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Effect of Addition of Bismuth Oxide, Zirconium Oxide Nanoparticles and Niobium Oxide Nanoparticles to Portland Cement on the Proliferation and Migration of Dental Pulp Stem Cells

Research Article

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Abstract

Introduction: Various pulp capping agents have been used for direct and indirect pulp therapies. In recent times, calcium silicate based cements have replaced calcium hydroxide as a pulp capping agent. Mineral Trioxide Aggregate was one of the most commonly used pulp capping agents. However, the presence of bismuth oxide as a radio opacifying agent added to certain disadvantages which led to the discovery of alternatives such as zirconium oxide and niobium oxide. Since the process of pulp dentin regeneration involves the action of dental pulp stem cells, the present study was conducted with the aim of analysing the effect of addition of three different radio opacifiers to Portland cement, namely zirconium oxide nano particles, niobium oxide nanoparticles and bismuth oxide on the proliferation and migration of dental pulp stem cells.
Materials and Method: The dental pulp stem cells were cultured and treated with the test drugs. An in vitro Bromodeoxyuridine (BrDu) cell proliferation assay and a wound healing scratch assay to assess the cell migration rate was performed.
Results: Dental pulp stem cells showed a higher rate of proliferation and migration when treated with Portland cement containing zirconium oxide nanoparticles and niobium oxide nanoparticles.
Conclusion: Zirconium oxide nanoparticles and niobium oxide nanoparticles could serve as an alternative to bismuth oxide as a radio opacifier in calcium silicate based cements.

Keywords: Calcium Silicate Cement; Dental Pulp Stem Cells; Niobium Oxide; Pulp Therapy; Zirconium Oxide.

Introduction

The regeneration of dentin pulp complex after an injury is a complex cellular and molecular process. The process involves a series of events such as migration, proliferation, adhesion and differentiation of dental pulp stem cells into odontoblast cells which eventually aids in dentinogenesis.[1] An ideal pulp capping agent should be able to modulate this process of healing for regeneration of the dentin pulp complex.[2, 3] Calcium hydroxide has been used in the past as an agent for pulp capping, but with the invention of tricalcium silicate based cements, MTA has become the material of choice. Studies have shown MTA to be more effective than calcium hydroxide in maintaining the pulp vitality in the long run after direct pulp capping.[4] Further, a previous study has shown the presence of tunnel defects in the dentin bridge formed under the influence of calcium hydroxide as a pulp capping agent thus failing to provide a hermetic seal.[5] The formation of dentin bridge by MTA at the site of pulp exposure has been depicted in In vivo and In vitro studies.[6] However, MTA contains bismuth oxide as a radio opacifier which reduces its compressive strength [7], promotes discolouration of teeth [8] and also reduces cell proliferation. [9]

The drawbacks of bismuth oxide used in MTA as a radio opacifier

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has led to discovery of alternative radio opacifying agents that do not hinder the activity of dental pulp stem cells. In order to improve the biocompatibility of calcium silicate based cements, other radio opacifying agents have been added to it as an alternative to bismuth oxide. Microparticles and nanoparticles of Zirconium oxide and Niobium oxide have been studied as an alternative radio opacifying agent in calcium silicate based cements with satisfactory results.[10] One such material which has been introduced into the market is Biodentine, which contains zirconium oxide and has shown to induce dental pulp stem cell proliferation. [11] Dental pulp stem cells play an important role in the regeneration of dentin pulp complex. Hence it is important to evaluate the bioactivity and biocompatibility of the materials used as a pulp capping agent.

Previously our team has a rich experience in working on various research projects across multiple disciplines Now the growing trend in this area motivated us to pursue this project [12-26].

The purpose of this study was to evaluate the effect of Portland cement with three different radio opacifying agents namely bismuth oxide, zirconium oxide nanoparticles and niobium oxide nanoparticles on the wound healing capacity of dental pulp stem cells in terms of proliferation and migration.

Materials And Method

Dental Pulp Stem Cells Culture

The cryopreserved dental pulp stem cells were maintained in essential culture medium containing alpha-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 5 × 10-5 M 2-mercaptoethanol and 10-4 ml-ascorbic acid 2-phosphate sesquimagnesium salt hydrate. The culture plate was incubated at 37°C. After 72 h, the culture medium was replaced with fresh one, and unattached cells were also removed. When 80-90% confluency was reached, cells were routinely sub cultured and counted using a microscope.

Test Drug Preparation

Portland cement: The Portland cement was mixed with distilled water at the liquid/powder ratio of 1:3 (mL:g). The mixtures were left for setting for 24 h then were sterilized by autoclave. The extraction ratio was 0.2 g/mL and conducted under 37°C for 24 ± 2 h with culture medium (pH 7.4). The collected extracts were regarded for the 100% extract.

Synthesis of ZrO2 Nanoparticles: About 2.58 g $ZrOCl_2 \cdot 8H_2O$ and 4.80 g urea were weighed and dissolved in 20.0 mL methanol under stirring to form a colourless solution. The solution was transferred to a 20 mL Teflon-lined stainless-steel autoclave, which was heated by 200°C and maintained for 20 h. The obtained product was post-treated with sulphuric acid solution (0.167 mmol) and then calcined at 645°C. The nanoparticle size was 74 nm which was tested by surface area analysis.

Preparation of Nb2O5 NPs: For this synthesis hydrated citric acid (99.5%), NbCl5 (Sigma-Aldrich 99%) and ethylene glycol (99.8%) was used. The resin decomposition was realized in a pre-calcination at 300°C for 4 hours. The material obtained was macerated and the obtained powders were calcined at temperatures between 500°C and 750°C obtaining oxides with fine nanoparticles.

Experimental Groups

The dental pulp stem cells treated with
Group I: 70% white Portland cement with 30% nanoparticulate zirconium oxide (ZrO2 nano)

Group II: 70% white Portland cement with 30% nanoparticulate Nb2O5 (Nb2O5 NPs)

Group III: 70% white Portland cement with 30% bismuth oxide.
Group IV: 70% white Portland cement
Group V: Cells without treatment

Each powder had been previously sterilized in ultraviolet light for 30 min. A ratio of 30% ZrO2 or 30% Nb2O5 NPs or 30% Bismuth oxide and 70% PC by weight were used for the analyses.

Wound Healing Assay

The wound healing assay of dental pulp stem cells were assessed by the scratch assay method. The cell density of "3 × 105 cells" was seeded into each well of a 24-well plate and incubated with complete medium at 37°C and 5% CO_2. After 24 h of incubation, the monolayer confluent cells were scrapped horizontally with sterile microtips. The debris was removed by washing with PBS. The cells were treated with test drugs by diluting with serum-free DMEM. The cells without treatment were used as the control. At 0 hrs and after 24 hrs of incubation, the images were photographed using phase contrast microscopy at ×40 magnification. To determine the migration rate, the images were analyzed using "image J" software, and percentage of the closed area was measured and compared with the value obtained at 0 h. An increase in the percentage of the closed area indicated the migration of cells. Experiments were performed in the triplicate manner and the data were recorded and analyzed statistically using SPSS.

In vitro Bromodeoxyuridine (BrDu) Cell Proliferation Assay

A 10 mM stock solution of BrdU was prepared by dissolving 3 mg of BrdU in 1 mL water. The 10 mM BrdU stock solution was diluted in cell culture medium to make a 10 µM BrdU labeling solution. About dental pulp stem cells (2500-100000 cells/well) in 100 µl medium was plated in 96-well plate and 100 µl of respective test substance was added and incubated for 72 hr. This was followed by addition of prepared 10 µl of 10X BrdU solution per wells. The cells were placed in an incubator for 24 hr. The plate was centrifuged at 300 g for 10 min and the medium was removed to obtain a suspension of cells. Then 100 µl/well of the fixing solution was added to each well at room temperature for 30 min. After the solution was removed, 100 µl of the prepared 1X detection antibody solution was added per well and placed at room temperature for 1 hour. The solution was discarded and plates were washed 3 times with PBS buffer. Next 100 µl/well of prepared HRP-conjugated secondary antibody solution was added and incubated at room temperature for 30 min. Then 100 µl TMB substrate was added and incubated for 30 min at room temperature. This was followed by addition of 100 µl stop solution, The

absorbance was read at 450 nm.

Statistical Analysis

Statistical analysis was done using one way analysis of variance (ANOVA) and post hoc by using SPSS software (version 22.0). Results were considered statistically significant if p value was less less than 0.05.

Results

The results showed a higher proliferation (Fig 1) of dental pulp stem cells when treated with portland cement containing zirconium oxide nanoparticles (78.4%) and niobium oxide nanoparticles (75.1%) when compared to the proliferation rate of dental pulp stem cells treated with portland cement alone and portland cement containing bismuth oxide particles (Table 1, fig 2). Figure 1 depicts the fluorescent microscopic images of the cell proliferation assay. Table 2 shows a higher migration of dental pulp stem cells as seen by the closure in the scratch assay (Fig 3, Fig 4) was observed for portland cement containing zirconium oxide nanoparticles (62.1%) and niobium oxide nanoparticles (63.5%) when compared to the migration rate of dental pulp stem cells treated with Portland cement alone and Portland cement containing bismuth oxide particles (Fig 4).

Table 1. Percentage of Brdu positive cells expressed as mean and standard deviation.

	PC	PC + ZrO_2 NPs	PC + Nb_2O5 NPs	PC + Bi_2O_3	Saline
Percentage of Brdu positive cells	45.2 +/- 3.4	78.4 +/- 2.4	75.1 +/- 5.2	60.4 +/- 3.9	10.1 +/- 0.98

Table 2. Cell migration as determined by wound healing scratch assay. Percentage of wound closure expressed as mean and standard deviation.

	PC	PC + ZrO_2 NPs	PC + Nb_2O_5 NPs	PC + Bi_2O_3	Saline
Wound healing assay (percentage closure)	30.8+/-2.4	62.1+/-4.9	63.5+/-5.1	45.9+/-3.8	15.6+/-1.3

Figure 1. Brdu cell proliferation assay photos taken using fluorescence microscope.

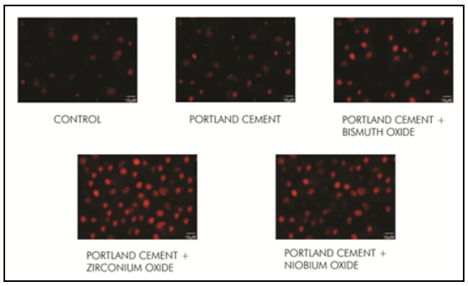


Figure 2. Graphical representation of the Brdu cell proliferation assay. The x axis represents the groups and the Y axis represents the percentage of positive cells which is directly proportional to the proliferation rate. *** p<0.001 and ** p<0.01 statistically significant with the control group, a p<0.001 and b <0.01 statistically significant with PC.

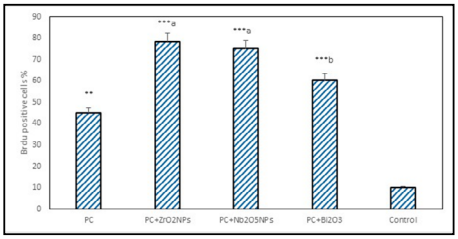


Figure 3. Invitro scratch assay images taken after 24hrs.

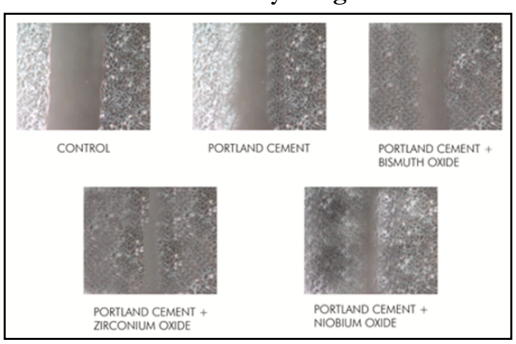
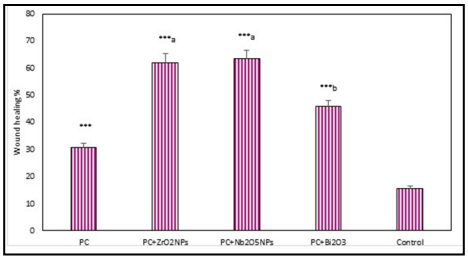


Figure 4. Graphical representation of the wound healing assay. The x axis represents the groups and the Y axis represents the percentage wound closure which is directly proportional to the cell migration rate. *** p<0.001 statistically significant with the control group, a p<0.001 and b <0.01 statistically significant with PC.



Discussion

Our institution is passionate about high quality evidence based research and has excelled in various fields [16, 27-36]

The results of the present study show that the proliferation and migration of dental pulp stem cells were maximum when treated with portland cement containing either zirconium oxide nanoparticle or niobium oxide nanoparticles as the radio opacifier as compared to Portland cement containing bismuth oxide as the radio opacifier. Portland cement is composed of tricalcium silicate ($3CaO \cdot SiO_2$), dicalcium silicate ($2CaO \cdot SiO_2$), tricalcium aluminate ($3CaO \cdot Al_2O_3$), and a tetra-calcium aluminoferrite (4CaO • Al2O3Fe2O3). In addition to these constituents, MTA contains additional bismuth oxide as the radio opacifier where as biodentin contains zirconium oxide as the radio opacifier. This study was designed to only examine the effect of the radio opacifier on the proliferation and migration of DPSCs. Hence portland cement was taken as the standard component and the radio opacifiers were altered.

The results of the present study are in accordance to the results of the study conducted by Silva et.al where zirconium oxide nanoparticles and zirconium oxide microparticles were added to Portland cement. The addition of these radio opacifying agents yielded a calcium silicate cement with a higher compressive strength and which was more biocompatible in terms of tissue inflammatory reaction as compared to a calcium silicate cement containing bismuth oxide.[37] Another study conducted by Silva et.al in 2017 has evaluated the influence of addition of zirconium oxide microparticles and nanoparticles and niobium oxide nanoparticles to Portland cement on the subcutaneous healing process in rats in comparison to MTA Angelus. The study has demonstrated an increased fibroblast proliferation and a faster regression of inflammatory reaction under the influence of calcium silicate cement containing either zirconium oxide nanoparticles/microparticles or niobium oxide nanoparticles. [38] A study comparing the coronal tooth discoloration caused by various endodontic materials has shown that all materials containing bismuth oxide elicit tooth discolouration in comparison to materials like EndoSequence and Biodentine.[39]

The inflammatory phase, regenerative phase and remodelling phase constitute the three phases of wound healing. [40] Vital pulp therapy involves the application of a pulp capping agent directly onto the exposed part of the pulp tissue which in turn maintains the vitality and function of the pulp. The initial inflammatory reaction if followed by migration of dental pulp stem cells to the site of injury and their differentiation to a new generation of odontoblast like cells which in turns helps to regenerate the dentin pulp complex. [41] They replace the lost odontoblast and synthesize and secrete tertiary dentin. [42] A study conducted by Katge et.al has shown the successful outcome after the use of MTA and Biodentine as a direct pulp capping agent with evident dentine bridge formation after a period of 6 months and 12 months. [43] It is important that the pulp capping agent must be both bioactive and biocompatible. Various chemokines affect the proliferation, migration and adhesion of cells. An increased migration and adhesion of human dental pulp stem cells was observed in the presence of Biodentine in a study conducted by Luo.et al. The study has also demonstrated an upregulation of Fibronectin, Intercellular Adhesion Molecule-1, Vascular Cell Adhesion Molecule-1 and Integrinb1 expression in human dental pulp stem cells in the presence of Biodentine. [11] A study conducted by Gomes Cornélio al et.al has shown a significant increase in mineralized nodule deposition in osteoblast cultures when niobium oxide was used as the radio opacifier in comparison with MTA plus and Biodentine.[44] Another study conducted by Mestieri.at al demonstrated greater viability of osteoblastic cells in the presence of Portland cement containing niobium oxide micro and nanoparticles in comparison to MTA. [45] These studies suggest a possible role of the radio opacifying agent used in calcium silicate cements on the activity of cells which in modulates wound healing which is also seen in our study.

Conclusion

The data obtained from the present study implies that zirconium oxide nano particles and niobium oxide nanoparticles show the highest rate of proliferation and migration of dental pulp stem cells. They could serve as an alternative to bismuth oxide in calcium silicate based cements used for pulp therapies to enhance the outcome.

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