Journal of Dentistry Indonesia

Volume 25
Number 2 *August*

Article 5

8-28-2018

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## Recommended Citation

Özen, B., S, S. S., Mousavi, A., Semeins, C. M., Tüzüner, T., İnce, E. B., – Schriks, M. C., van Strijp, A., & Bakker, A. D. The Importance of Storage Time for Human Dental Pulp Cells Isolation. J Dent Indones. 2018;25(2): 99-103

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*Journal of Dentistry Indonesia 2018, Vol. 25, No.2, 99-103*
*doi: 10.14693/jdi.v25i2.1227*

***ORIGINAL ARTICLE***

# The Importance of Storage Time for Human Dental Pulp Cells Isolation

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## ABSTRACT

**Objective:** To compare the importance of storage time and the tooth type for isolation of dental pulp cells (DPCs) from extracted human teeth. **Methods:** 35 human teeth were used in this study. The teeth were stored in phosphate buffered saline (PBS) after extraction and divided into two groups randomly according to the time elapsed between extraction and isolation. In group one, the isolation was performed within 2 hours and in the other group it was performed 24 hours after extraction. **Results:** No significant differences between isolation time and total cell counts (p=0.483) and between isolation time and viable cells (p=0.341). No significant differences between the first molar and the premolar related cell counts and viable cells, but both teeth groups showed significant higher viability and had higher total cell amounts than third molars after isolation. Statistically significant correlations were found between age of donors and viable cells and viability after 24 hours isolation time. **Conclusion**: The immediate isolation of DPCs is not necessary after the tooth extraction. The tooth can be stored in PBS at room temperature up to twenty four hours after the extraction without a significant reduction in cell viability and counts. The cells obtained from younger donors might have more chance for more viability even if storage time was extended. Premolars and first molars were better donors than the third molars for DPCs isolations and the high number of success revascularization rate in premolars with necrotic immature premolars might be because of their high cell viability potentials.

**Key words:** Dental pulp stem cells (DPSCs), storage time, isolation

How to cite the article: Bugra Özen, Salia Shabazi S, Arash Mousavi, Cor M Semeins, Tamer Tüzüner, Elif Bahar Tuna İnce, Martine CM van Gemert-Schriks, AJP van Strijp, Astrid D Bakker. The importance of storage time for human dental pulp cells isolation. J Dent Indones. 2018;25(2):99-103.

## INTRODUCTION

There are many areas of research for which dental stem cells are currently used. They offer potential for tissue regeneration in dentin, periodontal ligament and dental pulp. Even enamel tissue engineering has been suggested. Also the use of dental stem cells as sources of cells to facilitate repair of non-dental tissues such as bone and nerves have been introduced.[1,2]

Dental pulp stem cells (DPSCs) were discovered by Songatao Shi and Stan Gronthos in human dental pulp tissues of extracted third molars in year 2000.[3] Other type of human dental pulp-derived stem cells were later identified such as dental pulp of human exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), and human supernumerary tooth derived stem cells (SNTSCs).[4,5] These cells have all a common origin and are derived from neural crest cells, which provide mesenchymal stem cell-like properties.[6]

Among these stem cells, DPSCs are relatively easily achievable and have a high self-renewal capacity and proliferation activity. They are capable of differentiation into mesenchymal lineage cells including odontoblasts, osteoblasts, chondrocytes, adipocytes, and myocytes. Therefore, they present a gold standard for the repair of small body defects in low risk autologous therapeutic approaches.[2]

The results so far give good assumptions for the use of stem cells in clinical trials and the role they may have

in regenerative therapies .DPSCs may allow the root maturation in endodontically treated immature teeth with periradicular periodontitis or abscess.[7,8]

A growing number of studies have focused on the characteristics of DPSCs, development of isolation or growth strategies of these cells.[9,10] Characterization of these cells, and determination of their potentials in terms of specificity of regenerative response, may help new clinical treatment approaches. Such findings may supply an innovative and novel biologically based new generation of clinical materials and/or treatments for dental disease[10]. Research has shown that whenever biomaterials have been in contact with DPSCs, the proliferation and differentiation of the DPSCs into cells almost identical to odontoblasts has been induced.[11]

Many factors influence both the quantity and quality of DPSC tissue available for future clinical or bioengineering applications. One of the primary factors is the tooth type used for the isolation of DPSCs. It is important to evaluate which type of tooth offers the best DPSC property for the improvement of the clinical outcomes of the treatment. Impacted third molars were used in the original studies, also SHEDs have been used as an excellent source of cells.[12] Other studies reported DPSCs isolation from supernumerary teeth[8]. DPSCs from permanent and SHEDs have been compared, and SHEDs reported higher proliferation rates.[13] Determining the duration of time DPSCs remain viable and establishing standardized cell isolation and expansion protocols, are really important in isolation of DPSCs. Still, one of the practical issues for cell therapies using DPSCs is the potential degradation of the pulp tissue between the time of tooth extraction and DPSC isolation (storage time).[14]

Current methods of tooth storage and the viability of DPSCs have limitations. For in vitro experiments, the pulp cells sometimes cannot be isolated freshly after the extraction. Therefore, it is important for the researchers to find out the maximum storage time for the cells to remain viable. DPSCs are the small fraction of dental pulp cells that possess Mesenchymal Stem Cells (MSCs) characteristics. Thus, this study aimed to compare the importance of the storage time and tooth type (premolars or molars) with the highest cell viability for DPSCs and the heterogeneous mix population of dental pulp cells isolation from extracted human teeth.

## METHODS

Ethical approval was obtained to undertake this study from VU Medisch centrum Ethical Committee (2015.562). The protocol was followed according to S. Gronthos et al.[15] for the isolation of dental pulp stem cells. This method involves the enzymatic digestion of fresh samples of pulp. Different dentists were asked

1. *The pulp was cut into small pieces in 5ml HFF HFF= HBSS (Hank's Balance Salt Solution)+ 5% FBS*
2. *5ml cold HFF was added*
3. *Centrifuged 10 min at 400G*
4. *The supernatant was removed*
5. *Add 5 ml collagenase solution ( 3mg/ml PBS)*
6. *Incubated 1 hour in 37 $^0$ C and mixed every 15 min*
7. *Washed 2 times with 5 ml HFF*
8. *Centrifuged 10 min at 400G*
9. *The supernatant was removed and pellet was re- suspended with 1 ml clonogenic growth medium.*
10. *The cell suspension was passed through a 70 μm strainer*

**Figure 1.** The protocol of the study

to donate non-carious extracted teeth for this study. If the patient agreed to provide teeth that were being extracted, the teeth were stored in phosphate buffered saline (PBS). In this study, 35 extracted teeth (13 pairs premolars, 4 first molars and 5 third molars) were stored in PBS at room temperature after the extraction. The teeth were obtained from 10 female and 12 male patients between the ages 11 and 28 years old (mean age 15.8±4.9 years old). The teeth were randomly divided in two groups. The DPSCs and the heterogeneous mix population of dental pulp cells were isolated in one group two hours after the extraction and in the other group twenty-four hours after the extraction.

The teeth were cut in a way that crown and root are still connected. In the cabinet the crown is broken of the root and the pulp was removed from the root using K-files. For premolar tooth, as the pulp tissue volume is smaller than molars, one maxillary and one mandibular premolar pulp tissue were combined from the same donor to reach the same amount of pulp tissue with molars and these two premolars counted as one tooth. For that reason, two premolars, one from maxilla and the other from mandibula extracted from the same donor in one session were included to the study.

The protocol of the study followed by the further steps as shown in Figure 1.After the protocol was completed, the cells were counted with Muse cell counter in

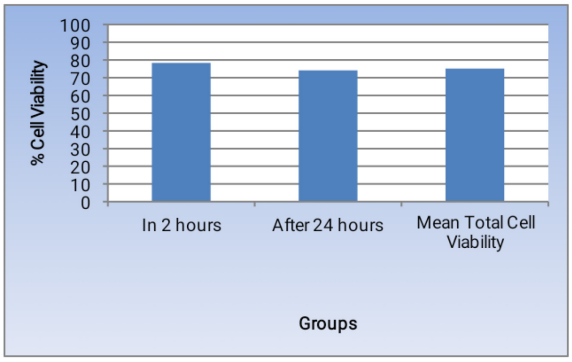


**Figure 2.** Cell viability in groups. There was no significant difference of the % cell viability between groups of storage.

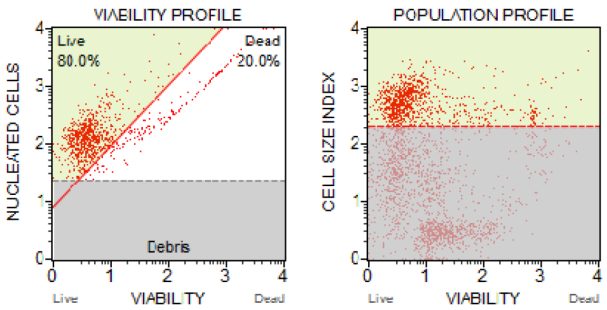


**Figure 3.** The viability profile and population profile of median according to cell counter.

order to evaluate the cell viability and storage time differences. Furthermore, the cells were seeded in 3 different densities into the 6 well-plates for further investigation.

**Statistical analysis**

Statistical analysis were performed with SPSS for Windows 17.0. The normality of data for continuous varibles (age, viable cell counts, total cell counts) were calculated with Shapiro Wilk test. Descriptive statistics were given as mean±sd (median; min-max) for continous variables, median (min-max) for viability values and ratios (%) as for genders when needed.

To determine the differences between isolation time periods regarding the viable cell and total cell counts, Mann Whitney U test was used. Kruskal Wallis, Mann Whitney U tests with Bonferroni correction were used for comparing the viable cell and total cell counts among tooth types. Viability and gender values were compared with Chi-square tests for both isolation time period and tooth types. Spearmen test was also used for obtaining correlation between age-viable cell counts, total cell counts and viability values. The confidence level was set as 95% for all tests.

## RESULTS

The mean of total cell viability of the samples was 75.4% and the median was 80%. The viability of the group which the cell was isolated in 2 hours was 78.3% and it was 74.1% in the after 24 hours group but there was no significant difference between the groups (p=0.341) (Figure 2). The median viability profile and population profile were shown in Figure 3. Moreover, there were no significant differences between the isolation time regarding the viable cells (p=0.443) and the total cell counts. (p=0.483).

There were no statistically significant differences between the premolar and the first molar teeth in regards to the viable cells (p=0.113) and the total cells

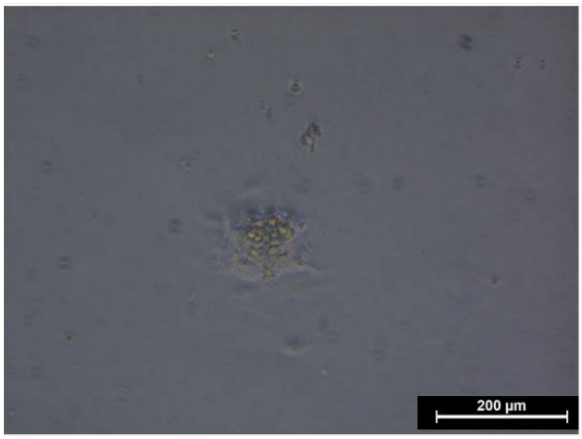


**Fig ure 4.** A colony of pulp stem cells in the CFU assay. The microscope: Leica DM-IL inverted microscope with photo setup (camera : DFC 7000T). The magnification: 10x

counts (p=0.089). Significantly, lower values were obtained in third molars when the premolar (p=0.016, p=0.012) and the first molar(p=0.014, p=0.014) were compared regarding the viable cells and the total cell amounts after isolation (respectively). However, there were no significant differences between the tooth types regarding the viability (p=0.306). Gender had no effect on the viability, viable cells and total cell amounts.

Finally, in the "two hours" isolation group, there were no significant correlations between age and viable cell (p=1;p>0.05), total cells (p=0.626;p>0.05), viability (p=0.682;p>0.05). On the other hand, statistically significant correlations were found in "after 24 hours" isolation group, between age and viable cell (r=-0.750;p=0.003;p<0.01), total cells (r=-0.786;p=0.001;p<0.01), and viability (r= 0.627;p=0.022;p<0.05).

Moreover, it was a successful method for the isolation of the DPSCs that the colonies of pulp stem cells were observed in the CFU assay for all groups. In general, more DPSC are situated at the center of the wells compared to the margins (Figure 4).

## DISCUSSION

This study evaluated the cell counts and cell viability in groups, where the isolation time was two hours after the extraction and twenty-four hours after the extraction. This research indicated that there is no need for immediate isolation even if when the tooth was stored at room temperature. This methodology enabled us to collect the teeth from all around of The Netherlands without considering cold-chain transportation and storage time. Furthermore by that way, the researchers could reach to different types of teeth from different age groups. In literature most of the researchers studied only the impacted third molars[16-18] and in contrast to our study they stored the teeth on ice pack and immediately transported them to the lab for sample processing[18]. It should be recognized that our approach differs somewhat from the recent literature in terms of the storage time, age of donors and the transportation temperature.

Dental pulp stem cells could play a key role in future regenerative treatments because of their high plasticity and multipotential capabilities[9,19]. However, for this strategy to become clinically possible, achievable methods for tooth storage and isolation should be developed. There are several studies which report the isolation methods and storage time of DPSCs[20]. It was pointed out by Eubanks et al that the time frame of storage did not affect the ability to isolate the DPSCs[14]. Their study describes that the overnight storage of extracted third molars in saline at $4^0$C was an accepted condition for the isolation and expansion of DPSCs. This study also confirms that there were no differences between storage time and cell counts and cell viability. Other studies also reported that immediate processing and storage after extraction might not influence the successful banking of DPSCs. The present study is consistent with other studies[21,22] that demonstrated a time dependent reduction in number of isolated DPSCs the heterogeneous mix population of dental pulp cells from extracted teeth as the storage time increased. Our data shows that there was no significant difference with different storage times but the number of isolated viable cells decreased by waiting twenty- four hours after the extraction. The protocol for isolating DPSCs was successful. The average viability of the cells was 75.4%. Some protocols recommend 4 $^0$C as the optimal storage temperature in order to prevent the reduction of DPSCs viability[14,15] and this study followed also the protocol of Gronthos.[15] As the teeth were selected from different dental clinics, there was no possibility to transport them at 4 $^0$C for this study. The extracted teeth were stored in PBS solution at room temperature after the extraction. Therefore, room temperature was used constantly to ensure no change in cell specific markers due to temperature changes during the storage. The viability of immediately isolated DPSCs and the heterogeneous mix population of dental pulp cells was 78.3% and the viability for the group isolated 24 hours after the extraction in room temperature was 74.1%. Therefore, this research determines that storage in room temperature in PBS solution is also an applicable option for maintaining teeth for DPSC isolation.

The DPSCs were used from isolated dental pulp and not from periodontal ligament or roots, and also only intact young pulp were included in order to limit the microbial contamination. It is well known that sterilization of extracted teeth is an important factor in determining the success of building DPSC cultures.[21] An interesting finding of this study was the correlation between age and viability & viable cells regarding isolation time. In the light of these findings, the cells obtained from younger donors might have more chance for more viability even if storage time was extended.

Another valuable result from our study was that the premolars and the first molars showed higher cell viability and counts after isolation than the third molars. Therefore, they might be a better candidate for DPSCs isolation. To our knowledge, there are no studies that compared these parameters earlier. In some case reports of pulp revascularization premolars showed high success rate of healing. The high number of success rate in premolars revascularization with necrotic immature premolars might be because of their high cell viability potentials.[23,24]

This study determined that the storage time of extracted teeth in room temperature in PBS, up to 24 hours after the extraction did not affect the cell viability or cell counts of DPSCs and the heterogeneous mix population of dental pulp cells . Additional studies are needed to evaluate these cells and their pulp healing and regenerative potentials.

## CONCLUSION

This study concluded that the procedure for isolation of the DPSCs and the heterogeneous mix population of dental pulp cells from extracted teeth should be started within 24 hours, but the cells obtained from younger donors might have more chance for more viability even if storage time was extended. Premolars and first molars were better donors than the third molars for DPSCs isolations and the high number of success revascularization rate in premolars with necrotic immature premolars might be because of their high cell viability potentials.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

## ACKNOWLEDGMENT

The first pilot part of the study was presented as an EAPD (2016) conference paper but this manuscript is the extended version of that conference paper with new parameters and more samples.

## REFERENCES

1. Bojic S, Volarevic V, Ljujic B, Stojkovic M. Dental stem cells--characteristics and potential. Histol Histopathol. 2014;29:699-706.
2. La Noce M, Paino F, Spina A, Naddeo P, Montella R, Desiderio V, De Rosa A, Papaccio G, Tirino V, Laino L. Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. J Dent. 2014;42:761-8.
3. 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
4. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003;100:5807-12.
5. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One. 2006;1:e79.
6. Sonoda S, Tomoda E, Tanaka Y, Yamaza T. Properties and Possibilities of Human Dental Pulp-Derived Stem Cells. Arch Stem Cell Res 2015;2: 1012.
7. Goldberg M. The Diversity of Pulp Stem Cells, Tools for Regenerative Biodentistry M J Dent 2016;1: 002.
8. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S. The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. J Endod. 2008;34:645-51.
9. Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. Trends Cell Biol. 2010;20:715-22.
10. Waddington RJ, Youde SJ, Lee CP, Sloan AJ. Isolation of distinct progenitor stem cell populations from dental pulp. Cells Tissues Organs. 2009;189:268-74.
11. Paranjpe A, Smoot T, Zhang H, Johnson JD. Direct contact with mineral trioxide aggregate activates and differentiates human dental pulp cells. J Endod. 2011;37:1691-5.
12. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch Oral Biol. 2012;57:1439-58.
13. Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. J Endod. 2010;36:1504-15.
14. Eubanks EJ, Tarle SA, Kaigler D. Tooth storage, dental pulp stem cell isolation, and clinical scale expansion without animal serum. J Endod. 2014;40:652-7.
15. Gronthos S , Arthur A, P. Bartold PM, Shi S. [ Vemuri M, Chase LG, RaoA MS (eds)] Method to Isolate and Culture Expand Human Dental Pulp Stem Cells Mesenchymal Stem Cell Assays and Applications. Methods in Molecular Biology. Humana Press, Springer, 2011;698:107-121.
16. Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, et al. Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: Implications in neovascularization, osteo-, adipo- and neurogenesis. Pharmacogenomics J. 2010;10:105–13.
17. Atari M, Barajas M, Hernández-Alfaro F, Gil C, Fabregat M, Ferrés Padró E, et al. Isolation of pluripotent stem cells from human third molar dental pulp. Histol Histopathol. 2011;26:1057–70.
18. Raoof M, Yaghoobi MM, Derakhshani A, Kamal-Abadi AM, Ebrahimi B, Abbasnejad M, Shokouhinejad N. A modified efficient method for dental pulp stem cell isolation. Dent Res J 2014;11:244-50.
19. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, Choung YH, Kim ES, Yang HC, Choung PH. Isolation and characterization of postnatal stem cells from human dental tissues. Tissue Eng. 2007;13:767-73.
20. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, Politis C, Lambrichts I, Bronckaers A. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell Tissue Res. 2013;353:65-78.
21. Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods. 2008;14:149-56.
22. Tirino V, Paino F, d'Aquino R, Desiderio V, De Rosa A, Papaccio G. Methods for the identification, characterization and banking of human DPSCs: current strategies and perspectives. Stem Cell Rev. 2011;7:608-1
23. Al-Ghamdi NS, Al-Nazhan S. Pulp revascularization of immature maxillary first premolar. J Conserv Dent. 2015;18:496-9.
24. Iwaya SI, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. Dent Traumatol. 2001;17:185–7.

(Received May 16, 2018 ; Accepted August 1, 2018)