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ORIGINAL ARTICLE

# The Growth of Dental Pulp Stem Cells in Portland Cement Micro-Environment

Shih-Min Wang[1], Chia-Hsuan Lee[2], Chih-Hung Lin[1], Gunng-Shinng Chen[3], Jiang-Chuan Liu[4], Chung-Hsing Li[3]

*[1]Graduate Institute of Dental Science, National Defense Medical Center, [2]School of Dentistry, National Defense Medical Center, [3]Division of Orthodontics, Dentofacial Orthopedics and Pediatric Dentistry, Department of Dentistry, Tri-Service General Hospital, School of Dentistry and Graduate Institute of Dental Science, National Defense Medical Center, [4]Department and Graduate Institute of Biology and Anatomy, National Defense Medical Center, Taipei City, Taiwan, Republic of China*

**Background:** Portland cement (PC), the base material of mineral trioxide aggregate has been applied *in vivo* and *in vitro* studies, and showed promising physical and mechanical properties on several cell lineages but human dental pulp stem cells (DPSCs), known as mesenchymal stem cells with their multipotency. Our study aims to evaluate the cytotoxicity of PC mixing with distilled water (Td) or normal saline (Tn) on DPSCs. **Materials and Methods:** DPSCs were isolated from pulp tissue and identified before viability assay. DPSCs were treated with the concentration of 100%, 50%, 25%, 12.5% and 6.25% extracts in Td and Tn. Cell viability was evaluate after 24 h, 48 h, 72 h and 7 days treating. Cells were analyzed for cell surface antigen expression by flow cytometry. The pH levels of extracts were detected by pH meter. Cell viability was determined with cell counting kit-8 assay. **Results:** Viability of Td and Tn showed the general trend that dropped slightly at 24 h, increased at 48 h then showed no statistical differences to control at 72 h. The viability of the concentration of 100% groups dropped gradually from 24 h to 72 h. Cell proliferation was improved in low concentration groups on 48 h and 72 h. On day 7, cell viability showed no statistical difference to control except Tn 50% (110.9%) and Tn 100% (84.0%). **Conclusions:** PC is probably a potential candidate for the use of pulp therapy, or further, a budding material for pulp regeneration.

Key words: Portland cement, dental pulp stem cell, cell viability, pulp therapy

## INTRODUCTION

Mesenchymal stem cells (MSCs) are known as postnatal stem cells and can be isolated from various human tissues such as adipose, bone marrow and skin.[1] Recently, novel therapeutic strategies have been progressing rapidly because of the applications of stem cells on regenerative medicine and cell-based therapies.[2-4] Dental pulp tissue is also a source of high-purity stem cells, which called dental pulp stem cells (DPSCs).[5,6] DPSCs are capable of self-renewal and can differentiate into various cell lineages. To date, DPSCs had been successfully induced into odontoblasts, osteoblasts, chondrocytes, myocytes, neurocytes, adipocytes, corneal epithelial cells, melanoma cells and induced pluripotent stems.[2]

Tricalcium silicate is the major component of Portland cement (PC), which is the base material of mineral trioxide aggregate (MTA).[7] MTA has been applied in endodontic treatment since the early 1990s such as pulp-capping, retrograde filling and perforation repair on account of its good seal ability, biocompatibility and other benefits.[8-10] PC showed promising physical and mechanical properties as well as MTA on different cell lineages and can be used as a safe pulp-capping material.[11,12] In addition, PC facilitated odontoblastic differentiation on human periodontal ligament fibroblast and human pulp cell.[13,14] However, MTA is a very expensive material, and PC is much more cheaper than MTA. It could provide the benefit in dental clinical service. According to CNS61 (Chinese National Standards) and ASTM150 (American Society for Testing and Materials), there are 5 major types of cement. Type 1 is the most common one for general uses. That is why we chose type 1. The composition of other types of cement is altered to change the specific property to meet the industrial needs. For example, in type 3 cement, the ratio of tricalcium silicate to dicalcium silicate is increased to inforce early intensity. This modification also increases the heat of hydration.[15]

Portland cement powder consists of mainly calcium oxide, which is highly hydrophilic that solidified when it contacts water. The setting process is caused by continuous hydration and hydrolysis reactions.[15] In an aqueous environment of different saline concentrations, PC absorbs water and releases

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Corresponding Author: Dr. Chung-Hsing Li, No. 161, Sec. 6, Minquan E. Rd., Neihu Dist., Taipei 114, Taiwan, Republic of China. Tel: 02-87923311 ext 12608; Fax: 02-87927147.
E-mail: chiyenchli@yahoo.com.tw

calcium hydroxide (CH) into the environment.[16] In other words, liquid used for mixing plays an important role during the reaction, as well the ratio of the liquid to the powder. Nowadays, there are a number of studies of PC *in vitro* and *in vivo*.[12-14,17,18] However, there is still no report on the effects of PC on DPSCs. In this study, we cultured DPSCs in different kinds of extract from type 1 PC mixed with distilled water (Td) or normal saline (Tn). The aim is to test the possibility of PC to be applied in pulp therapies by investigating the viability of DPSCs in the series concentration of extracts.

## MATERIALS AND METHODS

### Dental pulp stem cells isolation and culture

Teeth samples were collected with written informed consent from the donor undergoing tooth extraction for orthodontic treatment at Orthodontics and Dentofacial Orthopedics Division of Dental Department in Tri-Service General Hospital (TSGH). Our study was approved by the medical Ethical Committee of TSGH (099-05-143). Dental pulp tissue was taken out from pulp chamber and roots after cut crown off. The tissue was treated with dispase II (3 mg/mL) and collagenase I (4 mg/mL) for 40-60 min in 37°C water bath. The digested tissue was removed to a 6-cm dish flooded with essential culture medium (alpha-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL Streptomycin, 2 mM L-glutamine, $5 \times 10^{-5}$ M 2-mercaptoethanol and $10^{-4}$ M L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate). The dish 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 subcultured.

### Dental pulp stem cells identification (surface molecule characterization)

Cells were analyzed for cell surface antigen expression by flow cytometry (FACSCalibur™, BD Biosciences, CA, USA). In brief, for each cell marker, $5 \times 10^5$ cells were fixed with 4% paraformaldehyde at 4°C for 30 min. To determine the stemness, cells were then separately incubated with antibodies against CD44, CD73 and CD90 for positive makers of MSC, and incubated with antibodies against CD31, CD34 and CD45 for negative makers of hematopoietic stem cell (HSC). κ-isotype control served as a control (BD Biosciences Pharmingen, CA, USA). The resulting data were analyzed using the Cell Quest™ Pro software (BD Biosciences).

### Extracts of Portland cement

The extract is prepared according to ISO 10993.5 (International Organization for Standardization). Briefly, the commercial PC (Taiwan Cement Company, ROC) was mixed with distilled water (Td) normal saline (Tn) 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.

### pH level of Portland cement extract

Portland cement extract was obtained as described above at the liquid/powder ratio of 1:2, 1:3 and 1:4, the pH level of extracts were detected by pH meter (Jenco, CA, USA).

### Cell viability assay

Dental pulp stem cells were seeded in 96-well culture plate at the density of $4 \times 10^4/cm^2$. After 24 h, the medium was removed, and 100 μL extract (100%, 50%, 25%, 12.5% and 6.25%, Td and Td groups) was, respectively, added to each well. The culture plate was then incubated at 37°C. Cell viability assay was preformed after 24 h, 48 h, 72 h and 7 days culture by adding 10 μL cell counting kit-8 solution (Sigma, MO, USA) to each well. After the culture plate was incubated for 2 h, 10 μL of 10% sodium dodecyl sulfate solution was added for stopping the reaction. The plate was read by spectrometer at O.D. 450 nm to determine the viability.

### Statistical analysis

Statistical analysis was performed using Student's *t*-test and one-way ANOVA. The level of significance was set at $P < 0.05$. All data are expressed as means ± standard deviation.

## RESULTS

### Dental pulp stem cells identification

Flow cytometric analysis of cultured DPSCs revealed expression of MSC makers CD44 (99.55%), CD73 (99.40%) and CD90 (99.72%), but was negative for surface molecules of HSC markers CD31 (0.29%), CD34 (0.13%) and CD45 (0.28%) [Figure 1].

### pH level of Portland cement extracts

The pH level of extracts from all groups (PC mixed with distilled water, Td or normal saline, Tn in different liquid/powder ratio) increased compared with that of original medium after 24 h extraction [Figure 2]. The pH level of Tn extracts (1:2-8.91 ± 0.17, 1:3-9.13 ± 0.03, 1:4-8.98 ± 0.42) showed higher alkalinity than Td extracts (1:2-8.60 ± 0.42, 1:3-8.55 ± 0.37, 1:4-8.29 ± 0.22). However, there was no significant difference between groups.

### Cell viability assay

Dental pulp stem cells were exposed to the extracts at a series concentrations for 24 h, the viability of control was considered 100%. The viability of other groups were generally lower than control [Figure 3a]. At the concentrations of 6.25% (96.5%) and 50% (97.3%) in Tn, the viability showed

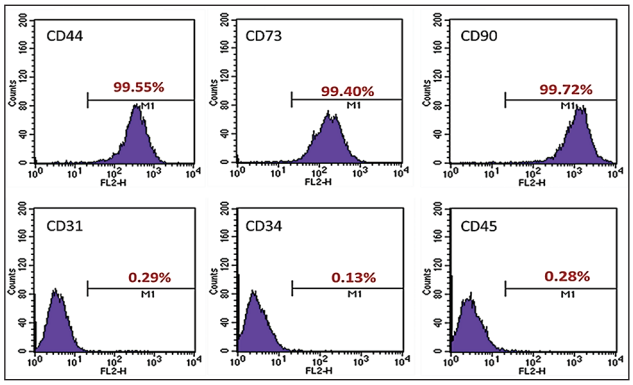


**Figure** 1: Flow cytometric analysis showed that high-purity dental pulp stem cells stained with positively selected mesenchymal stem cell markers and negatively selected hematopoietic stem cell markers

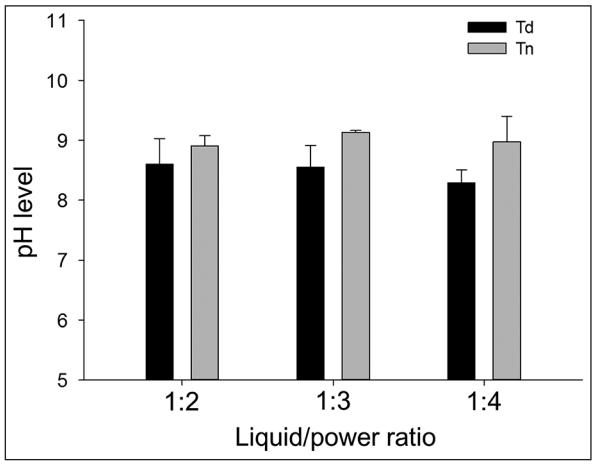


**Figure** 2: pH level of Portland cement (PC) extracts after 24 h extraction in different liquid/powder ratio. PC mixed with distilled water (Td) and normal saline (Tn) ($n = 3$)

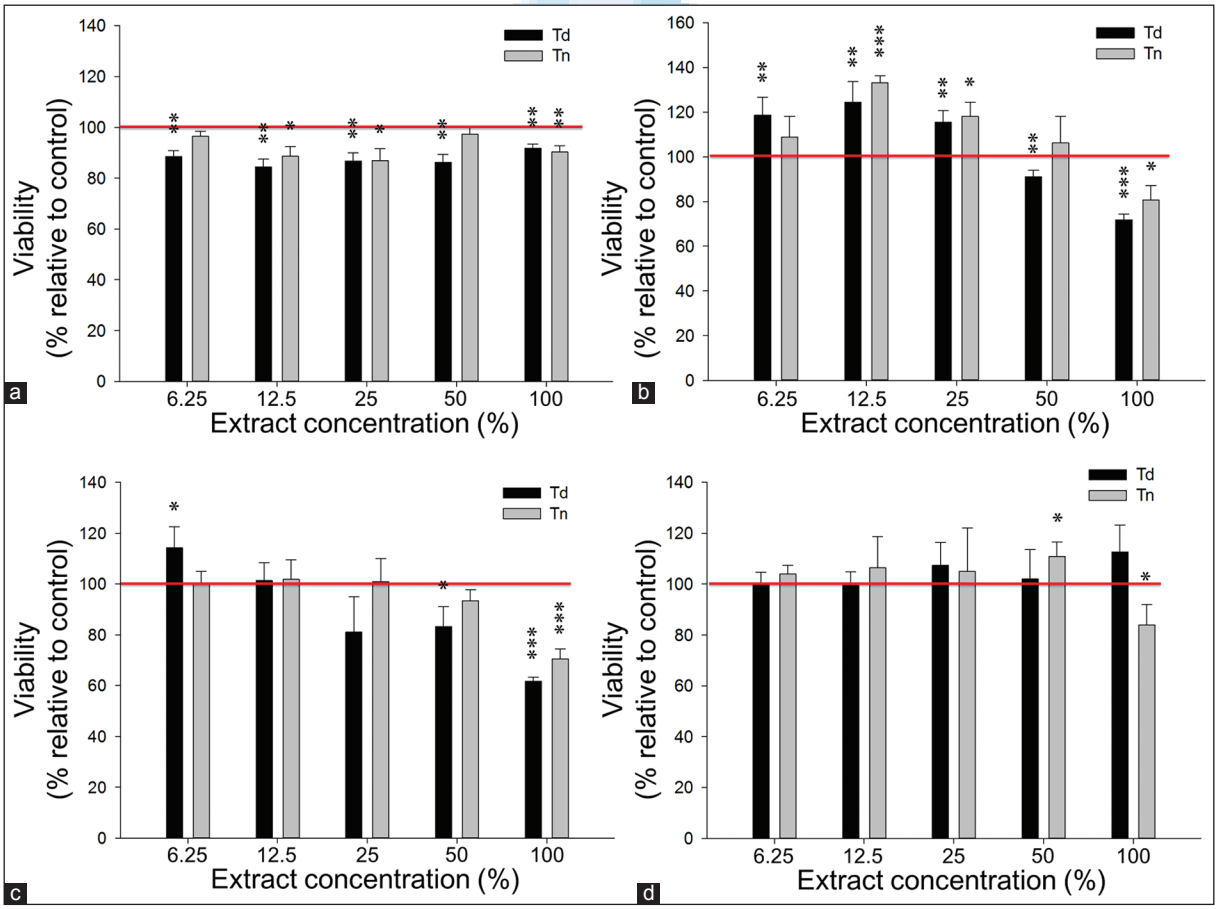


**Figure** 3: Viability of dental pulp stem cells on (a) 24 h, (b) 48 h, (c) 72 h and (d) 7 days were determined with cell counting kit-8 assay. Significant differences were determined using Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with control ($n = 4$.)

no statistical difference when compared with control. The viability in Td at the concentrations of 6.25%, 12.5%, 25%, 50%, 100%, and in Tn at the concentrations of 12.5%, 25%, and 100% (88.9%, 84.4%, 86.8%, 86.3%, 91.8%, 88.6%, 86.9% and 90.2%, respectively) were significantly lower than the viability of control.

At 48 h [Figure 3b], the viability in Td at the concentrations of 50% (91.0%), 100% (71.9%), and in Tn at the concentration of 100% (80.7%) were lower than control significantly while 6.25% (108.8%) and 50% (106.2%) were not significantly different from control. However, cell viability was significantly higher than control in Td at the concentrations of 6.25%, 12.5%, 25%, and at the concentrations of 12.5% and 25% in Tn (118.7%, 124.5%, 115.5%, 133.1% and 118.2%, respectively).

After 72 h treating [Figure 3c], only Td group at the concentration of 6.25% (114.2%) was significantly higher than control; the concentration of 50% (83.3%) was significantly lower than control. The viability in Td at the concentrations of 12.5%, 25%, and in Tn at the concentrations of 25%, 12.5%, 25%, 50% (101.3%, 81.1%, 100.0%, 101.9%, 100.9% and 93.4%, respectively), there was in the absence of statistical difference to control.

At day 7 [Figure 3d], the viability in Tn at the concentration of 50% (110.9%) was significantly higher than control; at the concentration of 100% (84.0%) was significantly lower than control. In Td at the concentrations of 6.25%, 12.5%, 25%, 50%, 100%, and in Tn at the concentrations of 6.25%, 12.5% and 25% (99.7%, 99.4%, 107.4%, 102.0%, 112.6%, 104.0%, 106.4% and 105.0%.) the viability showed no statistical difference to control.

In Figures 4-6, different concentrations of the same extract were compared. In Td, viability was all lower than 100% at 24 h [Figure 4a], decreased at the concentrations of 50% and 100% at 48 h, but increased at low concentrations [Figure 5]. At 72 h at the concentrations of 25%, 50%, and 100%, the viability reduced obviously [Figure 6]. In Tn at the concentrations of 12.5%, 25%, and 100% showed mild lower viability after 24 h treating [Figure 4b]. While viability rose at 48 h and maintained stable at 72 h except 100% [Figures 5 and 6].

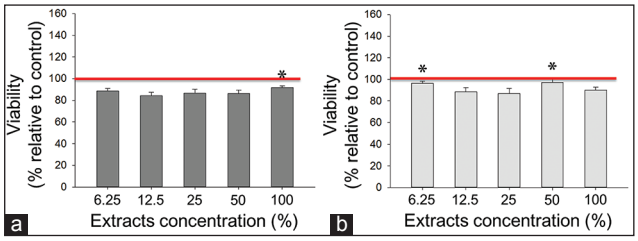


**Figure** 4: (a) Distilled water (Td) at 24 h. $^{*}P < 0.05$ when compared to 12.5%. (b) Normal saline (Tn) at 24 h. $^{*}P < 0.05$ when compared to 25%

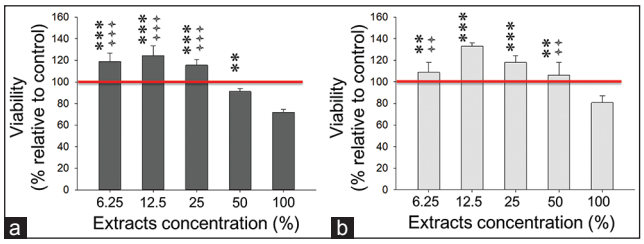


**Figure** 5: (a) Distilled water (Td) at 48 h. $^{**}P < 0.01$, $^{***}P < 0.001$ comparing to 100%, $^{***}P < 0.001$ comparing to 50%. (b) Normal saline (Tn) at 48 h. $^{**}P < 0.01$, $^{***}P < 0.001$ comparing to 100%, $^{**}P < 0.01$ comparing to 12.5%

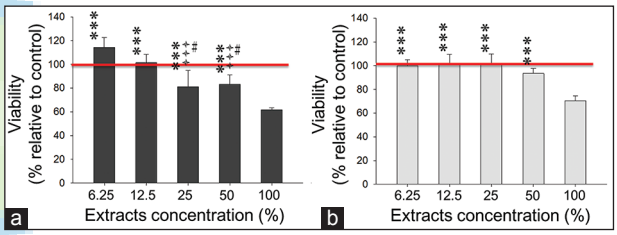


**Figure** 6: (a) Distilled water (Td) at 72 h. $^{***}P < 0.001$ when compared to 100%, $^{***}P < 0.001$ when compared to 6.25%, $^{\#}P < 0.05$ when compared to 12.5%. (b) Normal saline (Tn) at 72 h. $^{***}P < 0.001$when compared to 100% ($n = 4$)

## DISCUSSION

DPSC identification was performed by flow cytometry. Our results are consistent with the study in 2011 by Karbanová *et al.*,[19] which determined the cells we used were DPSCs. PC extracts showed the pH that ranged between 8.2 and 9.1 in the aqueous environment after 24 h incubation. During the setting process, CH is formed and then released from PC into solution, which becomes alkaline because of the dissociation of CH while extraction.[15] The low alkalinity (under pH 11.0) of CH solution is considered that it is possible to induce the formation of hard tissue and is not overly toxic.[20,21] Since the pH level showed no difference between different liquid/powder ratio and considering the manipulating process in PC mixing we choose the 1:3 liquid/powder ratio for viability tests.

An *in vitro* cytotoxicity test on extract of materials can be carried out on certain or different cell lines at the same time. This kind of cell culture technique was widely used for biocompatibility evaluation.[22] In our study, the viability of both Td and Tn showed the general trend that cell viability dropped slightly at 24 h, increased at 48 h then came back to the level, which has no statistical difference to control [Figure 3]. Only the viability at the concentration of 100% groups dropped gradually from 24 h to 72 h. Yoshino *et al.* reported that PC extract did not show cytotoxic activity over cultured periodontal ligament fibroblasts,[13] which lends support to our results. In Lee *et al.*'s research, the effects of

calcium phosphate cements on pulp cells were investigated, and both PC and MTA were compared.[11] The results show that the viability of PC and MTA were both significantly lower than control on day 1, however, PC group showed no difference to control on day 7, but MTA group showed no difference to control till day 14. In our study, cell viability of Td showed no statistical difference to control on day 7. These findings are in line with Lee *et al.*'s study.[11]

When different concentrations of each kind of extract were compared, the differences between each concentration are shown in Figures 4-6. Viability of 100% extract of both Td and Tn were apparently lower than other concentration. The most interesting part is that cell proliferation was improved in low concentration groups on 48 h and 72 h. As for day 7, cell viability of Td at 100% was even slightly higher than those at lower concentrations. Even though similar characteristics had been revealed in Kwon *et al.*'s study, which showed immortalized human gingival fibroblast had a relatively high proliferation rate in diluted extracts of zinc oxide-eugenol cements,[23] these results had never been found on DPSCs in previous studies. Nevertheless, such characteristics will be considered an economical and productive view of culturing cells.

Up to the present, we've known that PC is able to facilitate odontoblastic differentiation on dental pulp cells,[18] encourage hard tissue deposition[24] and show good biocompatibility. Several studies reported the use of PC in vital pulp therapy with considerable success in animal models.[12,25,26] In addition, two clinical case reports, which revealed that PC may be considered as an effective and economic substitute of MTA for primary molar pulpotomies in children.[27] However, the mechanisms of the effects of PC on DPSC and pulp cell *in vitro* and *in vivo* are still unclear. This is the first study of our project, which is designed to figure out how PC affects DPSCs.

Based on the present studies and our results, we suggest that PC is probably a potential candidate for the use of pulp therapy, or further, a budding material for pulp regeneration.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Hemmat S, Lieberman DM, Most SP. An introduction to stem cell biology. Facial Plast Surg 2010;26:343-9.
2. Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E. Mesenchymal stem cells in the dental tissues: Perspectives for tissue regeneration. Braz Dent J 2011;22:91-8.
3. Watson L, Elliman SJ, Coleman CM. From isolation to implantation: A concise review of mesenchymal stem cell therapy in bone fracture repair. Stem Cell Res Ther 2014;5:51.
4. Lau RL, Perruccio AV, Evans HM, Mahomed SR, Mahomed NN, Gandhi R. Stem cell therapy for the treatment of early stage avascular necrosis of the femoral head: A systematic review. BMC Musculoskelet Disord 2014;15:156.
5. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and *in vivo*. Proc Natl Acad Sci U S A 2000;97:13625-30.
6. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, *et al.* Stem cell properties of human dental pulp stem cells. J Dent Res 2002;81:531-5.
7. Torabinejad M, Smith PW, Kettering JD, Pitt Ford TR. Comparative investigation of marginal adaptation of mineral trioxide aggregate and other commonly used root-end filling materials. J Endod 1995;21:295-9.
8. Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. J Endod 1993;19:541-4.
9. Schmitt D, Lee J, Bogen G. Multifaceted use of ProRoot MTA root canal repair material. Pediatr Dent 2001;23:326-30.
10. Torabinejad M, White DJ. Tooth filling material and method of use. Google Patents; 1998.
11. Lee SK, Lee SK, Lee SI, Park JH, Jang JH, Kim HW, *et al.* Effect of calcium phosphate cements on growth and odontoblastic differentiation in human dental pulp cells. J Endod 2010;36:1537-42.
12. Holland R, de Souza V, Murata SS, Nery MJ, Bernabé PF, Otoboni Filho JA, *et al.* Healing process of dog dental pulp after pulpotomy and pulp covering with mineral trioxide aggregate or Portland cement. Braz Dent J 2001;12:109-13.
13. Yoshino P, Nishiyama CK, Modena KC, Santos CF, Sipert CR. *In vitro* cytotoxicity of white MTA, MTA Fillapex® and Portland cement on human periodontal ligament fibroblasts. Braz Dent J 2013;24:111-6.
14. Min KS, Kim HI, Park HJ, Pi SH, Hong CU, Kim EC. Human pulp cells response to Portland cement *in vitro*. J Endod 2007;33:163-6.
15. Neville AM. In: Adam M, editor. Properties of Concrete. 5th ed. Pitman London: Prentice Hall; 2012.
16. Fridland M, Rosado R. Mineral trioxide aggregate (MTA) solubility and porosity with different water-to-powder ratios. J Endod 2003;29:814-7.

17. Saidon J, He J, Zhu Q, Safavi K, Spångberg LS. Cell and tissue reactions to mineral trioxide aggregate and Portland cement. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;95:483-9.
18. Min KS, Lee SI, Lee Y, Kim EC. Effect of radiopaque Portland cement on mineralization in human dental pulp cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;108:e82-6.
19. Karbanová J, Soukup T, Suchánek J, Pytlík R, Corbeil D, Mokrý J. Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium. Cells Tissues Organs 2011;193:344-65.
20. Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. J Endod 1995;21:349-53.
21. Gordon TM, Ranly DM, Boyan BD. The effects of calcium hydroxide on bovine pulp tissue: Variations in pH and calcium concentration. J Endod 1985;11:156-60.
22. ISO Ducuments. ISO 10993-5. Biological evaluation of medical devices — Part 5: Tests for *in vitro* cytotoxicity. Geneva: International Standard; 2009.
23. Kwon JS, Illeperuma RP, Kim J, Kim KM, Kim KN. Cytotoxicity evaluation of zinc oxide-eugenol and non-eugenol cements using different fibroblast cell lines. Acta Odontol Scand 2014;72:64-70.
24. Holland R, de Souza V, Nery MJ, Faraco Júnior IM, Bernabé PF, Otoboni Filho JA, *et al*. Reaction of rat connective tissue to implanted dentin tube filled with mineral trioxide aggregate, Portland cement or calcium hydroxide. Braz Dent J 2001;12:3-8.
25. Wucherpfennig AL, Green DB. Mineral trioxide vs. Portland cement two biocompatible filling mate. J Endod 1999;25:308.
26. Menezes R, Bramante CM, Letra A, Carvalho VG, Garcia RB. Histologic evaluation of pulpotomies in dog using two types of mineral trioxide aggregate and regular and white Portland cements as wound dressings. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98:376-9.
27. Conti TR, Sakai VT, Fornetti AP, Moretti AB, Oliveira TM, Lourenço Neto N, *et al*. Pulpotomies with Portland cement in human primary molars. J Appl Oral Sci 2009;17:66-9.