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Original Article

Comparative evaluation of five different storage media and temperature effect on human periodontal ligament fibroblast viability

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Abstract

Introduction: Following dental avulsion, the immediate recommended treatment is tooth replantation to avoid adverse effects. Therefore, the tooth must be stored in a physiological storage medium to preserve the viability of the periodontal ligament fibroblast (PDLF) cells during transportation to dental office. The aim of this study was to determine the effect of several storage media in preserving the viability of human PDLF cells at different times and temperatures.

Materials & Methods: In this experimental study, the human PDL cells were obtained from the healthy extracted third molars and cultured in Dulbecco's Modified Eagle Medium (DMEM). The studied media were DMEM (10% FBS + 1% penicillin G Na (*10000 IU*) + 1% streptomycin (10 *mg*)), tap water, sterilized whole milk, zero fat milk and soy milk. After the cells had reached sufficient density in the plate, they were added to the experimental media and kept at 1, 2, 4 and 24 hours at 4° and 37°Centigrade. After incubation, the cell viability was determined by tetrazolium salt-based colorimetric (MTT) assay. The results were statistically analyzed using Kruskal–Wallis and post hoc Tests.

Results: Whole milk and DMEM showed significantly higher protective effect than other media. The viability of PDL cells had significant difference at 4°C compared to 37°C at 4 and 24 h in DMEM group and at 24 h in whole milk group ($p \leq 0.05$).

Conclusion: The results have suggested that the whole milk like DMEM have enough essential nutrients for PDLFs and have confirmed the hypothesis that the milk similar to HBSS or DMEM might be effective in preserving the PDLF cells.

Keywords: Periodontal ligament, Fibroblast, Milk

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بررسی اثر پنج نوع محیط نگهدارنده مختلف و دما بر حیات فیبروبلاستهای لیگامان پریودنتال انسانی

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چکیده

مقدمه: بدنبال خارج شدن دندان از حفره استخوانی روش درمانی پیشنهادی، جایگزین کردن سریع دندان است تا از عوارض جانبی آن پیشگیری شود. بنابراین دندان باید در یک محیط فیزیولوژیک مناسب قرار گیرد تا اینکه سلول‌های فیبروبلاست لیگامان پریودنتال دندان در طی انتقال به مطب دندانپزشکی سالم باقی بماند. هدف از این مطالعه بررسی تاثیر محیط های مختلف بر حفظ حیات سلول‌های فیبروبلاست لیگامان پریودنتال انسانی در زمان و دمای متفاوت بوده است.

مواد و روش ها: در این مطالعه آزمایشگاهی، سلول لیگامان پریودنتال انسانی از دندان های مولر سوم نهفته کشیده شده تهیه و سپس در محیط کشت(DMEM) Dulbecco's Modified Eagle Medium کشت داده شدند. محیط مورد آزمایش شامل: DMEM (FBS 10%، پنی سیلین G سدیم ۱٪ (10000 IU)، استرپتومایسین ۱٪ (10 mg) ، شیر بدون چربی ، شیر کامل و شیر سویا استریلیزه و آب شرب بوده است. پس از این که سلولها در پلیت به تراکم کافی رسید به محیط های آزمایشی اضافه شده و در زمانهای ۱، ۲، ۴ و ۲۴ ساعت، در دمای ۴ و۳۷ درجه سانتیگراد نگهداری و بعد از انکوبه شدن حیات سلولی به روش MTT by (tetrazolium salt-based colorimetric assay) ارزیابی شد. نتایج با استفاده از تست آماری Kruskal–Wallis و Post Hoc Tests آنالیز شد.

یافته ها: شیر کامل و DMEM تفاوت معنی داری نسبت به سایر محیط های آزمایشی نشان داده است. حیات سلولهای PDL تفاوت معنی داری در دمای ۴ درجه نسبت به دمای ۳۷ درجه سانتیگراد در زمانهای ۴ و ۲۴ ساعت در گروه DMEM و در زمان ۲۴ ساعت در گروه شیر کامل نشان داده است ($p\leq 0.05$) .

نتیجه گیری: نتایج نشان داده است شیر کامل همانند DMEM مواد مغذی کافی برای PDLFs داشته و این فرضیه را تقویت می کند که شیر همانند HBSS و یا DMEM در حفاظت از سلول های PDLFs میتواند مفید واقع شود.

واژگان کلیدی: لیگامان پریودنتال، فیبروبلاست، شیر

Introduction

Avulsion resulted from trauma is a complete displacement of a tooth from its alveolar socket. Following dental avulsion, replanting the tooth immediately is the recommended treatment to avoid adverse occurrences such as root resorption.[1, 2] Although immediate replantation has the best prognosis, it is impossible in most of the cases. Therefore, the tooth

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must be kept in a physiologic storage medium to preserve the viability of pulp and periodontal ligament fibroblast (PDLF) cells during transportation to the dental office. [3, 4] If the PDLF cells which are essential for healing the replanted avulsed tooth are damaged, the tooth may be lost because of the root resorption or ankylosis.[5] Many studies have been performed to find the ideal storage of avulsed tooth [2, 5, 6] including Hank's balanced salt solution (HBSS), water, milk, Dulbecco's Modified Eagle Medium (DMEM), saliva, Viaspan and saline. [5, 6] An ideal medium must have a physiological osmolality and neutral pH as well as must be stable at an appropriate temperature [7] and accessible for people. Saliva and saline are useful only for short periods. [8, 9] Tap water is inadequate due to its chlorine and low osmolality. [7, 8, 10] Viaspan, DMEM and HBSS are most suitable to maintain the viability of PDLF cells for longer period, [3, 5, 6] but these are so expensive and unusual. Milk which is cheap and available is a widely acceptable storage medium owing to its neutral PH, low bacterial content, some nutrient, physiologic osmolality [4] and growth factors for cells. [11, 12] The time period for maintaining cell viability of milk is controversial: some studies have shown that its effectiveness is for 3 hours [8, 13] but others have indicated that it is for up to 24 and 48 hours.

There is no consensus on other medium, especially, for HBSS. [5, 14-16] Souza et al. [17] have suggested that the milk is the best storage medium for up to 24 and 48 hours at 20°C and 37°C whereas Huang et al. [15] have expressed that the milk is not as effective as HBSS. However, Olson et al. [16] and Marino et al. [14] gained better results regarding to milk as a storage medium. The aim of this study was to compare the effectiveness of different temperatures (37°C and 4°C) and different storage media in various time periods on PDLFs viability.

Materials & Methods

This study was approved by Ethical Committee of Babol University of Medical Sciences (mubabol.rec.1393.148). In this experimental study, the PDLFs were obtained from extracted unerupted third molars which were surgically sterile. The PDLF of the third middle of the root was scraped and placed in culture flasks containing DMEM (ATOCEL Co., Wels, Austria), 10% fetal bovine serum (FBS) (ATOCEL Co., Wels, Austria), 1% penicillin G and 1% streptomycin.

The flasks were incubated at 37°C in a humidified atmosphere with 5% CO2 and the medium was exchanged every three days. Once confluent PDLFs were obtained, the medium was removed and the cells were trypsinized (Trypsin, ATOCEL Co., Wels, Austria) then transmitted to another flask (first passage). In addition, 4-7 passage cells were used for the experiments. Tetrazolium salt-based colorimetric (MTT) assay was used for cell viability evaluation.

MTT assay: PDLFs (12×10^3 cells per well) were seeded in twelve 96-well culture plates (Jet biofil, Sorfa, Germany) and incubated at 37°C with CO2 5%. Once confluency occurred after 48 hours, the DMEM was removed from the wells washed three times using phosphate-buffered saline (PBS) then filled with 100 µl of one of the selected medium: DMEM as positive control, tap water as negative control, pasteurized long-life whole milk (Kale, Amol, Iran), pasteurized long-life zero fat milk (Mihan, Tehran, Iran) and pasteurized long-life soy milk (Drinho, ACE canning corp, Malezi). Four plates were incubated at 37°C with CO2 5% and four plates were kept at 4°C in refrigerator. After 1, 2, 4 and 24 hours, the storage medium was removed, the wells were washed with PBS three times then stored with MTT solution (1 mg/ml), the plates were incubated at 37°C with CO2 5% up to 4 hours. After 4 hours, 50λ isopropanol (Isopropanol, Dow Chemical Company, Michigan, USA) was added to the wells. Cell viability was determined by measuring the differential optical density at 570 nm (630 nm as reference wavelength on a spectrophotometer). The cells were stored in DMEM used as positive control for cell growth.

Statistical analysis: The following results of the optical density are presented as Mean± S.E. Data were analyzed using the Kruskal–Wallis and post-hoc tests. A p-value<0.05 was considered as statistically significant.

Results

The viability of PDLF cells cultured with various storage media including tap water, whole milk, zero fat milk, soy milk and DMEM at 4°C or 37°C was determined using MTT assay after 1, 2, 4 and 24 hours (Figs. 1, 2 and 3). In the experiments carried out at 37°C, the efficacy of whole milk and zero fat milk was comparable to that of DMEM which was significantly higher than that of soy milk and tap water after 1 and 2 hours exposure to the storage media. After 24 hours, the whole milk as compared with other media showed a

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significantly higher protective effect ($p<0.05$). At 4°C, there were no significant differences among the groups at 1 hours. However, the cell viability in whole milk and zero fat milk was significantly higher than soy milk and tap water groups after 2 and 24 hours exposure. Nevertheless, the cell viability in all groups tends to decrease after 24 hours storage at 4°C.

Regardless of the other 3 storage media (DMEM, whole milk and zero-fat milk), generally there was no significant difference between soy milk and tap water media. Comparison of temperatures revealed that the ability of every storage medium in protecting the PDLF cell viability was not significantly different at 4°C compared to 37°C except for the whole milk and DMEM (after 4 hours and 24 hours storage in DMEM and 24 hours in whole milk).

On the other hand, there were no temperature-dependent (4°C compared to 37°C) differences in cell viability with zero fat milk and soy milk at different exposure time periods. In comparison to the other media, tap water represented the lowest efficacy in maintaining cell viability at both temperatures.

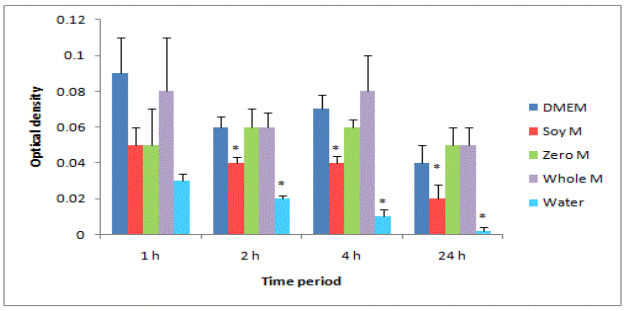


Figure 1. Cell viability of PDLFs after storage in five different storage media at 4°c, measured by MTT assay (* p < 0.05)

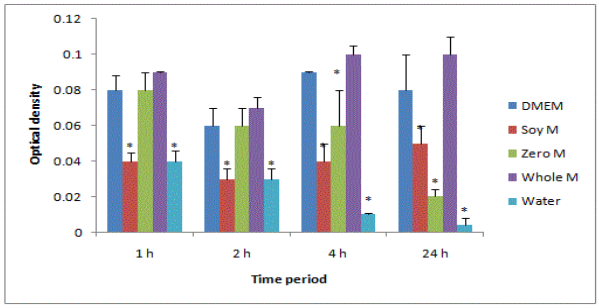


Figure 2. Cell viability of PDLFs after storage in five different storage media at 37°C, measured by MTT assay (* p<0.05)

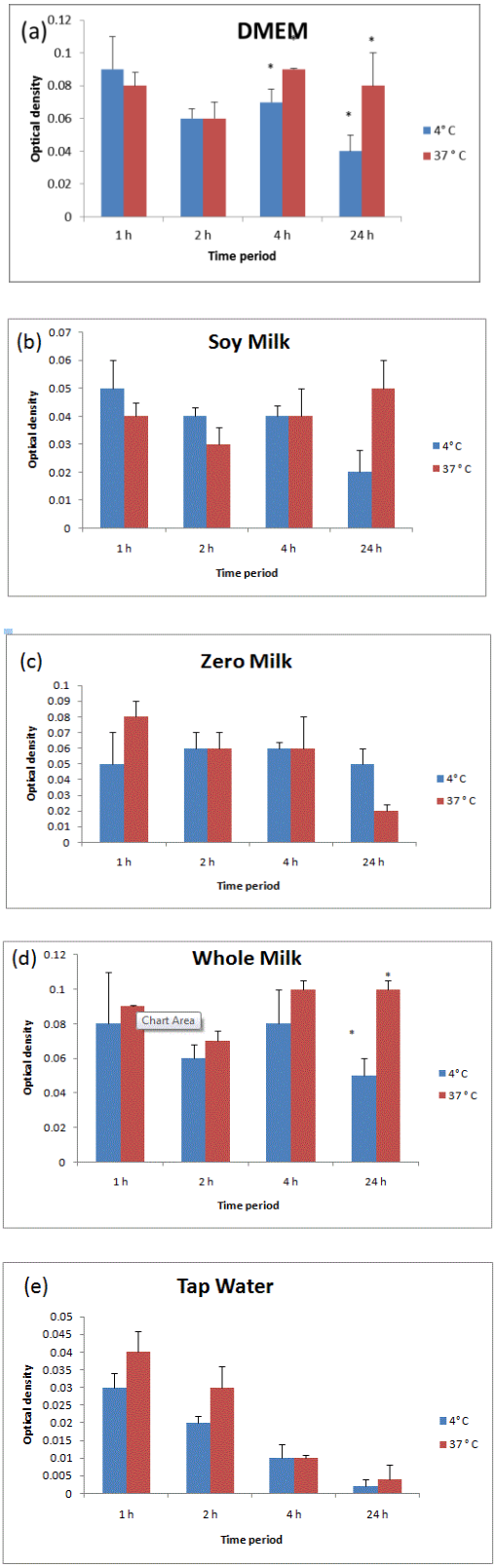


Figure 3. Comparison of cell viability of PDLFs stored in five different storage media at two different temperatures (4°c and 37°c) using the MTT assay. Error bars represent standard error (SE) of the mean of the optical density. (* p<0.05)

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Discussion

The primary outcome of this study was the confirmation of the equality or even the superiority of whole milk rather than other common studied media. The results have suggested that the whole milk like DMEM have enough essential nutrients for PDLFs and have confirmed the hypothesis that the milk similar to HBSS or DMEM might be effective in preserving the PDLF cells.

In the present study, the effectiveness of different media and storage conditions (temperature and time) in maintaining the viability of PDLFs was assey. Different studies have demonstrated that the storage time and temperature can affect the effectiveness of the storage media in maintaining the cell viability and was in agreement with this present study. [5, 8, 17, 18] Bağ et al. compared HBSS, milk and DMEM with each other found the decrease of RUNX2 and stability of other markers in the milk group compared to other groups, indicating that the milk has a better protective effect than others in supporting the result of the current study. [19] Since most traumatic patients with avulsed teeth receive dental treatment during first few hours after the accident, the storage times≤24 hours (1, 2, 4 and 24 hours) were chosen in the present study. Keeping the avulsed tooth warm or cold was another matter of concern, addressed in the current study by storing the PDLFs at two different temperatures. Some studies have demonstrated that the low temperature (e.g. 4°C, over the ice stored tooth) has the advantage of reducing cellular metabolism [20] and limiting bacterial growth, which might improve the prognosis of tooth replantation. [7, 21] On the other hand, the normal body temperature is 37°C, which can be provided by the patient's companion.

Hence, in this study, the PDLF cells were incubated at both 4°C and 37°C to evaluate the effects of storage temperature on the cell viability. The DMEM supplemented with 10% FBS which is commonly used in the PDLFs culture was applied as a positive control in the present study. The obtained results represented that the overall whole milk could protect PDLFs viability significantly better than the other two types of milk (zero fat milk and soy milk) at 37°C. Moreover, the cell viability became significantly better in whole milk and zero fat milk than in soy milk and tap water at 4°C, 2 hours and afterwards. Two common methods of cell viability measurements include MTT assay and try pan blue dye exclusion assay. For the try pan blue staining test has shown less sensitivity and no metabolic and physiological condition of the cells, [22] the MTT assay was applied for more sensitive measurement of PDLFs cell viability. [17]

The hypothesis that the colored substances may interfere with the absorbance reading and generate higher values in MTT assay was confirmed based on the findings of Smee et al.[23] and Chung et al.[24] For example, green color of HBSS or opacity of milk may have increased the absorbance values leading to overestimating results. For this reason, the wells were washed three times with PBS and filled with fresh medium each time after exposure to milk to avoid interference with the optical density readings. Furthermore, Souza et al. [17] and Chen et al. [21] confirmed the effectiveness of milk in the maintenance of fibroblast using the trypan blue assay as another method of cell viability assay. In this study, the cell viability tends to decrease in all groups during 24 hours with significant differences between 1 hours and 24 hours these results are in agreement with the findings of Olson et al. [16] and Marino et al. [14] who stated that the milk is effective for a short period of time. [8, 15] They also declared that the cold milk (4°C) is suitable for the preservation of the cell viability. [7, 25] de Souza et al. found that followed by whole milk and HBSS, the skimmed milk was the best storage medium, and the lower temperature (5°C) undermined the effectiveness of HBSS, favored skimmed milk and whole milk. [26] Tap water used as negative control at 4°C and 37°C in the current study indicated the lowest efficacy in protecting the PDLFs viability, especially after 2 hours storage time which is consistent with the previous studies. [21, 26, 27]

It demonstrated the importance of physiological osmolality and pH of the storage media. As mentioned earlier, in our study, the whole milk was more effective up to 24 hours in maintaining cell viability compared to the other storage media, especially tap water. There might be few explanations in favor of this positive result including having osmolality, physiological pH, some nutrients [28] and growth factors. [17] Milk is widely accepted as a storage media for short-term storage of avulsed tooth due to its availability and osmolality (270 mOs m/kg) and pH (6.5-7.2) very much close to the extracellular fluids. Although the whole milk at 37°C was the best efficient storage media up to 24 hours , it compared to the other three media had superior activity only up to 4 hours at 4°C (Fig 1).

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The systematic review and meta-analysis of Fagundes et al. aimed to verify the capacity of different storage media to preserve the viability of periodontal ligament cells in comparison to HBSS. This systematic review displayed that the herbal medicines and milk could demonstrate an alternative to HBSS, [29] which agrees with our results. In addition, Hiltz et al. [5] by using the trypan blue dye exclusion method suggested that the cell storage of room temperature in milk and HBSS was effective for 6 and 24 hours, respectively. Other studies of Olson et al. [16] and Garcia-Godoy et al. [30] have represented that the milk is effective only for a short period of time and loses its effect after 24 hours in vitro. Adeli et al. expressed that the green tea and milk than other studied media (DMEM, tap water and HBSS) had a good ability in preserving the PDLFs.[31] Because of the importance of mammalian cell incubation temperature, the effects of two different storage temperatures on PDLFs cell viability were evaluated in the present study.

It seems that the cells have a higher metabolism rate and nutritional needs in higher temperatures as well as that the low temperatures decline the cell swelling, enhance the cell viability and improve the cell recovery, [32] but the results of the current study have shown that the cells retain the same viability percentage at cool (4^{o}C) and warm (37^{o}C) temperature. Overall, the PDLFs cell viability tends to decrease via increasing the storage time from 2 hours to 24 hours regardless of the temperature (Figs. 1, 2).

There was a significantly difference between two temperatures at 4 and 24 hours in DMEM group and at 24 hours in whole milk group. The probable reason may be due to that depending on the intensity of the exposure, the cold stress can trigger a cell stress response, activate the apoptotic program and lead to necrosis through mechanisms such as the formation of ice crystals.[33] However, many of the genes induced by cold exposure (including a number of apoptosis-specific protein genes) do not increase after a heat shock during the period of thermal stress except during the cell stress response which results after rewarming. [34, 35] It seems that the tap water and soy milk contain poor nutritional and/or physiological constituents which make them capable of rendering a good storage; thus, the capacity of these media in maintaining cell viability is in a decreasing order (4^{o}C > 37^{o}C).

Nevertheless, the whole milk and DMEM have enough essential nutrients for PDLFs. In the present study, there was no difference between temperatures at every time period in terms of the efficacy of zero fat milk and soy milk. The nutritive value, osmolality and pH of the storage medium are the main factors influencing their efficacy in preserving the viability of PDLFs. Moreover, keeping the storage medium cold, which potentially should increase the cell viability by attenuating the basic metabolism, practically did not make such a huge difference, especially for whole milk.

Conclusion

The results showed that the whole milk and DMEM had enough essential nutrients for PDLFs and using cold whole milk (4^{o}C) compared to DMEM was the best suitable storage media. The findings would be useful for traumatic patients to preserve the avulsed tooth in suitable and accessible material until transmitted to office. Further research on cell viability is recommended using other technique.

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Conflict of interest: We declare no conflict of interest.

Authors' Contributions

The study was designed by Zahra Sadat Madani and Ebrahim Zabihi, and Zinat Sadat Rezaie defined the conceptual content of the research. The study data were collected by Zinat Sadat Rezaie and Zeinab Abedian. Statistical analysis and interpretation of data were accomplished by Zinat Sadat Rezaie. Preparation of manuscript was performed by Zinat Sadat Rezaie. Study supervision was performed by Ebrahim Zabihi.

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