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RESEARCH ARTICLE

In vitro proliferation of MSCs using mineral trioxide aggregate: A most recent material for *in situ* stem cells mobilization

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ABSTRACT

Background

Tissue/organ regeneration has been a major challenge before the scientific society in order to improve the functional benefit of the patients and dentistry has not been left untouched. The objective of endodontic therapy is to treat the affected tooth to become symptom free, without pathology and functional. Expansion of stem cell research has added a new paradigm to regenerative endodontic therapy. As far as dentistry is concerned number of stem cells is quite less and their application is often mimicked. Thus there is need to mobilize stem cells from other sources to increase the pool of regenerative cells. In endodontic therapy mineral trioxide aggregate (MTA) has been used since last few years in a myriad of clinical applications but so for its chemotactic and inductive effect on human mesenchymal stem cells (hMSCs) has not been demonstrated. Hence, the present study was aimed to find *in vitro* effect of ProRoot MTA on the proliferation of human bone marrow derived MSCs further to explore its clinical benefit in endodontic applications.

Materials and methods

Human bone marrow blood was collected in heparinized tubes and used for mononuclear cells (MNCs) isolation using Ficoll-paque density gradient medium. Cells were cultured *in vitro* and characterized for MSCs specific markers. After getting confluency cultured MSCs were subjected for MTT cell proliferation assay to assess the effect of MTA in long-term *in vitro* culture from day 0 to day 21.

Results

Significant increase in hMSCs proliferation was observed in both the groups (freshly coated MTA and 24 hours coated MTA) as compared to the controls from day 0 to day 21. Whereas, no significant difference was found between the two groups at each time of interval.

Conclusion

MTA is biocompatible vital material which significantly induces the proliferation of MSCs with no difference between material freshly prepared and incubated for 24 hours.

INTRODUCTION

Tissue/organ regeneration has been a major challenge before the scientific society in order to improve the functional benefit of the patients. Dentistry is not untouched with this and represents one of the major challenging areas for the development of new cellular based therapeutic strategies for better outcome. The objective of endodontic therapy is to treat the affected tooth to become symptom free, without pathology and functional. American Association of Endodontists' Glossary of Endodontic Terms (2012) defines regenerative endodontics as "biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex.". Expansion of stem cell research, in particular the discovery of MSCs has added a new paradigm and a possible answer for regenerative endodontic therapy. Any adult tissue with repair and/or regenerative capability is likely to harbor tissue-specific stem cells defined as cells that self-renew and retain sufficient proliferative and differentiation potential to repair and/or reconstitute a specific tissue. Various embryonic and adult sources of stem cells have been isolated and well characterized for their functional support (Pittenger et al., 1999; Bu¨hring et al., 2007; Da Silva Meirelles et al., 2006). However, in dentistry due to very less numbers of stem cells present in dental sources anchorage their study and need to find an alternative source in order to forecast utmost *in vitro* and *in vivo* remuneration.

hBM-MSCs is an easily available cell source and thought to be the basis for an extremely powerful "natural system for tissue repair" (Scipani et al., 2008; Phinney et al., 2007). MSC's have the ability not only to repair by trans-differentiation into the appropriate cell phenotype or to cell fusion, but also have the ability to secrete soluble factors that alters the tissue microenvironment (Prockop et al., 2007). Adult human MSCs have been reported to be non-immunogenic and immunomodulatory, which implies transplants into allogenic hosts without need of any immunosuppression (Majumdar et al., 2003).

However, before scheduling the cell based therapeutic move towards *in vivo*, there is need to conduct sufficient *in vitro* studies to prove their potential to engraft and home at the site of injury and survive in the residing microenvironment. In this context, materials used in endodontic therapy play a significant role in terms of being biocompatible and allowing tissue repair. A variety of materials such as zinc-oxide-eugenol, amalgam, calcium hydroxide, composite resin and glass ionomers and its derivatives have been used in managing the proliferation of regenerative cells (Torabinejad et al., 1996) but none of them have proven 100% safety and efficacy. An ideal material for treating radicular perforations and other dental problems should encompass the properties of non-toxicity, nonabsorbable, radiopaque, bactericidal, and should provide a seal against micro leakage from the perforation.

In recent years, Mineral trioxide aggregate (MTA) has evoked considerable interest exhibiting many of the ideal requisites in a myriad of clinical applications such as a single visit apexification, pulp capping and furcation repair. MTA is a FDA approved commercially available material that is almost universally utilized to seal the canal in all revascularization procedures wherein the material lies in contact with the blood clot. Roberts et al., (2008) have demonstrated that high pH (12.5) of MTA promotes growth of the cementum and bone, which in turns allows regeneration of the periodontal ligament around the site of injury. Several i*n vitro* studies have proved the biocompatibility of MTA in clinical trials (Oviir et al., 2006; Algendy et al., 2013). ProRoot MTA is available as gray and white forms and existing reports as a whole are equivocal regarding their biological response. (Roberts et al., 2008)

However, Hashem et al. (2008) have demonstrated that ProRoot white MTA has better sealing ability and can be used with/without matrix in regeneration of large furcation perforation. The international testing standards (ISO-10993) for cytotoxicity recommend extractions of material to be tested at 37°C for 24 hours. But clinical situations such as induced bleeding step in revascularization and placement of apical plug results in stem cells being released from their periapical niche (Lovelace et al., 2011) and may directly come in contact with MTA which is freshly prepared and not preset. Hence it is important to evaluate the effect of freshly prepared and 24 hours incubated MTA on cell proliferation to closely mimic their clinical situation. Therefore, the present study was undertaken to investigate *in vitro* effect of ProRoot white MTA on the proliferation of hBM-MSCs using MTT cell proliferation assay.

MATERIALS AND METHODS

All the experimental procedures were approved by Institutional Ethical Committee of Deccan College of Medical Sciences, Hyderabad, India.

Sample collection
Human bone marrow blood (hBM) was obtained by aspirated needle from the iliac crest by a physician from healthy donors after taking the informed consent. 15–20mL blood was collected into aspiration syringe loaded with 3,000 units of heparin to prevent clotting.

Mononuclear cells (MNC's) isolation
10mL anticoagulated hBM blood was diluted with 1X Phosphate Buffer Saline (PBS, Gibco) and layered on 10mL of density gradient medium Ficoll-Paque plus (clinical grade, Sigma Aldrich). Sample was centrifuged at 3000rpm at 20°C for 25min without applying a brake. Interphase buffy coat of MNCs between serum and erythrocyte layer was removed carefully and transferred into a fresh sterile tube and washed twice with Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) by stepwise centrifugation for 15 min at 5000rpm. Cells were titrated by gentle pipetting to remove cell clumps and subjected to cell viability testing and counting.

Cell counting and viability testing
MNCs isolated after density gradient media separation were checked for viability using trypan blue exclusion assay. Cell counting was performed by hemocytometer.

***In vitro* culture and characterization**
Isolated MNCs were cultured at 37°C and 5% CO_2 atmosphere as suspension in sterile tissue culture plates in medium containing serum. After 14days when cells reached to ~80% of confluency, trypsnization was done and cells were harvested by single cell suspension. Molecular analysis was performed to characterize the MSCs population using primers specific for CD90 (F-cgctctcctgctaacagtctt, R-caggctgaactcgtactgga), CD105 (F-cgtggacagcatggacc, R-gatgcaggaagacactgctg) and CD34 (F-gaccctgattgcactggtca, R-gttcacactggcctttccct) and GAPDH (F-ggcgatgctggcgctgagtac, R-tggttcacacccatgacga). For morphological characterization MSCs were cultured on collagen coated petri-dishes at 1.2 X 10^4 cells/cm^2 density in DMEM-F12 medium, supplemented with 10% Fetal calf serum (FCS, Invitrogen), 100U/mL Penicillin (Invitrogen), 100µg/mL streptomycin (Invitrogen), and 0.25 mg/mL amphotericin B (Invitrogen). Morphological characterization of cultured cells was done using phase contrast confocal microscopy (Carl Zeiss, Germany).

Preparation of ProRoot white MTA
100mg of ProRoot white MTA (Dentsply Endodontics, Tulsa, OK) was mixed with 35µl of sterile water under a laminar flow hood in a sterile container for approximately 1 minute. The thick creamy mixture of MTA was then painted onto the bottom of 96 well cell culture plate using sterile applicator brush to achieve a homogeneous coating. Two groups were created; one group was made after immediate coating and another after 24 hours of incubation at 37°C in 5% CO2 and 100% humidity.

MTT cell proliferation assay
1.5 x 10^4 cells/well were seeded on both groups of MTA coated wells in above described serum containing cell culture medium. Appropriate positive and negative controls were created for proper assessment of the results. Each experiment was done in triplicates in order to eliminate any technical error. Cultured MSCs were assessed after day 0, day 3, day 5, day 7, day 14 and day 21 for changes in their proliferation rate as compared to the cells seeded on uncoated wells.
Effect of MTA on mitochondrial function of the cultured cells was measured by a colorimetric assay as described by Mosmann (1983). Upon incubation with viable cells, the tetrazolium ring of MTT (pale yellow) is cleaved by cellular deydrogenases enzymes to convert the yellow water-soluble tetrazolium salt (MTT) into dark blue formazan crystals. MTT solution (500 µg/mL per well) was added to each plate and incubated to be solubilized with dimethylsulphoxide (DMSO, Sigma). The absorbance was taken at 570nm using a miroplate reader (BIO-RAD). At each experimental time period (day 0, day 3, day 5, day 7, day 14 and day 21), an MTT assay was conducted to assess the change in cell viability and proliferation.

Statistical analysis
Data was recorded as mean and standard deviation. Statistical analysis was performed by means of 't' test using SPSS software (version 20). p value ≤ 0.05 was considered to be significant.

RESULTS

In vitro proliferation of hBM-MSCs

MNCs when cultured *in vitro* as suspension showed multiplication of spherical shape of cells after 5-7 days having large nuclear and less cytoplasmic content representing typical feature of immature precursor cells (Fig. 1A). These cultured cells reached almost 80% of confluency after day 14 of culture. Further these cells when seeded on collagen coated dishes changed their morphology to polymorphic and spindle shaped representing MSCs features. Cells on monolayer started forming colonies after 3-5days and became confluent after 7-14days in culture (Fig. 1B).

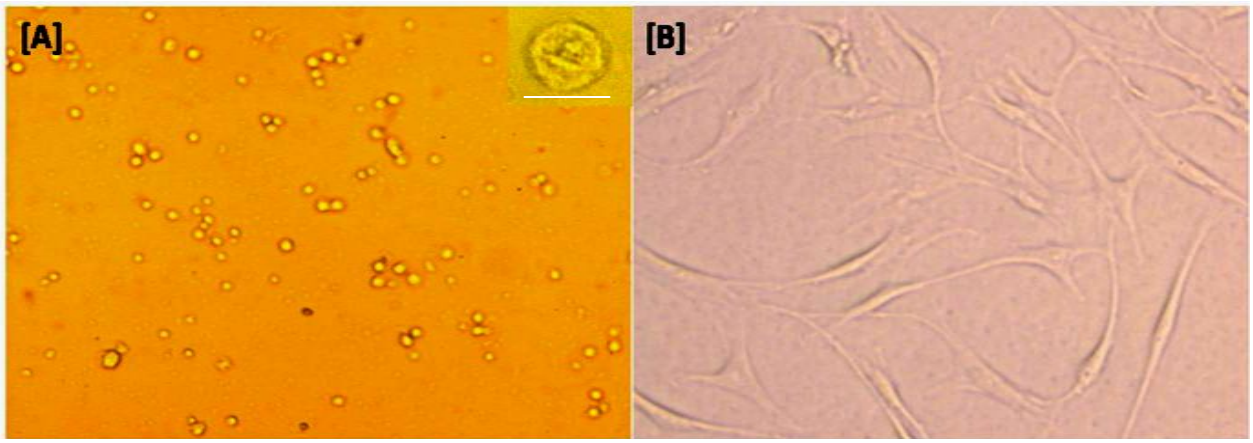

Fig. 1 *In vitro* proliferation of hBM-MSCs after day 7 as (A) suspension culture showing spherical and very small size (<10μm) cells having large nucleus and small cytoplasmic content representing feature of stem cells and (B) monolayer culture showing polymorphic, fibroblast-like morphology of cultured hMSCs

Characterization of cultured hMSCs

After 14days of initial plating of MNCs in suspension culture, cells were trypsinized and total cell RNA was isolated. RNA integrity was checked on 0.8% agarose gel after staining with ethidium bromide under UV. cDNA was synthesized and PCR was performed using primers specific for CD90, CD105 (positive for MSCs) and CD34 (negative for MSCs) in a thermocycler at 54-58°C annealing temperature for 35 cycles. Gene expression analysis showed strong positivity for CD90 and CD105 whereas was found negative for CD34 representing the presence of MSCs population in culture (Fig. 2).

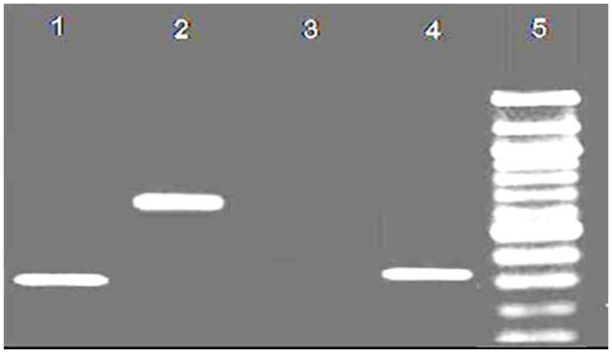

Fig. 2 Representative gel image showing gene expression pattern for human MSCs specific positive and negative markers (Lane 1: CD90 (142bp), Lane 2; CD105 (325bp), Lane 3: CD34 (202bp), Lane 4: GAPDH (150bp), and Lane 5: DNA ladder)

Effect of MTA on hMSCs proliferation

The coating of ProRoot white MTA of both the groups (freshly coated and 24 hours coated) was uniform (Fig. 3). The hMSC proliferation was assessed during a period of 21 days. An increase in cell viability was observed in both

groups studied at all intervals of the study. When hMSCs were grown in proliferation medium, cell proliferation rates were slightly decreased at day 3 and further increased significantly in the MTA group compared to the control group at 5 days, 7 days and continued to be so at 14 and 21 days (Fig. 4 and Fig. 5). No significant difference was observed between proliferation at day 0, day 3, day 5, day 7, day 14 and day 21 in either group. Similarly no significant difference existed between the two groups at each of the intervals tested (Fig. 6).

Fig. 3 Homogeneous coating of wells using MTA mixed with sterile water

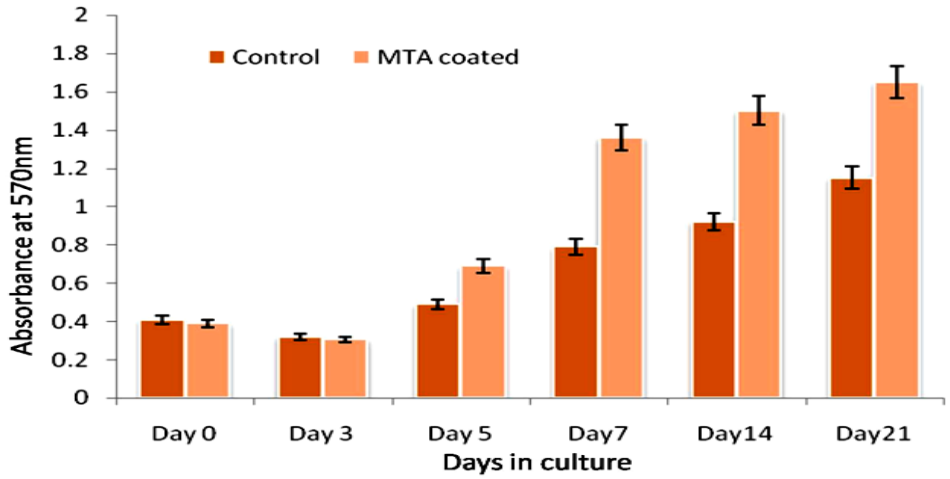

Fig. 4 Cell proliferation on MTA seeded with 1.5 x 10^4 cells/well at after incubation at 37°C in 5% CO2 and 100% humidity for 24 hours compared with control at 0, 3, 5, 7,14 and 21 days. Values indicate Optical density readings taken with elisa reader after MTT Assay. Results represent mean of readings in triplicate. $P<0.05$ at all time intervals

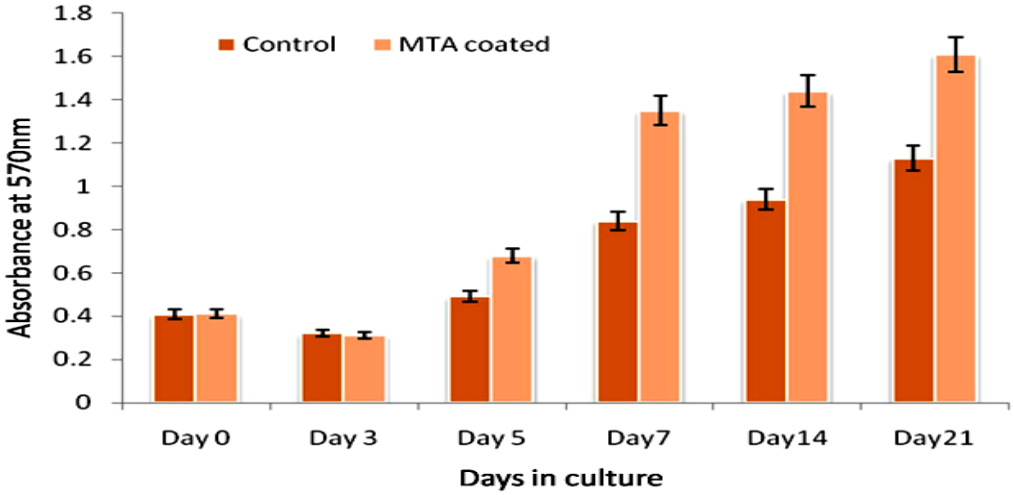

Fig. 5 Cell proliferation on freshly prepared MTA seeded with 1.5 x 10^4 cells/well compared with control at 0, 3, 5, 7,14 and 21 days ($P<0.05$)

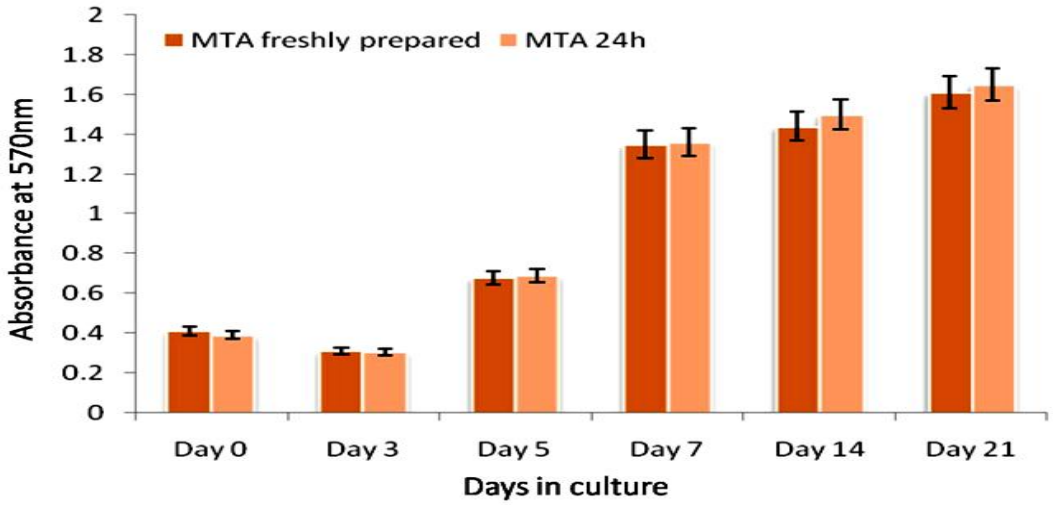

Fig. 6 Cell proliferation on freshly prepared MTA compared to MTA incubated for 24 hours (*P>0.05)*

DISCUSSION

In the last decade various biocompatible materials are being investigated in order to induce proliferation of stem cells in tissue/organ regeneration. As far as dentistry is concerned number of stem cells is quite less and their application is often mimicked. Thus there is need to mobilize stem cells from other sources to increase the pool of regenerative cells. In endodontic therapy MTA has been used since last few years in direct contact with tissues known to be niches for MSCs, but so far their chemotactic and induction effect have not been showed. In the current study, MTA was used for investigating its effect on the proliferation of hBM-MSCs under controlled proliferation environment. Significantly enhanced *in vitro* proliferation of hMSCs was observed after day 7 to day 21 as compared to the controls. This result was similar to the previous studies on other cell lines such as rat bone marrow cells, Saos-2 human osteoblast-like cells, and human alveolar bone cells (Nakayama et al., 2005, Pelliccioni et al., 2004 and Al-Rabeah et al., 2006). One explanation for this result may be the presence of calcium oxide in MTA which when mixed with water gets converted into calcium hydroxide and promotes cellular growth. Another possible explanation may be changes in ionic concentration within the cellular environment and plays a significant role for cellular proliferation and long-term survival. Freshly prepared MTA is alkaline in nature and its pH rises immediately after the material is mixed. Increase in pH of the culture medium provides an inducing factor for cell proliferation by providing increased ionic calcium levels in medium (McCullen et al., 2010). This may be due to some other factors also released from MTA during the period of time in culture and maintains the alkaline environment conducive for cell growth. This is an area that needs to be researched further.

De Deus (2005) has showed cytotoxic effect of MTA after 24 hours with growth of endothelial cells and inhibited proliferation as compared to the control groups. However at 48 hours and beyond the ProRoot MTA group have showed proliferation of endothelial cells similar to control indicating a decrease in the cytotoxic effects. The same result was obtained in the current study as cells proliferation rate was slightly decreased at day 3 and then significantly increased after day 5 to day 21 as compared to the control group and can be considered to be similar with previous studies (D'Anto et al., 2010).

CONCLUSION

Both the freshly prepared and 24 hour incubated MTA coated groups had no significant difference at all time points (day 0 to day 21) of the experiment. Thus if at all any difference does exist in the initial stages, this inhibitory effect is nullified by the day 5 and proliferation patterns continue in a similar manner. The study has also demonstrated the biocompatibility of MTA with hBM-MSCs and significant induction in proliferation, providing an evidence for its further application in regenerative endodontic therapy.

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