Am J Stem Cells 2026;15(1):13-26
www.AJSC.us /ISSN:2160-4150/AJSC0169871

Original Article

A bioactive composite of freeze-dried stem cells, porous microcarriers, and fibrinogen-thrombin gel for dental pulp tissue engineering

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Received November 8, 2025; Accepted February 4, 2026; Epub February 15, 2026; Published February 28, 2026

Abstract: Background: The aim of this study was to develop a novel biocompatible composite for the regeneration of damaged dental pulp tissue. Materials and Methods: To create the composite, porous microcarriers were loaded with freeze-dried bone marrow stem cells and embedded in a fibrinogen-thrombin gel. The regenerative potential of the composite was evaluated in both ectopic and orthotopic animal models of dental pulp injury. Results: The composite stimulated the migration and proliferation of host pulp cells via growth factors and cytokines secreted by freeze-dried bone marrow stem cells. Furthermore, the microcarrier-based scaffold created a three-dimensional microenvironment that preserved the paracrine activity of stem cells, promoting the effective regeneration of damaged or partially amputated dental pulp. Conclusion: This bioactive composite demonstrates significant potential for regenerative endodontics, facilitating the restoration of dental pulp. Further studies are needed to elucidate the specific role of paracrine factors from freeze-dried stem cells in improving pulp tissue regeneration.

Keywords: Freeze-dried bone marrow stem cells, three-dimensional microcarriers, fibrinogen-thrombin gel, decellularization of a tooth, pulp-dentin complex, tooth scaffold, regenerative endodontics

Introduction

The limited effectiveness of traditional endodontic approaches in restoring the full function of the tooth has led to a markedly increasing need in recent years for new, innovative therapeutic strategies. Pulp removal deprives the tooth of its natural immune defenses, sensory function, and ability to remodel dentin, thereby increasing the risk of fractures, fragility, and recurrent infections [1-5]. Recent advances in bioengineering may address this issue by creating functional bioengineered pulp, which could offset the limitations of traditional endodontic procedures [6]. For example, bioengineered pulp can restore the functional properties of natural tissue, including vascularization, innervation, and odontoblast formation. This will not only preserve the vitality of the tooth but also stimulate the growth of reparative dentin, support the local immune response, and ensure the long-term stability of the tooth [7-11]. Moreover, the development of bioengineered pulp opens new perspectives for personalized dentistry, where tooth restoration will be carried out taking into account the individual cellular and tissue characteristics of the patient, which minimizes complications and will improve clinical outcomes [12].

The fundamental principle of creating bioengineered pulp, as reported by many authors, is the use of the tissue engineering triad, which includes cellular components, biocompatible materials (scaffolds), and signaling molecules [13-15]. Various natural and synthetic materials are used as biocompatible scaffolds. In this paper, we will not characterize the scaffold materials used to create bioengineered teeth or pulp in detail, as they have been described in detail by many authors [16-19]. We note only that, according to the recommendations of the

https://doi.org/10.62347/RJPQ4886

American Society for Testing and Materials (ASTM F2150-19), the framework for bioengineered tissues and organs must provide support or serve as a means of delivering bioactive molecules or drugs, as well as promote the attachment, migration, and proliferation of transplanted cells [20-22].

In this study, during the creation of bioengineered pulp, we focused on stem cells. In modern regenerative dentistry, widely used stem cells include dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from the apical papilla (SCAP), induced pluripotent stem cells (iPSCs), as well as bone marrow-derived mesenchymal stem cells (BMSCs) [23-28]. Despite significant progress in this field, the creation of fully functional bioengineered pulp remains a challenging task. The key unresolved issues include the search for an optimal biocompatible material, the selection of cell sources capable of ensuring reliable vascularization and innervation, and the development of methods that allow complete integration of bioengineered pulp with the surrounding dental tissues [29-33].

In recent years, for the creation of bioengineered pulp, microcarriers made from various natural and synthetic materials have been effectively used together with stem cells, serving as a three-dimensional matrix for cell delivery and retention. They are capable of creating an optimal microenvironment, promoting the formation of blood vessels and nerve fibers, and gradually degrading, thereby allowing the newly formed pulp to integrate with the surrounding tissues [34, 35]. Many authors report that the use of biocompatible porous scaffolds together with stem cells can be safe and effective for the regeneration of pulp tissue and the restoration of tooth vitality. For example, the development of injectable matrices compatible with stem cells, growth factors, and other bioactive compounds has made it possible to create functional dental pulp capable of secreting dentin in both preclinical and clinical studies [36, 37].

Based on the results of these studies, it can be assumed that dental pulp regeneration using microcarriers in combination with stem cells is one of the key approaches in modern regenerative dentistry. However, despite obtaining encouraging results, the use of viable stem cells in pulp regeneration is limited by several factors, including the need for strict control of culture conditions, short storage periods, and risk of immune reactions, unwanted differentiation, and the possible formation of tumor-like structures [38]. These limitations emphasize the need to develop alternative approaches capable of ensuring safe and predictable pulp regeneration, one of which is the freeze-drying of cellular material.

At present, the technology of freeze-drying has achieved significant progress and is widely applied in pharmaceuticals, medicine, and biotechnology. As we described earlier [39], freeze-dried cells and tissues have been used in preclinical and clinical studies for many decades, including in the regeneration of bone tissue, cartilage, meniscus, human dermis, and other tissues [40-44]. Based on the above, we hypothesized that the creation of a biocompatible composite consisting of porous microcarriers loaded with freeze-dried stem cells and a fibrinogen-thrombin gel would make it possible to reproduce a biologically active environment that facilitates cellular repopulation and regeneration of damaged pulp, followed by the formation of a functional pulp-dentin complex. Unfortunately, the literature contains no reports on the use of freeze-dried stem cells in regenerative endodontics, which underscores both the relevance and novelty of our study, while at the same time complicating its scientific justification and planning. We believe that this study may attract the attention of the scientific community and initiate discussion on the possibility of applying freeze-dried stem cells for the restoration of functional, fully viable dental pulp.

Materials and methods

Animals and experimental design

The studies were conducted using both Lewis rats, domestic Turkish sheep and isolated mandibular teeth from porcine. Rats (n = 30), weighing 200-250 g, were acquired from the vivarium of Tbilisi State University (Tbilisi, Georgia) and maintained under controlled conditions at 24± 2°C with a 12-hour light-dark cycle, provided with rodent food and water ad libitum. Of the total number of rats, 10 were used as donors for bone marrow-derived stem cell isolation. These animals were euthanized by intraperitoneal administration of a lethal dose of thiopental. The remaining 20 rats were used as recipients for in vivo experiments.

All experimental procedures, except for euthanasia, were performed under general anesthesia induced by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (9 mg/kg).

Sheep (n = 6) weighing 15-20 kg were acquired from a certified breeding facility in Georgia and housed individually. Anesthesia was induced with intramuscular atropine (0.03-0.05 mg/kg) and diazepam (0.5 mg/kg), followed by ketamine (20 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Postoperative analgesia was provided via intramuscular ketoprofen (1 mg/kg) immediately after surgery and through the next 3 days. Animals were monitored for recovery from anesthesia to ensure the return of normal activity.

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The animal use protocol was reviewed and approved by the independent ethics committee of Tbilisi State University (Georgia). Porcine teeth used in this study were obtained from a licensed slaughterhouse as by-products of the food industry. No animals were euthanized specifically for research purposes. According to institutional and national regulations, the use of such materials does not require additional ethical approval.

Tissue collection

Twenty lower molars from porcine aged 8 months to 1 year, obtained at a slaughterhouse in Tbilisi, Georgia, were used to create a decellularized construct (slice). The extracted teeth were treated with phosphate-buffered saline (PBS) and disinfected with a 0.5% chloramine solution for 3 hours at a room temperature. The teeth were then rinsed with cold running water and sectioned longitudinally using a diamond-coated disc under water cooling. The sections were then thoroughly rinsed in PBS containing antibiotics (three times for 10 minutes) and decellularized.

Decellularization of tooth tissues

The decellularization process began with a freeze-thaw cycle. All tooth slices were placed in a freezer at -20°C. After 24 hours, the teeth were thawed at +4°C. The tooth slices, along with the pulp, were then placed in a 0.1% sodium dodecyl sulfate (SDS) solution and incubated for 24 hours at 37°C on a magnetic stirrer set at 100 rpm, and then, transferred to a 1% SDS solution and incubated under the same conditions for another 24 hours. Finally, the tooth slices were placed in a 10% SDS solution and incubated again for 24 hours at 37°C on a magnetic stirrer set at 100 rpm. After SDS treatment, the tooth slices were washed with PBS (3 times for 15 minutes) to remove residual SDS, then placed in 1% Triton X-100 solution (Sigma) and incubated for 4 hours at 37°C on a magnetic stirrer set at 100 rpm, and followed by washing with PBS (3 times for 15 minutes) to remove Triton X-100. Then, following the previously published decellularization method [45], the tooth slices were treated with DNase I (50-100 units/ml), RNase A (0.1 mg/ml) and PBS containing 5 mM $MgCl_2$, and incubated at 37°C on a magnetic stirrer at 100 rpm for 1 hour. After enzymatic treatment, the tooth slices were washed 3-5 times with PBS until the enzymes were completely removed. After washing, the decellularized tooth slices were freeze-dried in a Power Dry PL 6000 freeze dryer (Shenzhen, China) and stored at room temperature under sterile conditions until use.

Method for determining the depth of detergent penetration into dental tissues

Methylene blue was used to assess the depth of diffusion of 1% and 10% SDS solutions in dental tissues during decellularization at 24, 48 and 72 hours. We modified the previously described method for analyzing methylene blue dye penetration in human radicular dentin using different impregnation methods [46].

Samples of decellularized, longitudinally sectioned porcine teeth were immersed in 1% methylene blue for 20 minutes at room temperature. Afterwards, the samples were embedded in paraffin and longitudinal sections of 50-100 μm thickness were prepared to cover the entire tissue radius.

Quantitative determination of DNA in dental tissues

DNA was extracted from dental samples before and after decellularization using a commercial kit (G-spin Total DNA Extraction Mini Kit; iNtRON

Biotechnology, Inc., Seongnam, South Korea). Total DNA content was measured using a spectrophotometer at 260 nm (NanoDrop 1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Rat BMSCs isolation protocol and loading method onto the microcarrier

To isolate bone marrow-derived stem cells, rats of both sexes weighing 200-250 g were used in accordance with previously described protocols [47, 48]. The materials and reagents included porous microcarriers (Cultispher), calcium- and magnesium-free PBS, DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10-20% Fetal Bovine Serum (FBS), penicillin/streptomycin, 70% ethanol, and sterile instruments. All reagents were purchased from Sigma-Aldrich.

Animals were euthanized in accordance with ethical standards by intraperitoneal injection of a lethal dose of sodium thiopental (200 mg/kg). After treatment with 70% ethanol, the lower limbs were amputated, and following removal of muscle tissue, the femurs were dissected at the epiphyseal and diaphyseal regions. A 25G needle was inserted into the proximal end of the bone and flushed with a syringe filled with DMEM medium. The procedure was repeated 2-3 times until the bone became white. The resulting bone marrow suspension was filtered through 70 μm meshes to remove large fragments. The mononuclear cell fraction was isolated by density gradient centrifugation at 400×g for 10 minutes at room temperature using Ficoll-Paque Premium (GE Healthcare Bio-Sciences). After washing with PBS, the cells were centrifuged again at 200×g for 5 minutes at 24°C. The supernatant was discarded, and the pellet was resuspended in DMEM supplemented with 10-20% FBS and antibiotics. A small portion of the isolated stem cells was freeze-dried and stored under sterile conditions at room temperature until further use. Another portion of stem cells was prepared for co-cultivation with microcarriers. First, commercially obtained microcarriers were sterilized in an autoclave at 121°C for 15 minutes according to the manufacturer's recommendations. The sterilized microcarriers were then rehydrated in sterile PBS solution. Simultaneously, 2×10^6 BMSCs were suspended in 1-2 mL of medium. Sterile microcarriers (0.1 g) were combined with BMSCs in 12 wells of a Falcon 24-well Companion Plate (Falcon; Corning Life Sciences) and incubated at 37°C with 5% CO_2 and 90% humidity for 1 hour. The culture medium consisted of DMEM, 10% FBS (MilliporeSigma), 50 U/mL penicillin, and 0.05 mg/mL streptomycin. The plate was placed on an orbital shaker (110 rpm) inside the incubator, and the cells were co-cultured with microcarriers for 7 days. The culture medium was changed every 3 days. After cultivation, microcarriers loaded with BMSCs were stained with the red fluorescent dye PKH26 and 4',6-diamidino-2-phenylindole (DAPI) according to the manufacturer's instructions. Finally, the microcarriers loaded with BMSCs were subjected to freeze-drying in a lyophilizer and stored under sterile conditions at room temperature until further use.

Freeze-drying process of microcarriers loaded with rat BMSCs

The freeze-drying process was performed in two stages: deep freezing followed by thawing in vacuum [49]. Microcarriers loaded with rat BMSCs were freeze-dried using a lyophilizer (Heto PowerDry PL6000 Freeze Dryer; Sjia Lab, Shenzhen, China). The lyophilizer temperature was set to -40°C, and the vacuum was maintained at 10-15 Pa. The sublimation (thawing) phase lasted 18-24 hours, during which the chamber temperature was gradually increased to 15-20°C at a rate of 0.2°C/min and maintained for 6-8 hours. After freeze-drying, microcarriers containing BMSCs were placed in sterile disposable bags and stored under sterile conditions at room temperature until use.

The model of subcutaneous implantation of bioengineered dental constructs in rats

The bioengineered dental construct was prepared 2 hours prior to transplantation. Under sterile conditions, the decellularized, freeze-dried tooth slices were rehydrated in saline for 40 minutes. Simultaneously, microcarriers loaded with freeze-dried rat BMSCs were rehydrated in saline for 20 minutes and embedded in the fibrinogen component of commercially available Tisseel adhesive. The developed gel was prepared by adding 2 IU/ml thrombin. The resulting bioengineered pulp was immediately injected into the pulp chamber and root canals.

The slices, containing the bioengineered pulp, were then incubated at 37°C for 10 minutes to allow complete gel polymerization. The constructs were then transplanted subcutaneously into the animals, and the wounds were closed layer by layer using interrupted sutures.

Controlled pulp amputation and tissue restoration in an ovine model

Prior to bioengineered pulp transplantation, a model of partial, controlled pulp amputation was established in all animals. The teeth were polished and rinsed with 0.2% chlorhexidine gluconate solution. Coronal access cavities were prepared under constant water cooling using a high-speed diamond spherical bur (801L/012, DIA.TESSIN, Switzerland). Then, a partial pulpotomy was performed with a diamond conical bur with a round end (F850/014, DIA.TESSIN, Switzerland), removing approximately 7-8 mm of coronal pulp. Hemostasis was achieved by copious irrigation with sterile 0.9% saline solution until bleeding ceased. After pulpotomy, microcarriers containing freeze-dried rat BMSCs were rehydrated and gently mixed with a fibrinogen fraction (0.03 g per 0.2 ml). The resulting suspension was injected into the root canal using a 25-gauge needle under gentle pressure. A thrombin fraction was then injected through a new 25-gauge needle to initiate in situ fibrinogen polymerization and stabilize the cell-containing microcarriers. The root canal orifice was then sealed with a 2 mm thick layer of Biodentine™ (Septodont, USA), according to the manufacturer's instructions. After an initial curing period of approximately 12 minutes, the coronal restoration was completed using a light-cured resin-based composite (Asteria, Tokuyama Dental, Japan) following manufacturer's recommended protocol.

Postoperative care

All animals were maintained under standard laboratory conditions with a normal light-dark cycle and were provided with rodent food and water ad libitum. Rats were euthanized at 5, 14, 30 and 60 days postoperatively via intraperitoneal injection of ketamine and xylazine, followed by laparotomy, aortic rupture, and exsanguination. Sheep were euthanized at 10, 20, and 30 days postoperatively by intravenous administration of a lethal dose of sodium thiopental.

Scanning electron microscopy

The longitudinally sectioned tooth fragments, including the pulp, were immersed in a fixative solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer both before and after decellularization. After fixation, the specimens were dehydrated in a series of ethanol solutions of various concentrations, followed by immersion in a 1:1 mixture of 95% ethanol and isoamyl acetate for 10 minutes, and then in pure isoamyl acetate for 15 minutes. After the removal of the isoamyl acetate, the specimens were dried in a Tousimis Samdri-780 Critical Point dryer (Tousimis Research Corporation). The specimens were then sputtered-coated with gold and examined using a JEOL JSM-6510LW scanning electron microscope (JEOL, Ltd.).

Histopathological study

For histopathological analysis, tooth slice samples, as well as sheep tooth samples, were collected at various time points after transplantation, along with surrounding tissues, and fixed in 10% neutral buffered formalin. The samples were then embedded in paraffin and sectioned at 5 μm thickness. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome in accordance with the manufacturer's protocols. Immunostaining with antibodies against Ki-67 (incubation time 20 min; clone MM1; dilution 1:200; Leica Biosystems Newcastle Ltd.) and VEGF (incubation time 30 min; clone JH121; dilution 1:200; Abcam [ab2350]) was performed manually using the Novolink DAB Polymer Detection System (incubation time 20 minutes; cat. no. RE7260-CE; Leica Biosystems Newcastle Ltd.) in accordance with the manufacturer's instructions. Endogenous peroxidase activity was neutralized with Peroxidase Block reagent [3-4% (v/v) hydrogen peroxide; Novolink DAB Polymer Detection System; cat. no. RE7260-CE; Leica Biosystems Newcastle Ltd.]. Novocastra Protein Block was used as the immunohistochemical reagent (0.4% casein in phosphate-buffered saline with stabilizers, surfactant, and 0.2% Bronidox L as a preservative; Novolink DAB Polymer Detection System) to reduce non-specific binding of the primary antibody. The secondary antibody was rabbit anti-mouse IgG (< 10 μg/mL) in 10% (v/v) animal serum in Tris-buffered saline/0.1% ProClin™ 950 (Novolink DAB Polymer Detection System). All immunohistochemical reactions were performed at room temperature. The samples were examined under a light microscope. No quantitative scoring system was applied for histological evaluation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10.5.0 (GraphPad Software, Inc.). All quantitative data are presented as mean ± standard deviation (SD). Normality of data distribution was assessed using the Shapiro-Wilk test. DNA content was quantified at each time point following decellularization and compared between groups. Differences among three groups at the 24-hour time point were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Comparisons between the 1% and 10% SDS groups at 48 and 72 hours were performed using an unpaired two-tailed Student's t-test. A p-value of < 0.05 was considered statistically significant. Native tissue samples incubated under identical conditions without SDS treatment were used as controls. Since no decellularization reagents were applied, DNA content in control samples remained unchanged across all time points and is shown as a baseline reference at 24, 48, and 72 hours.

Results

All in vivo observations were descriptive in nature. The studies have shown that all animals tolerated the surgery and postoperative period well. Animals remained active and ate normally, without restrictions.

One-week post-transplantation, all rat (n = 20) have shown mild subcutaneous swelling at the transplantation site, consistent with a normal tissue response. The skin color at the wound site appeared light pink, with no signs of significant inflammation. The wound healed by primary intention in all rats. The absence of purulent discharge or necrosis of the wound edges indicated good biocompatibility of the decellularized dental slice material. A well-vascularized fibrous capsule was observed around the slice. Fibrin-thrombin gel degraded within 12 days and was gradually replaced by connective tissue with newly formed small vessels. During this period, a moderate number of macrophages and lymphocytes was observed, indicating progression to the remodeling phase. No acute inflammatory reaction was observed, further confirming the biocompatibility of the bioengineered pulp. For up to 20 days, the microcarriers were tightly surrounded by connective tissue, showing gradual degradation. During this period, BMSCs were virtually undetectable on the surface of the microcarrier. Hematoxylin and eosin, as well as Masson's trichrome staining, revealed that the newly formed fibrous tissue was rich in collagen fibers. Within one month, the fibrin matrix was partially lysed and replaced by young granulation tissue, containing newly formed vessels with a developing vascular network. In some areas, thin lines of mineralized matrix deposits (predentin or osteoid-like tissue) were observed near the slice walls. By four weeks, organized, vascularized, and partially mineralized tissue resembling fibrovascular tissue had formed, featuring a prominent capillary network (**Figure 1**).

The sheep studies have shown that the animals (n = 6) remained active and ate normally without restriction. One week after the transplantation of the bioengineered pulp, the crowns of the teeth appeared intact. Histological analysis revealed that 5-8 days after partial pulpotomy and filling of the pulp chamber and root canal with the bioengineered pulp, all sheep developed a small hematoma and lymphomacrophage infiltration. During this same period, the fibrin-thrombin gel underwent gradual resorption, forming numerous fibrin fibers with microcarriers positioned between them. After two weeks, the canal lumen was filled with basophilic pulp-like tissue containing newly formed vessels of varying diameters. Migration of stem cells from the microcarriers to the walls of the dentinal tubules was observed, and a smooth layer of young odontoblast-like cells lined dentin was observed on the canal walls. After three weeks, the fibrinogen-thrombin gel was completely lysed and replaced by connective tissue structure. At the border with dentin, a compacted cellular layer morphologically resembling early odontoblast-like cells was observed. This indicated a formation of organized, vascularized, and partially mineralized tissue resembling native pulp. After 4 weeks, the root canal was completely occupied with newly formed pulp-like tissue with newly formed vessels and a prominent capillary network. A homogeneous cell population, predominantly fibroblasts and odontoblast-like elements, was also observed.

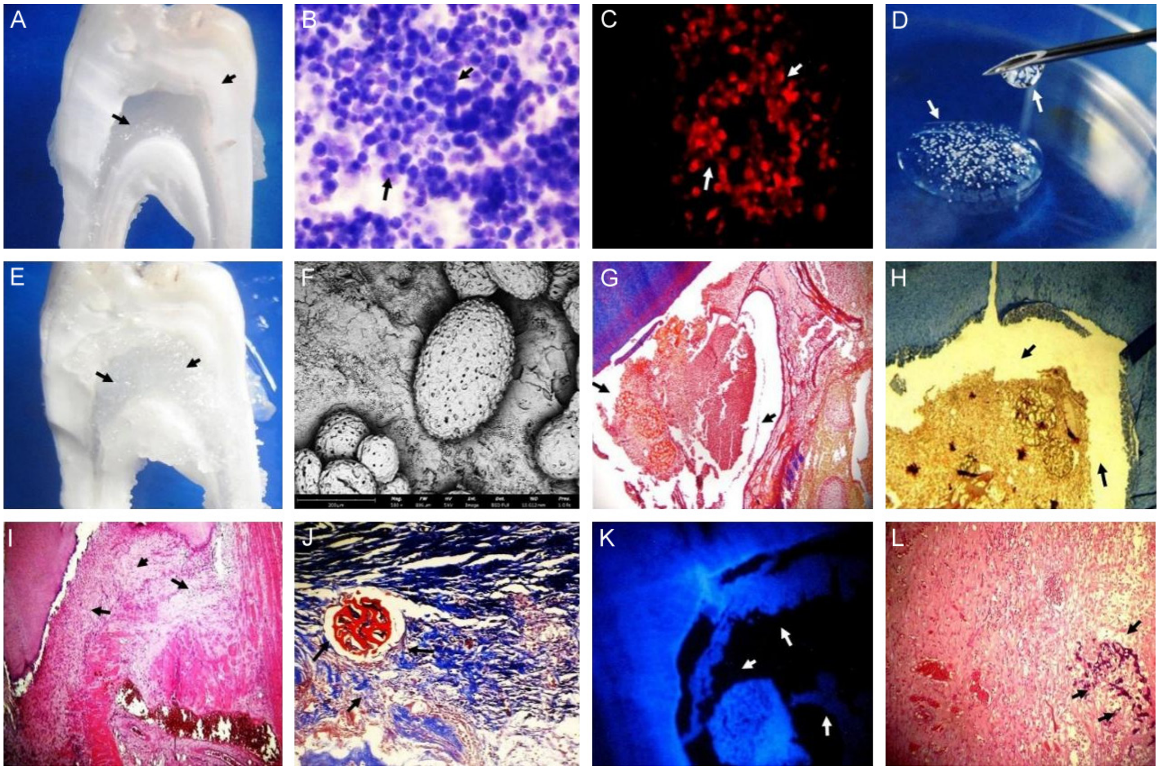

Figure 1. Formation of a bioengineered tooth construct and results of ectopic transplantation. A. Longitudinally dissected porcine tooth fragment after decellularization. B. Freeze-dried BMSCs after rehydration with Giemsa staining. C. Cultispher-loaded BMSCs. PKH26 Red Fluorescent cell linker staining. D. Cultispher-loaded BMSCs embedded in fibrinogen. E. Cultispheres with BMSCs and fibrinogen-thrombin gel placed into a canal lumen of tooth fragment. F. Cultispher-loaded BMSCs. Scanning electron microscopy (SEM). G, H. Cultispheres surrounded by fibrinogen-thrombin gel. H&E and Masson's trichrome staining. Magnification x 200. Observation period - 10 days. I. Established vascularized fibrous tissue with well-defined newly formed vessels. H&E staining. Magnification x 400. Observation period - 20 days. J. Cultispheres surrounded by fibrous tissue. Masson's trichrome staining. Magnification x 400. Observation period - 10 days. K. Fluorescent microscopy. Established fibrous tissue around the Cultisphere. DAPI staining. Magnification x 200. Observation period - 10 days. L. Cultisphere degradation process. H&E staining. Magnification x 400. Observation period - 15 days.

A continuous layer of mature odontoblast-like cells was observed along the canal walls, beneath which a continuous zone of new predentin was visible. The central part of the pulp-like tissue contained loose connective tissue with vessels, collagen fibers, and sparse macrophages. No necrotic changes were observed in the apical region. The histological examination also revealed the formation of a dentinal bridge. Immunohistochemistry studies demonstrated both high VEGF and Ki67 expression in the newly formed pulp-like tissue of the tooth indicating active angiogenesis and cell proliferation (**Figure 2**).

Discussion

At present, dental pulp stem cells (DPSC) are considered the most promising cells for pulp bioengineering due to their odontogenic potential, angiogenic properties, and immunocompatibility [50-52]. However, BMSCs remain a good alternative, particularly in cases where DPSC are unavailable [53-56]. With regard to induced pluripotent stem cells (iPSC), they represent a promising direction in regenerative dentistry; nevertheless, their application is limited. For example, there is a risk of teratogenicity and tumor formation, and the differentiation of iPSC specifically into odontoblast-like or pulp fibroblast-like cells is extremely challenging [57, 58]. Stem cells derived from adipose tissue (ASC) and stem cells from exfoliated human deciduous teeth (SHED) have also been used for pulp regeneration [59]. However, their use is constrained by several issues, such as incomarroplete odontogenic differentiation,

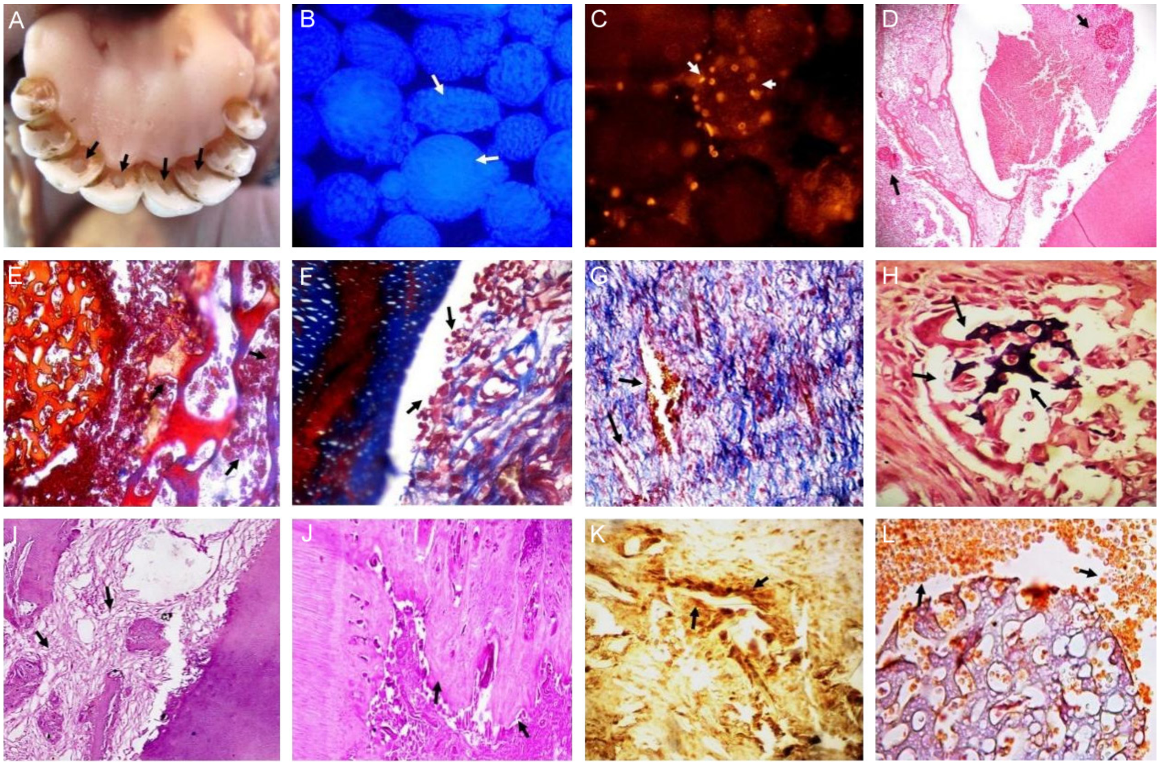

Figure 2. Controlled pulp amputation and tissue restoration in an ovine model. A. The process of controlled partial amputation of the dental pulp. B. Isolated microcarriers loaded with BMSCs stained with 4',6-diamidino-2-phenylindole (DAPI); C. PKH26 Red Fluorescent cell linker staining. D. Fibrinogen-thrombin gel undergoing gradual resorption, forming numerous fibrin fibers with microcarriers located between them. Observation period - 15 days. H&E staining. Magnification ×200. E. Migration of cells from the surface of microcarriers towards the dentin walls. Observation period - 20 days. Masson's trichrome staining. Magnification ×800. F. A compacted cell layer morphologically similar to early odontoblast-like cells observed at the dentin border. Masson's trichrome staining. Magnification ×800. G. Newly formed pulp-like tissue. Visible well-developed vessels. Masson's trichrome staining. Magnification ×1000. Observation period - 28 days. H. Microcarriers undergoing gradual resorption. H&E staining magnification ×800. Observation period - 12 days. I. Gradual filling of the canal lumen with newly formed pulp-like tissue. H&E staining. Magnification ×400. Observation period - 25 days. J. Formation of a dentin bridge with non-mineralized remains. H&E staining. Magnification ×800. Observation period - 30 days. K. VEGF expression in newly formed dental pulp-like tissue. L. Ki-67 expression in newly formed dental pulp-like tissue.

potentially insufficient vascularization of the regenerated tissue, and variability of outcomes depending on the source and quality of the cell material. In contrast, BMSCs are well studied and supported by an extensive database of clinical applications [60-64]. For instance, BMSCs secrete various paracrine factors, including cytokines and chemokines (interleukin-6 [IL-6], interleukin-8 [IL-8], and tumor necrosis factor alpha [TNF-α]), as well as growth factors (vascular endothelial growth factor [VEGF], transforming growth factor beta [TGF-β], platelet-derived growth factor [PDGF], among others) [65].

Currently, stem cell-based strategies for dental pulp regeneration have been investigated in both preclinical and clinical studies. For example, several authors have reported that the regeneration of vascularized, pulp-like tissue was achieved using stem cell transplantation strategies in animal models [66-68]. Clinical studies have likewise reported successful stem cell transplantation, showing favorable outcomes [69, 70]. However, we did not find studies concerning the use of freeze-dried stem cells for dental pulp regeneration. Therefore, in this study our focus was directed toward freeze-drying technology, which has been successfully applied for the preparation of freeze-dried plasma, erythrocytes, leukocytes, platelets, fibroblasts, as well as freeze-dried mesenchymal stem cells (MSC), secretomes, exosomes, liposomes [71-73], and other cellular products. In

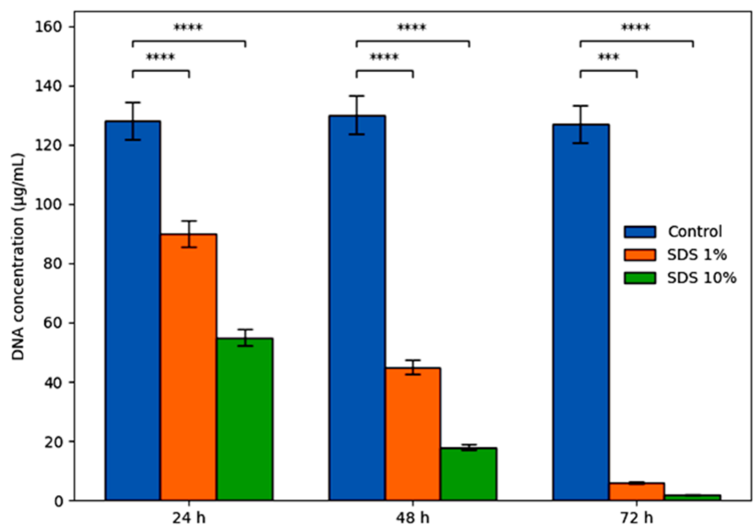

Figure 3. DNA concentration (μg/mL) in control and SDS-treated samples (1% and 10%) measured after 24, 48, and 72 h. Data are presented as mean ± standard deviation (SD), with SD corresponding to 5% of the mean value in each group (n = 5). Error bars indicate SD. Statistical significance between the control and SDS-treated groups at each time point is shown directly on the graph using asterisks ($P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$).

our previous work, we reported that following freeze-drying, BMSCs retained more than 53% of their paracrine factors [74]. Similar data were provided by other authors, who noted that after freeze-drying, most bone marrow stem cells retain their viability without the addition of protective substances [75, 76].

Our studies demonstrated that freeze-dried BMSCs, when transplanted into the root canal, act as biological stimulators of regeneration by creating a microenvironment favorable for vascular growth and invasion of the host's own cells. The results also showed that the primary function of freeze-dried BMSCs is not direct tissue replacement, but rather paracrine stimulation of regeneration through the preservation of biomolecules, extracellular vesicles, and structural components of the cellular matrix. These cells activated the migration and differentiation of host cells, thereby promoting neoangiogenesis and the formation of pulp-like tissue within the root canal. It is particularly important to highlight the role of the fibrinogen-thrombin gel in combination with microcarriers loaded with freeze-dried BMSCs, which creates a more functional bioengineering system for dental pulp-like tissue regeneration. Despite these encouraging results, more detailed and extensive studies are required to develop effective protocols for constructing bioengineered dental pulp. This prompted us to conduct a study of ectopic transplantation of a bioengineered dental construct into the backs of rats. This model allowed us to evaluate the biocompatibility and safety of microcarriers loaded with rat freeze-dried BMSCs, as well as to assess the host tissue response. However, one of the challenges in creating a bioengineered dental construct was the simultaneous, uniform, and complete removal of all cellular components from dental tissue. It is well known that enamel, dentin, cementum, and dental pulp differ in density, permeability, and structure, which complicates effective cell removal. Authors have reported that the density of porcine enamel ranges from 2.8 to 3.1 g/cm^3, dentin from 2.0 to 2.2 g/cm^3, and cementum from 1.9 to 2.1 g/cm^3 [77, 78]. Studies have shown that the number of cells removed during decellularization of longitudinal sections of porcine teeth using freeze-thaw cycles and SDS/Triton X-100 solutions depends on several critical factors: detergent concentration, stirring speed, fragment size, solution temperature, and exposure time. To achieve complete single-step removal of all cells from a longitudinally sectioned porcine tooth fragment, we optimized the decellularization method. The optimal SDS exposure time for complete cell removal was 72 hours (24 + 24 + 24) at 37.0°C with a magnetic stirrer speed of 100 rpm. The fragment was then incubated in 1% Triton X-100 (Sigma) for 4 hours at 37°C under the same stirring conditions. Subsequent treatment involved DNase I (50-100 U/mL), RNase A (0.1 mg/mL), and PBS containing 5 mM $MgCl_2$. Our studies demonstrated that the residual DNA content in fragments decellularized by this method did not exceed 2% of the initial amount. Quantitative analysis of residual DNA content demonstrated a time- and concentration-dependent effect of SDS treatment (**Figure 3**). After 24 hours, one-way ANOVA revealed significant differences between all experimental groups (control, 1% SDS, and 10% SDS; $P < 0.0001$). Residual DNA content was significantly lower in the 10% SDS group compared to the 1% SDS group at both 48 hours ($P < 0.0001$) and 72 hours ($P < 0.05$). Following 72 hours of decellularization, the residual DNA content did not exceed 2% of the initial value.

Detergent penetration analysis showed that after decellularization of longitudinal porcine tooth sections, methylene blue staining of decellularized areas was weak or absent, indicating effective removal of cells and nuclear material. Additional hematoxylin and eosin staining improved visualization of the extracellular matrix (ECM) and collagen fibers.

Our studies also demonstrated that microspheres loaded with BMSCs, when transplanted into root canals in pulp injury models, function as a fully-fledged three-dimensional scaffold. Their porous structure ensures high cell adhesion, while facilitating the controlled release of paracrine factors that play a crucial role in activating regenerative processes and restoring damaged pulp tissue. Consistent findings have been reported by other studies, emphasizing that stem cell-loaded microspheres are central to pulp bioengineering, functioning simultaneously as a three-dimensional scaffold and a biologically active microenvironmental niche. The introduction of such microspheres combined with stem cells into a biocompatible gel matrix forms a biologically active construct capable of mimicking the natural dental pulp niche and supporting structural and functional restoration.

Thus, the effective use of microcarriers loaded with freeze-dried BMSCs in combination with a fibrinogen-thrombin gel demonstrates significant potential for the regeneration of mechanically damaged or partially amputated dental pulp. This combination ensures high cell viability and differentiation, the formation of a three-dimensional matrix that supports the growth and integration of new tissue, as well as the local delivery of signaling molecules that promote the restoration of the functional pulp-dentin complex. The results obtained confirm the potential of this approach for the development of bioengineering strategies for pulp-dentin complex regeneration.

Conclusion

Our findings demonstrate the robust efficacy of microcarriers seeded with freeze-dried BMSCs, in conjunction with a fibrinogen-thrombin gel, for regenerating damaged dental pulp. The composite we developed, when introduced into the root canal, acted as a biological stimulator of regeneration by creating a microenvironment favorable for angiogenesis and invasion of the host's own cells. The results demonstrated that the primary function of freeze-dried BMSCs was not direct tissue replacement, but rather paracrine stimulation of regenerative processes through biomolecules, extracellular vesicles, and structural components of stem cells preserved after freeze-drying. These biologically active components activated the migration and differentiation of endogenous host cells, promoting vascular network formation and the development of pulp-like tissue within the root canal. Thus, the proposed composite shows significant potential for the further advancement of regenerative endodontic technologies. However, to refine strategies for bioengineered pulp formation and to enhance the effectiveness of the developed composite, further studies are required to elucidate the role of stem cell-derived paracrine factors

Acknowledgements

This research was funded by the Shota Rustaveli National Science Foundation of Georgia (SRNSFG) [grant number: YS-23-1748].

Disclosure of conflict of interest

None.

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