noted. On the contrary, in the buffy coat group, cells in the center and surrounding areas displayed active differentiation into chondrocytes and production of GAG. In the MSC+TGF-β1 group, all aspects of cartilage differentiation were active, while the surrounding cartilage was well differentiated, and the central part couldn't enter into the culture fluid, resulting in a necrotic cavity in the tissue center (Figure 1B). Analysis of these sections using the Bern score supported this observation. The cells constructed with high cell density showed the increased effect of Safranin-O staining in the buffy coat group and MSC+TGF-β1 group, which demonstrated a rounded cell shape and higher production of extracellular matrix (ECM) (Figure 1B). A significant difference in the Bern score among the buffy coat, MSC+TGF-β1, and MSC groups was noted. The mean score of MSC group was lower than that of the latter two groups, with significant statistical difference. The buffy coat group, however, had a lower mean score than the MSC+TGF-β1 group, but there was no statistical difference. It might be due to the large standard deviation and small sample size. (n ≥ 4; ** P=0.002; *** P<0.001)
From this, it can be seen that the MSC contained in buffy coat has a good differentiation ability and various growth factors that can promote the cartilage differentiation.

### 3.3 In vivo evaluation

At 4th week post-operation, the cartilage defect site was partially regenerated by white tissue. The HA group has significantly less regenerative tissue than the other groups. The buffy coat group and buffy coat+HA group regenerated tissue surfaces, covering the fibrous tissue. At 8th week, all the groups regenerated more tissue than at 4th week, and the regenerated tissue in the buffy coat group and buffy coat+HA group was closer to the surrounding normal cartilage (Figure 3A). Safranin-O staining showed that the HA group had a very thin cartilage regeneration, while the GAG content was very small and was separated from the subchondral bone at 4th week. Buffy coat group and buffy coat+HA group were also rich in GAG regeneration, whereas regenerated tissues were immature. At 8th week, the buffy coat group showed to be very mature, while the surface was somewhat degraded. A large amount of GAG was produced in the buffy coat+HA group, and the regenerated cartilage was mature as well. The chondrocytes were parallel to the articular surface, and the chondrocyte of the deep zone was perpendicular to the articular surface (Figure 3B).

The ICRS scores were significantly increased in all the groups over time. There was a statistically significant difference in the scores between the HA group and the buffy coat+HA group at 4th week (n≥5; **P=0.001). The average score in the buffy coat group was notably higher than that in the HA group, which was close to that in the buffy coat+HA group. At 8th week, there was a significant difference in the ICRS scores between the HA group and buffy coat group (n≥5; **P=0.004; ***P<0.001). The mean ICRS score was lower in buffy coat compared with buffy coat+HA group, while no significant difference was noted between these two groups (Figure 4). Therefore, it can be concluded that HA can remarkably improve the stability of buffy coat in the treatment of cartilage damage.

In Sirius Red staining, at 4th week, HA group showed a small, very fine collage, which was formed and arranged irregularly. In buffy coat group and buffy coat+HA group, there were numerous regenerated collagens, while the collagen fibers were very thin and irregularly arranged. In 8th week, the HA group had a small amount of collagen formed, and the fiber was thicker than that at 4th week, which was irregularly arranged. The buffy coat group and the buffy coat+HA group regenerated several collagens, and the fiber was also thick and grew perpendicular to the surface, demonstrating that the regenerated cartilage was very mature (Figure 5A). In immunohistochemical staining of type II collagen, at 4th week, the regenerated tissues in HA group were not stained, but buffy coat and buffy coat+HA group were stained slightly. At 8th week, regenerative tissue was partly stained in HA group, while regenerative tissue was remarkably stained in the buffy coat group and buffy coat+HA group (Figure 5B).

At 8th week post-operation, the content of GAG in the buffy coat group and buffy coat+HA group was significantly higher than that in the HA group, while there was a gap compared with normal cartilage (Figure 6). The standard deviation in the buffy coat group was very large, whereas the standard deviation in the buffy coat+HA group was small, and the regeneration of cartilage was found to be very stable (n=6;
**P=0.001; ***P<0.001). Therefore, it was found that buffy coat+HA greatly improved the stability of cartilage regeneration, thereby overcoming the problem of the buffy coat to fix to damaged cartilage.

**Discussion**

The results of the present study indicated that buffy coat is appropriate for cartilage regeneration. It contains several MSCs, growth factors, and cytokines. Therefore, it can differentiate into engineered cartilage without TGF-β1 in vitro. After addition of HA, it promotes the cartilage regeneration capability of buffy coat, and the problem that buffy coat is difficult to adhere to the cartilage defect could be solved as well.

In addition, TGF-β1 at the range of 0.1-25 ng/mL is often essential for proper regulation of MSC proliferation, differentiation, and ECM synthesis, while a higher concentration may inhibit the process [16,17]. All the values of TGF-β1 obtained are, in fact, lower than the expected ones. According to a research performed by Veselý et al. [18], FGF-2 is normally undetectable in serum or with very low values; and the experimental value obtained by the buffy coat was 1.326 ng/ml. In a recently conducted study on a rabbit, a low dose of FGF-2 (10 μg/mL) soaked in a highly porous scaffold showed improved healing of osteochondral lesions, whereas higher dose of FGF-2 (100 μg/mL) or scaffold without FGF-2 demonstrated an inverse response [19].

The chondrogenic differentiation of MSCs is induced by IGF-1, while it is enhanced when IGF-1 and TGF-β1 are used in combination. As the value of IGF-1 was found to be as low as 0.195 ng/ml in the present study, we hypothesized that such a low concentration of IGF-1 might not be able to suppress the chondrogenic differentiation of MSCs.

Moreover, PDGF released from platelets stimulates chemotaxis and mitosis of fibroblasts, synthesis of collagen, as well as remodeling of the extracellular matrix. In an experiment carried out by Schmidt et al. [20], the effect of PDGF increased proliferation of chondrocytes with concentrations ranging from 4.7 to 300 ng/mL with a maximum number of cells at 75 ng/ml. In the current research, the concentration of PDGF, 1.514 ng/ml, is neither very high nor very low than Schmidt et al.’s findings, thus it is reasonable to hypothesize that the PDGF present in the isolated buffy coat has enough potential for proliferation of chondrocytes.

Generally, VEGF is not found in adult human articular cartilage under physiological conditions and has been reported as one of the most significant factors that can cause arthritis. VEGF in the minimum amount can trigger the formation of a new blood vessel and increase blood flow to the site of injury, thereby protecting the proliferated MSCs [21]. Another growth factor, EGF stimulates epithelization and decreases the healing process, in which a high level of EGF causes thinning, loss of integrity, and degradation of the articular cartilage [22]. Since the concentration of EGF is insignificant in the isolated buffy coat, it is reasonable to hypothesize that the chances of losing the integrity and degradation of the articular cartilage are very low, while its’ presence can stimulate angiogenesis of endothelial cells and mitosis of mesenchymal stem cells.
Additionally, IL-1β and TNF-α are present in joints where cartilage is undergoing repair or regeneration due to injury or disease. In an in vitro study carried out by Schuerwegh et al. [23], it was uncovered that very high concentrations of IL-1 (100 ng/mL) and TNF-α induced apoptosis of bovine chondrocytes. In addition, no effect of IL-6 was noted on the function of cartilage with respect to chondrocyte apoptosis, viability, and proliferation. To our knowledge, inflammatory cytokines are inevitable after tissue injury or disease, hence, we hypothesized that the presence of very low concentration (0.219 ng/ml) of inflammatory cytokines by the buffy coat is negligible [23].

Furthermore, Behrendt et al. found that the effect of IL-10 was highly tangible at 100 pg/ml compared with 1000 pg/ml, which prevented posttraumatic cell death, matrix degradation, and chondrocyte dedifferentiation in mechanically injured cartilage [24]. The value obtained in the present research was as low as 1.969 ng/ml, which is near to the concentration of IL-10 reported by Behrendt et al. [24].

A number of previous studies found that IL-4 has an inhibitory effect on degradation of proteoglycans in the articular cartilage, and also reduces the variation in the production of proteoglycans that are visible in the course of OA [25]. The concentrations of IL-4 obtained in the current experiment was 1.115 ng/ml, which might inhibit the effect of degradation of injured cartilage.

Platelet-rich plasma (PRP) research has become more prevalent in recent years. It is a safe treatment for autologous blood products, containing high concentrations of platelets that can be treated by minimally invasive arthroscopy. It has also been reported that the repaired cartilage by PRP is fibrocartilage in nature, while the frequency and duration of treatment have not been reported yet [26,27]. Therefore, treatment by PRP needs to be further studied to eliminate the existing clinical barriers.

It is noteworthy that BMC contains a variety of biologically active molecules and cells, including platelets, lymphocytes, neutrophils, and monocytes, in addition to MSC. A number of scholars demonstrated that BMC is a product of PRP plus MSC. In fact, BMC and PRP contain different cytokines and growth factors. It was reported that BMC contains several platelets, total monocytes, and CD34 positive cells [28]. Cassano et al. [28] confirmed that BMC is rich in interleukin 1 receptor antagonist (IL-1ra), which is a natural IL-1 receptor antagonist, and it can treat autoimmune diseases and rheumatoid arthritis. Scholars also expressed that buffy coat is a BMC without red blood cell (RBC), that is, there is no RBC that hinders adhesion and proliferation of MSC, thus, buffy coat has a superior cartilage regeneration capability [10]. When buffy coat was extracted, we accidentally scraped numerous RBC samples, in which the quality of tissue-engineered cartilage was not eventually satisfactory. Horn et al. also reported that RBCs interferes with adhesion and proliferation of MSCs [27].

The concentration of MSC in bone marrow extracted from rabbit iliac crest was previously reported equal to 607.8/ml [9]. Thus, there are approximately 1216 MSCs and 4.6 × 10^6 MNCs in 2 mL of bone marrow. The MSC concentration in bone marrow is very low. Therefore, the same amount of MSCs cannot be inoculated, although the same number of MSCs and MNCs must be inoculated. After PGA plus HA, the buffy coat was easily fixed on the defected cartilage. We used PGA to cover cartilage defects, similar to a
membrane. And it was disclosed that cartilage regeneration is generally stable and the appearance is acceptable after coverage of PGA. Siciari et al. demonstrated that a cell-free PGA-HA scaffold has an appropriate cartilage regeneration effect [29].

The present in vivo study revealed that some individuals with poor cartilage regeneration had hyper osteogeny around the joints and had traumatic arthritis as well. These may be related to surgery or individual differences. However, the cartilage regeneration effect in buffy coat+HA group was found to be very satisfactory and stable. When the bone marrow was pumped out for a long period of time, the bone marrow was easily solidified in the syringe. When the buffy coat was extracted with such bone marrow, the buffy coat likely contained several RBCs or MSCs. The same situation was also observed when the ambient temperature was high.

However, the buffy coat was extracted by Ficoll gradient centrifugation, Ficoll couldn't be used in clinical practice. Thus, it is essential to develop a new method for extracting buffy coat over the Ficoll gradient centrifugation, which is the key to the application of buffy coat in the future.

In the current research, we developed a new method for extracting buffy coat without Ficoll. The buffy coat extracted by the new method and the buffy coat extracted by Ficoll had no difference in growth factor and cytokine content, and the MSC content was known as well. Therefore, our next study is to apply the new method to extract the buffy coat to regenerate cartilage for clinical application of buffy coat.

Conclusions

In summary, a number of components of buffy coat were herein studied, and it was revealed that there are several growth factors and cytokines that promote cartilage regeneration. The capability of buffy coat to produce cartilage was also demonstrated in in vitro and in vivo experiments. The problem that the buffy coat is difficult to fix onto the cartilage defect due to the liquid state was solved, and the cartilage regeneration effect in the buffy coat+HA group was verified as well. The clinical application of buffy coat is another main challenge in the treatment of cartilage defect.

Abbreviations

BMC: bone marrow concentration;

BMSC: bone marrow mesenchymal stem cell;

DMB: 1,9-Dimethylmethylene Blue;

ECM: extracellular matrix; PRP: Platelet-rich plasma;

FBS: fetal bovine serum;
EGF: epidermal growth factor;
ELISA: enzyme-linked immunosorbent assay;
FGF-2: fibroblast growth factor 2;
GAG: Glycosaminoglycan;
HA: hyaluronic acid;
ICRS score: International Cartilage Repair Society score;
IGF-1: insulin-like growth factor;
IL-1ra: interleukin 1 receptor antagonist;
IL-1β: interleukin-1β;
IL-4: interleukin-6;
IL-6: interleukin-6;
IL-10: interleukin-10;
LSD: least—significant difference;
MNC: mononuclear cell;
MSC: mesenchymal stem cell;
OA: osteoarthritis;
one-way ANOVA: one-way analysis of variance;
PDGF: platelet derived growth factor;
PGA: polyglycolic acid;
RBC: red blood cell;
TGF-β1: Transforming Growth Factor β1;
TNF-α: tissue necrosis factor α;
VEGF: vascular endothelial growth factor;
α-MEM: α-minimum essential medium;
Declarations

Acknowledgement

The author thank Prof. Xun Cui, Prof. Delai Qiu from Yanbian University and Prof. Lin Li, Prof. Bairi Cui from Yanbian Hospital for their help.

Authors’ contributions

Guo conducted experiments, prepared manuscripts, and participated in the design, coordination, review and editing of the study. Kumar contributed to the conception and supervision of the paper and made important suggestions for improving the manuscript. Guo and Kumar contributed equally to the study. Shouyong helped to draft the manuscript and drew a graphic abstract. Guangwen contributed to data interpretation and manuscript preparation. Longhao Jin was the study's instructor and helped design the experiment. All authors read and approve the final manuscript.

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Funding

This study was supported by the grant of the National Natural Science Foundation of China (81560353); China Postdoctoral Science Foundation (2016M601394); Jilin Postdoctoral Science Foundation, China; The Science and Technology Research Project of the Department of Education, Jilin, China.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate

The experimental procedure was approved by the Institutional Animal Care and Use Committee of Yanbian University (Approval No. 2015036).

Consent for publication

Not applicable.
Competing interests

The authors declare no competing interest.

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Figures
Figure 1

(A) shows the gross image after 1 and 21 days. a-c illustrate the image after cell inoculation, and there is basically no difference in size after the three groups are inoculated; d-f display the image after 21 days of culture. d shows that the size is small and the edges are blurred; the size of e and f is basically unchanged, and the edges are round and clear. The red liquid is chondrogenic defined medium. (B) depicts staining with Safranin-O after 21 days; g-i display magnification at X40, and j-l illustrate
magnification at X100. There is a limited red area around g, and a hole in the center. In h and i, the red dotted area is evenly distributed, and a hole appears in the central area of i.

Figure 2

Bern score of In vitro study after 21 days. There was a statistical gap between MSC and Buffy coat group. There was a statistical difference between MSC and MSC+TGF-β group (n≥4; *p<0.05; **p<0.01).
Figure 3

a-c show the gross image at 4th week post-operation; all levels of regenerated tissue can be observed. a is a small amount of tissue formed, and b and c are regenerated tissues filled within the defect area. d-f illustrate the gross image at 8th week post-operation. It can be seen that the regenerated tissues filled within the defect area. g-i display staining with Safranin-O at 4th week post-operation. The regenerated
tissue was evenly dyed dark red. j-i depict staining with Safranin-O at 8th week post-operation. Mature reproductive tissue can be observed as well.

Figure 4

ICRS scores at 4th and 8th weeks post-operation. The ICRS scores significantly increased in all the groups over time. There was a statistically significant difference in the scores between the HA group and the buffy coat+HA group at 4th week. At 8th week, there was a gap between the scores of HA group and buffy coat group, and the scores in HA and buffy coat+HA groups were larger (n≥5; **p<0.01; ***p<0.001).
Figure 5

a-c show Sirius Red staining at 4th week post-operation; a very fine regenerated collagen fiber (which is a mature collagen fiber) can be seen. d-f illustrate staining with Sirius Red method at 8th week post-operation. d depicts a very fine collagen fiber, whereas e and f can be seen mature, in form of a very coarse collagen fiber. g-i display immunohistochemistry of type-II collagen at 4th week post-operation. The regenerated tissues of the g and h were not stained, and i was stained with deep tissue and around
the chondrocytes. j-i show immunohistochemistry of type-II collagen at 8th week post-operation. In j, regenerative tissue was partly stained, while k and i were extensively stained.

![Graph showing GAG content](image)

**Figure 6**

It is the percentage of GAG content (dry weight) (n=6; **p<0.01; ***p<0.001).