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IZVORNI ZNANSTVENI RAD
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Plazma siromašna trombocitima kao dodatak fibroblastima kultiviranima u fibrinu obogaćenom trombocitima

Platelet-Poor Plasma as a Supplement for Fibroblasts Cultured in Platelet-Rich Fibrin

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Sažetak

Svrha: Svrha ove studije bila je procijeniti proliferaciju i adheziju mezenhimalnih stanica (3T3/HIH) u Eagleovu mediju koji je modificirao Dulbecco (DMEM), uz dodatak plazme siromašne trombocitima (PPP) u trombocitima obogaćenom fibrinskom (PRF) nosaču. **Materijal i metode:** Dobivena ljudska krv obrađena je u centrifugi, uzimajući u obzir jednadžbu G = 1,12 x Rx (RPM/1000)² da bi se dobili PRF i PPP. Analize stanične adhezije i rasta provedene su MTT testom u pločici s 96 jažica uz dodatak DMEM : PPP-a (90 : 10) tijekom 24 sata. Osim toga, PRF je nanesen na pločicu s 48 jažica i 10 x 10^4 stanice nasađene su na svaki PRF (n = 3) s 800 ul DMEM : PPP-a (90 : 10) te kultivirane sedam dana. Obavljena je histološka analiza i imunohistokemijsko bojenje za vimentin. **Rezultati:** Rezultati su analizirani dvofaktorskom analizom varijance u Stata12®. Uočeno je značajno smanjenje ($p < 0,05$) stanične adhezije u odnosu na FBS. No prikazano je slično svojstvo rasta stanica za 10-postotni PPP ($P > 0,05$). Kultura fibroblasta tijekom sedam dana u PRF-u ostvarena je uz dodatak 10-postotnog PPP-a, te je pokazala pozitivno bojenje za vimentin. Dakle, stanični dodatak PPP-a smanjio je početnu adheziju stanica, ali je podržao proliferaciju adheriranih stanica i njihovu vitalnost u PRF-u. **Zaključak:** Ova metoda je potencijalna klinička prednost za pružanje autolognog i prirodnog nosača za staničnu kulturu u samo jednom postupku, bez uporabe ksenogenih spojeva. Mogla bi poboljšati kliničke translacijske terapije na temelju upotrebe PRF kultiviranih stanica, promovirajući regenerativni potencijal za buduću uporabu u medicini i dentalnoj medicini.

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Ključne riječi
krvna plazma; tkivni inženjering; plazma bogata trombocitima; prianjanje stanica; kulture stanica

Uvod

Klinička uporaba fibrina obogaćenog trombocitima (PRF) za regenerativnu terapiju pokazala je da se mogu poboljšati biološki rezultati u parodontnoj (1) i endodontskoj (2) terapiji, ako djeluje uglavnom kao analog izvanstaničnoj matrici. Nedavno obavljeno nasumično kliničko ispitivanje revaskularizacije nezrelih stalnih zuba pokazalo je poboljšanje biološkog odgovora u skupini liječenih PRF-om, u usporedbi s konvencionalnom revaskularizacijom ili uporabom plazme bogate trombocitima (PRP) (2). Iako se čini da PRF povećava odgovor stanica pri revaskularizaciji, tkivo je sličnije reparatornom negoli regeneriranom, jer nije pokazalo taloženje dentina (3). Zato bi uporaba mezenhimalnih stanica (MC) nasađenih u PRF-u mogla poboljšati biološki kapacitet za regeneraciju tkiva. No mezenhimalne stanice koje su nasađene i kultivirane na ovom prirodnom nosaču teško je ostvariti, ponajprije zbog ksenogenskih dodataka koji se koriste u staničnoj kulturi i ekspanziji (4, 5).

Introduction

The clinical use of Platelet-rich fibrin (PRF) in regenerative therapies has shown the capacity to improve the biological outcomes in periodontal (1) and endodontic (2) treatments, acting principally as analogous to the extracellular matrix. In this way, a recent randomized clinical trial evaluating the revascularization of immature permanent teeth showed an improvement of biological response in groups treated with PRF instead of using conventional revascularization or using Platelet-Rich Plasma (PRP) (2). Although the PRF seems to increase the cells response in revascularization, the tissue formed in this way is more similar to repaired tissue than to regenerated tissue because it does not present dentin deposition (3). Thus, the use of mesenchymal cells (MC) seeded in the PRF could improve the biological capacity for tissue regeneration. However, the use of mesenchymal cells seeded and cultured in this natural scaffold is a difficult task, principally due to use of xenogeneic agents used in cell culture and expansion (4, 5).

Ostvarivanje optimalnih uvjeta za kulturu mezenhimalnih stanica izazov je za prijenos terapije temeljene na stanicama iz laboratorija u kliniku (6). Da bi mogli biti u kliničkoj uporabi, MC-i moraju biti izolirani i ekspandirani *in vitro* (7, 8). Razni mediji za kulturu, uključujući Dulbeccov modificirani Eagleov medij (DMEM), korišteni su za održavanje MC-a *in vitro*. Kako bi omogućili stanicama fiziološke uvjete, mediju za kulturu treba biti dodana kompleksna smjesa čimbenika rasta, proteina, ugljikohidrata i citokina (9). Fetalni goveđi serum (FBS) najčešće je korišten u te svrhe, unatoč rizicima povezanima s prijenosom zoonoze i patogena (7). Osim toga, životinjski (ksenogeni) proteini u FBS-u mogu biti inkorporirani u ljudske stanice, aktivirajući antigeni odgovor nakon implantacije. I proteini FBS-a koji ostaju inkorporirani u staničnoj citoplazmi čak i nakon uzastopnih ispiranja (9), mogu pokazati promjene u površinskim markerima koji mogu potaknuti promjene u staničnoj biologiji (7).

Venski krvni derivati (VBD) korišteni su kao alternativa tradicionalnim dodatcima kako bi se izbjegli rizici povezani sa ksenogenim serumima. VBD, kao i plazma obogaćena trombocitima, potanko su istraženi te je dokazano da mogu održavati MC kulture jednako dobro kao i FBS (9, 10). Uporaba koncentrata trombocita u regeneraciji tkiva može omogućiti djelotvornije cijeljenje i ubrzati proces reparacije (11). I protokoli za dobivanje PRP-a složeni su te zahtijevaju korištenje kemikalija koje su nužne za odgovarajuću obradu PRP-a (12). Istodobno, PRF je dokazan kao djelotvoran za endodontsku i parodontnu regeneraciju i može se dobiti jednostavnim protokolom bez dodataka kemikalija ljudskoj krvi, za razliku od PRP-a (12).

Tijekom centrifugiranja krvi za dobivanje PRF-a trombociti su aktivirani uz značajno oslobađanje čimbenika rasta – transformirajući čimbenik rasta beta I (TGF-bI), inzulinu sličan čimbenik rasta (IGF-I), trombocitni čimbenik rasta (PDGF-β) – koji su izdvojeni u fibrinskoj mreži tijekom procesa polimerizacije. Nedavne studije (13, 14) pokazale su da supernatant koji nastaje nakon dobivanja PRF-a, plazma siromašna trombocitima (PPP), može sadržavati neke čimbenike rasta. Osim toga, PPP je sposoban inducirati osteoblastnu diferencijaciju matičnih stanica iz parodontnog ligamenta *in vitro* (14) i obnavljanje koštanih defekata *in vivo* (13). Prema našim spoznajama, nema objavljene studije o PPP-u kao alternativi FBS-u kao dodatka DMEM-u. Uz to, nakon dobivanja PRF-a, PPP se uglavnom odbacuje. Zato je cilj ovog istraživanja bio procijeniti proliferaciju i adheziju mezenhimalnih stanica (3T3 / NIH) u DMEM-u i plazmi siromašnoj trombocitima u PRF nosaču.

Materijali i metode

Istraživanje

U prvom dijelu studije procijenjena je stanična adhezija i proliferacija u dvodimenzionalnom (2D) okružju gdje je stanicama dodan PPP. U drugom dijelu procijenjen je rast mezenhimalnih stanica nasađenih u PRF-u s PPP-om.

The identification of optimal condition for mesenchymal cells culture comprises a challenge for the transition of cell-based therapies from the bench to the bedside (6). To be clinically applied, MC must be isolated and expanded *in vitro* (7, 8). Ranges of culture media, including Dulbecco's Modified Eagle Medium (DMEM), have been employed for the *in vitro* maintenance of MC. To allow the cells remaining in physiological conditions, the culture medium should be supplemented with a complex mixture of growth factors, proteins, carbohydrates and cytokines (9). Fetal Bovine Serum (FBS) is the solution commonly applied for this purpose, despite present risks associated with transmission of zoonosis and pathogens (7). Besides, animal (xenogeneic) proteins that are present in FBS can be internalized into the human cells by activating antigenic response post-implantation. In addition, FBS-proteins remain internalized in cells cytoplasm even after successive washings (9) and may cause changes in the surface markers, which could induce alterations in cells biology (7).

Venous blood derivatives (VBD) have been developed as an alternative to traditional supplements to overcome the risks related to xenogeneic serums. Venous blood derivatives such as Platelet Rich Plasma have been widely studied, proving to be able to maintain MS culture as well as FBS (9, 10). The use of platelet concentrates in tissue regeneration can provide a more effective healing and can accelerate the repair process (11). However, the protocols for obtaining the PRP are complex and there is a need for incorporation of chemicals, which are indispensable for proper PRP processing (12). Unlike the PRP, the PRF has been shown to be efficient for endodontic and periodontal regeneration and can be obtained by a simple protocol, without any addition of chemicals to human blood (12).

During blood centrifugation, in order to obtain PRF, platelets are activated with a significant growth factor release - Transforming Growth Factor Beta I (TGF-β1), Insulin-Like Growth Factor I (IGF-I), Platelet-Derived Growth Factor Beta (PDGF-β) - which are sequestered in the fibrin network during the polymerization process. Recent studies (13, 14) have shown that the supernatant resulting from the obtained PRF, the Platelet-Poor Plasma (PPP), could contain some grow factors in its composition. In addition, the PPP is able to induce the osteoblastic differentiation of stem cells from periodontal ligament *in vitro* (14) and the repair of bone defects *in vivo* (13). To the best of our knowledge, there have been no reports evaluating PPP as DMEM supplementation as an alternative to FBS. Besides, after the PRF has been obtained, the PPP is generally discarded. Thus, the aim of this study was to evaluate the proliferation and adhesion of mesenchymal cells (3T3/NIH) in DMEM supplemented with Platelet-poor plasma in the PRF scaffold.

Materials and Methods

Study design

In the first part of the study, the initial cell adhesion and proliferation in a two-dimension (2D) environment was evaluated, where the cells were supplemented with PPP. In the second part, the maintenance of mesenchymal cells seeded in the PRF and supplemented with PPP was evaluated.

Dobivanje PRF-a i PPP-a

Vensku krv donirali su sami istraživači nakon odobrenja Etičkoga odbora broj 62282216.8.0000.5318. Uzorci su obrađivani u komori s laminarnim protokom neposredno nakon skupljanja krvi, u sterilnim uvjetima kako bi se spriječila kontaminacija. Protokol koji su razvili Choukroun i suradnici (15) primijenjen je za izolaciju PRF-a. Takav protokol oslanja se samo na centrifugiranje, uzimajući u obzir izračun gravitacijske sile (G-force) proizvedene na uzorcima krvi – G-force = 1,12 x. Radijus x (RPM / 1000): kako bi se postigao G-force jednak 400. Tako su uzorci krvi centrifugirani (1500 RPM-a) deset minuta na sobnoj temperaturi. Nakon toga je dio koji odgovara PPP-u lagano pipetiran i prebačen u dvomililitarske kriogene bočice i odmah stavljen u zamrzivač na ultra nisku temperaturu (-80 °C).

Stanična kultura

Fibroblasti 3T3/NIH uzgojeni su u DMEM-u (Cultilab®) uz dodatak 10-posto FBS-a (Cultilab®). Plastična posuda od 75 cm^3 sa stanicama prenesena je u inkubator (37 °C, 5 % CO_2). Nakon što je postignuta subkonfluentnost fibroblasta (80 %) isprani su fosfatno puferiranom fiziološkom otopinom (PBS) (Gibco®) kako bi se uklonili metaboliti stanica. Zatim je 5 ml 0,25-postotnog tripsina/EDTA (Gibco®) naneseno na stanice tijekom pet minuta na 37 °C. Za inaktivaciju tripsina korišteno je 5 ml standardnoga medija za kulturu. Stanična suspenzija stavljena je u epruvete od 15 ml i centrifugirana pet minuta na 1000 rpm (G-sila = 180). Na taj način uklonjen je supernatant, a preostao je stanični pelet (talog). Stanice su suspendirane u 3 ml standardnog medija, gdje je 20 uL uklonjeno za brojenje stanica u hemocitometru. Nakon brojenja je 2 x 10^4 stanica nasađeno (200 uL DMEM-a s FBS-om ili PPP-om) po jažici na pločici s 96 jažica. Skupine (n = 8) su se sastojale od sljedećih dodataka: DMEM : PPP (90 : 10) i DMEM : FBS (90 : 10) kao pozitivna kontrola; DMEM (100 %) je bio negativna kontrola.

Test stanične adhezije

Odmah nakon dodavanja različitih dodataka na pločicu s 96 jažica, stanice su inkubirane 24 sata. Nakon toga su DMEM i ostali dodatci uklonjeni s pločice, te su jažice isprane PBS-om. DMEM, s odgovarajućim dodatcima, pohranjen je u svaku jažicu (200 ul), sada s dodatkom MTT-a (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazolij) (0,5 mg/) (Sigma Aldrisch®) i održavan u kontaktu sa stanicama četiri sata (37 °C i 5 % CO_2). Nakon inkubacije medij je aspiriran i kristali formazana suspendirani su u 200 ul 10 % dimetilsulfoksida (DMSO) tijekom 15 minuta. Zatim je ploča stavljena na tresilicu pet minuta (150 rpm). Rezultati su procijenjeni spektrofotometrijom (univerzalni ELISA čitač – valna duljina od 540 nm) na kojoj je vrijednost apsorpcije očitana kao pokazatelj stanične proliferacije.

Test rasta stanica

Kako bi se procijenio rast stanica, 2 x 10^4 stanica stavljeno je na pločicu s 96 jažica kojima je na početku dodan 10-postotni FBS. Tako je potaknuta početna adhezija stanica s istim dodatkom (FBS). Nakon 24 sata stanične adhezi-

PRF and PPP Obtaining

Venous blood was donated by own researchers after approval by the institution's Research Ethics Committee Number 62282216.8.0000.5318. The samples were handled immediately after blood collection under sterile conditions and biosecurity to prevent contamination in a laminar flow hood. The protocol developed by Choukroun et al. (15) was applied for PRF isolation. Such a protocol relies just on centrifugation, considering the calculation of the force of gravity (G Force) produced on blood samples - G-Force = 1.12 x. Radius x (RPM / 1000): to achieve a resulting G-Force equal to 400. Thus, the blood samples were centrifuged (1,500 RPM) for 10 minutes at room temperature. After centrifugation, the portion corresponding to PPP was gently pipetted and transferred into 2 ml cryogenic vials and frozen immediately in ultra-freezer (-80C °).

Cell Culture

Fibroblast 3T3/NIH was cultured in DMEM (Cultilab®) supplemented with FBS (Cultilab®) 10%. A 75cm^3 culture flask containing cells was transferred to an incubator (37°C, 5% CO_2). After reaching fibroblasts sub confluency (80%), they were washed with phosphate buffered saline (PBS) (Gibco®), in order to remove cell-metabolites. Subsequently, 5 ml of 0.25% trypsin/EDTA (Gibco®) has been applied on cells for 5 minutes at 37°C. For trypsin inactivation, 5 ml of standard culture media has been used. The cell-suspension was placed in 15 ml falcon-like tubes and centrifuged for 5 minutes under 1000 rpm (G-force =180). Thus, the supernatant was removed, leaving just the cell pellet. The cells were suspended in 3 ml of Standard media where of 20 µL were removed for cell-counting in a hemocytometer. After counting, 2x10^4 cells were plated (200µl DMEM supplemented with FBS or PPP) per well in a 96 well plate. The groups (n=8) were comprised by the following supplements: DMEM: PPP (90:10) and DMEM: FBS (90:10) as the positive control; DMEM (100%) was the negative control.

Cell Adhesion Assay

Just after the addition of different supplements in the 96 well plates, cells were incubated for 24 hours. After the incubation period, DMEM + supplements were removed from the plate and the wells were washed with PBS. DMEM, with respective supplements, have been deposited in each well (200µl), now with the addition of MTT - (3- (4, 5-dimethylthiazol-2-yl) -2, 5 -diphenyl tetrazolium) - (0.5mg/ml) (Sigma Aldrisch®) and maintained in contact with the cells for 4h (37 ° C and 5% CO_2). Post incubation, the medium was aspirated and formazan crystals were suspended in 200µL of 10% dimethyl sulfoxide (DMSO) for 15 minutes. Then, the plate was placed on a shaker for 5 minutes (150 rpm). The results were assessed by spectrophotometry (Universal ELISA reader - wavelength of 540 nm), where the absorbance values were considered as an indicator for cell proliferation.

Cell Maintenance Assay

To evaluate the cell maintenance, 2x10^4 cells were plated in a 96 well plate. All the groups were initially supplemented with FBS 10% to promote the initial adhesion of cells with the same supplementation (FBS). After 24 hours of cell adhe-

je, DMEM + FBS uklonjeni su s pločice i stanice su isprane s 20 ul PBS-a. Zatim je medij promijenjen u adheriranim stanicama u DMEM : PPP (90 : 10) i praćene su dodatnih 24 sata. DMEM : FBS (90 : 10) služio je kao pozitivna kontrola, a DMEM (100 %) kao negativna. Provedeni MTT test je već opisan.

Stanična kultura u PRF-u s dodatkom PPP-a

PRF je dobiven nakon Choukrumova protokola i odložen u pločicu s 48 jažica. Zatim je 10 x 10^4 stanica nasađeno iznad svakog PRF nosača (n = 3) s 800 ul DMEM : PPP-a (90 : 10 %). Stanice su kultivirane u kontroliranim uvjetima (37 °C i 5 % $C0_2$) sedam dana. Medij je mijenjan svaka dva dana, pri čemu je PRF ispran PBS-om između promjena medija. Skupine su fiksirane u 4-postotnom formalinu tijekom 24 sata i uklopljene u parafin za histološku analizu. Histološki izbrusci dobiveni su po uzdužnoj osovini PRF-a. Obavljeno je hematoksilin-eozinsko bojenje i procijenjeno pod svjetlosnim mikroskopom.

Imunohistokemijska analiza

Da bi se identificirali nasađeni fibroblasti u PRF-u i razlikovali od krvnih stanica, obavljeno je imunohistokemijsko bojenje za vimentin (Vimentin Immunohistology Kit Sigma-aldrich®).

Statistička analiza

Rezultirajuće vrijednosti apsorpcije prikazale su normalnu raspodjelu, te su parametrijski analizirani jednosmjernom analizom varijance (jednosmjerna ANOVA), a zatim komplementarnim Bonferonijevim testom. Priznaje se pogreška tipa α od 5 posto. Statistička analiza provedena je u softveru Stata 12®.

Rezultati

Da bi se procijenio utjecaj PPP-a na staničnu adheziju, stanice su odmah nasađene u jažice s pomoću PPP-a. Tako je uočeno značajno smanjenje ($p < 0,05$) adhezije stanica nakon 24 sata u odnosu na skupinu kojoj je dodan FBS (slika 1.). U skupini stanica s DMEM-om (100 %) ($p < 0,05$) uočeno je da su neke stanice ostale vitalne i adherirane. Kako bi se procijenio rast stanica i osigurala slična stanična adhezija, stanice su čuvane 24 sata u jažicama, te je nakon toga dodan PPP. Uočen je sličan rast adheriranih stanica u PPP-u bez statičke razlike ($P > 0,05$) (slika 2.).

U drugom dijelu studije ispitivana je stanična kultura u PRF-u s dodatkom PPP-a. Nakon sedam dana kulture, fibroblasti kultivirani u PRF-u s dodatkom PPP-a, pokazali su značajan broj stanica distribuiranih u PRF-u. Hematoksilin-eozinskim bojenjem (slika 3.) uočene su stanice u vlaknima PRF-a, pretežno prožimajući područje zgrušane krvi. Slika 4. prikazuje pozitivno bojenje vimentinom za fibroblaste u značajnom broju stanica, potvrđujući da su fibroblasti. Stanice koje nisu obojene vimentinom (krvne stanice) također su rasle u PRF-u. Dakle, PPP je također podržao rast krvnih stanica koje su zaostale u PRF-u.

sion, DMEM+FBS were removed from the plate and washed with 20µl of PBS. Then, the media was changed in the adhered-cells for DMEM: PPP (90:10) and maintained for more 24 hours. DMEM: FBS (90:10) was the positive control and DMEM (100%) the negative control. The MTT assay was carried as described above.

Cell Culture in PRF Supplemented with PPP

The PRF was obtained following the Choukrum's protocol and deposited in a 48 well plate. Then, 10x10^4 cells were seeded above each of the PRF scaffolds (n=3) with 800 µl of DMEM: PPP (90:10%). The cells were cultured in a controlled environment (37°C and 5% CO_2) for 7 days. The medium was changed every two days, and the PRF was washed with PBS between medium changes. The groups were fixed in formalin (4%) for 24 hours and embedded in paraffin for histological analysis. Histological sections were performed in the long axe of the PRF. Hematoxylin-eosin staining was performed and evaluated under optical microscope.

Immunohistochemical Analysis

To identify the seeded fibroblasts into PRF and differentiate them from blood cells, immunohistochemical staining for Vimentin has been performed (Vimentin Immunohistology Kit Sigma-Aldrich®).

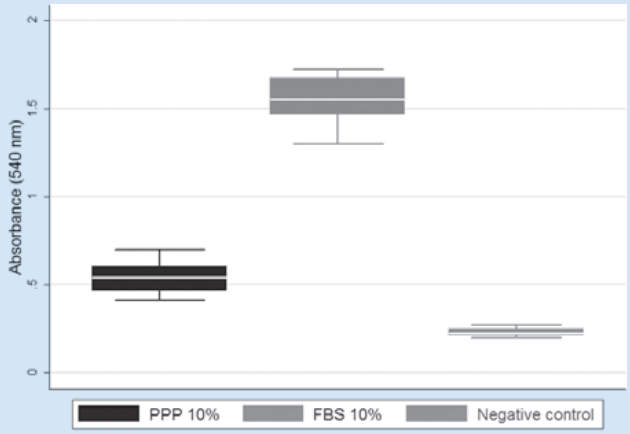
Statistical Analysis

The resultant absorbance values presented normal distribution, which was parametrically analyzed through the one-way analysis of variance (One-way ANOVA) followed by Bonferroni complementary test. A type I error, α error of 5%, occurred. The statistical analysis was performed using the Stata 12® software.

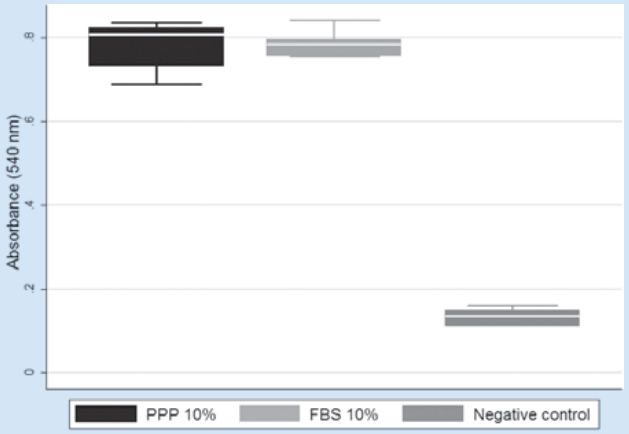
Results

To evaluate the influence of PPP on the cell adhesion, the cells were immediately seeded in the wells with the PPP. Thus, a significant decrease ($p<0.05$) of cells adhesion after 24 hour in relationship to the group supplemented with FBS (Figure 1) was observed. However, it was more elevated in the cells with DMEM (100%) ($p<0.05$) showing that some cells remained viable and adhered. To evaluate the cell maintenance, the cells were maintained for 24 hours in the wells to provide similar cell adhesion. After this, the cells received the PPP. Therefore, a similar ability of cell-maintenance for PPP in adhered cells without statistical difference ($P>0.05$) (Figure 2) was observed.

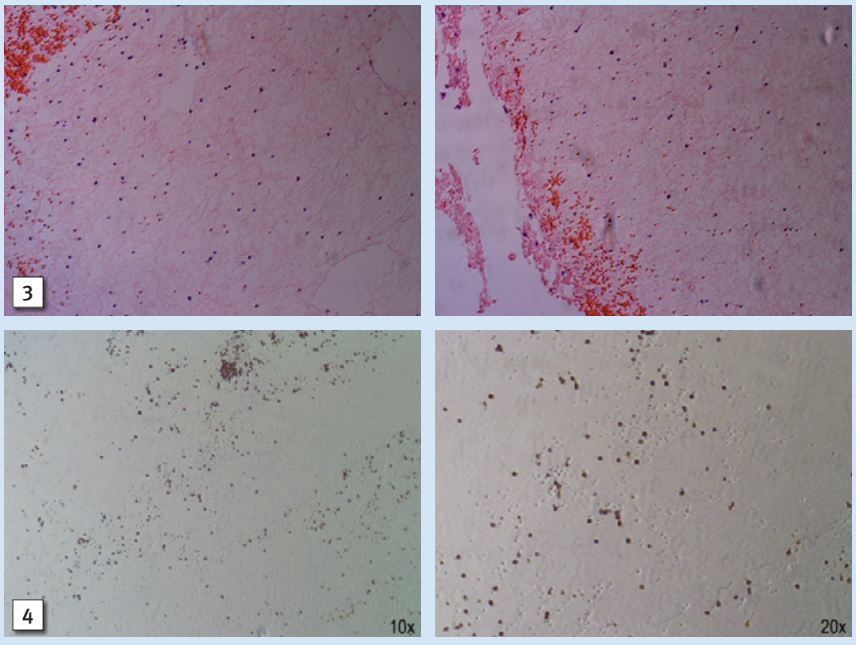
In the second part of the study, the cell culture in PRF supplemented with PPP was evaluated. After 7 days of culture, the fibroblasts cultured in PRF and supplemented with PPP showed an expressive number of cells distributed in the PRF. In hematoxylin-eosin staining (Figure 3) it was possible to observe the cells into the fibers of PRF, principally permeating the region of clotted blood. Figure 4 shows the positive fibroblast staining for Vimentin in a significate number of cells, confirming to be fibroblasts. Besides, the cells which were not stained for vimentin (the blood cells) were also maintained into the PRF. Thus, the PPP was also able to maintain the blood cells that remained in the PRF.



Slika 1. Stanična adhezija PPP-a 10 %
Figure 1 Cell adhesion of PPP 10%



Slika 2. Stanično održavanje PPP-a 10 %
Figure 2 Cell maintenance of PPP 10%



Slika 3. Kultura fibroblasta sedam dana u PPF-u s dodanim PPP-om 10 %
Figure 3 Fibroblasts cultured for 7 days in PPF supplemented with PPP 10%
Slika 4. Imunohistokemijsko bojenje vimentinom PRF-a s dodanim PPP-om 10 %
Figure 4 Vimentin Immunohistochemically in PRF supplemented with PPP 10%

Rasprava

PPP i PRF korišteni u ovoj studiji dobiveni su prema jedinstvenom Choukrounovu protokolu za dobivanje PRF-a. (15). Sastoji se od suspenzije s trima fazama, od čega je PRF srednja frakcija, a crvene krvne stanice donja (16). Supernatant, poznat kao PPP, dio je gornje frakcije koja se odbacuje nakon dobivanja PRF-a, unatoč tvrdnjama u literaturi da PPP sačinjava čimbenike rasta slične kao i u PRF-u (13, 14). Ova je studija prva koja je dobila u jednom protokolu PRF koji se koristi kao prirodni nosač, te PPP kao prehrambenu dopunu kulturi stanica u PRF-u. Dakle, PPP korišten kao nutritivni dodatak za kultiviranje 3T3/NIH u PRF-u, pokazao je svojstvo održavanja stanične vitalnosti. Krvne stanice koje su bile zarobljene u PRF-u tijekom centrifugiranja također su rasle u nosaču tijekom tog razdoblja.

Choukrumov protokol nastao je kako bi se dobio autologni biomaterijal koji može potaknuti cijeljenje tkiva nakon kirurškog zahvata (16). Dugo godina je PRP bio proučavan

Discussion

The PPP and the PRF used in this study were obtained following the single protocol proposed by Choukroun et al. (15). Such a protocol results in a three phase-composed suspension, from which the PRF comprises the middle fraction and the red blood cells the lower portion (16). The supernatant, known as PPP, comprises the upper fraction, which is discarded after the PRF has been obtained, despite the literature reporting that the PPP possesses a growth factor profile similar to PRF (13, 14). In this context, this study was the first one to obtain the PRF used as natural scaffold and the use of PPP as nutritional supplement to cells culture in PRF in a single protocol. Thus, PPP used as nutritional supplement for 3T3/NIH cultivation in the PRF showed the ability to maintain the cellular viability. Besides, blood cells trapped in the PRF during the centrifugation were maintained in the scaffold during this period.

The Choukrum's protocol has been developed to obtain an autologous biomaterial which is able to induce tissue heal-

i korišten za reparaciju koštanih i parodontnih defekata (1, 17), a nedavno i za revaskularizaciju u endodontskoj terapiji (2). No dobivanje PRP-a zahtijeva ksenogene dodatke, kao što je goveđi trombin koji potiče koagulaciju i oslobađanje čimbenika rasta iz trombocita (16). Osim toga, ksenogeni dodatci mogu biti rizični kad je riječ o zdravlju pacijenata zbog mogućeg stvaranja protutijela protiv čimbenika V i XI, koji mogu uzrokovati koagulopatije (11, 16). PRF je bogat čimbenicima rasta, trombocitima i fibrinom, a oni imaju velik potencijal za kliničku primjenu (9, 10). No istraživanje PPP-a, koje je dobiveno od PRF-a, slabo je istraženo, bez obzira na prisutnost važnih biomolekula (13, 14). Rezultati ove studije pokazuju da 10-postotni PPP osigurava sličan rast stanica u 2D okružju, što omogućuje njegovu uporabu kao staničnog dodataka PRF-u, iako je početna adhezija u 2D-u smanjena u PPP grupi.

Smanjenje adhezije u stanicama s VBD-om raspravljeno je u nekoliko studija (18, 19). VBD dodatci smanjuju ekspresiju proteina adhezije u stanicama kojima je dodan humani serum i humani lizat trombocita. (18, 19). *Microarray* test pokazao je smanjenje od 90 gena koji su u korelaciji sa staničnom adhezijom (18). To može objasniti smanjenje stanične adhezije s dodatkom PPP-a. Iako se nakon adhezije stanica pokazalo slično svojstvo FBS-a da održava staničnu vijabilnost kao i 10-postotnim PPP-om. Dakle, 10-postotni PPP alternativni je dodatak koji bi mogao zamijeniti fetalni goveđi serum u regenerativnim terapijama. VBD poboljšava potencijal MC-a za translacijske terapije na staničnoj osnovi, jer omogućuje dobivanje svih komponenti (stanica i čimbenika rasta) iz pacijentova tijela, smanjujući imunosne probleme (16). Osim toga, PRF može biti prirodan nosač koji prima prehrambeni PPP dodatak umjesto ksenogenih dodataka poput FBS-a. Dakle, pacijentova krv djelovala bi kao izvor prirodnog nosača i prehrambenih čimbenika rasta prijeko potrebnih za održavanje i ekspanziju MC-a. Trenutačni rezultati pokazuju mogućnost krvi da služi kao materijal koji može biti i nosač i dodatak dobiven jednostavnim centrifugiranjem. Bojenje vimentinom korišteno za identifikaciju fibroblasta nasađenih u PRF-u, potvrdilo je svojstvo prianjanja i rasta stanica u prirodnom nosaču s dodatkom 10-postotnog PPP-a kao zamjene 10-postotnom FBS-u. Značajan broj fibroblasta obojen je vimentinom, a krvne stanice zarobljene u PRF-u tijekom centrifugiranja nisu obojene, pokazujući mogućnost 10-postotnog PPP-a da održava te ljudske stanice vitalnima. PPP osigurava značajnu količinu trombocitnih čimbenika rasta (PDGF - AB) transformirajući čimbenik rasta-β1 (TGF-β1) te drugih bioaktivnih molekula (9, 10, 13), što može objasniti rezultate prema kojima 10-postotni PPP omogućuje sličnu proliferaciju stanica u usporedbi s kontrolnom skupinom.

Nekoliko se studija koristilo koncentriranom krvi kao dodatkom kulturi stanica s dodatnim drugim hranjivim sastojcima kako bi održali vitalnost stanica. Isaac i suradnici (9) obavili su filtraciju nakon dodavanja 10-postotne ljudske plazme u DMEM, a PRF frakcija primijenjena je kao dodatak. DMEM – nisko glukozni medij s dodatkom 10-postotne autologne krvne plazme (20 ng/ml): β fibroblastni čimbenik rasta (βFGF) i endotelni čimbenik rasta (EGF) s L-glutamiing after surgical procedures (16). For many years, the PRP has been studied and applied for repair of bone and periodontal defects (1, 17) and recently it has been applied in revascularization endodontic treatments (2). However, xenogeneic agents, such as bovine thrombin, are required to obtain the PRP in order to induce coagulation and, consequently, growth factors release from the platelets (16). Besides, xenogeneic agents could pose risks to patients' health due to their ability to generate antibodies against factor V and XI, which could cause coagulopathies (11, 16). The PRF is rich in growth factors, platelets and fibrin, thus having high potential for clinical application (9, 10). However, the study of PPP, which is obtained of PRF, has been poorly investigated even though it has a high range of important biomolecules (13, 14). The results of this study demonstrate that PPP 10% provided similar cell-maintenance in 2D environment, which enables their use as cell-supplement in PRF, although the initial adhesion in 2D has been decreased in the PPP group.

The decrease of adhesion in cells supplemented with VBD was discussed in few studies (18, 19). The VBD-supplements seem to decrease the expression of protein adhesion by cells supplemented with human serum and human platelet lysate (18, 19). The microarray assay showed a decrease of 90 genes correlated with the cell adhesion (18). In this way, a decrease of cell adhesion in PPP supplement can be explained. However, after cell adhesion, a similar ability to FBS in the ability to maintain viable cells for PPP 10% was shown. Thus, the PPP at 10% seems to be an alternative viable to replace the fetal bovine serum in regenerative therapies. VBD have shown to improve the MC potential for translational cell-based therapies, since they allow the obtaining of all components (cells and growth factors) from the patient's body, thus reducing immunological problems (16). Besides, PRF could act as a natural scaffold receiving a nutritional PPP supplementation instead of xenogeneic agents such as FBS. Thus, the patient's blood would act as source for a natural scaffold and nutritional growth factors supplementation, indispensable for MC maintenance and expansion. The present results show the possibility of blood to provide a material able to serve as scaffold and supplement by the simple step-centrifugation. The vimentin staining was used to identify the fibroblast seeded in PRF, confirming the capability to adhere and be maintained into the natural scaffold using the PPP 10% as supplement to substitute FBS 10%. A significant amount of fibroblast cells was stained by vimentin, while the blood cells trapped in PRF during the centrifugation were not stained by vimentin, showing the possibility of PPP 10% in maintaining the viability of those human cells. The PPP provides a significant amount of platelet-derived growth factor (PDGF-AB), transforming growth factor-β1 (TGF-β1) and other bioactive molecules (13) (9, 10) and this can explain the results that showed that 10% PPP provided similar cell proliferation when compared to control group.

Few studies have used concentrated blood in order to supplement cell culture and, in addition, other nutrients were added to blood concentrates to maintain the cell viability. Isaac et al. (9) carried out a filtration after the addition

nom također je primijenjen u istraživanju Lina i suradnika (10), te su dobiveni dobri rezultati s krvnim koncentratima. No taj pristup onemogućuje pojedinačno promatranje stvarne uloge koncentrirane krvi u proliferaciji MC-a. Postoje i studije koje procjenjuju metode koje se temelje na remećenju trombocitne membrane ciklusima smrzavanja i odmrzavanja kako bi se poboljšala potencijalna dopuna. No takva tehnika zahtijeva uporabu heparina koji će spriječiti zgrušavanje koncentrata u mediju za kulturu (5). U ovom kontekstu, nedavna studija istražila je PPP i druge VBD-e u kombinaciji s 10 ng/ml rekombinantnog ljudskog epidermalnog čimbenika rasta (rhEGF) u adipocitnoj matičnoj stanici (20). Na ovaj su način slični rezultati dobiveni za svojstvo proliferacije i diferencijacije MC-a nadopunjenim derivatima krvi (PRP, PPP i humani serum) u usporedbi s FBS-om. Dodavanje egzogenih čimbenika rasta PPP-u omogućilo je zadovoljavajuće rezultate u adipocitnim matičnim stanicama. No čini se da PPP sam osigurava dovoljnu prehrambenu dopunu u usporedbi s FBS-om.

Iako metoda ispitana u ovoj studiji zahtijeva invazivni postupak vađenja pacijentove krvi, ovaj se postupak smatra manje invazivnim od tehnika koje se koriste presadcima donora. Osim toga, čini se da je ovoj metodi klinička prednost pružanje autolognog i prirodnog nosača s njihovim dodatkom za kultiviranje stanice samo u jednom postupku, bez uporabe ksenogenih dodataka. To bi moglo poboljšati potencijal kliničkih translacijskih terapija koje se temelje na korištenju kultiviranih stanica PRF-a, potičući regenerativni potencijal za buduću uporabu u nekoliko područja medicine i dentalne medicine.

Zaključak

Dodavanje 3T3/NIH stanica plazmi siromašnoj trombocitima smanjilo je početnu adheziju stanica, ali je uspjelo održavati staničnu proliferaciju slično kao fetalni goveđi serum. Osim toga, moglo se održati stanice vitalnima u PRF-u s PPP-om koji je korišten kao dodatak. Dakle, može se dobiti prirodni nosač i stanični dodatak iz krvi u jednom koraku centrifugiranja, za uporabu u regenerativnim terapijama.

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Sukob interesa

Nije bilo sukoba interesa.

of 10% human plasma in DMEM; a PRF fraction has been applied as an additional supplement. DMEM-Low Glucose medium supplemented with 10% autologous blood plasma (20 ng/ml): β fibroblast growth factor (βFGF) and endothelial growth factor (EGF) plus L-glutamine has, also, been applied in the study of Lin et al. (10), which has obtained good results with blood concentrates. However, it is impossible to individually observe the actual role of blood concentrated in MC proliferation. Besides, there are studies evaluating methods based on platelet membrane disruption by freeze-thaw cycles to improve the potential supplementation. However, such a technique requires the use of heparin to prevent coagulation of the concentrate in the culture medium (5). In this context, a recent study evaluated PPP, and other VBD, combined with 10 ng/ml recombinant human epidermal growth factor (rhEGF) in adipogenic stem cell (20). In this way, similar results have been observed regarding the proliferation and differentiation ability of MC supplemented with blood derivatives (PRP, PPP and human serum) compared to FBS. The addition of exogenous growth factors to PPP has provided satisfactory results in adipocyte stem cells. However, the PPP alone seems to provide a sufficient nutritional supplementation compared to FBS.

Although the method investigated in this study has the disadvantage because it requires blood collected from the patient, it is considered to be less invasive than techniques using grafts from donor sites. Besides, the use of this method seems to be clinically advantageous since it provides an autologous and natural scaffold with their respective supplement for cell culture in only one procedure, without using xenogeneic compounds. This could improve the potential of clinical translational therapies based on the use of PRF cultured cells, promoting the regenerative potential for future use in several areas of medicine and dentistry.

Conclusions

The supplementation of 3T3/NIH cells with Platelet-Poor Plasma decreased the initial cell adhesion but was able to maintain the cell proliferation similar to the Fetal Bovine Serum. Besides, the cell viability in PRF with PPP used as supplement was ensured. Thus, it was possible to obtain a natural scaffold and the cell supplements from the blood through a single centrifugation step for use in regenerative therapies.

Author Contributions

L.A. Chisini and M.C.M. Conde contributed to conception, design, experimentation, literature review, analysis, interpretation and article writing. F.F. Demarco, M.C.M. Conde and L.A. Chisini drafted and critically revised the manuscript; S.A. Karam, T.G. Noronha, L.R.M.Sartori and A.S. San Martin contributed to experimentation, literature review and paper writing. All authors gave their final approval and agreed to be accountable for all aspects of research.

Conflict of Interest

None declared

Abstract
The aim of this study was to evaluate the proliferation and adhesion of mesenchymal cells (3T3/NIH) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Platelet-Poor Plasma (PPP) in a Platelet-Rich Fibrin (PRF) scaffold. Human blood was obtained and processed in a centrifuge considering the equation G=1.12xRx (RPM/1000)² to obtain PRF and PPP. Cell adhesion and maintenance analyses were performed by MTT assays in a 96 well plate with supplemented DMEM: PPP (90:10) for 24 hours. Besides, the PRF was deposited in a 48 well plate and $10x10^4$ cells were seeded above each PRF (n=3) with 800µl of DMEM: PPP (90:10) and cultured for 7 days. Histological analysis and the immunohistochemical staining for Vimentin were performed. Results were analyzed by one-way ANOVA in Stata12®. A significant decrease ($p<0.05$) of cells adhesion in relationship to FBS was observed. However, a similar ability of cell-maintenance for PPP 10% was observed ($P>0.05$). Fibroblasts culture for 7 days in PRF supplemented with PPP 10% was possible, showing positive staining for Vimentin. Therefore, PPP cell supplementation decreased the initial adhesion of cells but was able to maintain the proliferation of adhered cells and able to support their viability in PRF. It seems that this method has many clinical advantages since it provides an autologous and natural scaffold with their respective supplement for cell culture by only one process, without using xenogeneic compounds. This could improve the potential of clinical translational therapies based on the use of PRF cultured cells, promoting the regenerative potential for future use in medicine and dentistry.

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