Archives of Oral Biology 82 (2017) 302–309

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Research paper

Regenerative capacity of allogenic gingival margin- derived stem cells with fibrin glue on albino rats' partially dissected submandibular salivary glands

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ARTICLE INFO

Keywords:
Gingival margin-derived stem cells
Autologous fibrin glue
Submandibular salivary gland
Proliferating nuclear cell antigen
Alpha smooth muscle actin
Histomorphometry

ABSTRACT

Objective: To evaluate the possible regenerative effect of allogenic gingival margin-derived stem cells (GMSCs) with or without autologous fibrin glue on partially dissected submandibular salivary glands of albino rats.
Methods: Forty rats were randomly divided into four equal groups. Group I, where no operation was performed, was considered the negative control. Group II rats were considered the positive control and were subjected to a rectangular cut on the outer surface of the center right of the submandibular salivary gland and received no other treatment. Groups III and IV rats were handled as those in group II, but the cut areas of group III were filled with fibrin glue and the cut borders of group IV were injected with 1×10^5 cell/ml GMSCs and then glued with fibrin glue. Five animals from each group were euthanized at the end of the first postoperative week, while the remaining animals were euthanized at the end of the second postoperative week, i.e., end of the experiment.
Results: Regeneration of ductal, acinar, and myoepithelial cells was better in group IV. A two-way ANOVA for proliferating cell nuclear antigen and α-smooth muscle actin revealed an overall significant difference between the different groups ($P < 0.05$). In addition, an LSD post hoc test for multiple comparisons revealed a significant difference between each two groups. An independent sample *t*-test revealed significant differences between time periods for groups II, III, and IV, but there were no significant differences between the time periods for group I.
Conclusion: Injecting GMSCs at the cut borders and gluing the cut area with autologous fibrin glue ameliorates the regeneration of partially dissected submandibular salivary gland better than using fibrin glue alone.

1. Introduction

Saliva is very important for maintaining the health of the oral cavity, and consequently, any disease affecting salivary glands will affect the amount of saliva secreted and the oral health. Hyposalivation and xerostomia can be very annoying problems that decrease the patient's quality of life (Thomson, Lawrence, Broadbent, & Poulton, 2006). Hyposalivation is evident in some diseases such as Sjögren's syndrome and ectodermal dysplasia and as a side effect of some drugs (Berk, 2008; Nordgarden, Storhaug, Lyngstadaas, & Jensen, 2003). It could be associated with certain medical conditions such as in the case of head and neck malignancies treated by radiotherapy (Jensen et al., 2010).

Damage to the salivary glands in the abovementioned situations is almost permanent, and the only approved treatment is symptomatic. Artificial saliva, sialagogues, and improvements in lifestyle failed to manage the condition completely (Vissink et al., 2010). Moreover, sialagogues have many adverse effects such as excessive tearing, chills, flushing, dizziness, excessive sweating, stuffy nose, voice change, nervousness, tremor, and diarrhea (Jellema, Slotman, Doornaert, Leemans, & Langendijk, 2007). Therefore, recent research in this field aims to find an effective regenerative therapy for salivary gland disorders to completely overcome the problem of xerostomia.

Mesenchymal stem cells (MSCs) are multipotent adult stromal cells that have several advantages as a recent therapeutic option. These advantages include minimum donor-site morbidity compared to auto grafts, superiority of regenerating damaged tissues without forming fibrous tissue, and low risk of disease transmission and autoimmune rejection. Therefore, MSCs represent a valuable resource for tissue engineering as it has the capability to self-renew at a high rate of proliferation and differentiate into multiple cell lineages including ectodermal, mesodermal, and endodermal cells (Grover, Bhardwaj, & Gupta, 2014; Hashemian, Kouhnavard, & Nasli-Esfahani, 2015).

During general dental treatments, the gingiva is frequently resected from the region overlying the retromolar area and alveolar ridges and

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http://dx.doi.org/10.1016/j.archoralbio.2017.06.030
Received 25 March 2017; Received in revised form 22 June 2017; Accepted 23 June 2017
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can often be routinely obtained as a discarded biological sample. In 2009, Zhang et al. were the first to characterize human gingiva-derived MSCs (GMSCs), which exhibited clonogenicity, self-renewal, and a multipotent differentiation capacity similar to that of bone marrow-derived MSCs (BMSCs) (Zhang et al., 2009). GMSCs display a stable morphology, do not lose their MSC characteristics with extended passaging, and proliferate faster than BMSCs. The availability of gingival connective tissue with minimal complications for the donor makes it a good source of MSCs for cell-based regenerative therapies (Mitrano et al., 2010).

Fibrin is a major component of extracellular matrix microenvironment. It has been reported that fibrin enhances angiogenesis of wound healing *in vitro* and *in vivo* (Feng, Tonnesen, Mousa, & Clark, 2013). Fibrin glue has been widely accepted as a cell delivery vehicle to improve the therapeutic effects of MSCs. It has been proved that fibrin glue provides many advantages for MSCs because MSCs in fibrin glue successfully suppress immune reactions, thus protecting themselves from oxidative stress (Kim et al., 2013). In addition, fibrin glue reduces blood loss, protects against bacterial infection, and accelerates wound healing (Buchta et al., 2004). Mehanna et al. (2015) investigated the effect of BMSCs and their conditioned media (CM) topically delivered in fibrin glue on chronic wound healing in rats. They concluded that albeit without true acceleration of wound closure, the topically applied BMSCs and their CM through fibrin vehicle could effectively improve the quality of healed skin in chronic excisional wounds.

In this context, GMSCs may enable the reconstruction of partially dissected submandibular salivary gland in rats. The primary outcome was to evaluate the possible regenerative effect of allogenic GMSCs with or without autologous fibrin glue on partially dissected submandibular salivary glands of albino rats. If the primary outcome was achieved, the secondary one was to indirectly test the function of the regenerated salivary gland with α-smooth muscle actin (α-SMA). The null hypothesis was that there was no effect of GMSCs in combination with fibrin glue on the regenerative capacity of partially dissected submandibular salivary glands in albino rats.

2. Materials and methods

Forty pathogen-free albino rats, weighing 200–250 g, were selected. All experimental procedures were performed under the protocol approved by the Ethical Committee of Faculty of Dentistry, Mansoura University, and the animal house unit of Nile Center for Experimental research, Mansoura city, Egypt. The rats received water ad libitum and a standard pelleted diet and were kept in a 12 h light/dark cycle.

2.1. Study design

The sample size was determined using G* Power 3.1.9.2 software. The test family was F test, the statistical test was ANOVA (fixed effects, special, main effects, and interactions), and the type of power analysis was to compute the required sample size, given the following: α, power, and effect size. The input parameters were effect size, 0.50; α error probability, 0.15, and power (1-β error probability), 0.80. The numerator degree of freedom was 10, and the number of groups was 4. The total sample size was approximately 40.

The rats were randomly divided into four equal groups. Group I, where no operation was performed, was considered the negative control. Group II rats served as the positive control and were subjected to a rectangular cut by a custom-made tissue punch of dimensions 5 mm width × 8 mm length × 1.5 mm depth on the outer surface of the center of the right submandibular salivary gland (Fig. 1A) and received no treatment. Groups III and IV rats were handled as those in group II; however, the cut areas of group III were filled with autologous fibrin glue, whereas the cut borders of group IV were injected with 1×10^5 cell/ml GMSCs, and the cut areas were glued with fibrin glue. Five animals from each group were euthanized at the end of the 1st week, while the remaining animals were euthanized 2 weeks postoperatively, at the end of the experiment. All surgical steps were performed in the Surgical Unit of Nile Center for Experimental Research, Mansoura city, Egypt.

2.2. Isolation and culture of GMSCs

All *in vitro* steps were performed in the Stem cell Unit of Nile Center for Experimental research, Mansoura city, Egypt. Gingival stem cells were isolated according to a protocol described by Zhang et al. in (2009). Briefly, rat tissue samples were collected from clinically healthy gingiva after euthanization of four pathogen-free albino rats weighing 80–150 g. The samples were treated aseptically and incubated overnight at 4 °C with dispase (2 mg/ml; Sigma-Aldrich) to separate the epithelial and lower spinous layer. Then the remaining connective tissues were minced into 1–3 mm^2 fragments and digested at 37 °C for 2 h in sterile phosphate-buffered saline (PBS) containing 4 mg/ml collagenase IV (Worthington Biochemical). The samples were then grown in Dulbecco's Modified Eagle's medium Ham's F-12 (DMEM-F 12) with 10% fetal bovine serum (FBS) and 1% streptomycin, penicillin, and amphotericin. The explants were maintained in the incubator at 37 °C and 5% CO_2 humidified air. Confluence was reached 80% on days 14–21 of cell cultures, after which the cells were trypsinized with trypsin and subcultured (Fig. 1B).

2.3. Characterization

The immunophenotype was determined by flow cytometry. A total of 5×10^5 gingival cells were incubated with individual fluorescein isothiocynate (FITC)-conjugated primary monoclonal antibodies in 100 μl PBS for 20 min in the dark at room temperature. The primary antibodies used were CD45, CD90, CD105, and CD106. Cells were then diluted in 2 ml PBS/bovine serum albumin, centrifuged, and resuspended in 200 μl of 4% paraformaldehyde. Acquisition and analysis were performed using a BD Accuri C6 flow cytometer and BD Accuri C6 software program.

2.4. Preparation of GMSCs for injection

When the cell confluence reached the required number for the experiment, plastic syringes of 100 IU were loaded with stem cell suspension. Each syringe was prepared to contain 0.1 ml of PBS carrying 1×10^5 GMSCs.

2.5. Preparation of autologous fibrin glue

Rat blood was obtained, and plasma was separated. Thrombin reagent was added to the plasma, and concurrently, the plasma coagulated to form a solid fibrin web.

2.6. Histological and immunohistochemical evaluation

Specimens were fixed in 10% neutral buffered formalin for 24 h and then were prepared for routine histological examination by hematoxylin and eosin (H & E) staining. Moreover, immunohistochemical staining was performed using proliferating cell nuclear antigen (PCNA) to express the nuclei of cells during the DNA synthesis phase of the cell cycle and α-SMA to characterize the myoepithelial cells.

2.7. Evaluation of immunostaining

Slides were photographed using ToupView® digital camera installed on an Olympus® microscope with 1/2 photo adaptor, using X objective. Twenty images of 10 cm width and 5 cm length with a resolution of 300 dpi for each group were analyzed using the Fiji ImageJ processing package software (https://fiji.sc/). PCNA index was analyzed using the

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