EFFECT OF TEMPERATURE AND SIX STORAGE MEDIA ON HUMAN DENTAL PULP CELLS

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ABSTRACT

Aim*: The viability of human dental pulp cells (hDPCs) can affect the long-term prognosis of replanted avulsed teeth. When immediate replantation of an avulsed tooth is not possible, the cells should be incubated in a physiological storage medium instantly to maintain their biological activity. The ability of different storage media to preserve human periodontal ligament fibroblasts(PDLF) viability has been previously evaluated, Few studies have showed the effect of different storage media and temperature on the viability of to hDPCs in vitro. The aim of this study was to evaluate the efficacy of the storage media and temperature on the viability of hDPCs.*

Material and methods*: hDPCs which were obtained from extracted immature human teeth were kept at DMEM, Hanks' buffered salt solution (HBSS), 0.9% saline, saliva, milk (Fresh milk, Bright dairy, China), or tap water (negative control) (n = 5) under 4°C and 37°C for 1, 2, 4, 8 or 24 h, respectively (n = 5). This study was designed to measure hDPCs activity by CCK-8 assay.*

Results*: At 4°C and 37°C, milk, HBSS, and DMEM were absolutely superior storage media in maintaining the viability of hDPCs than tap water (the negative control), saliva, and saline at every storage period (P < 0.001).*

Conclusion*: HBSS was the best storage medium, followed by Milk and DMEM. HBSS, Milk and DMEM can be indicated for the conservation of hDPCs up to 4h.*

Keywords*: Avulsed tooth, Human dental pulp cells, CCK-8, HBSS.*

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Introduction

Tooth Avulsion is considered as one of the most serious dental injures, with a proportion of 0.5-3% in the permanent teeth and 7-21% in the primary dentition[1, 2]. Avulsions of permanent teeth occur most often in 7-10-year old children, because their resilient alveolar bone provides only minimal resistance to extrusive forces[3, 4]. Tooth avulsion represented severe pulpal and periodontal injuries. Dental pulp is the specialized tissue responsible for maintaining tooth viability, composed of blood and lymph vessels nerves and various populations of multipotent cells. When tooth is damaged or avulsed, pulp is exposed to a plethora of environment stimuli. And pulp necrosis and consecutive infection are the main causes of early tooth loss[5, 6].

In mature teeth, pulp necrosis is predictable and it is strongly recommended to receive an endodontic treatment. But in immature teeth, there is a chance of revascularization. The success of revascularizations depends on the diameter of the apical foramen and the length of the root. Immature teeth with a large open apex and short roots seem to be more conducive to the successful treatment of pulp revascularization[7, 8]. For the preschoolers and schoolchildren, most of avulsion happened in maxillary incisor, which make the development of incisor roots stop and unfinished[9, 10]. Therefore, the apical foramen of the avulsed teeth is much bigger, which will provide the chance for maintaining the viability of dental pulp.

When the avulsion happened, a prompt and correct emergency management is very important for

the prognosis. Although it has been shown that immediate replantation was the best choice to save the tooth, it is not practical in most situations. If this is not possible, avulsed tooth should be stored in a suitable medium and replanted as soon as possible. The use of an appropriate transport or storage medium is vital in maintaining the viability of the Dental pulp cells(hDPCs), therefore improving the long-term prognosis and survival of avulsed teeth[11, 12]. The ideal storage medium should preserve cell vitality, adherence and clonogenic capacity and it should be readily available or easily accessible at the site of an accident[13].

Numerous investigations demonstrated that the extra-alveolar storage conditions played critical role in the viability of the PDL cells. Several storage media including coconut water, DMEM, HBSS and milk have been proved to be associated with favorable healing outcomes. Considering the practicalities, the costs and the lack of availability to the general public, milk remains the most convenient, cheapest and readily available solution in most situations which also being capable of keeping PDL cells alive. Thus, milk is the suitable storage medium for avulsed teeth that cannot be replanted immediately or very soon after the avulsion[14, 15]. Little data was available on the viability of Dental pulp cells(DPCs) after storage in the storage or transport media. A study showed that a special cell culture medium (SCCM) was able to maintain pulp cell viability better than HBSS[16]. However, commonly used storage medium for PDL cells such as HBSS, DMEM and milk was not investigated for hDPCs. Our study was designed to evaluate the efficacy of the storage media on the viability of hDPCs in vitro., which were obtained from extracted immature human teeth.

Materials and methods

Preparation of hDPCs culture

Human dental pulp cells were cultured using an explant technique[17-20]. Human dental pulp tissues were obtained from the premolars of healthy patients (14 years old) undergoing orthodontic treatments with informed consent. Teeth were cleaned and kept with phosphate-buffered saline solution (PBS, pH = 7.4). Then teeth were transferred aseptically to the laboratory and sectioned horizontally at 1 mm below the cementoenamel junction by using sterilized dental fissure burs. Pulp tissue was gently collected, cut into small pieces with a blade, and cultured in a 60mm Petri dish containing DMEM with 10% fetal bovine serum (FBS) (Gibco), penicillin (100U/ml) and streptomycin (100μg/ml). Cultures were maintained in a humidified atmosphere of 5% CO2 at 37 °C until cell outgrowth was confluent. Then cells were detached with 2.5g/l trypsin (Gibco) and transferred into new dishes for continued growth. Cells from the fourth to sixth passage were used in this study. All of the procedures were under an informed consent approved by Human Studies Committee of Shanghai Tenth People's Hospital.

Cell viability assay

hDPCs were counted using a hemocytometer and seeded in 96-well culture plates (2×104 cells per well). The plates were then incubated at 37°C with 5% CO2 for 24h to allow the cells to adhere. On the day of treatment, the culture media was drained from each well and washed with PBS for three times. Then, the cells were incubated with 100μl of DMEM, Hanks' buffered salt solution (HBSS), 0.9% saline, saliva, milk (Fresh milk, Bright dairy, China), or tap water (negative control) (n = 5) under 37°C for 1, 2, 4, 8 or 24 h, respectively (n = 5). To examine the viability of the cells, the storage solutions were replaced by cell counting kit-8(CCK-8) solution from CCK-8 kit (Dojindo Kagaku Co, Kumamoto, Japan), and the plates were incubated at 37°C and 4 °C for 2 h. Cell viability was determined by measuring the optical density at a wavelength of 450 nm.

Statistical analysis

The results were stored in an Excel file and SPSS 21.0 (IBM, Chicago, IL, USA) software was used for statistical analysis. One-way ANOVA, complemented by Scheffe test was used to analyze the data. The results were represented as mean±SD from three independent experiments. $P<0.05$ was considered statistically significant.

Results

hDPCs were cultured in different storage media including tap water, saliva, saline, milk, HBSS and DMEM under 37°C and 4 °C. hDPCs viability was determined by CCK-8 assay at 1, 2, 4, 8 and 24 h for each tested medium. The mean absorbance values, which represent hDPCs viability for each tested medium, temperature, and for storage periods, are shown in Fig. 1 (37°C) and Fig. 2 (4°C). At both temperatures, Milk, HBSS, and DMEM were absolutely superior storage media in maintaining the viability of hDPCs than tap water (the negative con-

trol), saliva, and saline at every storage period ($P < 0.001$).

Tap water treatment showed a significant decrease of cell viability in 2h and had almost no viable cells present after 4h, while saliva and saline maintained certain cell viability for up to 8 h. However, almost all the hDPCs died in saliva and saline after 24h. Compared with tap water, saliva and saline, storage in milk, HBSS and DMEM showed significantly better efficacy on maintaining cell viability at each time periods ($P<0.01$). when hDPCs were stored in milk, HBSS and DMEM, no significant difference in cell viability was found in 1h, 2h and 4h ($P > 0.05$), while a little reduction was appeared in 8h ($P<0.01$) (Fig. 1 and 2).

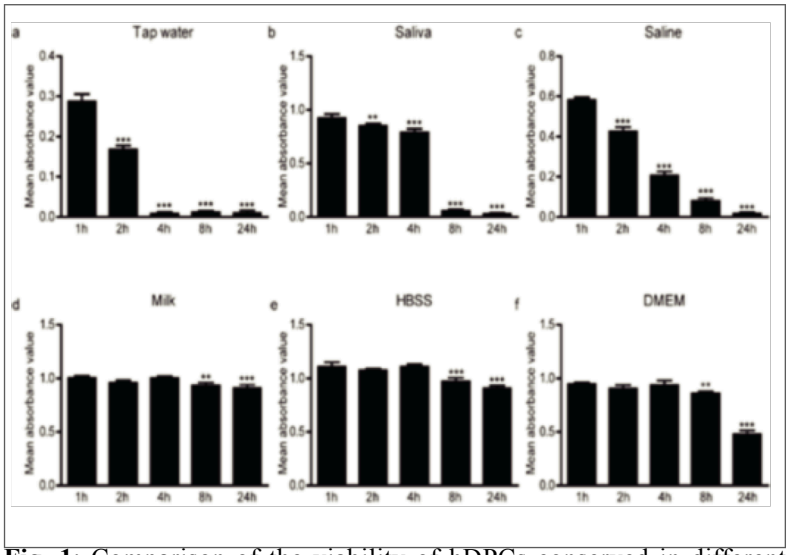

Fig. 1: Comparison of the viability of hDPCs conserved in different media at 37°C. The viability was represented by absorbance values, obtained from CCK-8 assay. Statistical analysis of the data was accomplished using one-way ANOVA, complemented by the Scheffe test (*P<0.05, **P<0.01, ***P<0.001). (**a**) tap water solution, (**b**) saliva, (**c**) saline, (**d**) milk, (**e**) Hanks' buffered salt solution (HBSS), (**f**) Dubelco's modified Eagle's media (DMEM).

Interestingly, milk and HBSS storage performed higher efficacy in maintaining cell viability than DMEM over the whole time period, especially at 24h. In 24h, milk and HBSS performed significant advantage on cell storage than DMEM ($P<0.05$).

Discussion

Avulsion of teeth usually happens in the anterior teeth and among young children, accounts for one third of all traumatic injuries in boys and one fourth of all injuries in girls[21]. Pulp necrosis and root resorption are major complications after avulsions, and endodontic treatment is strongly recommended for mature permanent teeth. However, for avulsed immature teeth, revascularization therapy may be a promising alternative, offering advantages for root development and reinforcement. Studies have targeted pulp revascularization in replanted and transplanted teeth[7, 22]. It is showed that pulp revascularization in replanted teeth without extroral endodontic treatment would be beneficial, new blood vessels grew and necrotic pulp tissue was replaced by new tissue elements. Root continued to develop and usually resulted in the deposition of calcified tissue with the root canal, which might strengthen the existing root structures and increase the potential for long-term retention of the teeth[23-25]. The prognosis of revascularization is better in replanted immature teeth with a wide-open apical foramen and short roots[5, 8]. It is possible that a few vital pulp cells remains at the apical end of the root canal, which might proliferate into the newly formed matrix and differentiate into odontoblasts stimulated by Hertwig's epithelial root sheath cells[26, 27].

Pulp revascularization is influenced by the extraoral storage conditions and the stage of root development. Studies demonstrated that teeth with an open apical foramen > 1.1mm had an increasing likelihood of natural revascularization, approximately 18% to 34% in teeth with immature roots[7, 8]. A multivariate statistical analysis revealed that pulp revascularization was more frequent in teeth with shorter distances from the apical foramen to the pulp horns. Furthermore, even short storage in unphysiologic conditions- i.e. in dry conditions or in saline or saliva- decreased the rate of revascularization to 10-30% while it was 60% after immediate replantation[8]. Considering that immediate replantation is not practical in most situations, avulsed teeth should be stored and transported in an appropriate solution before replantation. Thus, we expected to identify a unique medium both beneficial for hDPCs, in order to provide alternative treatment choice after replantation and get a long-term prognosis of avulsed teeth, especially for immature permanent teeth. Cell culture media maintain vitality and viability of cells.

Varies of media have been proposed and tested for the storage of avulsed teeth, and several storage media have showed favorable ability in maintaining the viability of PDL cells, but hDPCs were rare explored.

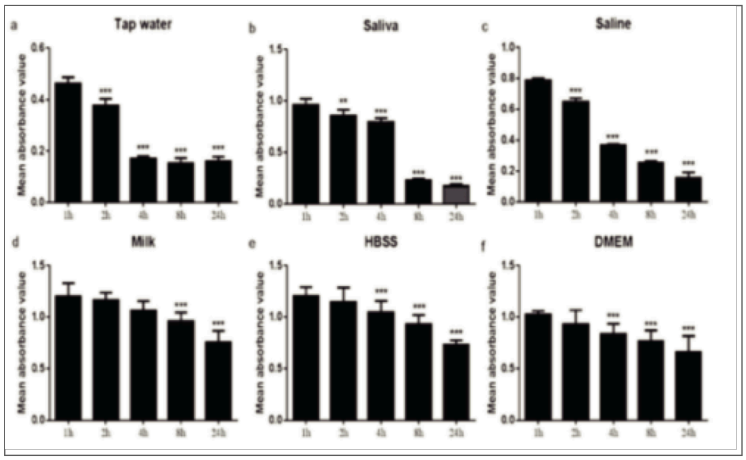

Fig. 2: Comparison of the viability of hDPCs conserved in different media at 4°C. The viability was represented by absorbance values, obtained from CCK-8 assay. Statistical analysis of the data was accomplished using one-way ANOVA, complemented by the Scheffe test (*P<0.05, **P<0.01, ***P<0.001). (**a**) tap water solution, (**b**) saliva, (**c**) saline, (**d**) milk, (**e**) Hanks' buffered salt solution (HBSS), (**f**) Dubelco's modified Eagle's media (DMEM).

In our previous study, the viability of PDL fibroblasts in tap water, saliva, saline, milk, HBSS and DMEM storage was tested[28]. These media were also used for the current study to identify the best storage medium for hDPCs.

In this study, we cultured hDPCs in the six different media and detected the viability at 1h, 2h, 4h, 8h and 24h, respectively. As observed for hDPCs, milk, HBSS and DMEM were more effective storage media for avulsed teeth while saliva and saline were less effective. Tap water, acted as a negative control, showed the lowest efficacy. Nutrients might be the important factor that influenced viability of hDPCs, which are sufficient in milk, HBSS and DMEM but poor in tap water, saliva and saline. In addition, there was little difference in viability of hDPCs cultured in milk, HBSS and DMEM within 4h but the viability in all the groups decreased after 4h. The results indicated that hDPCs could be stored in appropriate media such as milk, HBSS and DMEM less than 4h in emergencies. HBSS and milk showed the best effect on maintaining the viability on hDPCs for 24h. Considering the practicalities and lack of ready availability, HBSS and DMEM were less than ideal. As milk remains the most convenient, cheapest and readily available solution in most situations, it might be the best choice for avulsed teeth in the extra-alveolar period, especially for children in undeveloped rural areas of China who may encounter dental trauma.

Revascularization may be a promising treatment in avulsed immature teeth, as an alternative to apexification. A case study demonstrated that the choice of treatment for avulsed immature teeth diagnosed with pulp necrosis should be evaluated carefully, favorable conditions of replantation, including no delay in replantation and use of a favorable storage medium, may indicate pulp revascularization therapy[33]. The present research is limited to the viability of pulp cells after storage in vitro. It cannot be concluded from this study whether the maintenance of viability of pulp cells is advantageous for a revascularization. Long-term clinical research needs to be conducted.

The last hypothesis regarding temperature was partially accepted because low storage temperature had a better effect than 37°C, only on the ability of milk to maintain the viability of hDPCs. The lowest temperature favored Milk and undermined the effectiveness of HBSS. It is possible that the lower temperature slowed the decline of the milk's pH[3], limiting bacterial growth and preventing milk from souring[30], maintaining a more physiological environment for cell survival. This result corroborates with previous studies[8, 26,], which determined that cooled milk is better suited for the preservation of cell viability. Regarding HBSS, it is possible that at 37°C there are more disposable nutrients from HBSS, maintaining cellular metabolism and the reversion of tetrazolium salts in formazan crystals.

Conclusion

In conclusion, despite limitations of the current study, the present experiments indicate that at both temperatures HBSS was the best storage medium, followed by Milk and DMEM. Based on the favorable results obtained in this study, HBSS can be recommended as a suitable storage medium for avulsed teeth 4°C is adequate for storing the avulsed teeth in HBSS, DMEM, or milk in vitro.

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