

A simple method of tooth regeneration by bone marrow mesenchymal stem cells in albino rats

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SUMMARY

The present study explored regeneration of tooth by adult bone marrow mesenchymal stem cells without co-culture of the oral epithelium. One male Wistar albino rat (8 w) was used for the preparation of bone marrow mesenchymal stem cells and six male rats (5 w) for stem cell transplantation. One maxillary incisor tooth was extracted and a socket was prepared. Adult bone marrow in femora and tibiae was collected and mesenchymal cells were cultured in minimal essential medium containing 10% heat-inactivated foetal bovine serum and were sub-cultured. Mesenchymal cells had long processes: star-shaped and gradually changing to spindle-shape and showing a tendency to adhere to tissue culture plastic. The mesenchymal stem cells were transplanted into the prepared sockets with and without scaffold (poly lactic-co-glycolic acid). On the 14th day after transplantation, in the rat with the scaffold, radio-opacity was observed at the site of transplanted socket. The density of the radio-opaque substance was comparable to that of an adjacent natural tooth. The margins of the stem cell tooth were as well defined as that of an adjacent natural tooth. The stem cell tooth was shorter, but at the third month the root of

the unerupted tooth was almost as long as that of a natural tooth. In the rat without the scaffold, the density of the stem-cell tooth was comparable to that of a rat with the scaffold but its length was smaller. The margins of the stem-cell tooth were ill defined as compared to that of a natural tooth. The macroscopic morphology of the stem cell tooth was comparable to that of a natural tooth but was smaller and its consistency was firm, resistant and hard. The apex was pointed, height 5 mm, and its colour was pearly white and it was conical, almost resembling an incisor tooth. Confocal laser scanning microscopy of the stem-cell tooth revealed it to have long processes, enclosing a narrow canal as dentinal tubules and a nucleus as an odontoblast.

Key words: Bone marrow – Mesenchymal stem cells – Tooth regeneration

INTRODUCTION

The regeneration of a functional tooth is a promising therapeutic strategy. Tooth regeneration has been possible with dental stem cells (Mantesso and Sharpe, 2009), periodontal ligament stem cells (Trubiani et al., 2008), and bone marrow mesenchymal stem cells (Li

et al., 2007). Mesenchymal stem cells residing in bone marrow are one of the most widely studied and clinically important populations of adult stem cells. The present study explores the regeneration of tooth structure by adult bone marrow mesenchymal stem cells without co-culture of the oral epithelium, which is of clinical importance for human therapeutic purposes. This study also compares tooth regeneration with and without a bioscaffold.

MATERIALS AND METHODS

Seven male Wistar albino rats of suitable health status for the experiments were used. The rats were maintained with inherent physiology, ecology, behaviour, and minimum stress. One rat (8 w, 140 g) was used for the preparation of the bone marrow mesenchymal stem cells. Six rats (5 w, 130, 125, 130, 128, 130, and 135 g) were used for stem cell transplantation.

Socket preparation

To extract the maxillary incisor, six rats were anesthetized by intraperitoneal injection of ketamine (0.3 ml of diluted stock to 100 g young rats) followed by xylazine (0.9 ml of diluted stock to 100 g young rats). Ten minutes later, each rat was laid down on its back with its mouth open for extraction of the tooth. One maxillary incisor from each rat was extracted using a modified oral spatula (dental elevator). Then, a haemostatic forceps was used to remove the tooth from the oral cavity. After the extraction, tissue approximation was done and a suture was placed at the extracted site to close the socket. Aseptic procedures were followed throughout the surgery. The rats were allowed to recover and were maintained in individual cages, with normal nutrition. Extraction of the entire complete tooth was confirmed by intra-oral peri-apical radiographs. The rats were observed during feeding and drinking. Post-operative analgesics and antibiotics were given. Wound healing was observed. The suture was removed after three days and irrigation was done with saline.

Collection of adult bone marrow mesenchymal cells for culture

One rat was anaesthetized by intraperitoneal injection of lignocaine (5 mg/100 g body weight). Ten minutes later, the rat was laid on its back. The skin over its fore limbs and hind limbs was aseptically cleaned.

Femora and tibiae were dissected out free of muscles and soft tissues. Both ends of the bones were cut and the marrow was slowly flushed out into a small aseptic glass container with culture medium at one end of the bone using a sterile 18-gauge needle. Bone marrow cells were thus suspended in minimal essential medium (MEM) containing 10% heat-inactivated foetal bovine serum (FBS).

Culture and subculture of mesenchymal stem cells

Fifteen ml of rat mesenchymal stem cell growth minimal essential medium (MEM) containing 10% heat-inactivated foetal bovine serum was pipetted into a 75 cm square plastic flask. Bone marrow cells were rapidly drawn into the lower half of a vial in a 37°C water bath for one minute. Then the cells were resuspended in the vial by gently pipetting them five times with a two-ml pipette. Following this, the cell suspension (one ml) was pipetted from the vial into the flask containing 15 ml of mesenchymal stem cell growth medium. The flask was capped and shaken gently to distribute the cells evenly. The flask was placed at 37°C, in a 5% CO₂ humidifier incubator. The cap was loosened to allow gas exchange and the culture was left undisturbed for the next 24 hours after incubation. The medium was changed after 24 hours and then every two days until the cells had reached 60% confluence. The culture was maintained in a 75-cm square tissue flask for 12 days. Subculture was performed on the 13th day when the cells had reached 85-95% confluence to trypsinize them at room temperature. Then, the cells were seeded for another three days after subculture to form homogeneous colonies.

Transplantation of the cultured mesenchymal stem cells

Six rats were anaesthetized by intraperitoneal injection of lignocaine (5 ml/100 g body weight) twenty-one days after eruption of the tooth, the tooth-extracted and socket-prepared. Group A of three rats was transplanted with scaffold (Size, length = 4.5 mm). The scaffold contained poly lactic-co-glycolic acid (PLGA). The bioscaffold was obtained from Bioscaffold International Pvt. Ltd. Singapore. The polymer quality permitted strong mesenchymal cell adhesion and was constructed with macrochannels and micropores in such a way that it gave less space and more nutrition. Group B of three rats was transplanted with-

out scaffold. An incision was made on the tooth-extracted site. The scaffold was dissolved with normal saline in a Petri dish and allowed 10 minutes to gel, and the gel was then seeded into the socket under aseptic conditions. In the Group A rats, the cavity of the socket was cleaned with a needle and the scaffold was placed in the socket above which the mesenchymal stem cells were injected with a syringe. Then, the mouth of the socket was closed, after tissue approximation, using superglue (cyanoacrylate). In the Group B rats, the cavity of the socket was cleaned with a needle and the mesenchymal stem cells were injected directly into the socket. Then, the mouth of the socket was closed, after tissue approximation, using superglue. The wound was allowed to heal.

RESULTS

Culture observations

Observation on the 4th day of culture. Among the mixed population of cells, only a few mesenchymal cells were present. The mesenchy-

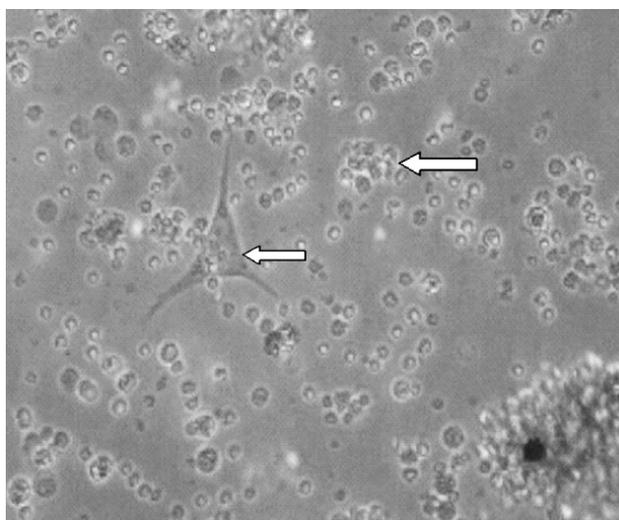


Fig. 1. Observation on the 4th day of culture. Short arrow indicates mesenchymal stem cell. Long arrow indicates mixed cell population.

Radiological observations

On 14th day after transplantation, intra-oral peri-apical radiographs were taken to observe the effect of the transplantation of stem cells. In the Group A rats, radio-opacity was found at the site of the transplanted socket. This was compared with the check radiograph, taken prior to transplantation. At the site of the empty socket, a radio opaque substance was present. The density of the radio

mal cells had eccentric nuclei and processes. The mesenchymal cells were star-shaped and the other mixed cells were round (Fig. 1). The ratio between mesenchymal cells and the mixed population cells was small. The mesenchymal cells showed a tendency to adhere to the tissue culture plastic

Observation on the 6th day. Mesenchymal cells had begun to grow and their long processes extended among the mixed cells (Fig. 2). The ratio between the mesenchymal cells and mixed population cells increased.

Observation on the 8th day. Clusters of mesenchymal cells with long processes, a nucleus and a nucleolus were present. The ratio between mesenchymal cells and the mixed population cells had markedly increased. The mesenchymal cells had gradually changed from star- to spindle-shaped.

Observation during the subculture study. Homogeneous adult mesenchymal cells were obtained. On the 14th day, multiple colonies of mesenchymal cells were present, which were thickly populated: about 15,000 cells per cm². There was linear increase in the number of colonies and mesenchymal cells (Fig. 3).

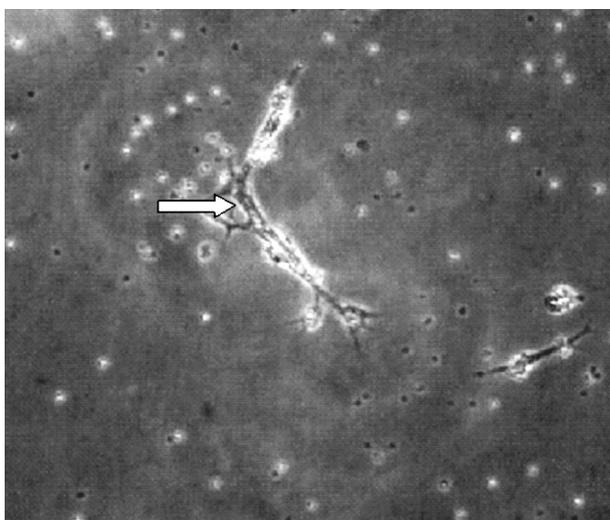


Fig. 2. Observation on the 6th day of culture arrow indicates mesenchymal stem cells with long process.

opaque substance was comparable to that of an adjacent natural tooth. The margins of the stem cell tooth were as well defined as that of an adjacent natural tooth (Fig. 4). The stem-cell tooth was shorter. The scaffold was seen to be a powerful tool for tissue engineering and acted as a template for cell aggregation. A radiograph of a stem-cell tooth taken on the 14th day revealed a short root, but the one taken in the third month showed an unerupt-

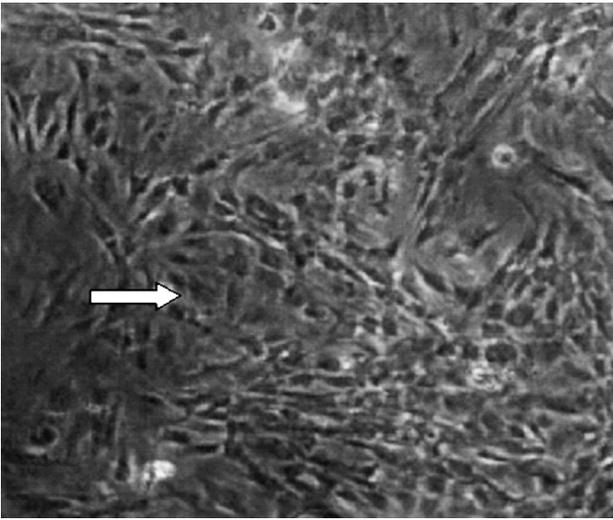


Fig. 3. Observations after subculture indicate colonies of mesenchymal stem cells.

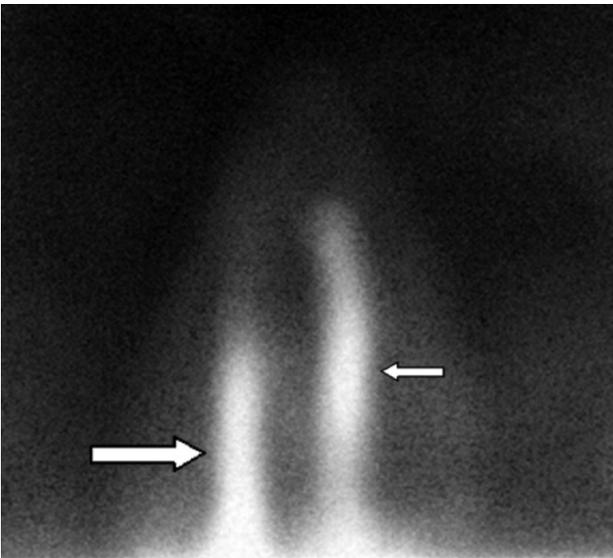


Fig. 4A.

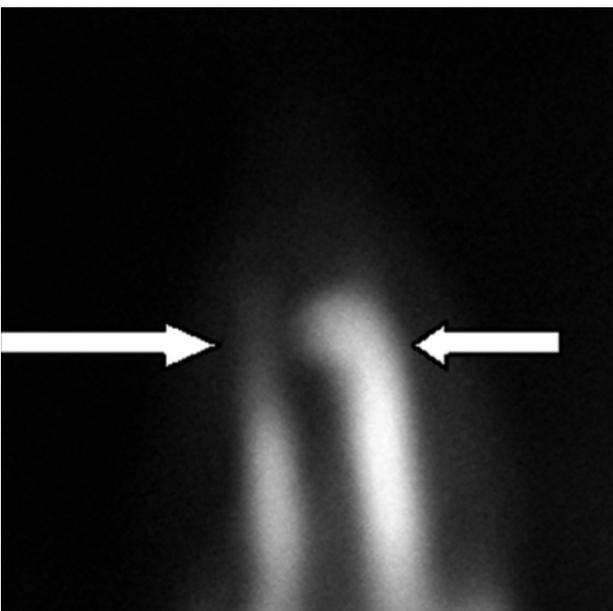


Fig. 4B. Radiological observation on the 14th day. Short arrow indicates natural tooth. Long arrow indicates stem cell tooth. A is with scaffold and B is without scaffold.

ed tooth with a root almost as long as an adjacent tooth (Fig. 5). In the Group B rats a radio opaque substance was also present at the site of the socket. This was compared with the check radiograph taken prior to transplantation of the stem cells. At the site of the empty socket, a radio opaque substance was present. The density of the radio opaque substance formed was comparable to that of an adjacent natural tooth. The density of the stem-cell tooth was comparable to that of a Group A rat but its length was smaller. The margins of the stem cell tooth were ill defined as compared to that of an adjacent natural tooth (Fig. 5B).

Macroscopic observations

After two weeks, one rat was sacrificed to observe the structure within the socket. The socket was carefully opened with a fine forceps. The morphology of the structure grown was comparable to that of a natural tooth but was smaller. Its consistency was firm, resistant, not soft and resembled that of a hard tissue. The apex was pointed and the base adhered to the base of the socket. Its height was 5 mm, and its colour was pearly white. The shape was conical and resembled the morphology of an incisor tooth.

Confocal laser scanning microscopic observations

The stem-cell tooth grown within the socket had long processes, which when traced to the bottom were slightly dilated, resembling a nucleus. The processes extended from the apex of a cell. Each process enclosed a narrow canal similar to dentinal tubules (Fig. 6), and enclosed a nucleus at the bottom, like an odontoblast (Fig. 7). Each cell body contained cytoplasmic organelles, lacked endoplasmic reticulum, but contained a granule-like substance, microtubules and micro filaments.

DISCUSSION

In the present study, adult bone marrow stem cells were taken from a Wistar albino rat, cultured, and transplanted into tooth sockets prepared in six other rats. The findings of the present study, as regards the culture of mesenchymal stem cells, were comparable to those of Raimondo et al. (2006), Prockop et al. (1997), Poliseti et al. (2010) and Kassis et al. (2006); in subculture of the stem cells to those of Friedenstein et al. (1970); in radiological

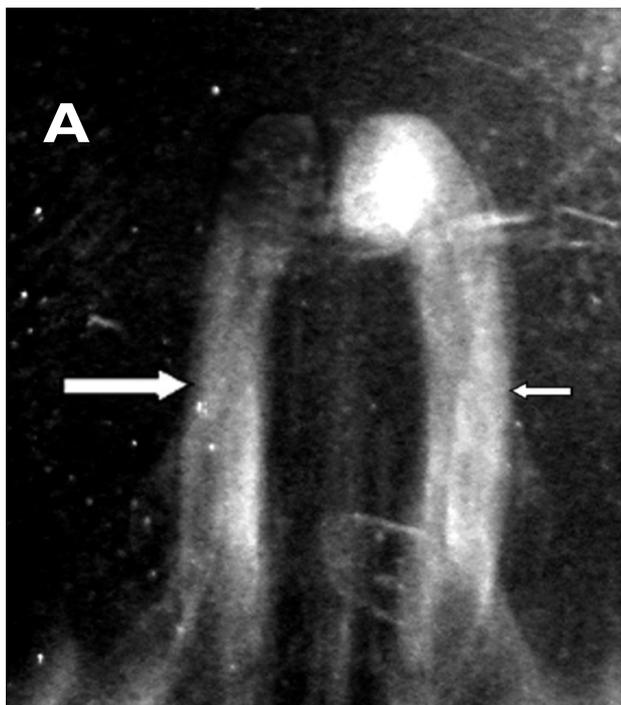


Fig. 5A. Radiological observation on the 3rd month. Short arrow indicates natural tooth. Long arrow indicates stem cell tooth. A is with scaffold and B is without scaffold.

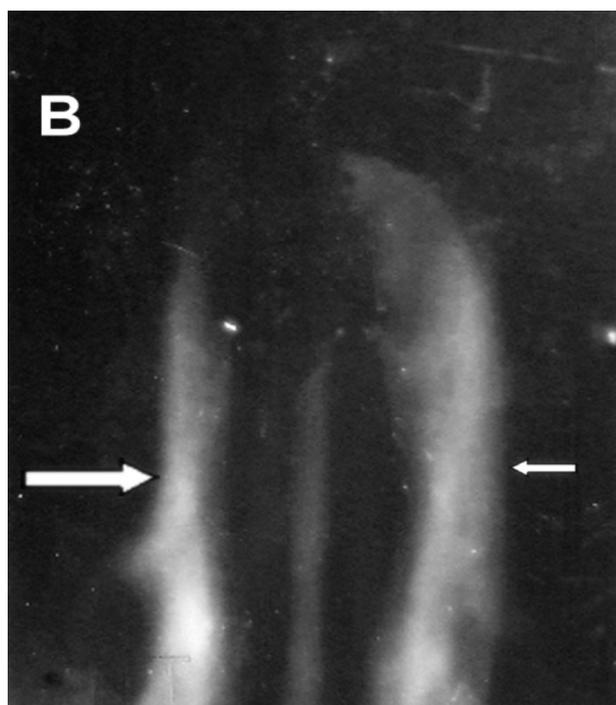


Fig. 5B.

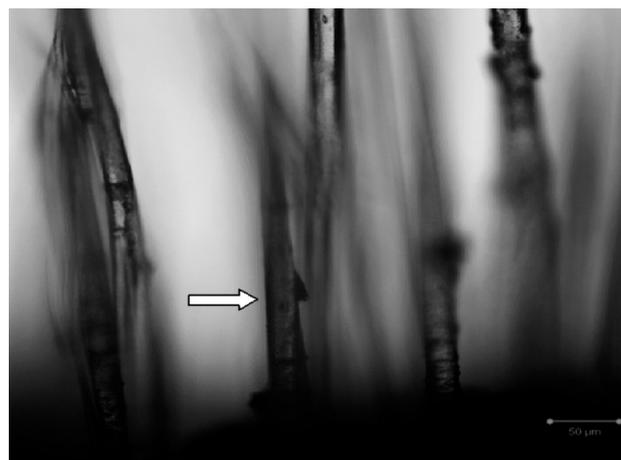


Fig. 6. Observation under confocal laser scanning microscopy indicates a narrow canal similar to dentinal tubules.

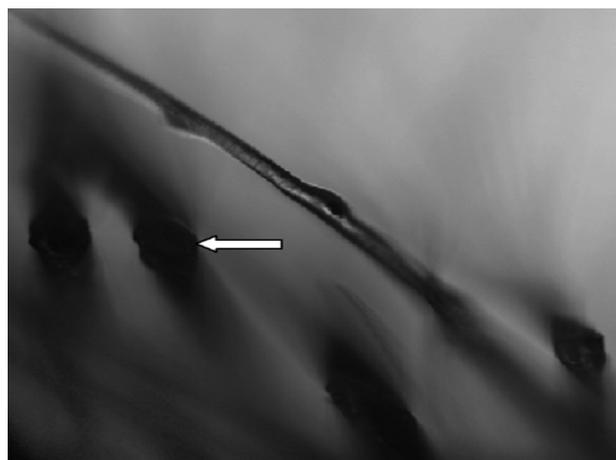


Fig. 7. Observation under confocal laser scanning microscopy indicates a narrow canal enclosing a nucleus at the bottom similar to odontoblast.

studies to those of Langer and Vacanti (1993), Koc et al. (2008) and Duailibi et al. (2008); in macroscopic study with those of Tucker et al. (1998), and in confocal laser scanning microscopy to those of Marzuki and Masudi (2008). The mesenchymal stem cells differentiated into odontoblasts and secreted several molecules, which promoted an extracellular mineralization matrix (Tucker et al., 1998). The root of the stem-cell tooth was shorter than that of natural teeth because it was only two weeks old but had grown to an almost equal length of the natural tooth in the third month because it had reached its full growth time, and it would erupt at its time span.

This procedure is uncomplicated and does not entail any epithelio-mesenchymal interaction, signaling, growth factors, transcription factors, regulatory genes, bioengineering, developmental biology, enzymes, stains, or controls.

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