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Viability of fibroblasts in a novel probiotic storage media

E Çaglar[1], N Sandalli[1], OO Kuscu[1], MA Durhan[2], R Pisiriciler[2], E Ak Calıskan[2], B Kargul[3]

[1]Department of Paediatric Dentistry, School of Dentistry, Yeditepe University; [2]Department of Histology, School of Dentistry, Marmara University; [3]Department of Paediatric Dentistry, School of Dentistry, Marmara University, Istanbul, Turkey

Correspondence to: Esber Caglar, Yeditepe University, Faculty of Dentistry, Dept of Paediatric Dentistry, Bagdat cad 238, Goztepe, 34728 Istanbul, Turkey
Tel.: + 90 216 3636044/323
Fax: +90 216 3636211
e-mail: caglares@yahoo.com

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Abstract – A number of storage media have been investigated as to their ability to maintain the viability of the periodontal ligament (PDL) cells and thus to permit longer extra-alveolar periods prior to replantation of avulsed teeth. The aim of the present *in vitro* study was to evaluate the number of viable PDL cells of avulsed teeth treated by Hank's Balanced Salt Solutions (HBSS), saline, a novel probiotic solution and milk. Thirty-six freshly extracted single-rooted human teeth with closed apices were divided into one of the four experimental groups and two control groups ($N = 6$ each). The positive and negative controls corresponded to 0 min and an 8-h dry time respectively. Following extraction, the coronal 3 mm of PDL tissue was scraped with a #15 scalpel to remove cells that might have been damaged. The experimental teeth were dried for 30 min followed by a 45 min immersion in one of the four experimental media. Each experimental tooth, after drying and soaking, was incubated for 30 min with a 2.5 ml solution of 0.2 mg ml^{-1} of collagenase CLS II and a 2.4 mg ml^{-1} solution of dispase grade II in phosphate buffer saline (PBS). The cells were then labelled with 0.4% Trypan blue for determination of viability. The teeth stored in positive control demonstrated the highest number of viable PDL cells followed in rank order by HBSS, saline, *Lactobacillus reuteri* solution and milk. There was no significant difference in the number of viable PDL cells between HBSS, milk, *L. reuteri* solution and saline. Within the parameters of this study, it appears that probiotic may be able to maintain PDL cell viability as HBSS, milk, or saline.

Traumatic injuries to newly erupted permanent anterior teeth are common during childhood (1, 2). Teeth that are replanted immediately after avulsion usually show excellent healing and have been found to have a good prognosis (3). The prognosis of replantation of traumatically exarticulated teeth is very much influenced by the conditions under which the teeth have been kept before replantation. Recently, when a tooth is accidentally avulsed; a number of storage media have been investigated as to their ability to maintain the viability of the periodontal ligament (PDL) cells and thus to permit longer extra-alveolar periods prior to replantation. Until 1970s, saliva was recommended as the most suitable storage medium, but there were no studies where saliva was compared with other possible storage media. Blomlof and Otteskog (4) were the first investigators to compare the storage effect of milk with that of saliva on human PDL cells. In the beginning of the 1980s, further related research supported the early findings that milk was found to be superior to saliva as a storage media with respect to number of viable cells, cell size, ability to recover and heal experimentally produced wounds (5, 6).

Currently, the suggested storage media are; milk, saliva, either in the vestibule of the mouth or in a container into which the patient expectorates, physiological saline, cell culture media (7, 8). Water is the least desirable storage medium because the hypotonic environment causes rapid cell lysis and increased inflammation on replantation (9). It should be noted that prefer order is changable regarding trauma protocols printed in the last decade. Milk may keep the cells viable for up to 6 h. Saliva is suitable for up to 2 h, while saline will maintain cells for 1 h (10, 11).

Hank's Balanced Salt Solutions (HBSS) or Viaspan® are cell culture media in special transporters and have been found successful in preserving the viability of the PDL fibres for extended periods (10, 12). Viaspan® is locally sold in some US states. Save a tooth®, a HBSS, was introduced into market in 1995. Previously, it had American Dental Association (ADA) certificate; however, it is not sealed by ADA today. In some US states, such as Wisconsin, Save a tooth® is recommended to be in Pediatric Emergency kits. Another HBSS, EMT Tooth Saver®, is sold in US & Canada. Beside their positive biological effects, HBSS and Viaspan® are presently considered impractical as they are not available where injury is likely to occur and not widely sold in the international market.

Currently, there are reports of new storage media for avulsed teeth. Pearson et al. (13) evaluated milk substitutes and stated that a powder form infant formula served better results in maintaining the viability of human PDL cells on avulsed teeth than other milk substitutes and milk itself. Propolis also seemed to be a

new alternative storage media, in maintaining viable PDL cells on simulated avulsed teeth (14).

Natural solutions may also be easily achieved to obtain a better surrounding for avulsed teeth. Changes in the environment imposed by injury disturb the homeostasis and lead to endogenous infections or susceptibility to exogenous infections. The resident oral microflora is diverse, being comprised of species with differing nutritional (saccharolytic, proteolytic, secondary feeders), atmospheric (aerobic, anaerobic, facultative, micro-aerophilic, capnophilic) and physico-chemical (pH, co-factors) requirements (15). Besides, septic complications represent frequent causes of infections following injuries and operations. Regarding elimination of pathogenic members of the oral cavity in dental trauma cases, a new method such as probiotic approach can be investigated. Probiotic bacteria are live microbial food supplements that may benefit the host by influencing the balance between the many species of the commensal flora both in oral cavity and the intestinal tract (16, 17).

A number of potential benefits arising from the use of probiotics have been proposed, including increased resistance to infections (18, 19). Recently, administration of different probiotic strain in an acute organ injury model has mostly reduced bacterial translocation and cellular damage (20). The beneficial role of *Lactobacillus reuteri* ATCC 55730 in oral health, general health, immunomodulation and disease protection has been observed and described in a number of studies (21–26).

Regarding the above statements, a storage media with probiotics should be investigated if is to be considered to combat dental infections arising dental trauma. Therefore, the aim of the present *in vitro* study was to evaluate the number of viable PDL cells of avulsed teeth by HBSS, saline, a novel probiotic *L. reuteri* solution and milk.

Materials and methods

Patients received both oral and written information about the study and signed a consent form prior to handling their extracted teeth. Thirty-six second premolars extracted for orthodontic reasons were included in the study after obtaining informed consent. Teeth extracted from patients with moderate-to-severe periodontal disease or with extensive caries were excluded. The teeth were extracted as atraumatically as possible and washed in sterile saline solution to eliminate residual blood. Following extractions, the coronal 3 mm of PDL tissues was scraped with a #15 scalpel to remove cells that may have been damaged. Freshly extracted single-rooted human permanent second premolar teeth with closed apices were divided into one of the four experimental groups and two control groups (positive and negative) consisting of six samples each. The positive and negative controls corresponded to 0 min and an 8-h dry time respectively. The experimental teeth were dried for 30 min (during the present time interval, the coronal PDL cells were curetted) followed by a 45-min immersion in one of the four media: *L. reuteri* probiotic solution [five drops of the probiotic drop (BioGaia Reuteri drops®; BioGaia AB, Stockholm, Sweden)], containing freeze-dried *L. reuteri* DSM 17938 ($\geq 1 \times 10^8$ CFU/5 drops) and *L. reuteri* ATCC PTA 5289 ($\geq 1 \times 10^8$ CFU/5 drops) suspended in oil where the daily intake was 0.15–0.20 g/5 drops at a single occasion) in 10 ml saline (prepared according to manufacturers' instructions), HBSS, saline and milk. After extraction, the positive control teeth were immediately treated with dispase and collagenase. The negative control teeth were bench-dried for 8 h, with no follow-up storage solution time, and then placed in the dispase and collagenase. Each experimental tooth, after drying and soaking, was incubated for 30 min in 15 ml falcon tubes with a 2.5 ml solution of 0.2 mg ml^{-1} of collagenase CLS II (Biochrom, Berlin, Germany) and a 2.4 mg ml^{-1} solution of dispase grade II (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffer saline (PBS). After incubation, 50 μl of foetal bovine serum (Biowest, Nuaille, France) was added to each tube. All tubes were then centrifuged for 4 min at 1000 r.p.m. The supernatant was then removed with sterile micropipettes, and the cells were labelled with 0.4% Trypan Blue (Sigma-Aldrich) for determination of viability, according to Polverini & Leibovich (24). The number of viable protective least significant difference PDL cells were counted under a light microscope with a haemocytometer at 20× magnification and analysed. The number of viable cells was obtained by the following mathematical equation: unstained cell count (viable cell) × the dilution of the cell suspension × 10^4/number of squares of the hemocytometer counted (14, 27).

Statistical analysis of the data was accomplished using Nonparametric ANOVA complemented by Kruskal–Wallis Test and Dunn's Multiple Comparisons Test.

Results

Table 1 shows the ANOVA results among the groups. The results showed that positive control was a more effective storage media than other groups. Positive control was found to be significantly better than the others. There was only a statistically significant difference between positive control and negative control ($P < 0.001$). There were no statistically significant differences between other storage media ($P > 0.05$). When *L. reuteri* solution was compared with other storage media, there was no significant difference ($P > 0.05$). Table 2 presents the median of all groups. The teeth stored in positive control demonstrated the highest number of viable PDL cells followed in rank order by HBSS, saline, *L. reuteri* solution and milk. There was no significant difference in the number of viable PDL cells between HBSS, milk, *L. reuteri* solution and saline ($P > 0.05$). All

Table 1. ANOVA demonstration of viable periodontal ligament cells for groups

Groups (*N*: 6 each)	Sum of ranks	Mean of ranks
Positive control	201.00	33.500
Negative control	21.000	3.500
HBSS	120.00	20.000
Saline	122.00	20.333
Lactobacillus reuteri solution	128.00	18.286
Milk	74.000	14.800

Table 2. The Median, Minimum and Maximum number of viable periodontal ligament cells for different storage medium per number of square of the hemocytometer

Groups (*N*: 6 each)	Median	Minimum	Maximum
Positive control	16 580 000	11 100 000	28 550 000
Negative control	31 000	10 000	66 000
HBSS	1 387 500	386 000	2 415 000
Saline	1 256 000	412 000	1 364 000
Lactobacillus reuteri solution	996 000	655 000	2 380 000
Milk	880 000	644 000	1 046 000

experimental groups were significantly lower than positive controls ($P < 0.001$).

Discussion

Successful replantation of avulsed teeth is dependent upon the prevention or limitation of replacement root resorption. At this point, treatment of such injuries must be geared towards early re-establishment of PDL cellular physiology. In 1955, Hammer (28) first addressed the importance of PDL cell viability prior to replantation when he demonstrated that the length of survival of a replanted tooth is directly correlated with the amount of viable periodontal membrane. Regarding the critical extraoral dry times for PDL cell viability and storage media should be used on the avulsed tooth are the key points to prevent the unpredictable sequelae of inflammatory root resorption or replacement resorption postreplantation.

The critical extraoral dry times before PDL cell damage occurring has been a controversial topic in dental traumatology. Andreasen & Hjørting-Hansen (29) and Martin and Pileggi (14) and showed that teeth replanted within 30 min had a better success rate than those that were extraoral for longer periods of time before replantation. Andreasen (30) and Soder et al. (31) have shown that at 2-h dry time, no vital PDL cells remain. In the current investigation, a 30-min dry time was chosen, as this seems to be a critical time at which damage has been done to many PDL cells. It should be noted that the storage media are primarily to maintain the viability of the cells and that infection is a secondary consideration. Several protocols deal with the preferred immersion medium for tooth storage before reimplantation, which permit the PDL cells to overcome the trauma and lack of nutrition and enable tooth reimplantation. The PDL fluid supplies the tooth with the nutrition necessary for the PDL cells to survive. The PDL remaining on the root after injury is dependent on a supply of vital metabolites. Cell destruction begins when these metabolites are withheld. To preserve optimal cell metabolism, the supply should be renewed within 60 min from the time of injury. If these cells survive, they will catalyse the reproduction of new cells, which can differentiate and reinstate the supporting tissues. The main philosophy of this survival may involve prevention of protein synthesis in the bacterial cell, encouraging the action of fibroblasts and healing of connective tissue, which contributes to the recovery of the PDL after injury (32). Regarding current gastrointestinal developments, the intestinal epithelial cells are critical for maintaining normal intestinal homoeostasis through several protective defence mechanisms, including barrier function formed by tight junctions, antibacterial substances synthesis and active involvement in innate and adaptive immunity. Probiotics facilitate intestinal epithelial homoeostasis through a number of biological responses, including promoting proliferation, migration, survival, barrier integrity, antimicrobial substance secretion and competition for pathogen interaction with epithelial cells (33, 34). To enhance antibacterial and anti-inflammatory activities of the intestinal epithelium, probiotics stimulate cytoprotective protective protein synthesis and secretion, including heat shock protein, defensin, angiogenin and mucin by intestinal epithelial cells (35).

In the present study, the teeth stored in positive control demonstrated the highest number of viable PDL cells followed in rank order by HBSS, saline, *L. reuteri* solution and milk. However, there was no significant difference in the number of viable PDL cells between HBSS, milk, *L. reuteri* solution and saline.

It was previously confirmed that in periodontal pockets treated with beneficial bacteria, subgingival re-colonization of periodontopathogens was delayed and reduced, as was the degree of inflammation. Radiologically, the healing of a periodontal pocket after scaling and root planing seems better when beneficial bacteria are applied (36). There is evidence to show that periodontal pathogens, when in contact with periodontal tissues, may invade epithelial cells, endothelial cells and fibroblasts, prompting evasion of host immune defence mechanisms and disease progression. It is not known if probiotic species interfere with the metabolism and/or growth of the putative periodontal pathogens, thus affecting host–microbe interactions (37). However, there are no studies currently defining direct relationship betweend pdl cells and probiotics. In the present study, the probiotic mediawas not worse than the accepted media. *Lactobacillus reuteri* solution seems to be a favourable storage media in case of dental trauma. Regarding dental trauma, a probiotic storage media can be achieved in two ways: (i) *as a culture concentrate*: culture concentrations sold in markets can be added to milk and saline, (ii) *inoculated into milk:* directly use probiotic milk as a storage media (16). Numerous studies have tried to assess the optimum storage media for PDL cell viability and preservation (38–41). Cvek et al. (42) found that avulsed teeth soaked in an isotonic saline for 30 min before replantation showed less resorption than those stored dry between 15 and 40 min. Lindskog et al. (43) in an *in vivo* study wit monkeys compared saliva with milk and concluded that saliva was less suitable than milk because of its low osmolality and higher risk for bacterial contamination. Matsson et al. (44) examined extracted dog's teeth stored dry for 15, 30, or 60 min and then replanted, versus teeth stored dry for the same dry times but also soaked in HBSS for 30 min before replantation. These researchers found a significantly less resorption in the HBSS-soaked teeth for all dry times. Hiltz & Trope (45) found that human lip fibroblasts retained the most viability at 168 h in Viaspan

versus HBSS, which kept 76% of the fibroblasts vital for 96 h. They also showed at 6 h that milk was comparable to HBSS or Viaspan. Sigalas et al. (12) found that water had a detrimental effect on the cells, whereas culture medium and HBSS preserved significantly more viable cells than the other experimental contact lens solutions and Gatorade. Martin and Pileggi (14) investigated Propolis 50% and Propolis 100% and propolis was found to be an alternative to HBSS, milk, or saline.

In terms of storage time periods, past studies have used time frames of 30 min (42, 43) or 45 min (46). The current study stored the teeth in the experimental storage solution for 45 min. This time period was chosen as it allows for comparison with previous investigations.

In the dental literature, various techniques have been used to quantify the number of viable PDL cells. Reinholdt et al. (47) used a stepwise trypsinization procedure by exposing samples to trypsin three consecutive times for 20 min each. Patil et al. (48) used a stepwise trypsinization procedure and fluorescein diacetate as a new staining technique for determining the viability of PDL cells in simulated avulsion injuries. In the present study, to minimize the exposure of cells to active trypsin and to preserve maximum cell viability, the root surface was treated with collagenase and dispase grade II, as it was previously performed by Pileggi et al. (46) and Martin and Pileggi (14). This procedure allowed rapid cell retrieval and maintained maximum cellular integrity, as was demonstrated by the positive control samples.

Our study has some obvious limitations. First, the number of patients is quite limited. Increasing the number of patients would certainly increase the statistical power of this study. Second, the amount of trauma given during the extraction might be an important determinant of PDL cell viability. In this study, the same surgical resident performed all extractions and we believe that this would help minimize those variations.

Probiotics has never been tested for its potential benefits on PDL cells of an avulsed tooth. The present study compares a novel probiotic storage media with HBSS, saline and milk in terms of PDL cell viability. *Lactobacillus reuteri* solution was not significantly different from HBSS, milk and saline. Further *in vitro* and *in vivo* studies should be conducted with variable dry times and longer storage times to determine a standard formulation for therapeutic use.

Conclusion

In conclusion, probiotics may be suitable transport media for avulsed teeth, but further research is warranted using the commercially available products. Within the parameters of this study, it appears that probiotic may be able to maintain PDL cell viability as HBSS, milk, or saline. The collagenase and dispase assay appears to be a viable method for evaluating PDL cell viability.

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