Carrier selection for bone marrow mesenchymal stem cells transplantation in periodontal regeneration: calcium alginate or fibrin gel?

Zhen Tan[1], Qing Zhao[2], Ping Gong[1,3], Jinchao Zhang[1], Dapeng Liao[1], Quan Yuan[1], Na Wei[1]

[1]Oral Implant Center, West China College of Stomatology, Sichuan University, Chengdu, China
[2]Department of Orthodontics, West China College of Stomatology, Sichuan University, Chengdu, China
[3]State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China

Corresponding author:
Ping Gong, PhD
West China College
of Stomatology
Sichuan University
No. 14, Third Section
Renmin Nan Road
Chengdu, Sichuan
610041, China
Phone: +86-28-85503579
Fax: +86-28-85582167
E-mail:
dentistgong@hotmail.com

Abstract

Introduction: The aim of the present study is to find a more suitable carrier for bone marrow mesenchymal stem cells (BMSCs) transplantation to promote periodontal regeneration.
Material and methods: Bone marrow mesenchymal stem cells were cultivated in the presence of calcium alginate or fibrin glue. Cell viability was investigated using Vi-CELLTM after incubation for 30 and 60 min at room temperature. Cell proliferation was investigated with the MTT method. Cell adhesion and morphology were detected by scanning electron microscopy after co-culture with fibrin glue or alginate gel for 24 and 48 h. The osteogenic differentiation marker (ALP) of BMSCs was measured as well.
Results: The viability rate of BMSCs declined after cells were mixed with both calcium alginate and fibrin gel. Both carriers inhibited cell proliferation and spreading, but these effects became weaker with time. ALP expression in BMSCs was not significantly different between the calcium alginate group and a control group, but increased after incubated with fibrin glue.
Conclusions: Considering these factors, the potential immunological rejection risk of fibrin glue, and the advantages of calcium alginate in simple manipulation and lessened interference, calcium alginate might be more suitable as a cell-carrier for bone marrow mesenchymal stem cells transplantation in periodontal regeneration.

Key words: calcium alginate, fibrin glue, bone marrow mesenchymal stem cells (BMSCs).

Introduction

For the most part, cell carriers are involved in stem cell therapy and tissue engineering. They provide a microenvironment where stem cells temporarily exist, and exert strong influences on cell viability, proliferation and biological function. Therefore, cell carriers are directly linked to the efficacy of stem cell transplantation.

Bone marrow mesenchymal stem cells (BMSCs) are a possible cell source for tissue engineering, in particular for applications in periodontal tissue regeneration [1, 2]. Although the underlying mechanism by which BMSCs promote periodontal regeneration remains unknown, some scholars

speculated that BMSCs could accelerate periodontal regeneration by differentiation into three types of periodontal cells (osteoblasts, cementoblasts, and periodontal ligament fibroblasts). These cells are required for periodontal tissue regeneration. Therefore, the desired features of potential tissue engineering materials include not only enhancing cell's survival, promoting cell proliferation and function, and proper biodegradation, but also maintaining the multi-lineage differentiation potential of BMSCs. The optimal cell carrier cannot alter the differentiation state of transplanted BMSCs.

Calcium alginate and fibrin gel have been widely used in recent years as cell carriers. Alginate is an anionic polysaccharide found in seaweed. An appealing feature of alginate is that it undergoes gelation under physiological conditions in the presence of a small concentration of certain divalent cations, such as Ca2+, Ba2+, and Sr2+, through ionic interactions between the carboxylic group located on the polymer backbone and the cation. Fibrin glue (FG), a composite of fibrinogen and thrombin, is well known for its hemostatic function in preventing bleeding by forming blood clots. Calcium alginate and fibrin gel keep cells in place after seeding and improve the immediate mechanical properties of the graft.

The aim of the present work was to find a more suitable carrier for BMSCs transplantation to promote periodontal regeneration by comparing the effects of calcium alginate and fibrin gel on the biological activity of BMSCs derived from beagle dogs.

Material and methods

After receiving approval from the Committee of Research Facilities for Laboratory Animal Science, Sichuan University, three male beagle dogs weighing 5 to 10 kg and aged 6-10 months were used in this study.

Isolation and development of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells were obtained from healthy adult canine bone marrow aspirates and were purified by density gradient centrifugation and anchoring culture.

Bone marrow aspirates of 1 ml were drawn out from the tibias of each animal under sodium pentobarbital (40 mg/kg) anesthesia with an injector containing 0.1 ml heparin (3000 U/ml). Then, the bone marrow aspirates were washed three times with phosphate buffered saline (PBS) and suspended in modified eagle's medium of the α type (α-MEM, with 10% fetal bovine serum, 0.05 units/ml penicillin, and 0.05 mg/ml stre-ptomycin).

The suspension was then put into an equal volume of Percoll separating medium (1.079 g/ml) and centrifuged at 2000 r/min for 20 min. The ivory white cell layer was taken out and rinsed with PBS twice, and then suspended in α-MEM. These cells were cultured in a culture flask at 37°C in a humidified atmosphere with 5% CO_2. One day after seeding, floating cells were removed, and the medium was replaced with fresh medium. Thereafter, attached cells were fed with fresh medium every 3 days. Passages were performed when cells reached 90-95% confluence. Cells of passage 3 were used for transplantation. The cells were harvested with trypsin plus EDTA and then washed with PBS [3, 4].

Preparation of cell Carrier

Sodium alginate and calcium chloride were obtained from Sigma (St. Louis, MO, USA) and Kelong Chemicals Ltd (Chengdu, China), respectively. Gels were prepared with the concentration of sodium alginate (1% concentration) and calcium chloride (0.1% concentration). Equal volumes of calcium chloride solution and sodium alginate dispersion were thoroughly mixed.

One milliliter of bovine thrombin (Sigma Aldrich, St Louis, USA) was mixed with 10 mg/ml bovine fibrinogen (Sigma Aldrich, St Louis, USA) solution in 0.9% NaCl. Mixing was carried out in a 24-well tissue culture plate by injecting equal volumes (0.2 ml) of each component [5].

Cell viability assay

Bone marrow mesenchymal stem cells from the 3rd passage were suspended in phosphate buffered saline (PBS) at a density of 2×10^6 cells/ml. The cell suspension was added into calcium alginate gel or fibrin gel. A negative control was represented by a cell suspension with no cell delivery vehicle in it. After incubation at room temperature for 30 or 60 min, the samples were flushed with PBS three times, and cell viability in each group was determined by Vi-CELL[TM] (Beckman Coulter Co, USA), which automates the widely accepted trypan blue cell exclusion method. Statistical differences between groups were determined using a non-parametric rank sum test. Value $p < 0.05$ was considered significant.

Cell proliferation assay

Bone marrow mesenchymal stem cells from the 3rd passage were suspended in α-MEM with 15% fetal bovine serum (FBS) at a density of 2×10^4 cells/ml. The cell suspension was seeded on 24-well plates and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 24 h. Then, calcium alginate gel and fibrin gel were added into the culture

medium proportionally and well mixed. After the allotted time, each well was rinsed with PBS and an MTT test was carried out. The absorbance of each sample was determined at a wavelength of 492 nm using a spectrophotometer. All the cell culture assays were repeated twice on four samples per group. One-way Analysis of Variance (ANOVA) was used to test for differences between these groups. Value $p < 0.05$ was considered significant.

Morphology of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells were seeded onto the culture plates and cultured for 24 h under standard culture conditions. Cell delivery vehicles (calcium alginate gel and fibrin gel) were then added into the culture system and thoroughly mixed. After a 24 or 48 h in culture, the samples were fixed in 3% glutaraldehyde and prepared for scanning electron microscopy (SEM) observation.

Measurement of induced alkaline phosphatase activity of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells from the 3rd passage were suspended in α-MEM with 15% fetal bovine serum (FBS) at a density of 2×10^4 cell/ml. The cell suspension was seeded on 24-well plates and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 24 h. At that time, calcium alginate gel and fibrin gel were added into the culture medium proportionally and well mixed. The medium was replaced with fresh medium every 3 days. Both carrier materials were added to the 24-well plates at the same time. After nine days, each well was rinsed and then incubating the cells with 5 mmol/l p-nitrophenyl phosphate (pNPP) in 50 mmol/l glycine, 1 mmol/l

Table I. BMSCs viability rates after co-culture with cell carrier for 30 and 60 min

	30 min [%]	60 min [%]
Control group	93.33 ±1.91	68.83 ±4.61
Alginate group	80.03 ±1.23	67.10 ±1.45
Fibrin group	79.80 ±2.82	65.25 ±3.53
Value p	0.024	0.334

Date are given as mean ± SD percentages, n = 4

Table II. BMSCs proliferation after co-culture with cell carrier for 24 and 48 h

|	24 h	48 h
Control group	0.2845 ±0.0085*	0.5390 ±0.0164*
Alginate groups	0.2548 ±0.0062	0.4593 ±0.0244
Fibrin group	0.2443 ±0.0099	0.4650 ±0.0102
Value p	0.00 (F = 25.128)	0.00 (F = 24.534)

Date are given as mean ± SD, n = 4
* SNK test showed a significant difference between control group and other groups at the two time points ($p > 0.05$)

$MgCl_2$, pH 10.5, at 37°C for 30 min. The reaction was stopped by adding 0.1 M NaOH. The yellow-colored pNP was measured spectrophotometrically at 405 nm. Then, the amount of pNP was determined using an external standard curve of pNP and expressed as nmol/min mg protein [6]. Statistical analysis was performed using Student's t-test. A significance level of $p < 0.05$ was used.
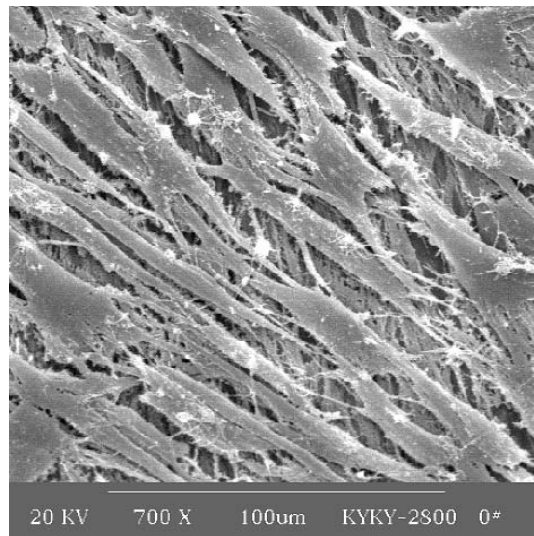
Results

Isolation and development of bone marrow mesenchymal stem cells
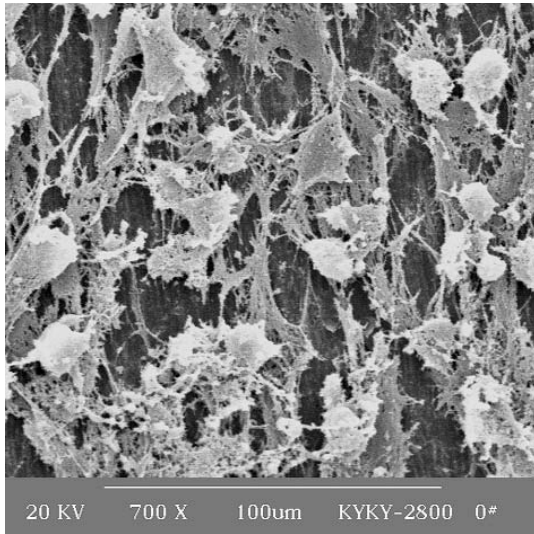
Bone marrow mononuclear cells were isolated using density gradient centrifugation. Nonadherent
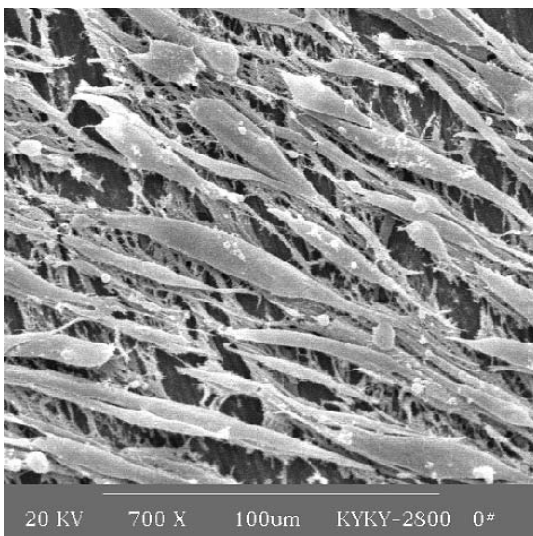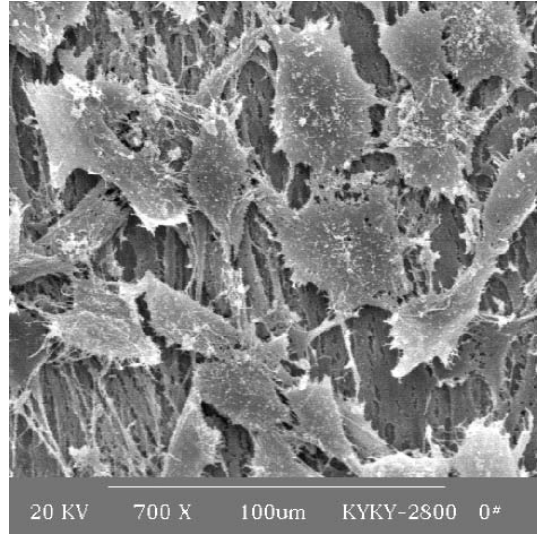

Figure 1. SEM images of the BMSCs in the control group after culturing for 24 h (×700)
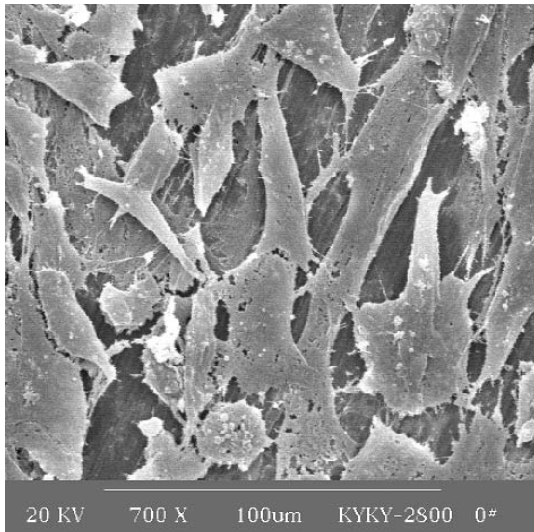

Figure 2. SEM images of the BMSCs in the control group after culturing for 48 h (×700)
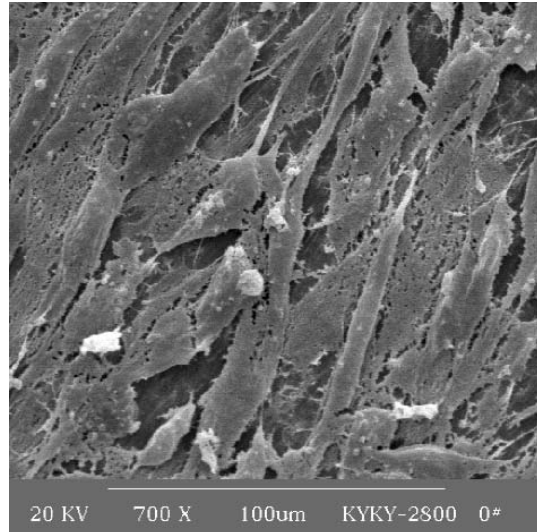
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Figure 3. SEM images of the BMSCs after co-culturing with alginate gel for 24 h (×700)

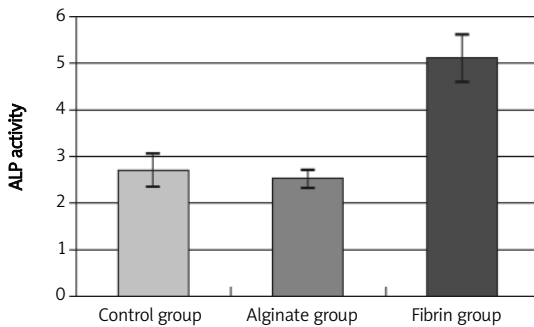

Figure 4. SEM images of the BMSCs after co-culturing with alginate gel for 48 (×700)

Figure 5. SEM images of the BMSCs after co-culturing with fibrin glue for 24 h (×700)

Figure 6. SEM images of the BMSCs after co-culturing with fibrin glue for 48 h (×700)

Figure 7. Alkaline phosphatase (ALP) activity at day 9

cells were removed by a change of medium at 24 h. Bone marrow mesenchymal stem cells isolated by density gradient centrifugation were cultured for 5 to 7 days until individual colonies with fibroblast-like morphology formed. Flow cytometric analyses showed that the 3rd passage of BMSCs was CD44-positive (95%) but CD34-negative.

The effect of cell carrier on viability of bone marrow mesenchymal stem cells

The viabilities of the three groups of BMSCs cultured at room temperature after 30 and 60 min are shown in Table I. The results of the non-parametric rank sum test were as followed: after 30 min, the viabilities of the alginate group and the fibrin group were significantly lower than the control

group (p = 0.024), but there was no significant difference (p = 0.773) between the alginate group and the fibrin group. After 60 min, there was no significant difference among the three groups (p = 0.334).

The effect of cell carrier on proliferation of bone marrow mesenchymal stem cells

The proliferation of BMSCs cultured after 30 and 60 min are shown in Table II. One-way ANOVA analysis revealed that after both 24 and 48 h, there was a significant difference between the control group and the alginate or fibrin groups (p < 0.05), but there was not a significant difference between the alginate group and the fibrin group (p > 0.05).

The effect of cell carrier on morphology of bone marrow mesenchymal stem cells

The morphologies of BMSCs cultured on titanium discs in the three groups after 24 and 48 h are shown, respectively, in Figures 1-6. After 24 h, the cells in the test groups were poorly spread, and cell shrinkage was found. Some cells were rounded. Most of the cells had an irregular morphology. After 48 h, the number of cells increased. Multi-layer growth of BMSCs was observed in some areas. The shape of the BMSCs became relatively regular. The cells spread well and possessed many cell processes.

Effect of cell carrier on alkaline phosphatase of bone marrow mesenchymal stem cells

After co-culturing with fibrin glue for 9 days, the ALP activity increased by approximately twofold relative to the untreated control group. There was no statistical difference in ALP activity between the alginate gel group and the control group. The alginate gel could not alter the differentiation state of BMSCs (Figure 7).

Discussion

The main function of a cell vehicle material in tissue engineering is to provide a microenvironment for transplanted cells. It functions in much the same way as the body's own extracellular matrix, allowing normal cellular and physiological processes to occur. Many attempts have been made to produce and select suitable cell carrier that can be used in tissue engineering applications. Fibrin glue and calcium alginate gel are two kinds of injectable systems that have been commonly used for the delivery of stem cells.

Fibrin gel mimics the final stage of the physiological coagulation cascade, in which fibrinogen molecules are cleaved by thrombin, converted into fibrin monomers and assembled into fibrils, eventually forming fibers in a network

structure independent of the patients' coagulation process. This property also makes fibrin glue a global surgical sealant, and it has been widely used as an adhesive in plastic and reconstructive surgery [7]. This gel could have many advantages as a cell delivery vehicle in terms of biocompatibility, biodegradation, hemostasis and antibacterial activity. Many researches have been conducted to explore the feasibility of fibrin gel for in situ delivery of stem cells in the regeneration of bone, cartilage, and cardiovascular tissue, and in the treatment of chronic trauma [8-13]. However, fibrin gel demonstrates drawbacks in some aspects, including mechanical strength and moldability [14]. Additionally, allogenic FG might possibly lead to the development of immune-mediated adverse effects, including coagulapathies or anaphylactic shock. Furthermore, as a blood product, fibrin gel may transmit diseases such as AIDS and hepatitis B. Because it lacks the risks mentioned above, autologous or animal fibrinogen has gained more and more attention in recent years.

Calcium alginate gel has also been used in many biomedical applications, including cell transplantation and drug delivery. Recently, it has been widely employed in the regeneration of bone, cartilage and nerve tissue [15-17]. Nevertheless, alginate itself has a very slow degradation rate. Gamma-irradiation was applied to decrease the average molecular weight of alginate polymer chains (< 50 kDa) and increase its biodegradation rate in vivo, which potentially allows for renal clearance. Other methods (e.g., partial oxidation of alginate by oxidants) have also proven to be very effective in the degradation of high molecular weight alginates [18-20].

In this experiment, based on tests of the residual ratio, growth curve, appearance change and differentiation of the BMSCs, we compared the biological effects of fibrin glue and alginate gel on BMSCs.

The present results revealed that the viability rates of BMSCs in both fibrin and alginate groups decreased after being away from normal culture conditions. The viability of these groups was lower than in the control group. We speculate that the main reason for this is the polymerization and cross-linking process for the gel, which is harmful to cell vitality. After 60 min, no significant differences in BMSCs viability were detected among the three groups, which was probably because the cells in PBS would be affected by the external environment relatively easily with time.

The results of the present study were somewhat different from previous reports [5]. We believe the main reasons might be as follows. First, the tests were done under different conditions. Second, the different fibrinogen and thrombin concentrations used could result in different cell behaviors. Many

researchers have demonstrated the influences of the concentration and ratio of these two factors on implanted cells. The shape, migration speed, gene expression, secretion ability of fibroblasts could be affected by fibrinogen and thrombin concentrations [21]. Andrade *et al.* discovered that different fibrinogen and thrombin concentrations caused the secretion ability of pancreatic islets to change significantly under glucose stimulation [22]. Different ratios of the two contents also have effects on the structure of the gel, which can reduce the mobility of the cells and decrease the effectiveness of tissue engineering therapy. Thus, the desired ratio and concentration will be different for the different cell lineages [23].

The results of this research also suggest that both cell carrier materials could inhibit the proliferation of BMSCs. In addition to the reasons mentioned above, we speculated further that the formed gel might act as the major barrier to the provision of nutrition to BMSCs.

The SEM images show that the cells in the test groups were crowded, and their morphologies recovered gradually after incubation with cell carriers for 48 h. Generally, cell morphology is an indicator of cell activity. Therefore, this morphology change suggests that the cellular migratory behavior of these cells was promoted.

The alkaline phosphatase activity of BMSCs increased after incubation with fibrin glue. This study suggests that canine BMSCs underwent osteogenic differentiation when cocultured with fibrin glue sealant. We speculate that the residual growth factors in the fibrin glue were responsible for osteogenic differentiation of BMSCs. On the other hand, calcium alginate could not influence the ALP expression of BMSCs significantly. This means that the BMSCs embedded in calcium alginate could maintain multilineage differentiation potential and accelerate periodontal regeneration by differentiation into osteoblasts, cementoblasts, and periodontal ligament fibroblasts.

In conclusion, this study reveals that although calcium alginate and fibrin gel are often used as delivery systems for living cells, both cell carriers can influence viability and inhibit cell proliferation and the spreading of BMSCs, these negative effects become weaker over time. At the same time, fibrin glue can induce osteogenic differentiation of BMSCs, which is not favorable for periodontal regeneration although it could adhere to the bodies tissue and be more stable in a wound. Considering these factors, together with the potential immunological rejection risk of fibrin glue and the advantages of calcium alginate, in which include simple manipulation and lessened interference, we conclude that calcium alginate is more suitable for BMSCs transplantation to induce periodontal regeneration.

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