Plazma siromašna trombocitima kao dodatak fibroblastima kultivirana u fibrinu obogaćenom trombocitima

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

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 Dulbecco modificiran Eglov medij (DME), 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 govedi serum (FBS) najčešće je korišten u te svrhe, unatoč rizicima povezanim s prijenosom zoonoze i patogene (7). Osim toga, životinjski (kenogeni) proteini u FBS-u mogu biti inkorporirani u ljudske stanice, aktivirajući antišteriodove odgovor nakon implantacije. I proteini FBS-a koji ostaju inkorporirani u stanično citoplazmičko 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 ranjivim dodatcima kako bi se izbjegli rizici povezani sa ksenogenim serumima. VBD, kao i plazma obogaćena trombocitima, potakno su istraženi te je dokazano da mogu održavati MC kulture jednako dobro kao i FBS (9, 10). Uporaba koncentrata trombocita u regeneracioni tkiva može omogućiti djelotvornije cijeljenje i ubrzanje procesa 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 – transformiračući čimbenik rasta beta I (TGF-β1), inzulinsku sličan čimbenik rasta (IGF-I), trombociti čimbenik rasta (PDGF-β) – koji su izdvojeni i 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 osteoblastna 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 plazi siromašnoj trombocitima u PRF nosaču.

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 (DME), 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 (12).

Venous blood derivatives (VBD) have been developed as an alternative to traditional supplements to overcome the risks related to xenogeneic sera. 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 1 (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 growth 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 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.

**Materijali i metode**

Istraživanje

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

Vensku krv donirali su sami istraživači nakon odobre-nja Etičkoga odbora broj 62282216.8.0000.5318. Uzorci su obradivani u komori s laminarnim priborom neposredno na-kon skupljanja krvi, u sterilnim uvjetima kako bi se spriječi-la kontaminacija. Protokol koji su razvili Choukroun i surad-nici (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 dvo-militarske kriogene bočice i odmah stavljen u ultra nisku temperaturu (-80 °C).

Stanična kultura

Fibroblasti 3T3/NIH uzgojeni su u DMEM-u (Cultilab®) uz dodatak 10-posto FBS-a (Cultilab®). Plastićna po-suda od 75 cm³ sa stanicama prenesena je u inkubator (37 °C, 5 % CO₂). Nakon što je postignuta subkoncentnus fibro-blasta (80 %) isprani su fosfatno puferiranom fiziološkom otopinom (PBS) (Gibco®) kako bi se uklonili metaboliti sta-nica. Zatim je 5 ml 0,25-postotnog tripsina/EDTA (Gibco®) nanesenno na stanice tijekom pet minuta na 37 °C. Za inakti-vaciju tripsina korišteno je 5 ml standardnoga medija za kul-turu. 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 10⁴ stanica nasadeno (200 ul) DMEM-a s FBS-om ili PPP-om) po jažici s 96 jažica. Skupe-nine (n = 8) su se sastojale od sljedećih dodataka: DMEM : PPP (90 : 10) i DMEM : FBS (90 : 10) kao pozitivna contro-la; DMEM (100 %) je bio negativna kontrola.

Test stanične adhezije

Odmah nakon dodavanja različitih dodataka na plošti cu s 96 jažica, stanice su inkubirane 24 sata. Nakon toga su DMEM i ostali dodaci uklonjeni s plošte, te jažice isprane PBS-om. DMEM, s odgovarajućim dodacima, pohranjen u svaku jažicu (200 ul), sada s dodatkom MTT-a (4,5-di-metylizol-2-il) -2,5 -difeniltetrazolij) - (0,5mg/ml) (Sigm-a Aldrich®) i održavan u kontaktu sa stanicama četiri sata (37 °C i 5 % CO₂). Nakon inkubacije mediji je aspiriran i kristali formazana suspendirani su u 200 ul 10 % dimetilsulfoksid (DMSO) tijekom 15 minuta. Zatim je ploča stavljena na tre-slicu pet minuta (150 rpm). Rezultati su procijenjeni spek-trofotometrijom (univerzalni ELISA čitač – valna duljina od 540 nm) na kojoj je vrijednost asporpcije očitana kao poka-zatelj stanične proliferacije.

Test rasta stanica

Kako bi se procijenio rast stanica, 2 x 10⁴ staniča stav-ljeno je na pločicu s 96 jažica kojima je na početku dodan 10-postotni FBS. Tako je potaknuta početna adhezija stani-ca s istim dodatkom (FBS). Nakon 24 sata stanične adhezi-

PRF and PPP Obtaining

Venous blood was donated by own researchers after ap-proval by the institution’s Research Ethics Committee Num-ber 62282216.8.0000.5318. The samples were handled im-mediately 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 ap-plied for PRF isolation. Such a protocol relies just on centrif-ugation, considering the calculation of the force of gravity (G Force) produced on blood samples - G-Force = 1,12 x. Radi-xus 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 (-80°C ).

Cell Culture

Fibroblast 3T3/NIH was cultured in DMEM (Cultilab®) supplemented with FBS (Cultilab®) 10%. A 75cm² culture flask containing cells was transferred to an incubator (37°C, 5% CO₂). 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 sus-pended in 3 ml of Standard media where of 20 µL were re moved for cell-counting in a hemocytometer. After counting, 2x10⁶ 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-dimethyl thiazol-2-yl) -2, 5 -diphenyl tetrazolium) - (0,5mg/ml) (Sig ma Aldrich®) and maintained in contact with the cells for 4h (37 °C and 5% CO₂). Post incubation, the medium was aspirated and formazan crystals were suspended in 200µL of 10% dimethyl sulphoxide (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, 2x10⁴ 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 DMEM : FBS (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 žačica. Zatim je 10 x 10^4 stanica nasadeno iznad svakog PRF nosača (n = 3) s 800 ul DMEM : PPP-a (90 : 10 %). Stanice su kultivirane u kontroliranim uvjetima (37 °C i 5 % CO₂) sedam dana. Medij je mijenjan svakog dva dana, pri čemu je PRF ispran PBS-om između promjena medija. Skupine su fiksirane u 4-postotnom formalinu tijekom 24 sata i uklonjene u parafin za histološku analizu. Histološki izbrusci dobiveni su po uzdužnom osovinu PRF-a. Obavljeno je immunohistokemijsko bojenje i procijenjeno pod svjetlosnim mikroskopom.

Immunohistokemijski analiza

Da bi se identificirali nasadeni fibroblasti u PRF-u i razlikovali od krvnih stanica, obavljeno je immunohistokemijsko 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 Bonferronijevim testom. Priznaje se pogreška α = 0.05. Statistička analiza provedena je u softveru Stata 12®.

Rezultati

Da bi se procijenio utjecaj PPP-a na staničnu adheziju, stanice su odmah nasadene u žačice 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 žačicama, te je nakon toga dodan PPP. Uočen je sličan rast adheriranih stanica u PPP-u bez statističke 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 značajnu broj stanica distribuiranih u PRF-u. Hematoksin-eozinski bojenjem (slika 3) uočene su stanice u vlaknima PRF-a, pretežno prozirajući 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 krivih stanica koje su zaostale u PRF-u.

sion, DMEM+ FBS were removed from the plate and washed with 20ul 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, 10x10⁴ cells were seeded above each of the PRF scaffolds (n=3) with 800 ul of DMEM: PPP (90:10%). The cells were cultured in a controlled environment (37°C and 5% CO₂) 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.
Rasprava

PPP i PRF korišteni u ovoj studiji dobiveni su prema jedinstvenom Choukrounovu protokolu za dobivanje PRF-a (15). Sastoji se od suspendije s trima fazama, od čega je PRF srednja frakcija, a crvene krvene stanice donja (16). Supernatant, poznat kao PPP, dio je gornje frakcije koja se odbacuje nakon dobivanja PRF-a, unatoč tvrdnjama u literaturi da PPP sadržava čimbenike slične kao i u PRF-u (13, 14). Ova 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, pokažao je svojstvo održavanja stanične vitalnosti. Krvne stanice koje su bile zarobljene u PRF-u tijekom centrifugiranja također su raste u nosaču tijekom tog razdoblja.

Choukrounov 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 Choukroun’s protocol has been developed to obtain an autologous biomaterial which is able to induce tissue heal-
Plazma siromašna trombocitima u fibrinu

Chisini i sur.

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Chisini i sur.

rasta (\(\beta\) fibroblastni čimbenik)

plazme u DMEM, a PRF frakcija primijenjena je kao dodat-ki

obavili su filtraciju nakon dodavanja 10-postotne ljudske

stojcima kako bi održali vitalnost stanica. Isaac i suradnici

dodatkom kulturi stanica s dodatnim hranjivim sa-

ni PPP omogućuje sličnu proliferaciju stanica u usporedbi s

10, 13), što može objasniti rezultate prema kojima 10-postot-

benika rasta-1) te drugih bioaktivnih molekula (9,

β

bljene u PRF-u tijekom centrifugiranja nisu obojene, poka-

broj fibroblasta obojen je vimentinom, a krvne stanice zaro-

nja i rasta stanica u prirodnom nosaču s dodatkom 10-po-

ii dodatci mogu biti rizični kad je riječ o zdravlju pacijena-

ta zbog mogućeg stvaranja protutijela protiv čimbenika V i

XI, koji mogu uzrokovati koagulopatije (11, 16). PRF je bo-

gat čimbenicima rasta, trombocitima i fibrinom, a oni ima-

ju velik potencijal za kliničku primjenu (9, 10). No istraživa-

nje 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 PPP-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 dodaci smanjuju ek-

Presprijenu adheziju u stanicama kojima je dodan hu-

mani 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 sta-

nične adhezije s dodatkom PPP-a. Iako se nakon adhezije sta-

nica pokazalo slično svojstvo FBS-a da održava staničnu vi-

jabilnost kao i 10-postotnim PPP-om. Dakle, 10-postotni

PPP alternativni je dodatak koji bi mogao zamijeniti fetalni

govedi serum u regenerativnim terapijama. VBD poboljšava

potencijal MC-a za translacijske terapije na staničnoj osno-

vi, jer omogućuje dobivanje svih komponenti (staniča i čim-

benika rasta) iz pacijentova tijela, smanjujući imunosne pro-

bleme (16). Osim toga, PRF može biti prirodan nosač koji

primu prehrambeni PPP dodatak umjesto ksenogenih doda-

taka poput FBS-a. Dakle, pacijentova krv djelovala bi kao

izvor prirodnog nošača i prehrambenih čimbenica rasta pri-

jeko potrebnih za održavanje i ekspanziju MC-a. Trenutačni

rezultati pokazuju mogućnost krvi da služi kao materijal ko-

ji može biti i nosač i dodatak dobiven jednostavnim centri-

fugiranjem. Bojenje vimentinom korišteno za identifikaciju

fibroblasta nasadenih u PRF-u, potvrdilo je svojstvo prihan-

anja i rasta stanica u prirodnom nosaču s dodatkom 10-po-

stotnog PPP-a kao zamjene 10-postotnom FBS-u. Značajan

broj fibroblasta obojen je vimentinom, a krvne stanice zaro-

bljene u PRF-u tijekom centrifugiranja nisu obojene, poka-

zujući mogućnost 10-postotnog PPP-a da održava te ljudske

stanične vitalnima. PPP osigurava značajnu količinu trombo-

citrinih čimbenika rasta (PDGF - AB) transformirajući čim-

benik rasta-B1 (TGF-β1) te drugih bioaktivnih molekula (9,

10, 13), što može objasniti rezultate prema kojima 10-postot-

ni PPP omogućuje sličnu proliferaciju stanica u usporedbi s

kontrolnom skupinom.

Nekoliko se studija koristilo koncentriranom krvi kao

dodatkom kulturi stanica s dodatnim drugim hranjivim sa-

stojcima 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 doda-

tak. DMEM – nisko glukožni medij s dodatkom 10-postotne

autologne krvne plazme (20 ng/ml): β fibroblastni čimbenik

rasta (βFGF) i endotelni čimbenik rasta (EGF) s L-glutami-

ing after surgical procedures (16). For many years, the PRP

has been studied and applied for repair of bone and peri-

donatal defects (1, 17) and recently it has been applied in re-

vascularization endodontic treatments (2). However, xenoge-

nic 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, xe-

nogeneic 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 po-

tential for clinical application (9, 10). However, the study of

PPP, which is obtained of PRF, has been poorly investi-

gated even though it has a high range of important biomol-

eules (13, 14). The results of this study demonstrate that

PPP 10% provided similar cell-maintenance in 2D environ-

ment, which enables their use as cell-supplement in PRF, al-

though 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. How-

ever, after cell adhesion, a similar ability to FBS in the abil-

ity 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 compo-

nents (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 supple-

mentation 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-centrifu-

gation. The vimentin staining was used to identify the fibro-

blast 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 main-

taining the viability of those human cells. The PPP provides a

significant amount of cell-maintenance PPP, which is obtained

of PRF; although the initial adhesion in 2D has been decreased in the

PPP 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

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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 xenogenic compounds. This could improve the potential of clinical translational therapeutics 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 sketched and critically reviewed the manuscript; S.A. Karam, T.G. Noronha, L.R.M. Sartori and A.S. San Martin pridonijeli su eksperimentiranju, pregledom literature i pisanjem članka. Svi autori dali su konačnu potvrdu za sadržaj i usuglasili se za odgovornost za sve aspekte ovog rada.

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=1.12xR by R (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 10x10⁴ 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 xenogenic components. 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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