BJOS
Brazilian Journal of Oral Sciences

Original Article

Volume 23
2024
e240327

Human venous blood derivatives as fetal bovine serum substitute for fibroblast culture cells in a fibrin construct

Luiz Alexandre Chisini[1*], Marcus Cristian Muniz Conde[2], Sarah Arangurem Karam[3], Rodrigo Varella de Carvalho[1], Sandra Beatriz Chaves Tarquinio[4], Flávio Fernando Demarco[3]

[1] Graduate Program in Dentistry, Department of Restorative Dentistry, School of Dentistry, Federal University of Pelotas, Pelotas, Brazil.

[2] Graduate Program in Dentistry, Department of Restorative Dentistry, School of Dentistry, Federal University of Pelotas, Pelotas, Brazil.

[3] Professional Master's in Health in the Life Cycle, Catholic University of Pelotas, Pelotas, RS, Brazil.

[4] Diagnostic Center for Oral Diseases, School of Dentistry, Universidade Federal de Pelotas, Brazil.

Corresponding author:
Professor Luiz Alexandre Chisini
Professor – Graduate Program in Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil. 457, Gonçalves Chaves st., Room 502, Pelotas - RS - Brazil ZIP: 96015-360
Phone: +5551 997618703
E-mail: alexandrechisini@gmail.com

Editor: Altair A. Del Bel Cury

Received: July 06, 2022
Accepted: Apr 12, 2023

Aim: Venous blood derivatives (VBDs) have been suggested as substitutes for Fetal Bovine Serum (FBS) to improve the clinical transition of cell-based therapies. The literature is not clear about which is the best VBDs substitute. The present study aimed to evaluate the influence of VBDs on cell viability and describe a new method to seed these cells in a 3D Platelet-Rich Fibrin (PRF). **Methods:** Blood was processed to obtain Platelet-Poor Plasma from PRF (P-PRF), Human Serum (HS), Platelet-Poor Plasma from PRP (P-PRP), activated-PRP (a-PRP), and Platelet lysate (PL). Cells were supplemented with each VBD at 10% and FBS at 10% was the control. Cell viability (fibroblast 3T3/NIH) test was evaluated with MTT assay in two ways: i) cell-seeded and expanded with VBD; ii) cell-seed with FBS and expanded with VBD. To seed the Fibrin construct, cells were suspended in PBS and dropped into the blood sample before performing Choukroun's protocol for PRF. Constructs were cultured for 7 days in VBD supplements and FBS. Histological and Immunohistochemical analysis with vimentin was performed. Cell viability was analyzed by one-way ANOVA. **Results:** VBD's production time was very heterogeneous. Cells expanded in HS and a-PRP has grown faster. VBD-supplemented culture media provided cell culture highly sensible to trypsin/EDTA 0.25%. Cells seeded and expanded with VBD presented viability comparable to FBS in HS, a-PRP, and P-PRP ($p>0.05$) and lower in P-PRF and PL groups ($p<0.05$). The viability of cell seed with FBS and expanded with VBD was similar between P-PRF, a-PRP, PL, and FBS ($p>0.05$) and lower in HS and P-PRP ($p<0.005$). PRF-seeded cells showed a positive expression of vimentin and were able to maintain all cells supplemented with VBD. **Conclusion:** VBD supplements were able to maintain fibroblast cells in 2D and 3D cultures. The new method of the fibrin-cell construct was efficient to insert the cells into the fibrin network.

Keywords: Blood. Fibrin. Blood platelets. Platelet-rich fibrin.

Introduction

Behind an optimal scaffold for cell seeding, regenerative therapies depend on specific reagents providing nutrients and growth factors needed for cell expansion *in vitro*[1-7]. Fetal bovine serum (FBS), a xenogeneic product, has been universally applied as supplementation for *in vitro* cell expansion[8,9]. The use of xenogeneic agents for *in vitro* cell expansion is considered a critical obstacle to cell-based therapy (C-BT) clinical transition[8,10]. Due to the potential for pathogen transmission and cell internalization of animal proteins, FBS replacement by human venous blood-derived (VBD) has been proposed as an alternative to overcome such barriers[8,10-15].

Human blood appears to be a plentiful source for the creation of a fully autologous and biologically active scaffold made from the fibrin network[16] providing additional supplementation for cell *ex vivo* expansion[17]. VBDs have been proposed as alternatives to FBS to enhance the clinical translation of cell-based therapies. However, the literature is unclear as to which VBDs substitute to provide the best properties for cell expansion[10]. Despite the limited number of studies that make direct comparisons between available VBDs, several VBDs have been suggested as substitutes for FBS; however, most of them present xenogeneic compounds, such as bovine thrombin to promote platelet activation[8-10]. Platelet Poor Plasma (PPP), or platelet-rich plasma (PRP) byproduct, has been recently tested as a supplement for cell *in vitro* expansion[18]. Moreover, human serum (HS) and activated PRP (a-PRP) have been proposed as FBS-substitute due to the growth factors released during platelet activation[8,19]. Most of the growth factors released during coagulation are derived from platelets' α-granules[7,20]. To disrupt the α-granules, Platelet-Rich Fibrin (PRF) is carried out using a protocol based on membrane lysis brought about by gravitational force[8]. The mechanical spin breaks the platelet membranes releasing the bioactive molecules retained on α-granules. Although all of these VBDs have been developed, the literature is not clear whether any of these serums have a greater potential for maintaining cells cultured in different environments[10].

PRF is a second-generation platelet concentrate composed of a natural fibrin network obtained after one-step centrifugation of whole blood venous, without the need for thrombin or $CaCl_2$ addition for platelet activation[21]. PRF provides favorable properties for cell adhesion and proliferation due to the growth factors (GF) trapped in the fibrin network during centrifugation[22]. PRF has been safely administered in humans showing promising results for bone, periodontal, and pulp regeneration[21-29]. Therefore, strategies that provide a construct with cells already present inside the PRF could favor faster tissue repair[30]. Researchers reported the difficulty of seed cells inside PRF as the main limitation of using PRF as natural scaffolds[22,31,32]. Two strategies have been reported in the literature: the first one relies on seeding cells over intact PRF surface; the second one is based on chopping PRF, after isolation, to improve homogenous cell seeding[33-35].

Despite many possibilities presented in the literature[8,9], to the best of our knowledge, no study has compared various supplements derived from human blood, especially in a three-dimensional environment provided by scaffolds. Thus, the presented study aimed to evaluate the influence of five venous blood derivatives as Fetal Bovine Serum substitutes on cell viability and describe a new method to seed these cells in a 3D Platelet-Rich Fibrin.

Material and Methods

The present study was approved by the research ethics committee of the Faculty of Dentistry of the Federal University of Pelotas, under protocol 1,989,692. The flow chart of the study is displayed in Figure 1.

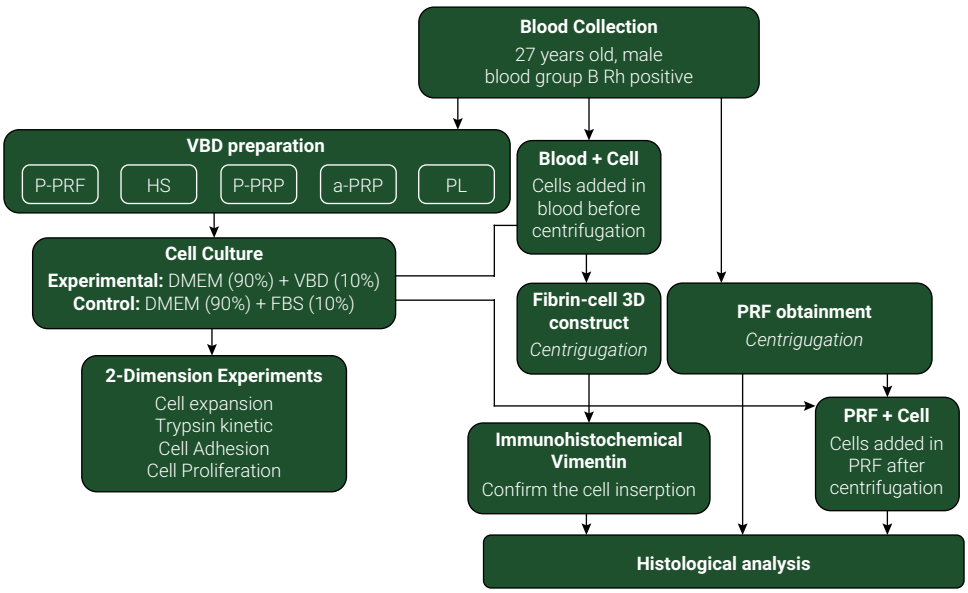


Figure 1. Diagram Flow of study.

Blood samples: Whole human blood (400ml) was obtained from a single healthy donor, male, 27 years old, blood group B, Rh-positive, performed through blood donation for Pelotas Regional Blood Center (HEMOPEL), which performed a battery of biological tests following the standards of National Health Surveillance Agency (HIV, hepatitis B, and C, syphilis, Malaria, hemoglobin screening/iron management, Chagas disease). The material was transported following the same rules and processed immediately at the School of Dentistry of the Federal University of Pelotas (UFPel). Blood samples have been handled in a laminar flow cabinet to obtain the following VBD:

- **Platelet-Poor Plasma from PRF (P-PRF):** PRF has been produced according to Choukroun et al.[21]. Briefly, whole blood samples were taken and centrifuged for 10 minutes under 400G. Blood centrifugation resulted in a three-layered suspension where the topmost comprises P-PRF.

- **Human Serum (HS):** Whole isolated blood was stored, without coagulating inducers, at 4 °C overnight to allow blood clotting. Thus, the blood clot was centrifuged (3000rpm – 5 min). The supernatant was the HS.

- **Platelet-Poor Plasma from PRP (P-PRP):** 10ml of blood was collected in vacuum tubes with sodium citrate 3.8% and centrifuged (2400rpm – 10 min)[22]. The platelet concentrate and acellular plasma were transferred to a new vial anticoagulant-

-free to perform additional centrifugation (3600 rpm – 15 min) to obtain PRP[22]. 2/3 of the whole supernatant volume has been collected and stored.

- **Activate-PRP (a-PRP):** one-third of the plasma, resulting from PRP protocol, received 100 µl of $CaCl_2$ (20% weight/volume) for every 1 ml of PRP and stored (4°C overnight) for platelet activation[19,36]. Thus, further centrifugation (1500rpm – 5 min) was performed and the resulting supernatant was stored.
- **Platelet lysate (PL):** New samples of a-PRP were performed. However, before the final centrifugation, three cycles of freezing (-80°C) and thawing (37°C) were performed. Thus, the final centrifugation was performed (1500g for 5 minutes) to obtain PL[36].

Every one of the isolated VBDs was stored at -80°C until use.

Cell Expansion: Immortalized mouse fibroblast cell line (3T3/NIH) were seeded ($5x10^6$) and expanded in $25mm^3$ culture flasks containing 5ml of Dulbecco's Modified Eagle Medium (DMEM – Cultilab®) supplemented with 1% antibiotics (10,000 IU/mL of penicillin G and 10,000 mg/mL of streptomycin) and 10% of one of the previously isolated VBD, as follows:

- G1 = DMEM/HS (90/10);
- G2 = DMEM/P-PRF (90/10);
- G3 = DMEM/a-PRP (90/10);
- G4 = DMEM/PL (90/10);
- G5 = DMEM/P-PRP (90/10);
- G6 (control) = DMEM/FBS (90/10).

Cells could grow for 30 days in a controlled environment (37°C and 5% CO_2). During this period, every 24 hours a digital image was registered in each experimental group. The culture medium was changed every 48h. As soon as any cell culture reached 80% subconfluence, cells were disaggregated and ¼ of whole-cell content was maintained in culture. Trypsin/ethylenediaminetetraacetic acid (Trypsin/EDTA) at 0.25% (Invitrogen®) has been applied for cell detachment in all performed experiments.

Trypsin kinetic: To investigate adhesive characteristics, fibroblasts under the five experimental culture supplements were cultured until a confluence of 70%–80% was reached. Thus, a concentration screening, 0.25% (1x) to 0.00333% (75x), has been performed in the third passage. The medium was removed, cells rinsed in PBS (GIBCO) and trypsin-EDTA was added. For 5 minutes, every minute, a digital image was registered (Nikon Eclipse TI-S®), in the same region of the culture flask, for each experimental group.

Cell Viability of cell-seeded and expanded with VBD: This experiment aimed to investigate whether the use of VBDs could change the initial adhesion of cells and their subsequent proliferation. So, previously FBS-expanded cells have been disaggregated and suspended in 10 ml of DMEM/FBS 10%. The cell suspension was distributed in seven tubes and centrifuged for 5 minutes under 1000 rpm. The supernatant

was removed, and cells were suspended (3ml) in DMEM containing one of six VBD previously isolated (G1 to G6). Thus, $2x10^4$ cells (200 µl) were seeded in a 96-well plate (n=8). Post incubation the culture media was removed and a PBS solution (200µL) containing 0.5mg/ml MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – Sigma Aldrich®) added to each well and kept for 4h (37°C and 5% CO_2). MTT solution has been aspirated and the formazan crystals were diluted in 200µL of dimethylsulfoxide (DMSO), and cell viability was evaluated in a spectrophotometer reader (540 nm). Every test has been performed in triplicate and independent experiments. DMEM/FBS was a positive control and Serum-free DMEM for negative control.

Cell viability of cell seed with FBS and expanded with VBD: This experiment aimed to evaluate only the proliferation of cells already initially adhered in a 96-well plate with FBS. Thus, cells cultured in DMEM/FBS were seeded (1×10^4 cells) and cultured in a 96-well plate for 24h. Post-incubation, basal media has been removed and DMEM+VBD (G1 – G6) has been added to the cell culture. Then, the cells were again incubated for 24 hours. Post incubation, the culture media was removed and MTT evaluation has been carried out as described in the previous section. Every test has been performed in triplicate and independent experiments. DMEM/FBS was a positive control and Serum-free DMEM for negative control.

Cell seeding in PRF: the cells previously expanded in different VBDs were used to seed PRF. Immediately after blood collection, a PBS suspension (100µl) containing $10x10^4$ cells/ml was dropped into the blood sample before performing Choukroun's PRF protocol[21]. The containing-cell PRFs were cultured in 48 well plates containing 800 µl of medium corresponding to each experimental group. As positive controls, $10x10^4$ cells were seeded directly over the PRF surface. Cell-free PRF was the negative control. The cell seeded PRFs (n=3) have been cultured in 48 well plates containing 800 µl of DMEM: VBD (90:10) in each experimental group for seven days to observe cell growth. Cell-free PRF has been considered a negative control. The cultural mediums were changed every three days. Post incubation, cell-seeded PRF were fixated (4% paraformaldehyde - 24h), and histological sections (5µm) were made in the PRF-long ax. Obtained histological sections (hematoxylin-eosin) were observed under an optical microscope to observe cell distribution inside PRF. To confirm if cells observed inside the PRF did not come from the volunteer's organism, immunohistochemical staining for Vimentin (Vimentin Immunohistology Kit Sigma-aldrich®) was performed. The experiment was realized in triplicate.

Statistical analysis: The data were submitted to the Shapiro-Wilk normality test and analyzed for one-way ANOVA with Bonferroni complimentary test. Statistical significance was assigned when $p<0.05$. We used the software Stata 12® to conduct the analysis.

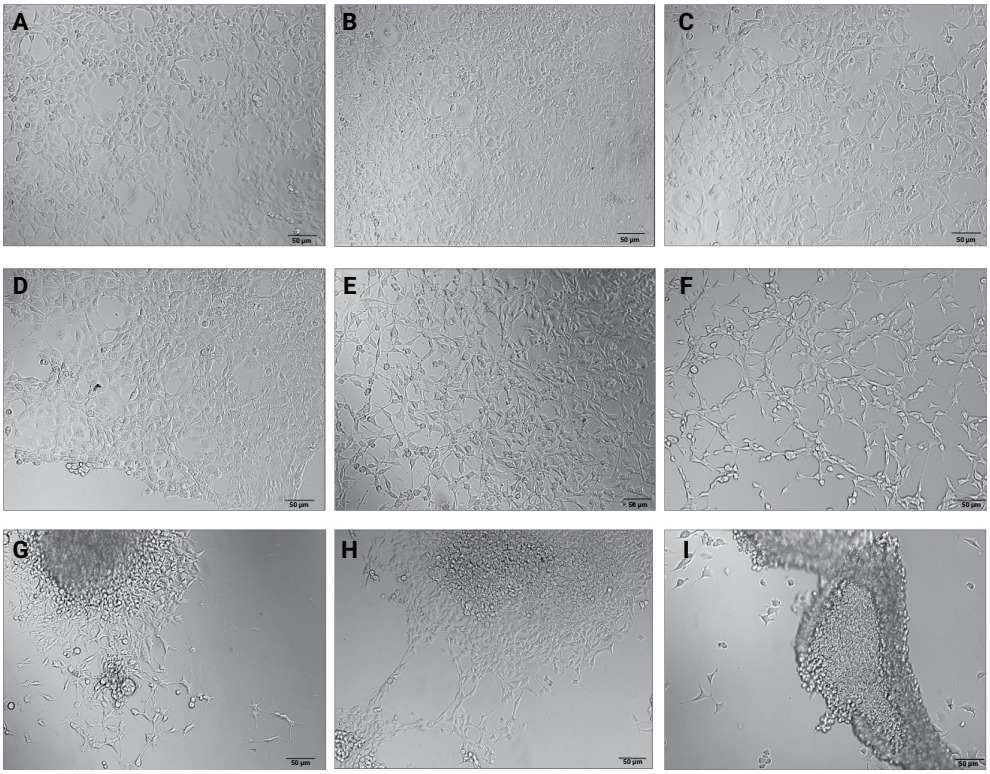
Results

Production of VBD supplements: the five produced VBD yielded volumes ranging from 3ml to 9ml from 20ml blood (Table 1). Besides, the VBD's production time was very heterogeneous. While a few minutes were needed to obtain HS, some hours were required to produce PL. The PL presented the lowest income and the longest production time. P-PRP has the highest yielding and HS required the shortest production time.

Table 1. The income of VBD-supplements and time required for production.

	HS	P-PRF	a-PRP	PL	P-PRP
Income (ml)	8	6	3	3	9
Time spent (minutes)	10	15	50	95	30
Days Required	2	1	2	2	1

Cell expansion: Cells expanded in HS and a-PRP have grown faster, reaching sub-confluence nine times in 30 days. Meanwhile, cells in P-PRF, PL, and P-PRP attained sub-confluence six times. Positive control (FBS) reached sub-confluence fifteen times in the same period. All tested VBD provided a fibroblast-like morphology for cells after 30 days in culture (Figure 2). HS, P-PRF, and P-PRP provided cells and arrangements quite similar to FBS (Figure 2-A, 2-B, 2-C, and 2-D). a-PRP and PL provided cells with less evident cytoplasmic processes, more spindle-shaped and stellate with well-defined narrow cell elongations (Figure 2-E and 2-F). Cell clusters were observed in a-PRP and PL (Figure 2-G and 2-H) as well as the medium coagulation in the a-PRP, PL, and P-PRP. However, this fact not interfered with the cell culture and the capacity of cell maintenance.



Legend: FBS (A), HS (B), P-PRF (C) and P-PRP groups; a-PRP (E), PL (F) with starry growth. Cluster formation area in PL (G) a-PRP (H). Cell-detached plate area (I). a-PRP and PL provided cells with less evident cytoplasmic processes, more spindle-shaped and stellate with well-defined narrow cell elongations.
Figure 2. Fibroblasts morphology and cell arrangement in VBD supplemented DMEM.

Trypsin/EDTA concentration screening: VBD-supplemented culture media provided cell culture highly sensible to trypsin/EDTA 0.25% (Figure 3), presenting immediate cell-disaggregation in a-PRP, PL, and P-PRP. A gradual reduction in trypsin concentration allowed us to observe an increase, at least 2.5-fold, in the time required for cell disaggregation. To observe trypsin kinetics like the FBS-supplemented group, a 50x dilution had to be performed. One minute in trypsin/EDTA 0.05% associated with mechanical breakdown was sufficient to detach cells expanded in VBD. The effect of trypsin/EDTA 0.05% is shown in Figure 4. hPL and a-PRP provided a detachment in clusters requiring more intense movements during the mechanical breakdown.

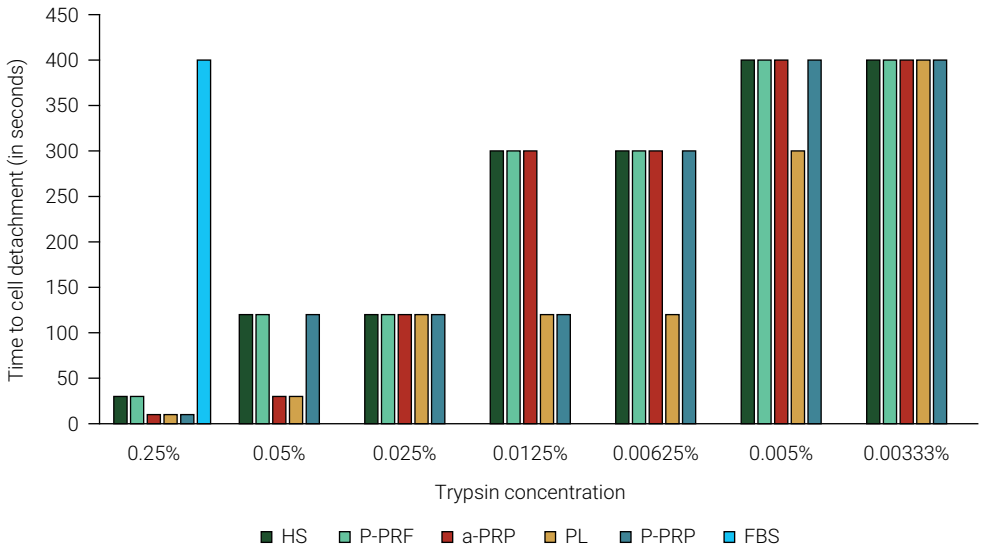


Figure 3. Trypsin/EDTA-Sensibility to 3T3/NHI fibroblasts supplemented with VBD. Trypsin has been diluted in PBS.

Cell Viability of cell-seeded and expanded with VBD serum: cell viability was comparable to positive control in HS, a-PRP, and P-PRP ($p > 0.05$), while those cells in P-PRF and PL presented lower viability rates than the positive control (FBS) ($p<0.05$) (Figure 5).

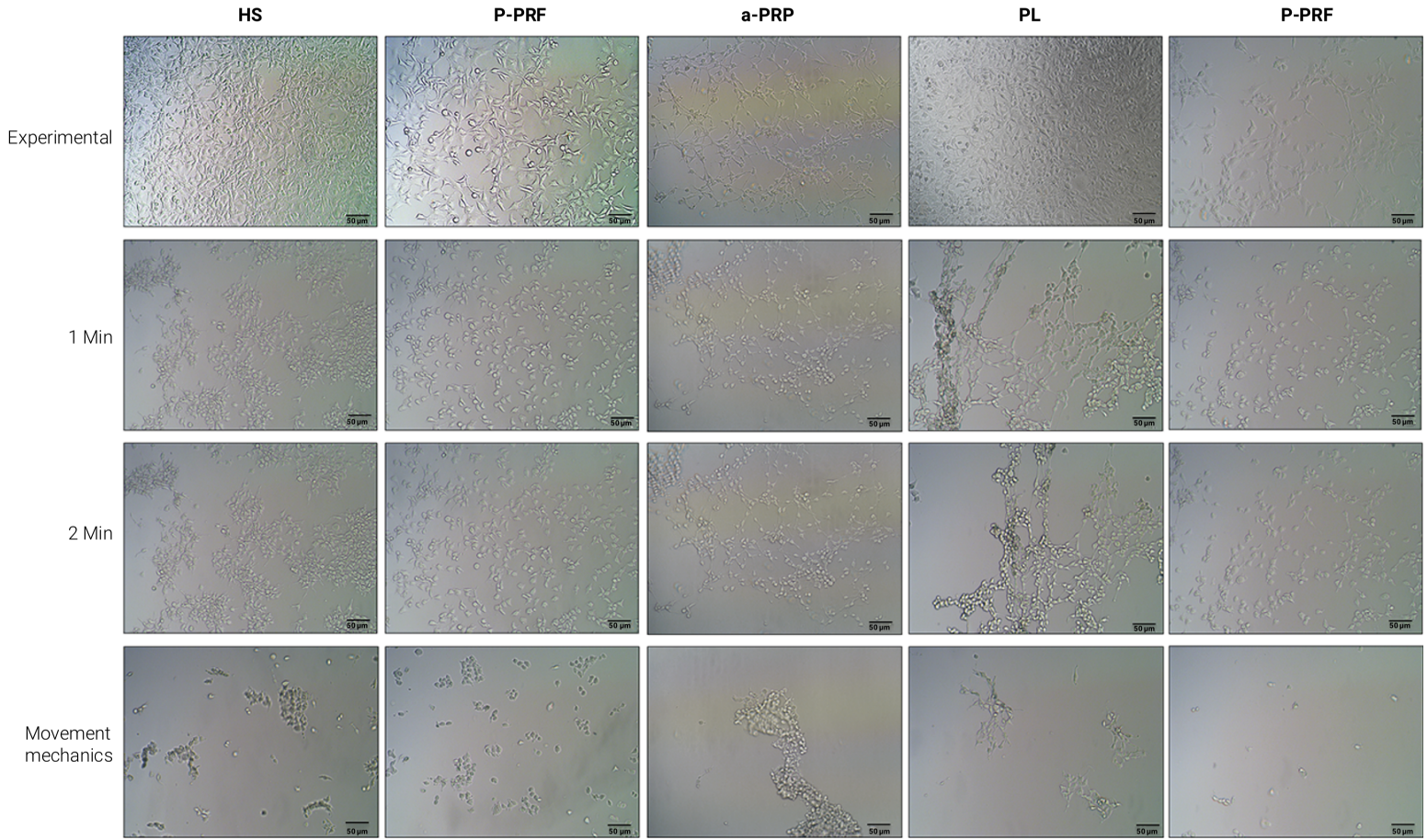


Figure 4. VBD-Cell disaggregation in trypsin/EDTA 0.05%.

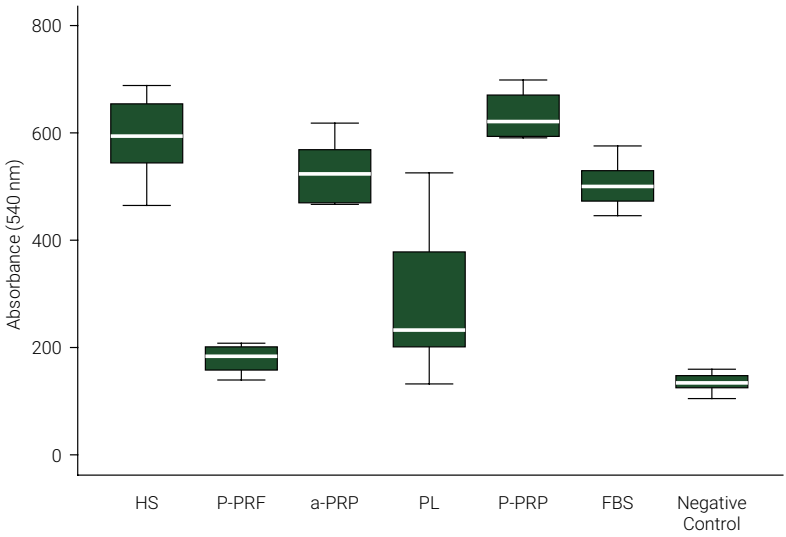


Figure 5. Cell viability of cell-seeded and expanded with VBD supplements (24 hours after the seeding with VBD-supplements.

Cell viability of cell seed with FBS and expanded with VBD serum: such test showed that P-PRF, a-PRP, and PL provided cell viability comparable to FBS ($p > 0.05$) while HS and P-PRP reduced proliferation rates (Figure 6).

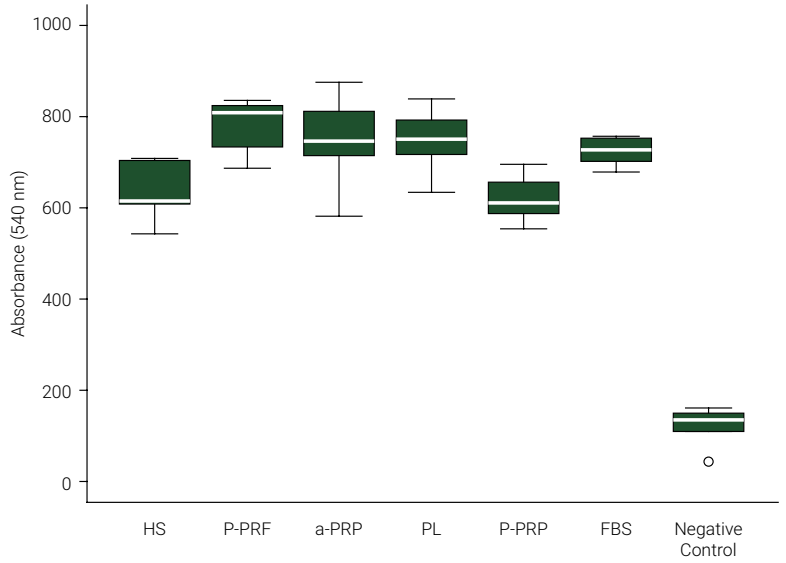


Figure 6. Cell viability of cell-seed with FBS and expanded with VBD supplements.

Cell seeding in PRF: The addition of cells in blood was performed before performing the centrifugation protocol and the analyses were performed after the PRF centrifugation. Histological analysis showed an eosin-pink fibrin network, with a dense number of fibers, inside PRF (Figure 7). The largest number of cells was found in the

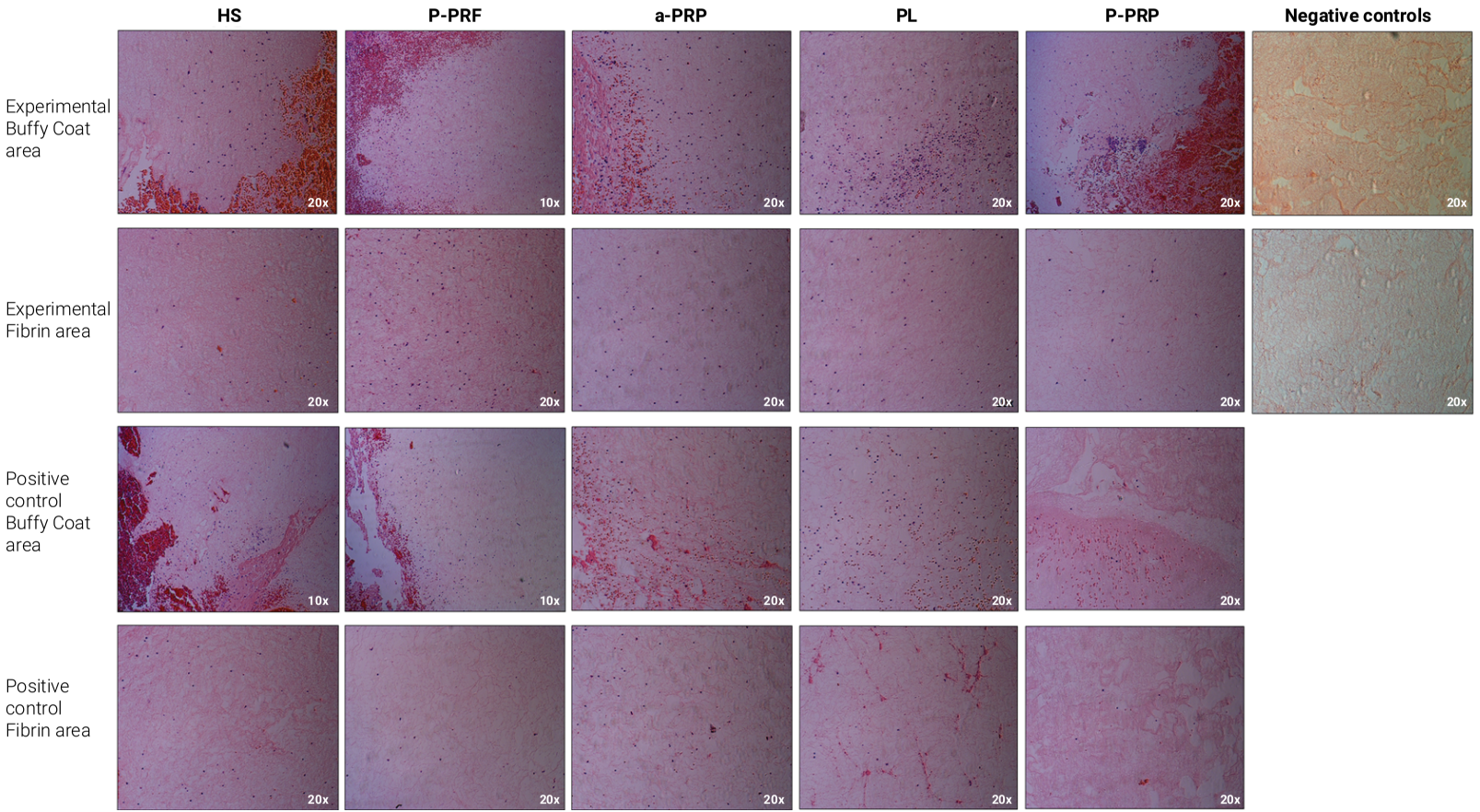


Figure 7. Fibroblasts cultured for 7 days in fibrin-cell 3D construct and respective control groups.

PRF border named “buffy coat”. Some cells were observed at the edges of the fibrin clot and others permeating the fibrillar network but in the proximal portions “buffy coat”. Besides, cells were found in PRF edges and permeating the fibrillar network. P-PRF, PL, and a-PRP provided better cell maintenance in a 3D environment. The seeded cells showed a positive expression of vimentin (Figure 8). Thus, was observed a large amount of fibroblast permeating the white blood cells deposited principally in the “buffy coat” region. In the general form, fibroblasts were in the same region of the blood cells.

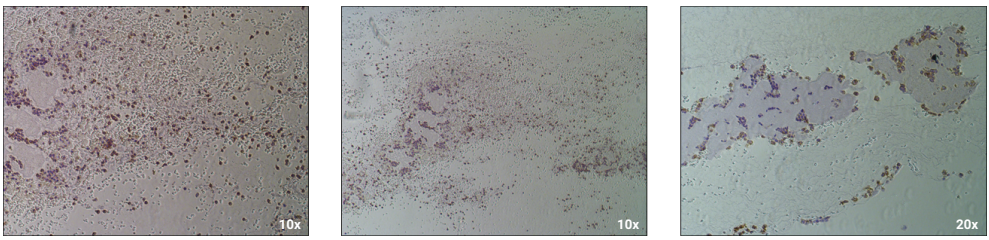


Figure 8. Vimentin Immunohistochemically in fibrin-cell 3D construct.

Discussion

The application of FBS as a cell supplement has been reported as a challenge to reaching the clinical translation of regenerative therapies[5,30]. In this study was possible to observe that all tested VBD-provided cells presented fibroblast-like morphology and presented the potential to be applied as a xeno-free alternative for *in vitro* cell expansion. Platelet activation is the main way to release growth factors, from platelet alpha-granules, which support cell adhesion and proliferation (18). Our findings show that cell-seeded and expanded with HS, a-PRP, and P-PRP presented similar viability to the FBS-serum group even as P-PRF, a-PRP, and PL provide similar viability of the FBS-serum group when seed with FBS and expanded with VBD serum. Moreover, our method of seeding these cells in a 3D Platelet-Rich Fibrin presented favorable results despite the cells being concentrated in the “buffy coat” region.

PRF has been extensively used in dentistry for a wide range of applications due to its excellent biocompatibility and regenerative properties[37]. PRF is derived from the patient’s blood and contains a high concentration of platelets, growth factors, and cytokines that can promote tissue regeneration, angiogenesis, and wound healing. As such, PRF has been investigated as a scaffold material for various dental procedures, including periodontal regeneration, implantology, and oral surgery[21,37,38]. PRF is a second-generation platelet concentrated possessing growth factors released from platelet activation induced by centrifugation[21], which allows for a natural fibrin network (fibrin coagulation) without the addition of exogenous, chemical, or xenogeneic, compounds[21]. PRF has been widely studied for pulp, bone, and periodontal regenerations promoting efficient healing and regeneration[21,22,25]. Due to the above-mentioned characteristics, PRF is reported to be quite similar to connective extracellular matrix turning PRF into a natural scaffold able to stimulate either, cell adhesion, proliferation, migration, and differentiation[1,25,32,39]. However, PRF comprises a dense and gelatinous solid, which makes cell seeding inside PRF a hard task[33,34]. The integrity of the fibrin

network comprises a key feature to apply PRF as a scaffold thus, achieving cell insertion into PRF without destruction of the fibrin network is a current challenge[33-35]. Here, the addition of cell-containing suspension in blood before centrifugation allowed for cell insertion into PRF. Histological results showed fibroblasts inside the PRF network, mainly in the "buffy coat" region. In the fibrin-peripheral areas, was observed few cell colonization, mainly in the control groups. Thus, was possible to obtain a fibrin-cell construct by a simple method. The fibroblast insertion has been confirmed by vimentin immunohistochemical analysis. Besides, the cells were successfully maintained for seven days with different FBS-substitutes.

All tested VBD provided cells with a high sensibility to trypsin/EDTA (0.25%), which can reflect lower cell adhesion. These results can be explained due to the possibility of VBD providing a down-regulation in genes related to cell adhesion. Bieback et al.[40] analyzed, by microarray assay, the genome of adipose stem cells cultured in HS and a-PRP supplemented medium, showing an underregulated expression of 90 genes related to cell adhesion. Corroborating, we observed that HS cell-seed with FBS and expanded with VBD serum reduced the viability. In the same way, Kocaoemer et al.[41] related a lack of adhesion in cells expanded in a-PRP, principally in high cell densities. DMEM contains calcium ions, which are required for cell adhesion (12). The lack of cell adhesion may also be related to the reaction established by the calcium of the culture medium with the molecules involved in the process of blood coagulation and could explain the reduction of PL observed in the present study.

The medium gelation was observed when a-PRP, PL, and P-PRP were added to DMEM. The calcium present in the culture medium induces fibrinogen activation through components of the coagulation cascade present in the plasma (12). Notwithstanding that P-PRF is a supplement with its origin in the plasma, medium gelation has not been observed, since the total activation of coagulation components occurs during centrifugation to obtain PRF (20). On the other hand, the serum does not present such molecules required for medium gelation, corroborating the results observed in our study. The HS comprises plasma depleted down coagulation proteins responsible for coagulation and due to this medium gelation did not happen when HS has been applied as supplementation. In this way, several studies have been adding heparin to prevent medium gelatinization (20, 21). Although it is possible to obtain human heparin, found studies have applied porcine heparin as an anticoagulant (21). Walenda et al. (22) proposed the use of a PL-supplemented gelatinized medium for cell culture, showing higher cell proliferation for cells growing into the gelatin. Besides, the cells in medium gelatinized did not change biological proprieties such as immunophenotype and multipotency (22). The increase in PL concentration provided an elevation of gelatin density, allowing for its use as a natural scaffold (12). In the present study, all tested VBD were applied at 10% and the gelatinization observed here presented very low density. Thus, the cells did not present three-dimensional growth as proposed by Walenda et al (22). To create a proper matrix for three-dimensional growth, PL must be applied in concentrations higher than 30% (12).

To improve the release of biomolecules, platelet membrane lyse has been also performed by freeze-thaw cycles[8,9]. PL is one of the most investigated supplements for

in vitro cell expansion[8] however, PL had not yet been compared to a-PRP. In the present study, PL and a-PRP provided similar cell viability when cell-seed with FBS and expanded with blood supplements, but HL presented low viability when the cells were seeded and expanded with supplements. In general, both supplements present similar obtaining methods applied to isolate PL and a-PRP, which is completely different from that for HS obtainment[9]. The protocol for HS follows the same principles applied to FBS. FBS is acquired from the coagulated heart blood of the bovine fetus. The blood clot is centrifuged and filtered. Despite this, HS provided lower cell proliferation than FBS, while cell adhesion and growth pattern were comparable in both, FBS and HS. A decrease in cell viability of seeded and cultured cells with supplements (possibly due to a decrease of cell adhesion) was observed for P-PRF; however, when the cells were seeded with FBS and after cell adhesion (24hs) the medium was changed to medium supplemented with VBD supplements cell induced by P-PRF was comparable to FBS, a-PRP, and hPL.

P-PRF and PL provided cell viability lower than FBS when seeded and cultivated with VBD serum. In this way, lower cell proliferation has been expected in these groups when 3D culture (PRF) was done. However, the opposite was observed. An elevated number of cells was observed in P-PRF and PL. Differences in cell behavior in 2D and 3D cultures have been related[42] and it had not been investigated in cells supplemented with VBD. Maybe the decrease in adhesion properties not interfered significantly when the cells are seeded in the PRF; it could be investigated in future reports to explain the mechanisms governing such phenomena.

Despite all tested VBD serums providing cells with fibroblast-like morphology and able to 2D and 3D cell expansion, the a-PRP presented excellent properties regarding cell expansion and viability in different environments (2D and 3D). However, their production is more complicated compared to other serums (i.g., HS, P-PRF, and P-PRP) expanding more time to be produced. On the other hand, the P-PRF, which presents a simple protocol obtention and demands less time to be produced exhibited good properties mainly regarding morphology and 3D growth but had a slower expansion. Similarly, the HS presents the simplest protocol to obtain adequate cell expansion, morphology, and viability properties. However, such serum showed a smaller amount of cells when cultured in the PRF scaffold. In this context, a-PRP, P-PRF, and HS were the VBD serums that showed the most promising results.

In the present study, the principle to promote *in vitro* cell expansion with different VBD supplements has described creating a 3D fibrin-cell construct. We use fibroblasts because they are present in most tissues of the buccal cavity and play a critical role in wound healing and tissue repair. When tissue damage occurs, fibroblasts migrate to the site of injury and produce extracellular matrix (ECM) components such as collagen, elastin, and proteoglycans[1]. These ECM components provide structural support to the tissue and facilitate cell migration, proliferation, and differentiation, ultimately leading to tissue regeneration[1]. Understanding the fibroblast behavior in PRF scaffolds supplemented with VBD supplements can provide insights into the mechanisms by which these scaffolds promote tissue regeneration and can guide the development of new therapies for regenerative therapies. Thus, the 3T3/NIH fibroblasts are cells established from cultures of

mouse embryos possessing a well-characterized and controlled behavior in culture, removing the variable behavior of primary culture cells. The results from the present study are in accord with those described in the current literature and can contribute to providing valuable information for cell culture with VBD supplements. Furthermore, cell insertion into the PRF can be a valuable technique that can be easily used in regenerative therapies. However, the clinical transition has required the replication of these results with human cells.

Conclusion

In conclusion, the five VBD supplements described in the present study have been able to maintain mesenchymal cells in 2D and 3D cultures. Considering the initial analysis performed in the present study, a-PRP, P-PRF, and the HS were the most promissory serums. Moreover, the proposed method for cell insertion into PRF is a promisor, despite the peripheral region still having a few cells. The results could be interpreted with caution and could be confirmed with the use of human cell lineages.

Funding

The work was supported by the CNPq - Brazil.

Disclosure Statement

Ethical approval: The Ethics Committee of Dentistry College of Federal University of Pelotas approved this project (number of 1,989,692).

Clinical Relevance: Human Venous Blood Derivatives presented interesting properties to be used as Fetal Bovine Serum-substitutes for culture cells and to support cells in the fibrin construct.

Conflict of Interest: The authors report no conflict of interest.

Data availability

Datasets related to this article will be available upon request to the corresponding author.

Authors contribution

Luiz Alexandre Chisini, contributed to the conception and design, donating blood for the experiments, performing the experimentation, analysis, and interpretation, drafted and critically revising the manuscript; **Marcus Cristian Muniz Conde** contributed to the conception and design, experimentation, interpretation, drafted and critically revised the manuscript; **Sarah Arangurem Karam** contributed to the conception and design, experimentation, interpretation, drafted and critically revised the manuscript; **Rodrigo Varella de Carvalho** contributed to drafted and critically revising the manuscript; **Sandra Beatriz Chaves Tarquinio** contributed to experimentation, drafted and critically revised the manuscript; **Flávio Fernando Demarco** contributed to critically revised the manuscript.

References

1. Conde MC, Chisini LA, Demarco FF, Nör JE, Casagrande L, Tarquinio SB. Stem cell-based pulp tissue engineering: variables enrolled in translation from the bench to the bedside, a systematic review of literature. Int Endod J. 2016 Jun;49(6):543-50. doi: 10.1111/iej.12489.
2. Chisini LA, Conde MC, Alcázar JC, Silva AF, Nör JE, Tarquinio SB, et al. Immunohistochemical Expression of TGF-β1 and Osteonectin in engineered and Ca(OH)2-repaired human pulp tissues. Braz Oral Res. 2016 Oct 10;30(1):e93. doi: 10.1590/1807-3107BOR-2016.vol30.0093.
3. Demarco G, Kirschnick L, Watson L, Conde M, Demarco F, Chisini L. What is the clinical applicability of regenerative therapies in dentistry? Rev Gauch Odontol. 2017;65(4):359-67. doi: 10.1590/1981-86372017000200113112
4. Conde MC, Chisini LA, Grazioli G, Francia A, Carvalho RV, Alcázar JC, et al. Does cryopreservation affect the biological properties of stem cells from dental tissues? a systematic review. Braz Dent J. 2016 Oct-Dec;27(6):633-40. doi: 10.1590/0103-6440201600980.
5. Jonsdottir-Buch SM, Gunnarsdottir K, Sigurjonsson OE. Human embryonic-derived mesenchymal progenitor cells (hES-MP Cells) are fully supported in culture with human platelet lysates. Bioengineering (Basel). 2020 Jul;7(3):75. doi: 10.3390/bioengineering7030075.
6. Aussel C, Busson E, Vantomme H, Peltzer J, Martinaud C. Quality assessment of a serum and xenofree medium for the expansion of human GMP-grade mesenchymal stromal cells. PeerJ. 2022 May;10:e13391. doi: 10.7717/peerj.13391.
7. Burnouf T, Strunk D, Koh MB, Schallmoser K. Human platelet lysate: replacing fetal bovine serum as a gold standard for human cell propagation? Biomaterials. 2016 Jan;76:371-87. doi: 10.1016/j.biomaterials.2015.10.065.
8. Bieback K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. Transfus Med Hemother. 2013 Oct;40(5):326-35. doi: 10.1159/000354061.
9. Hemeda H, Giebel B, Wagner W. Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. Cytotherapy. 2014 Feb;16(2):170-80. doi: 10.1016/j.jcyt.2013.11.004.
10. Chisini LA, Conde MCM, Grazioli G, Martin ASS, Carvalho RV, Nör JE, Demarco FF. Venous blood derivatives as FBS-substitutes for mesenchymal stem cells: a systematic scoping review. Braz Dent J. 2017 Nov-Dec;28(6):657-68. doi: 10.1590/0103-6440201701646.
11. Mannello F, Tonti GA. Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells. 2007 Jul;25(7):1603-9. doi: 10.1634/stemcells.2007-0127.
12. Dolley-Sonneville PJ, Romeo LE, Melkoumian ZK. Synthetic surface for expansion of human mesenchymal stem cells in xeno-free, chemically defined culture conditions. PLoS One. 2013 Aug;8(8):e70263. doi: 10.1371/journal.pone.0070263.
13. Haque N, Kasim NH, Rahman MT. Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells. Int J Biol Sci. 2015 Feb;11(3):324-34. doi: 10.7150/ijbs.10567.
14. Barro L, Nebie O, Chen MS, Wu YW, Koh MB, Knutson F et al. Nanofiltration of growth media supplemented with human platelet lysates for pathogen-safe xeno-free expansion of mesenchymal stromal cells. Cytotherapy. 2020 Aug;22(8):458-72. doi: 10.1016/j.jcyt.2020.04.099.
15. Liau LL, Hassan MNFB, Tang YL, Ng MH, Law JX. Feasibility of human platelet lysate as an alternative to foetal bovine serum for in vitro expansion of chondrocytes. Int J Mol Sci. 2021 Jan 28;22(3):1269. doi: 10.3390/ijms22031269.

16. Pasztorek M, Rossmanith E, Mayr C, Hauser F, Jacak J, Ebner A, et al. Influence of platelet lysate on 2D and 3D amniotic mesenchymal stem cell cultures. Front Bioeng Biotchnol. 2019 Nov 15;7:338. doi: 10.3389/fbioe.2019.00338.

17. Mujawar S, Iyengar K, Nadkarni S, Mulherkar R. Expansion and characterization of cells from surgically removed intervertebral disc fragments in xenogen-free medium. J Biosci. 2020;45:108.

18. Koellensperger E, Bollinger N, Dexheimer V, Gramley F, Germann G, Leimer U. Choosing the right type of serum for different applications of human adipose tissue-derived stem cells: influence on proliferation and differentiation abilities. Cytotherapy. 2014 Jun;16(6):789-99. doi: 10.1016/j.jcyt.2014.01.007.

19. Pham PV, Vu NB, Pham VM, Truong NH, Pham TL, Dang LT, et al. Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. J Transl Med. 2014 Feb;12:56. doi: 10.1186/1479-5876-12-56.

20. Harrison P, Cramer EM. Platelet alpha-granules. Blood Rev. 1993 Mar;7(1):52-62. doi: 10.1016/0268-960x(93)90024-x.

21. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part IV: clinical effects on tissue healing. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006 Mar;101(3):e56-60. doi: 10.1016/j.tripleo.2005.07.011.

22. Kawase T. Platelet-rich plasma and its derivatives as promising bioactive materials for regenerative medicine: basic principles and concepts underlying recent advances. Odontology. 2015 May;103(2):126-35. doi: 10.1007/s10266-015-0209-2.

23. Parihar AS, Narang S, Dwivedi S, Narang A, Soni S. Platelet-rich fibrin for root coverage: a plausible approach in periodontal plastic and esthetic surgery. Ann Afr Med. 2021 Jul-Sep;20(3):241-4. doi: 10.4103/aam.aam_31_20.

24. Starzyńska A, Kaczoruk-Wieremczuk M, Lopez MA, Passarelli PC, Adamska P. The growth factors in advanced platelet-rich fibrin (A-PRF) reduce postoperative complications after mandibular third molar odontectomy. Int J Environ Res Public Health. 2021 Dec;18(24):13343. doi: 10.3390/ijerph182413343.

25. Conde MCM, Chisini LA, Sarkis-Onofre R, Schuch HS, Nör JE, Demarco FF. A scoping review of root canal revascularization: relevant aspects for clinical success and tissue formation. Int Endod J. 2017 Sep;50(9):860-74. doi: 10.1111/iej.12711.

26. Angerame D, De Biasi M, Kastrioti I, Franco V, Castaldo A, Maglione M. Application of platelet-rich fibrin in endodontic surgery: a pilot study. G Ital Endod. 2015;29(2):51-7. doi: 10.1016/j.gien.2015.08.003.

27. Chisini L, Grazioli G, Francia A, San Martin AS, Demarco FF, Conde MC. Revascularization versus apical barrier technique with mineral trioxide aggregate plug: a systematic review. G Ital Endod. 2018;32(1):9-16. doi: 10.1016/j.gien.2018.03.006.

28. Yoshpe M, Kaufman AY, Lin S, Ashkenazi M. Regenerative endodontics: a promising tool to promote periapical healing and root maturation of necrotic immature permanent molars with apical periodontitis using platelet-rich fibrin (PRF). Eur Arch Paediatr Dent. 2021 Jun;22(3):527-34. doi: 10.1007/s40368-020-00572-4.

29. Pavani MP, Reddy KRKM, Reddy BH, Biraggari SK, Babu CHC, Chavan V. Evaluation of platelet-rich fibrin and tricalcium phosphate bone graft in bone fill of intrabony defects using cone-beam computed tomography: a randomized clinical trial. J Indian Soc Periodontol. 2021 Mar-Apr;25(2):138-43. doi: 10.4103/jisp.jisp_621_19.

30. Chisini LA, Conde MCM, Grazioli G, Martin ASS, Carvalho RV, Sartori LRM, Demarco FF. Bone, periodontal and dental pulp regeneration in dentistry: a systematic scoping review. Braz Dent J. 2019 Mar-Apr;30(2):77-95. doi: 10.1590/0103-6440201902053.

31. Kumar N, Prasad K, Ramanujam L, K R, Dexith J, Chauhan A. Evaluation of treatment outcome after impacted mandibular third molar surgery with the use of autologous platelet-rich fibrin: a randomized controlled clinical study. J Oral Maxillofac Surg. 2015 Jun;73(6):1042-9. doi: 10.1016/j.joms.2014.11.013.

32. Chisini LA, Karam SA, Noronha TG, Sartori LRM, San Martin AS, Demarco FF, et al. Platelet-poor plasma as a supplement for fibroblasts cultured in platelet-rich fibrin. Acta Stomatol Croat. 2017 Jun;51(2):133-40. doi: 10.15644/asc51/2/6.

33. Gassling V, Douglas T, Warnke PH, Açil Y, Wiltfang J, Becker ST. Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering. Clin Oral Implants Res. 2010 May;21(5):543-9. doi: 10.1111/j.1600-0501.2009.01900.x.

34. Chen Y, Niu Z, Xue Y, Yuan F, Fu Y, Bai N. Improvement in the repair of defects in maxillofacial soft tissue in irradiated minipigs by a mixture of adipose-derived stem cells and platelet-rich fibrin. Br J Oral Maxillofac Surg. 2014 Oct;52(8):740-5. doi: 10.1016/j.bjoms.2014.06.006.

35. Sun CK, Zhen YY, Leu S, Tsai TH, Chang LT, Sheu JJ, et al. Direct implantation versus platelet-rich fibrin-embedded adipose-derived mesenchymal stem cells in treating rat acute myocardial infarction. Int J Cardiol. 2014 May;173(3):410-23. doi: 10.1016/j.ijcard.2014.03.015.

36. Mojica-Henshaw MP, Jacobson P, Morris J, Kelley L, Pierce J, Boyer M, et al. Serum-converted platelet lysate can substitute for fetal bovine serum in human mesenchymal stromal cell cultures. Cytotherapy. 2013 Dec;15(12):1458-68. doi: 10.1016/j.jcyt.2013.06.014.

37. Choukroun J AF, Schoeffler C, Vervelle A. Une opportunité en paro-implantologie: le PRF. Implantodontie. 2001 Jan:42:55-62.

38. Dohan Ehrenfest DM, Bielecki T, Jimbo R, Barbé G, Del Corso M, Inchingolo F, et al. Do the fibrin architecture and leukocyte content influence the growth factor release of platelet concentrates? An evidence-based answer comparing a pure platelet-rich plasma (P-PRP) gel and a leukocyte- and platelet-rich fibrin (L-PRF). Curr Pharm Biotechnol. 2012 Jun;13(7):1145-52. doi: 10.2174/138920112800624382.

39. Karam S, San Martin A, Mazzetti T, Conde M, Chisini L, Demarco F. [Cryogenic treatment to increase the amount of macropores in plasma rich in fibrin used as scaffold in tissue engineering]. ROBRAC. 2017;26(77):14-9. Portuguese.

40. Bieback K, Ha VA, Hecker A, Grassl M, Kinzebach S, Solz H, et al. Altered gene expression in human adipose stem cells cultured with fetal bovine serum compared to human supplements. Tissue Eng Part A. 2010 Nov;16(11):3467-84. doi: 10.1089/ten.TEA.2009.0727.

41. Kocaoemer A, Kern S, Klüter H, Bieback K. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells. 2007 May;25(5):1270-8. doi: 10.1634/stemcells.2006-0627.

42. Jung JP, Bache-Wiig MK, Provenzano PP, Ogle BM. Heterogeneous Differentiation of Human Mesenchymal Stem Cells in 3D Extracellular Matrix Composites. Biores Open Access. 2016 Jan;5(1):37-48. doi: 10.1089/biores.2015.0044.