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Kvantitativna analiza probiotskih medija za pohranu izbijenih zuba

A Quantitative Analysis of a Probiotic Storage Media for Avulsed Teeth

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Sažetak

Svrha istraživanja bila je u uvjetima *in vitro* ispitati potencijal različitih medija za pohranu izbijenog zuba kao što je, primjerice, probiotski jogurt (*Bifidibacterium animalis* DN 173010) u usporedbi s Hankovom slanom otopinom (HBSS), fiziološkom otopinom i mlijekom te ocijeniti njihov potencijal u održavanju vitalnosti stanica parodontnog ligamenta (PDL). **Materijali i metode:** Trideset i šest jednokorijenskih ekstrahiranih zuba sa zatvorenim korijenima podijeljeno je u šest eksperimentalnih grupa (N = 6). Izvađeni su atraumatski i isprani fiziološkom otopinom kako bi se uklonila zaostala krv. Nakon ekstrakcije sa svakog zuba uklonjena su tri milimetra koronalnog dijela stanica parodontnog ligamenta kako bi se uklonile potencijalno oštećene stanice. Pozitivna i negativna kontrola obavljene su odmah te ponovno nakon osmosatnog sušenja. Nakon ekstrakcije uzorci u pozitivnoj kontroli odmah su tretirani dispazom i kolagenazom. Uzorci u negativnoj kontroli osušeni su i ostavljeni osam sati te su nakon toga tretirani dispazom i kolagenazom, bez prethodnog tretiranja određenom otopinom. Broj vitalnih PDL stanica prebrojen je svjetlosnim mikroskopom s pomoću hemocitometra (20 x povećanje). Rezultati su analizirani nanoparametrijskom ANOVA-om te Kruskal-Wallisovim i Dunnovim testom višestruke usporedbe. **Rezultati:** U pozitivnoj kontroli zabilježeni su znatno bolji rezultati negoli u ostalim skupinama. Uočena je statistički značajna razlika između pozitivne kontrole i ostalih testiranih grupa (p = 0,000). Zubi pohranjeni u otopini imali su u pozitivnoj kontroli najviše vitalnih PDL stanica, a zatim slijede stanice pohranjene u jogurtu, HDSS-u, fiziološkoj otopini i mlijeku. **Zaključak:** *Bifidibacterium animalis DN 173010* dobra je alternativa za privremeno pohranjivanje izbijenih zuba zbog mnoštva vitalnih PDL stanica. Probiotici su se pokazali kao medij pogodan za transport izbijenih zuba, ali je potrebno daljnje istraživanje kako bi se odredili komercijalno dostupni proizvodi za pohranu takvih zuba.

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Ključne riječi
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Uvod

Traumatske ozljede mladih trajnih inciziva vrlo su česte kod djece (1, 2). U slučaju avulzije dobra prognoza i cijeljenje ovise o imedijatnoj replantaciji zuba. Vrsta medija u koju se pohranjuje zub itekako utječe na dugoročnu prognozu replantiranog zuba. Naime, medij za pohranu održava vitalitet parodontnog ligamenta te produžuje vrijeme do replantacije (3). Pohranjivanje izbijenog zuba u adekvatni mokri medij može sačuvati vitalitet PDL stanica, što je ključno za replantaciju (4). U novim istraživanjima kao mediji za pohranu navode se mlijeko, sojino mlijeko, slina, fiziološka otopina, kultura stanica, probiotik, propolis te kokosova voda (3, 5 – 10).

Introduction

Traumatic injuries to newly erupted permanent anterior teeth are common during childhood (1-2). Avulsion is a traumatic scenario where immediate replantation leads to excellent healing and good prognosis. The type of storage media used is a significant factor that can affect the long-term prognosis for replanted teeth. The storage media maintains the viability of the periodontal ligament cells and thus permits longer extra-alveolar periods prior to replantation (3). Maintenance of the avulsed tooth in an adequate wet medium that can preserve the vitality of the periodontal ligament cells that remain on root surface is the key to success of replantation (4). Current research favors milk, soy milk, saliva,

Bakterije su prirodno okružje u slučaju izbijenog zuba. Probiotske bakterije mikrobni su dodatci prehrani te koriste domaćinu zato što utječu na usklađivanje mnogo vrsta komenzalnih flora u ustima i probavnom traktu (11 – 12). Nedavno je dokazano da u slučaju traume probiotici *L. reuteri* DSM 17938 i *L. reuteri* ATCC PTA 5289 održavaju vitalitet PDL stanica (3). Važno je da bezopasni mikroorganizmi kao, primjerice, sojevi laktobacila ili bifidobakterija okupiraju prostor u biofilmu koji bi inače bio prekriven patogenima (13). Bifidobakterija, kao anaerobna bakterija u lumenu crijeva najvažnija je za održavanje normalne probavne flore (14).

Od kasnih osamdesetih godina mnogo je mliječnih proizvoda koji sadržavaju bifidobakteriju i prodaju se u velikom broju zemalja, pa su obavljena mnoga istraživanja o preživljavanju i pozitivnim učincima *Bifidobacterium* DN-173 010 u probavnom traktu (15). Caglar i suradnici. (16) istaknuli su da ta bakterija može održavati zdravlje tako što pomaže sačuvati mikrobiološku floru u ustima te potiče oralne obrambene čimbenike, kao što je peroksidaza koja smanjuje kiselost bakterija. Svrha ovoga istraživanja *in vitro* bila je procijeniti potencijal probiotskog jogurta (*Bifidibacterium animalis DN 173010*) kao medija za pohranu izbijenog zuba u usporedbi s HBSS-om, fiziološkom otopinom i mlijekom u očuvanju vitaliteta PDL stanica na simulirano avulziranim zubima.

Materijali i metode

Pacijentima je objašnjena svrha istraživanja te su svi potpisali informirani pristanak. Izvađeno je 36 jednokorijenskih drugih trajnih premolara sa zatvorenim apeksom. Zubi su podijeljeni u četiri eksperimentalne grupe i dvije kontrolne (pozitivna i negativna). U svakoj je bilo po sedam uzoraka. Izuzeti su svi zubi izvađeni zbog opsežnog karijesa ili umjerenog do opsežnog parodontitisa. Zubi su izvađeni atraumatski i isprani fiziološkom otopinom kako bi se uklonila preostala krv. Nakon ekstrakcije sastrugana su tri milimetra koronalnog PDL-a skalpelom broj 15 da bi se skinule oštećene stanice. Pozitivna i negativna kontrola odgovarale su vremenski, a sušenje je trajalo 8 sati. Zubi u pozitivnoj kontroli odmah su nakon ekstrakcije tretirani dispazom i kolagenazom, a oni u negativnoj stavljeni su u vlažni medij i sušeni osam sati te nakon toga tretirani dispazom i kolagenazom.

Ekstrahirani zubi sušeni su 30 minuta (istodobno su kiretažom uklonjene PDL stanice u koronalnom dijelu korijena) te 45 minuta uronjeni u jedan od četiri medija: u probiotski jogurt koji sadržava *Bifidibacterium animalis DN 173010 (*Activia, Danone, Luleburgaz, Turska*),* u HBSS, fiziološku otopinu i mlijeko. Svaki zub je nakon sušenja i namakanja 30 minuta bio pohranjen u 15-mililitarskim epruvetama Falcon koje su sadržavale 2,5ml 0,2 mg ml^{-1}otopine kolagenaze CLS II (Biochrom, Berlin, Njemačka) i 2,4 mg ml^{-1} otopine dispaze II (Sigma-Aldrich, St.Louis, MO, SAD) u fiziološkoj

physiologic saline, cell culture media, probiotic media, propolis, coconut water as storage media (3,5-10)

Host bacteria are a natural surrounding for avulsed teeth. 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 (11-12). Recently a host probiotic, *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289, have been shown to preserve vitality of periodontal ligament (PDL) cells in case of dental trauma (3). The key event is that harmless microorganisms, such as strains of lactobacilli or bifidobacteria, can occupy a space in a biofilm that otherwise would be colonized by a pathogen (13). Bifidobacteria are the predominant anaerobic bacteria within the intestinal lumen and play a critical role in maintaining the equilibrium of the normal intestinal flora (14).

Since the late 1980s, a range of dairy products containing bifidobacteria has been marketed in a number of countries worldwide and studies have been performed to validate the survival and positive effects of *Bifidobacterium* DN-173 010 within the gastrointestinal tract (15). Caglar et al. (16) stated that *Bifidobacterium* may also have a role in maintaining health by promoting a microbiological balance in the oral cavity and oral defense factors, such as the peroxidase system may inhibit the acidogenicity of bacteria. The aim of the present *in vitro* study was to investigate the potential of a storage medium, probiotic yogurt (*Bifidobacterium animalis DN 173010*) in comparison with Hank's balanced salt solution (HBSS), saline and milk in maintaining viable periodontal ligament (PDL) cells on simulated avulsed teeth.

Materials & Methods

Patients received both oral and written information about the study and signed a consent form prior to handling their extracted teeth. Thirty-six 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 seven samples each. 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 The positive and negative controls corresponded to 0 minutes and an 8-hour dry time, respectively. 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.

The experimental teeth were dried for 30 minutes (during the present time interval, the coronal PDL cells were curetted) followed by a 45 min immersion in one of the four media: *Bifidibacterium animalis DN 173010* containing probiotic yogurt (Activia, Danone, Luleburgaz, Turkey), HBSS, saline and milk. Each experimental tooth, after drying and soaking, was incubated for 30 min in 15 ml falcon tubes with

otopini s fosfatnim prahom (PBS). Poslije inkubacije u svaku je epruvetu dodano 50 µl fetalnoga goveđeg seruma (Biowest, Nuaille, Francuska). Nakon toga sve su epruvete centrifugirane četiri minute na 1000 r.p.m. Centrifugirana tekućina odvojena je sterilnim mikropipetama, a stanice su obojene 0,4-postotnim Trypan Blueom (Sigma-Aldrich, St.Louis, MO, SAD) kako bi se odredio njihov vitalitet prema Polveriniju i Leibovichu (17). Svjetlosnim mikroskopom i hemocitometrom (20 x povećanje) prebrojene su vitalne PDL stanice s najmanjom razlikom u vitalnosti. Njihov broj dobiven je sljedećom matematičkom jednadžbom: broj neobojenih stanica (vitalne stanice) **x** razrjeđenje otopine **x** 10^4/broj prebrojenih kvadratića u hemocitometru (3,10,18).

Statistička analiza podataka obavljena je ANOVA-om te Kruskal-Wallisovim i Dunnovim testom mnogostrukih usporedba.

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 fetal 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 labeled with 0.4% Trypan Blue (Sigma-Aldrich, St.Louis, MO, USA) for determination of viability, according to Polverini & Leibovich (17). The number of viable protective least significant difference PDL cells were counted under a light microscope with a hemocytometer at 20× magnification and analyzed. The number of viable cells was obtained by the following mathematical equation: unstained cell count (viable cell) X the dilution of the cell suspension X 10^4 /number of squares of the hemocytometer counted (3,10,18). Statistical analysis of the data was accomplished using Nonparametric ANOVA complemented by Kruskal-Wallis Test and Dunn's Multiple Comparisons Test.

Rezultati

U pozitivnoj kontroli zabilježene su znatno više vrijednosti vitalnih stanica – srednja vrijednost iznosila je 16,580.000 (tablica 1.). Uočena je statistički značajna razlika između pozitivne i negativne kontrole ($p < 0,001$). Osim u pozitivnoj kontroli, zubi pohranjeni u jogurtu s probiotikom *Bifidibacterium animalis DN 173010* imali su najveći broj vitalnih stanica (N: 1, 830. 000), a zatim su slijedili oni u HBSS-u, fiziološkoj otopini i mlijeku. Unatoč broju vitalnih stanica, nije bilo statistički značajne razlike između vitalnih stanica pohranjenih u sve četiri otopine ($p > 0,05$). U svim eksperimentalnim skupinama dobiveni su statistički značajno manji rezultati nego u pozitivnoj kontroli ($p < 0,001$).

Results

The positive control was found to have a median of 16580000 viable cells and was significantly better than the others, (Table 1) There was only statistically significant differences between positive control and negative control ($p<0.001$). Besides positive control, the teeth stored in *Bifidibacterium animalis DN 173010* containing yogurt demonstrated the highest number of viable PDL cells (N:1 830 000) followed in rank order by HBSS, saline and milk. However there was no significant difference in the number of viable PDL cells between HBSS, milk, *Bifidibacterium animalis DN 173010* containing yogurt and saline ($p>0.05$). All experimental groups were significantly lower than positive controls ($p<0.001$).

Tablica 1. Kvantitativna analiza utjecaja probiotika na preživljavanje PDL stanica
Table 1 A quantitative analysis of probiotics on PDL cell survival

Grupa • Groups (N:6 each)	Median	Minimum	Maximum
Pozitivna kontrola • Positive control	16580000	11100000	28550000
Negativna kontrola • Negative control	31000	10000	66000
HBSS	1387500	386000	2415000
Slina • Saline	1256000	412000	1364000
Bifidibacterium animalis DN 173010 u probiotičkom jogurtu • *Bifidibacterium animalis DN 173010* containing yogurt	1 830 000	560 000	2 640 000
Mlijeko • Milk	880000	644000	1046000

Rasprava

Prevencija, odnosno limitiranje resorpcije korijena replantiranog zuba uvelike ovisi o ranoj uspostavi normalne stanične fiziologije PDL stanica nakon dentalne traume. Vrijeme do replantacije i medij u kojemu je zub pohranjen vrlo su važni za održavanje vitaliteta stanica. Blomlof je bio pionir u istraživanju medija za pohranu izbijenih zuba te je među prvima istražio učinak mlijeka i ljudske sline na PDL stanice u simuliranim uvjetima (19 – 22). Dokazano je da mlijeko šest sati može održati vitalitet stanica, slina dva sata, a fiziološka otopina jedan sat (23). Hank's Balanced Salt Solu-

Discussion

The prevention or limitation of replacement root resorption is the key point in early reestablishment of PDL cellular physiology in case of dental trauma. The critical extraoral dry time for PDL cell viability and the nature of storage media play a very special role at this cellular balance. Blomlof, a pioneer in storage media research, was one of the first to investigate the effect of milk with that of saliva on human PDL cells in simulated conditions (19-22). Milk may keep the cells viable for up to 6 h (hours), saliva may keep them up to 2 h while saline for 1 h (23). Hank's Balanced Salt Solutions

tions – HBSS (Save a tooth®, EMT Tooth Saver®) ili Viaspan® mediji su za pohranu stanica dostupni na tržištu u Sjevernoj Americi. Dokazano je da u duljem razdoblju održavaju vitalitet PDL stanica (24). Ti mediji uglavnom su tema mnogih istraživanja (25 – 31), ali je u nekoliko njih istaknuto da zub na suhome ne smije ostati dulje od 30 do 45 min (3, 10, 30, 32 – 36). Razmak od 45 minuta izabran je zato što se može uspoređivati s ranijim istraživanjima. Treba napomenuti da medij za pohranu isključivo služi za održavanje vitaliteta PDL stanica te da se infekcija smatra sekundarnom pojavom (3). PDL tekućina opskrbljuje zub tvarima potrebnima za preživljavanje PDL stanica. Preostale PDL stanice koje se zateknu na zubu nakon što je izbijen, ovise o dostupnim metabolitima. Njihovo razaranje počinje čim oni nestanu. Kako bi se održao optimalni metabolizam stanica, dostupnost metabolita trebalo bi obnoviti 60 minuta nakon nezgode. Ako te preostale stanice prežive, potaknut će reprodukciju novih koje će se diferencirati u novo vezivno tkivo. Moramo istaknuti da je pritom za preživljavanje tih stanica najvažnija prevencija sinteze proteina u bakterijskim stanicama, poticanje fibroblasta te cijeljenje vezivnog tkiva. Svi ti čimbenici pridonose cijeljenju PDL nakon ozljede (37).

Probiotici pomažu crijevnoj epitelnoj homeostazi nizom bioloških funkciji, kao što su pomoć u proliferaciji, migraciji, preživljavanju i održavanju integriteta barijere, izlučivanje antimikrobnih supstancija te interakcija s patogenom umjesto epitelnih stanica (38, 39).

U ovom istraživanju zubi pohranjeni u probiotski jogurt imali su najviše vitalnih PDL stanica, a zatim su slijedili oni u HBSS-u, fiziološkoj otopini i mlijeku. Unatoč tomu u rezultatima nije bilo statistički značajne razlike. Ovaj rezultat u skladu je s onima u istraživanju Caglara i suradnika (3) koji su istaknuli da je probiotik *L. reuteri* jednako učinkovit kao i ostali mediji za pohranu. U dosadašnjim istraživanjima dokazano je da u parodontološkim džepovima tretiranima korisnim bakterijama nastaje odgođena rekolonizacija subgingivalnih parodontnih patogena pa je manji i stupanj upale (40). Ovaj učinak može se usporediti s protektivnim djelovanjem probiotskog medija za pohranu izbijenog zuba.

Broj vitalnih PDL stanica održan je uporabom kolagenaze i dispaze II, kako bi se smanjio učinak aktivnog tripsina te održala maksimalna vitalnost stanica. Ova procedura omogućila je reaktivaciju stanica i održala maksimalni stanični integritet, kao što je i dokazano na uzorcima pozitivne kontrole (3, 10, 41).

Zaključak

Probiotik *Bifidibacterium animalis DN 173010* može se upotrijebiti kao alternativa za privremenu pohranu izbijenih zuba zbog većeg broja vitalnih PDL stanica. Dakle, probiotici mogu poslužiti kao transportni medij za izbijene zube, ali potrebno je daljnje istraživanje kako bi se ispitali svi takvi proizvodi dostupni na tržištu.

Sukob interesa

Nije bilo sukoba interesa.

(HBSS) (Save a tooth®, EMT Tooth Saver®) or Viaspan® are cell culture media introduced to North America. They have been successful in preserving the viability of the PDL fibers for extended periods (24). Whereas storage media was a topic of research interest (25-31), the critical extraoral dry time of PDL had been investigated in several studies (3,10,30,32-36) where time frames of 30 min or 45 min are appreciable. Forty-five minute time period was chosen as it allows for comparison with previous investigations. It should be noted that the storage media are primarily used to maintain the viability of the cells and that infection is a secondary consideration (3). 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 minutes from the time of injury. If these cells survive, they will catalyze the reproduction of new cells, which can differentiate and reinstate the supporting tissues. 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 (37).

Probiotics facilitate intestinal epithelial homeostasis through a number of biological responses, including promoting proliferation, migration, survival, barrier integrity, antimicrobial substance secretion, and competition for pathogen interaction with epithelial cells (38-39).

In the present study, the teeth stored in positive control demonstrated the highest number of viable PDL cells followed in rank order by *Bifidibacterium animalis DN 173010* containing yogurt, HBSS, saline and milk. However, there was no significant difference among them. This result is in accordance with Caglar et al. (3) where *L reuteri* was found to be as efficient as the latter storage media. 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 (40). This might have a protective storage media effect of current probiotic species.

The number of viable PDL cells have been detected through root surface treated by collagenase and dispase grade II to minimize the exposure of cells to active trypsin and to preserve maximum cell viability. This procedure allowed rapid cell retrieval and maintained maximum cellular integrity, as was demonstrated by the positive control samples (3,10,41).

Conclusion

In conclusion, *Bifidibacterium animalis DN 173010* seems to be an alternative for the temporary storage of avulsed teeth due to high number of viable PDL cells. Probiotics may be a suitable transport media for avulsed teeth, but further research is warranted using the commercially available products.

Conflict of interest

The authors deny any conflicts of interest.

Abstract

Aim: The aim of the present *in vitro* study was to investigate the potential of a storage medium, probiotic yogurt (*Bifidibacterium animalis DN 173010*) in comparison with Hank's balanced salt solution (HBSS), saline and milk in maintaining viable periodontal ligament (PDL) cells on simulated avulsed teeth. **Materials and methods:** Thirty-six freshly extracted single-rooted human teeth with closed apices were divided into six experimental groups (N=6). 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 were scraped with a #15 scalpel to remove cells that may have been damaged. The positive and negative controls corresponded to 0 minutes and an 8-hour dry time, respectively. 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. The number of viable protective least significant difference PDL cells were counted under a light microscope with a hemocytometer at 20x magnification and analyzed. Statistical analysis of the data was accomplished using Nonparametric ANOVA complemented by Kruskal-Wallis Test and Dunn's Multiple Comparisons Test. **Results:** Positive control was found to be significantly better than the others, there were statistically significant differences between positive control and other test groups (p=0.000). The teeth stored in positive control demonstrated the highest number of viable PDL cells followed in order by probiotic yogurt, HBSS, saline and milk. **Conclusion:** *Bifidibacterium animalis DN 173010* seems to be an alternative for the temporary storage of avulsed teeth, due to high number of viable PDL cells. Probiotics may be suitable transport media for avulsed teeth, but further research is warranted using the commercially available products.

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Key words
Tooth Avulsion; Tissue Preservation; Periodontal Ligament; Milk; Hanks Balanced Salt Solution; Saliva; Probiotics

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