Journal of Oral Biosciences 57 (2015) 124–130

Contents lists available at ScienceDirect

Journal of Oral Biosciences

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

Original Article

# The effects of enzymatically synthesized glycogen on the pulpal healing process of extracted teeth following intentionally delayed replantation in mice

Angela Quispe-Salcedo, Hiroko Ida-Yonemochi, Hayato Ohshima *

*Division of Anatomy and Cell Biology of the Hard Tissue, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuo-ku, Niigata 951-8514, Japan*

ARTICLE INFO

*Article history:*
Received 9 June 2014
Received in revised form
16 January 2015
Accepted 21 January 2015
Available online 24 February 2015

*Keywords:*
Dental pulp
Odontoblast
Tooth replantation
ICR mice
Wound healing

ABSTRACT

*Objectives:* Glucose uptake plays a crucial role in early tooth morphogenesis and size determination. Recently, enzymatically synthesized glycogen (ESG), with the characteristics of natural glycogen (a major storage form of glucose), has been developed. This study aimed to elucidate the effectiveness of ESG on the pulpal healing process following intentionally delayed tooth replantation in mice.
*Methods:* The upper first molar was extracted, immersed in phosphate buffered saline (PBS) or ESG (5000 kDa) solution (1 mg/mL) for 60 min, and then replanted. Immunohistochemistry (for nestin, osteopontin, and Ki-67), TUNEL assay, and reverse transcription-polymerase chain reaction were performed at different time points.
*Results:* Increased apoptosis occurred in the dental pulp of mice from both treatment groups at Day 7, followed by active cell proliferation at Day 14 and tertiary dentin and/or bone-like tissue deposition at Day 21, in the PBS group. In contrast, active cell proliferation and coronal immunoreaction for nestin occurred around Day 10, and hard tissue deposition were observed at Day 14, in the ESG group. The mRNA expression of genes encoding *dentin sialophosphoprotein* and *nestin* first reappeared in the ESG group at Day 5, while expression levels of *alkaline phosphatase* and *osteopontin*, as well as *Cd11c*, tended to increase from Day 3 in both groups, and that of the stem cell marker, *octamer-binding transcription factor Oct3/4*, greatly enhanced at Day 1, particularly in the ESG group.
*Conclusions:* ESG improved the pulpal healing process of extracted teeth following intentionally delayed replantation, although both ESG and PBS may induce the formation of bone-like tissue.


## 1. Introduction

Tooth replantation, a therapeutic method in which the avulsed tooth is repositioned in its original socket, has become widely utilized in clinical dentistry, with highly successful results [1]. However, this procedure interrupts the nerve and vascular supply to the pulp tissue, with subsequent degeneration of the pulpal cells [2]. Cases showing successful pulp regeneration, reinnervation, and revascularization have been reported, in humans [3–5] and in experimental animals [1,2,6–11]. However, the occurrence of relentless inflammatory reactions in the dental pulp leads to bone-like tissue formation in the pulp space of the replanted tooth [1,2,8–10]. Thus, the pulpal response to tooth replantation can be divided into tertiary dentin and/or bone-like tissue formation [1,2,6–11]. Although the mechanisms that determine the divergent regeneration process remain unclear, they may be directly linked to the death/survival of odontoblast-lineage cells, such as fully differentiated odontoblasts and/or odontoblastic progenitor cells [12]. In a previous study, the lack of a properly oxygenated medium decided the death of odontoblast-lineage cells. In addition, the occlusal forces during and/or after replantation negatively affect its outcome [10], as does the presence of bacteria, on the root surface and associated with the blood clot within the socket [13]. Management of these risk factors and establishment of a suitable environment in the alveolar socket are key factors determining the pulpal healing pattern of a replanted tooth.

Glycogen, a highly branched α-D-glucan containing α-1,4 and α-1,6 linkages, is a major storage form of glucose in many organisms, from bacteria to primates [14]. The main functions of glycogen are to supply energy in muscle and to release glucose to the bloodstream from the liver [15,16], but it has been reported to play additional roles [16,17]. Recently, an *in vitro* method was developed

* Corresponding author. Tel.: +81 25 227 2812; fax: +81 25 227 0804.
*E-mail address:* histoman@dent.niigata-u.ac.jp (H. Ohshima).

http://dx.doi.org/10.1016/j.job.2015.01.003
1349-0079/

for synthesizing glycogen from starch using isoamylase (EC 3.2.1.68), glycogen branching enzyme, and amylomaltase (EC 2.4.1.25); the product is referred to as enzymatically synthesized glycogen (ESG) [18,19]. ESG was shown to be the same as natural glycogen in terms of molecular shape, size, and weight, branch frequency, and chain length [16,18,20]. ESG shows various health benefits, including prolonging the survival of tumor-bearing mice, stimulating the immune system, and a dietary fiber-like action [19,21–23]. However, the effects of ESG on dental tissues remain unknown.

In a recent study, we successfully established a mouse model for the evaluation of therapeutic reagents in the replantation of teeth [24]. The major advantage of this model resides is the ease of replicating tooth replantation, allowing a clearer understanding of dental pulp dynamics during the healing process. The present study aimed to elucidate the effects of ESG on this process during intentionally delayed replantation in mice.

## 2. Materials and methods

### 2.1. Tooth replantation

Eighty-one Crlj:CD1 (ICR) mice (3 weeks old) were used in this study. The animals were divided into two groups on the basis of the treatment of their extracted teeth prior to replantation, as follows: (1) using phosphate buffered saline (PBS) and (2) using a 1 mg/mL solution of ESG (5000 kDa); these are hereafter referred to as the "PBS group" and "ESG group", respectively. ESG was supplied by Ezaki Glico Co., Ltd. (Osaka, Japan). The upper-right first molar of each animal was extracted with a pair of dental tweezers and then repositioned in its original socket, following immersion in PBS or ESG solution for 60 min. This procedure was performed under deep anesthesia, induced by intraperitoneal (IP) injection of chloral hydrate (maximum dose=350 mg/kg). The alveolar sockets were not treated following tooth extraction, and part of the resultant blood clot was removed before replantation. No treatment, such as fixation of the tooth or the relief of occlusion, was performed following replantation.

### 2.2. Tissue preparation

Material was collected from groups of three to six animals, on Days 7, 10, 14, and 21 after tooth replantation. On each of these days, the animals were transcardially perfused with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), under deep anesthesia; the maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 h at 4 °C. Following decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) for 4–6 d at 4 °C, specimens were processed for embedding in paraffin, and tissue blocks were sagittally sectioned at a thickness of 4 μm. Sections were mounted on adhesive silane-coated glass slides (Matsunami Glass Ind., Osaka, Japan) and processed for hematoxylin and eosin (H&E) staining and immunohistochemistry.

### 2.3. Immunohistochemical and apoptosis analyses

For immune peroxidase staining, sections were processed according to the EnVision system (Dako Japan, Tokyo, Japan) and the avidin–biotin–peroxidase complex method (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA, USA), using a mouse anti-rat nestin monoclonal antibody (1:100; Chemicon International, Temecula, CA, USA; catalog number: MAB353), a rabbit anti-mouse osteopontin (Opn) polyclonal antibody (1:3000; Cosmo Bio, Tokyo, Japan; catalog number: LSL-LB-4225), and a rat anti-mouse Ki-67 monoclonal antibody (1:100; Dako Japan; catalog number: M7249). Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore, Billerica, MA, USA). These procedures were previously described in detail [25,26].

### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the dental pulp tissue of replanted teeth at 0 d (immediately after immersion; $n$=4 [PBS]+4 [ESG]), 1 d ($n$=4 [PBS]+4 [ESG]), 3 d ($n$=4 [PBS]+3 [ESG]), 5 d ($n$=4 [PBS]+4 [ESG]), 7 d ($n$=4 [PBS]+3 [ESG]), and 14 d ($n$=4 [PBS]+4 [ESG]) after tooth replantation, using the TRIzol system (Life Technologies, Carlsbad, CA, USA); cDNA was synthesized using the SuperScript First-Strand Synthesis System (Life Technologies). The sequences of the PCR primer pairs used for amplifying cDNAs encoding *β-actin*, *dentin sialophosphoprotein* (*Dspp*), *nestin*, *alkaline phosphatase* (*Alp*), Opn, *cyclin D1*, *caspase 3*, *Cd11c*, and the *octamer-binding transcription factors Oct3/4A* and *Oct3/4B* are listed in Supplementary Table 1. The PCR protocol involved 30 or 35 amplification cycles of denaturation at 94 °C for 1 min, annealing at 58 °C or 60 °C for 1 min, and extension at 72 °C for 1 min. Amplified DNA fragments were separated by electrophoresis in 2% agarose gels. The relative density of each band in comparison with the *β-actin* PCR product was determined from monochrome photographs using the Image J software, version 1.45 (www.imagej.nih.gov).

### 2.5. Cell counting and statistical analysis of Ki-67- and TUNEL-positive cells

The number of Ki-67- or TUNEL-positive cells in the coronal and root pulp of each specimen was calculated (using a $3.4 \times 10^4$ grid). Data are presented as the mean and standard deviation of each group. The number of cells in the coronal and root pulp, and between the two groups, was compared at Days 7, 10, 14, and 21 after tooth replantation, by Student's $t$-test and one-way analysis of variance multiple comparisons adjusted by the Bonferroni correction, using the SPSS 16.0J software (SPSS Japan, Tokyo, Japan).

## 3. Results

### 3.1. Control

In untreated control teeth, coronal odontoblasts showed pseudostratified features, including the presence of blood capillaries in the odontoblast layer (Fig. 1A). Nestin immunostaining was exclusively detected in the odontoblast and subodontoblastic layers (Supplementary Fig. 1A).

### 3.2. Histological evaluation of the dental pulp by H&E staining and nestin immunohistochemistry

In the PBS group, the dental pulp showed degenerative features and the blood capillaries completely disappeared from the pulpal tissue at Day 7 after tooth replantation. The dental pulp contained eosinophilic amorphous matrices, including many erythrocytes and scattered inflammatory cells (Fig. 1B). Most cells in the coronal pulp lacked nestin immunoreactivity at this stage (Supplementary Fig. 1B), whereas ascending nestin-positive filamentous structures were observed in the apical one-third or two-thirds of the root pulp (data not shown). At Day 10, large areas of cell debris-derived matrix were observed in the pulpal tissue, surrounded by ascending nestin-positive filamentous structures (Fig. 1D), while scattered