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Basic responses of mesenchymal stem cells exposed to bovine biomaterial and platelet rich fibrin

Respostas básicas de células-tronco mesenquimais expostas ao biomaterial bovino e em fibrina rica em plaquetas

Respuestas básicas de las células madres mesenquimales expuestas a biomaterial bovino y fibrina rica en plaquetas

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Janaína Lima Heymovski
ORCID: https://orcid.org/0000-0002-0586-0814
Universidade Positivo, Brazil
E-mail: janaheymovski@gmail.com
Moira Pedroso Leão
ORCID: https://orcid.org/0000-0003-3724-911X
Curityba Biotech, Brazil
E-mail: moirapedroso@gmail.com
Jeferson Luis de Oliveira Stroparo
ORCID: https://orcid.org/0000-0003-1094-530X
Universidade Positivo, Brazil
E-mail: jef_stroparo@hotmail.com
Sabrina Cunha da Fonseca
ORCID: https://orcid.org/0000-0001-5280-1238
Universidade Federal do Paraná, Brazil
E-mail: sabrina.cfonseca@hotmail.com
Lisley Janowski Spisila
ORCID: https://orcid.org/0000-0002-3978-3650
Universidade Federal do Paraná. Brazil
E-mail: lisleyjspisila@gmail.com
Carla Castiglia Gonzaga
ORCID: https://orcid.org/0000-0001-6374-1605
Universidade Positivo, Brazil
E-mail: carlacgonzaga2@gmail.com
Victoria Cruz Cavalari
ORCID: https://orcid.org/0000-0002-0232-7328
Curityba Biotech, Brazil
E-mail: vitoria_cavalari@hotmail.com
Rafaela Araújo Mendes
ORCID: https://orcid.org/0000-0002-3774-6354
Universidade Positivo, Brazil
E-mail: rafaela.mendes@live.com
Denis Roberto Falcão Spina
ORCID: https://orcid.org/0000-0002-3040-9515
Instituto Federal de Educação Ciência e Tecnologia do Paraná, Brazil
E-mail: drfspina@gmail.com
Eduardo Discher Vieira
ORCID: https://orcid.org/0000-0002-4311-1486
Curityba Biotech, Brazil
E-mail: eduardo.discher@outlook.com
Roberto da Rocha Leão Neto
ORCID: https://orcid.org/0000-0002-1379-7819
Universidade Positivo, Brazil
E-mail: leaorep@gmail.om
Leonel Alves de Oliveira
ORCID: https://orcid.org/0000-0003-2235-4443
University of Brasilia, Brazil
E-mail: profleonel2@gmail.com
Célia Regina Cavichiolo Franco
ORCID: https://orcid.org/0000-0003-2525-7788
Universidade Federal do Paraná, Brazil
E-mail: crcfranc@terra.com.br

Tatiana Miranda Deliberador
ORCID: https://orcid.org/0000-0003-4076-4905
Instituto Latino Americano de Pesquisa e Ensino Odontológico, Brazil
E-mail: tdeliberador@gmail.com
João César Zielak
ORCID: https://orcid.org/0000-0003-3393-3491
Universidade Positivo, Brazil
E-mail: jzielak2@gmail.com

Abstract
Scaffolds and their interaction with mesenchymal stem cells are objects of study in bioengineering and tissue repair. Mechanisms such as proliferation, viability, and cytotoxicity are essential for the development of therapies. The present study analyzed the influence of platelet-rich fibrin (PRF) in viability, cytotoxicity, and proliferation of stem cells from human exfoliated deciduous teeth (SHED) exposed to bovine biomaterial surfaces. The studied groups were divided and analyzed as follows: (S) only SHED as control Group; (SB) SHED + biomaterial; (SBP) SHED + biomaterial + PRF. Analyses of cells seeded in 24-well plates were performed after 24, 48 and 72 hours. Individual groups were subjected to viability, cytotoxicity and cell proliferation tests using neutral red, MTT and crystal violet, respectively; and in the 72-hour group, scanning electron microscopy (SEM) was performed to record cell ultra-morphology. Data were submitted to statistical analysis by two-factor ANOVA with a significance level of 5%. The results demonstrated a better performance in the viability/cytotoxicity and proliferation of stem cells for the group (SBP) in comparison to the group (SB) and the group (S). The applied statistical tests showed that the biomaterial factor, time, and interaction between them led to results with statistical significance. SHED submitted to bovine biomaterial were more viable, proliferative and with lower citotoxicity when associated with PRF. PRF seemed to activate the metabolism of stem cells in culture, indicating that such an association can bring an effective benefit in clinical outcome.
Keywords: Mesenchymal stem cells; Biomaterial; Platelet-rich-fibrin; Bone regeneration; Cell proliferation; Regenerative medicine.

Resumo
Arcabouços e a suas interações com as células-tronco mesenquimais são objetos de estudo em bioengenharia e reparo de tecidos. Mecanismos como proliferação, viabilidade e citotoxicidade são fundamentais para o desenvolvimento de terapias. O presente estudo analisou a influência da fibrina rica em plaquetas (PRF) na viabilidade, citotoxicidade e proliferação de células-tronco mesenquimais de dentes decíduos humanos esfoliados (SHED) em contato com biomaterial de origem bovina. Os grupos estudados foram divididos e analisados da seguinte forma: (S) apenas SHED como Grupo controle; (SB) SHED + biomaterial; (SBP) SHED + biomaterial + PRF. As análises das células semeadas em placas de 24 poços foram realizadas após 24, 48 e 72 horas. Grupos individuais foram submetidos a testes de viabilidade, citotoxicidade e proliferação celular usando vermelho neutro, MTT e cristal violeta, respectivamente, e no grupo de 72 horas foi realizada a microscopia eletrônica de varredura (MEV) para registrar a ultra morfologia celular. Os dados foram submetidos à análise estatística por ANOVA de dois fatores com nível de significância de 5%. Os resultados demonstraram um melhor desempenho na viabilidade / citotoxicidade e proliferação de células-tronco para o grupo (SBP) em relação ao grupo (SB) e ao grupo (S). Os testes estatísticos aplicados mostraram que o fator biomaterial, o tempo e a interação entre eles deram origem a resultados com significância estatística. As SHED submetidas ao biomaterial bovino se apresentaram mais viáveis, proliferativas e com menor citotoxicidade quando associadas à PRF. A PRF pareceu ativar o metabolismo das células-tronco em cultivo, indicando que tal associação pode trazer um benefício efetivo nos desfechos clínicos.
Palavras-chave: Células-tronco mesenquimais; Biomaterial; Fibrina rica em plaquetas; Regeneração óssea; Proliferação celular; Medicina regenerativa.

Resumen
Marcos y tus interacciónes con las células madres mesenquimales son objetos de estudio en bioingeniería y reparación de tejidos. Mecanismos como la proliferación, la viabilidad y la citotoxicidad son fundamentales para el desarrollo de terapias. El presente estudio analizó la influencia de la fibrina rica en plaquetas (PRF) sobre la viabilidad, citotoxicidad y proliferación de células madre mesenquimales de dientes primarios humanos exfoliados (SHED) en contacto con biomateriales bovinos. Los grupos estudiados se dividieron y analizaron de la siguiente manera: (S) solo SHED como grupo de control; (SB) SHED + biomaterial; (SBP) SHED + biomaterial + PRF. Se realizaron análisis de células sembradas en placas de 24 pocillos después de 24, 48 y 72 horas. Los grupos individuales se sometieron a pruebas de viabilidad, citotoxicidad y proliferación celular utilizando rojo neutro, MTT y violeta cristal, respectivamente, y en el grupo de 72 horas, se realizó microscopía electrónica de barrido (SEM) para registrar la morfología celular. Los datos se sometieron a análisis estadístico mediante ANOVA de dos factores con un nivel de significancia del 5%. Los resultados demostraron un mejor desempeño en la viabilidad / citotoxicidad y proliferación de células madre para el grupo (SBP) en comparación con el grupo (SB) y el grupo (S). Las pruebas estadísticas

aplicadas mostraron que el factor biomaterial, el tiempo y la interacción entre ellos dieron lugar a resultados con significación estadística, las SHED sometidas a biomaterial bovino fueron más viables, proliferativos y con menor toxicidad cuando se asociaron con PRF. PRF parecio activar el metabolismo de las células madres en cultivo, lo que indica que tal asociación puede aportar un beneficio efectivo en los resultados clínicos.
Palabras clave: Células madres mesenquimal; Biomaterial; Fibrina rica en plaquetas; Regeneración ósea; Proliferación celular; Medicina regenerativa.

1. Introduction

Tissue engineering is the use of a combination of multidisciplinary approaches with the aim to improve or replace biological tissues (Huawei *et al.* 2019) and it is highly applied in reconstructive surgery procedures such as bone augmentation. The essential elements needed are osteoconductive scaffolds, osseoinductive growth factors and progenitor cells.

Autologous mesenchymal stem cells from the adjacent tissues to the bone, such as periosteum and bone marrow, represent the first source of undifferentiated cells for bone reconstruction. However, mesenchymal stem cells can be obtained from other sources, such as adipose, stromal fraction of bone, dental pulp tissues (Strauer *et al.,* 2008) and stem cells from human exfoliated deciduous teeth (SHED) (Miura *et al*., 2003), for use in regenerative medicine for bone tissue repair. Nevertheless, SHED represent a viable source for mesenchymal stem cells obtained, once the young human being has twenty opportunities for collection, because of the deciduous teeth natural process of exfoliation. Another important element to be considered is cell interaction with osseocondutor mineralized biomaterials when acting the basic components of scaffolds and play a fundamental role in bone tissue engineering (Huawei *et al.,* 2019). Xenogenic sources, such as bovine, are extensively (Precheur, 2007) due to their ultrastructural characteristics, which can drive the development of new bone tissue. In addition to that, osteoconductive materials are biocompatible of a bone neoformed structure (Moraschini *et al.,* 2015).

Finally, the mesenchymal stem cells need signalizing specific growth factors and cytokines signaling to promote transduction, gene transcription and protein synthesis, modulating chemotaxis, migration, proliferation, and cell differentiation.

The autologous platelet-rich fibrin (PRF) is an additional scaffold and can act as a continuum medium between cells and the biomaterial surface. The PRF possesses supraphysiologicalgrowth factors concentrations, glycoproteins with adhesives properties, and contains live and functional platelets and leukocytes and a variety of cell compounds like immunoglobulins and ligand molecules (Miron et al., 2017; Oliveira et al., 2020). Based on these properties, the PRF can promote induction proliferation and differentiation of osteoprogenitor cells and improve bone regeneration in reconstruction surgeries (He et al., 2009; Dohan et al., 2009).

Thus, the present study aims to qualitatively evaluate the influence of PRF in the levels of cytotoxicity, viability, and proliferation status of mesenchymal stem cells associated with a bovine bone grafting biomaterial.

2. Methodology

2.1 Individuals and ethical aspects

The present study observed all ethical standards for scientific research with humans in conformity with the Declaration of Helsinki (World Medical Association Recommendation 2013). The mesenchymal stem cells were obtained from a twelve-year-old donor after the signed an informed consent form. A healthy tooth, without caries, with complete rhizolysis, was used at the time of natural tooth exfoliation. The blood donation (single donor, thirty-year-old) for PRF preparation followed the same ethical steps. The Research Ethics Committee of Universidade Positivo approved the study under number

3.6665.172/2019. The methodology herein presented followed de Oliveira *et al.* (2020), Lisboa *et al.* (2021) and Stroparo *et al.* (2021).

2.2 Stem Cell Culture

After oral prophylaxis, the exfoliated tooth was inserted in a container with sterile medium (DMEM [Dulbecco's Modified Eagle's Medium] + 10% Fetal Bovine Serum + 1% streptomycin/penicillin + 1% amphotericin). Explant cells process and primary culture was obtained.

All cells were kept in a CO_2 incubator at 37ºC with the same medium. The cells were cultivated until they reached 80% confluence at the 4^{th} passage, observed in the inverted microscope. Trypsin was used and 4×10^4 cells were seeded in three wells (24-well plate) for each group, at 24, 48 and 72 hours. Mesenchymal stem cells were characterized by Dominici protocols (2006). This methodology was describeb by Lisboa *et al.* (2021) and Stroparo *et al.* (2021).

2.3 Obtaining the PRF matrices by centrifugation

A healthy and non-smoker adult voluntarily donated a total of 80 ml of blood, collected in 9,0 ml sterile polystyrene vacuum tubes (Greiner Bio-One, Brazil) for PRF production. Blood samples immediately transferred to fixed-angle centrifuge FibrinFuge25 (Montserrat, Brazil), centrifuged with 200 × g for 10 minutes, in conformity with de Oliveira *et al.* (2020). The methodology can be seen in Figure 1.

2.4 Experimental groups

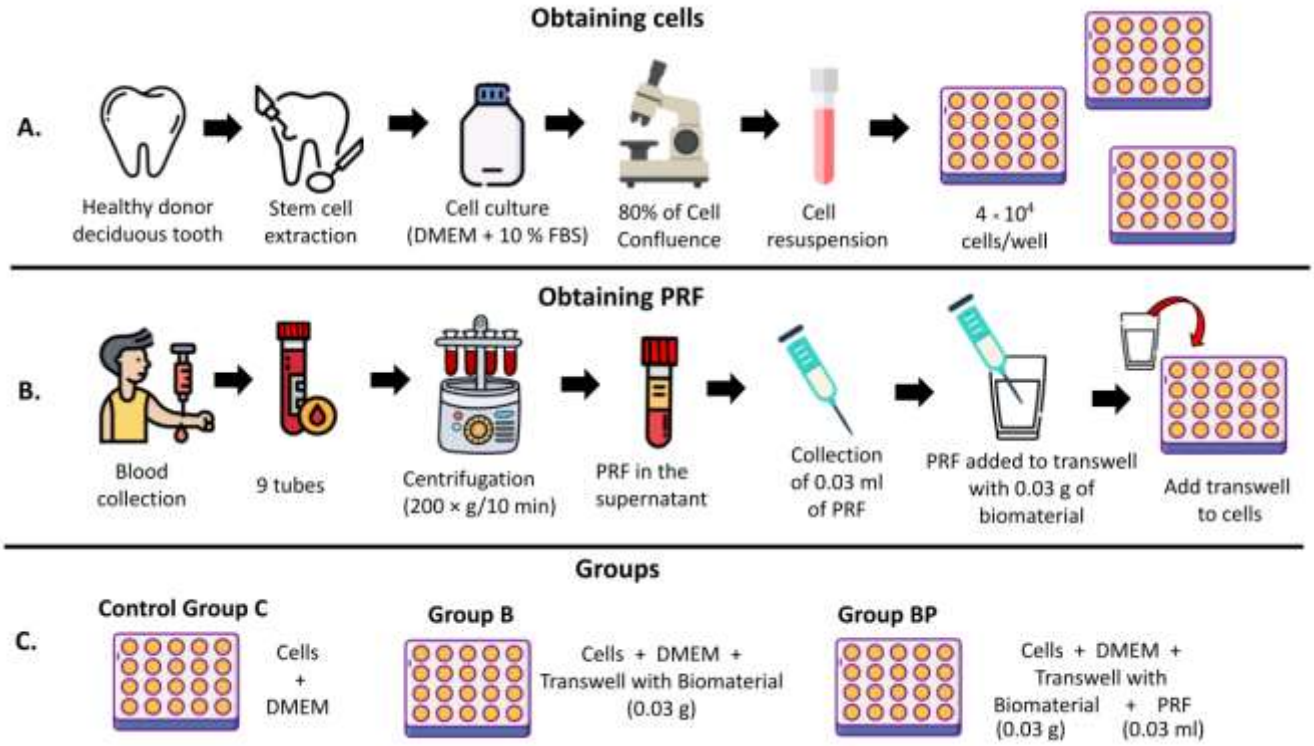
Cells from culture flasks were washed with PBS at 37ºC, treated with trypsin and resuspended with 1 ml of culture medium; a small aliquot was counted in Neubauer chamber and 4×10^4 cells that were plated in the experiment wells. In vitro tests were performed on 24-well plates (Greiner, Germany), in doble triplicates. The groups were divides as follows.

1) Group C (Control, SHED only): in the wells of the control group, the SHED was seeded and maintained only in supplemented DMEM.

2) Group B (SHED + Biomaterial): wells containing SHED received a 33.6 mm² transwell insert, with pores of 3 µm in diameter (Easy Strainer, Greiner Bio-One, Kremsmünster, Austria), with an amount of 0.03 g of biomaterial (Lumina Bone®, Critéria, São Carlos, SP, Brazil). The inserts were put into the wells and were submerged in the DMEM culture medium.

3) Group BP (SHED + Biomaterial + PRF): wells containing SHED received the transwell insert, with the amount of 0.03 g of biomaterial (Lumina Bone®, Critéria, São Carlos, SP, Brazil) associated with a fragment of PRF (0.03 ml). The inserts were put into the wells and were submerged in the DMEM culture medium.

Figure 1 - Methodology and division in groups.



Methodology used for the acquisition of stem cells from deciduous tooth (A); and acquisition of platelet-rich fibrin (PRF) (B); division of experimental groups (C). Group C = Control (SHED only). Group B = SHED + Biomaterial. Group BP = SHED + Biomaterial + PRF. Source: Authors; image icons made by Freepik; Monkik, Smalllikeart, Eucalyp; Flat Icons; photo3idea-studio from www.flaticon.com.

2.5 Cell Viability - Neutral Red

The Neutral Red method was performed according to the methodologies described by Borenfreund and Puerner (1985), Chaim *et al.* (2006) and Fotakis and Timbrell (2006). At 24, 48 and 72 hours of culture, the cells were incubated with 37.5 µl of a neutral red stock solution (1100 µg / ml, Sigma-Aldrich / Merck, Darmstadt, Germany) diluted in PBS, and then diluted in 337.5 µl of the culture medium, thus obtaining a final concentration of 100 µg / ml of neutral red. The absorbance was read in a microplate reader (540 nm, Biotek, Winooski, USA).

2.6 Cellular Cytotoxicity – MTT

The MTT method was performed according to the methodology described by Mosmann (1983), Reilly *et al.* (1998), and Fotakis and Timbrell (2006). At 24, 48 and 72 hours of culture, the cells were incubated with 37.5 µl of a 5 mg / ml MTT stock solution (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich / Merck, Darmstadt, Germany), in 337.5 µl of culture medium.

The Formazan crystals were dissolved in 200 µl of DMSO, then the contents of the wells were removed, and the absorbance was read in a microplate reader (BIOTEK, Winooski, USA) at a wavelength of 570 nm.

2.7 Cell proliferation – Crystal Violet Assay

After the period of 24, 48 and 72 hours of culture, the cells were fixed with 2% paraformaldehyde for 30 minutes, washed 2 times with PBS, stained with 300 µl of the 0.2% violet crystal solution in 5% ethanol, for 5 minutes, and then washed 10 × with PBS. The dye was then eluted with 200 µl of the 33% acetic acid solution in ultrapure water (15 minutes), according to Gillies *et al.* (1986).

The absorbance reading was performed in a microplate reader (BIOTEK, Winooski, USA) at a wavelength of 570 nm.

2.8 Scanning Electron Microscopy Analysis

The PRF clot fragments obtained by 200 × g / 10 minutes were separated and conditioned in plastic tubes that were free of additives (Greiner Bio-One, Brazil). Immediately after PRF production and after 24, 48 and 72 h at 37°C, the specimens were sectioned (body, buffy coat 3 of 12 proximal sediment) and fixed in 2% glutaraldehyde solution and 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.2. After being fixed, they were washed in 0.1 M sodium cacodylate buffer, post-fixed in osmium tetroxide (1%) in sodium cacodylate buffer, then the specimens were dehydrated for 15 minutes in solutions with increasing concentrations of acetone (30%, 50%, 70%, 90% and 3x 100%). Afterwards, the specimens were dried to the critical point with CO_2 and metalized with gold. For analyzes by scanning electron microscopy (Vega3 - Tescan Orsay Holding, Kohoutovice, Czech Republic), the images (20 to 20000 × of magnification) were analyzed by using GIMP 2.10 software (Cockroach Labs, USA). For the morphometric study the diameter of twenty fibers was evaluated using the digital planimeter of Autocad software (Autodesk, USA).

2.9 Statistical analysis

The data used for the different tests of cell viability, cytotoxicity and cell proliferation were analyzed statistically by two-way ANOVA and Tukey's test with a significance level of 5%.

3. Results

3.1 Cell Viability – Neutral Red

Despite being non-toxic, in 24 h the Group B samples showed a lower viability than the control, with results between 38 to 58%; while in the Group BP the viability was close to 60 %. In 48 h, the experimental groups showed an improvement in viability in relation to the 24 h test, with viability between 40 to 70% in Group B, and viability greater than 60% in Group BP. The results within 72 h showed even more improvement in comparison to 24 and 48 h, while Group (B) showed viability greater than 50%, the Group BP presented viability greater than 70%, with results surprisingly higher than the control. The results are shown in Figure 2 (1A, 1B, 1C).

The means and standard deviations for the groups that were evaluated indicated statistically significant differences for the biomaterial factor ($p = 0.001$) and time ($p < 0.001$). The double interaction was statistically significant ($p = 0.037$), as shown in Table 1.

Table 1 - Means ± standard deviations for cell viability values using the Neutral Red method for different groups and times.

Group	Time (h)		
	24	48	72
C	0.6768 ± 0.1527 Aa	0.6855 ± 0.1277 Aa	0.6893 ± 0.0394 Aab
B	0.4688 ± 0,0655 Ab	0,5310 ± 0.0708 ABab	0.6190 ± 0.0587 Ab
BP	0.5237 ± 0.0864 Bab	0.6267 ± 0.0891 Bb	0.7772 ± 0.0489 Aab

Group C = Control (SHED only). Group B = SHED + Biomaterial. Group BP = SHED + Biomaterial + PRF. In the line, values followed by the same capital letters are statistically similar ($p > 0.05$). In the column, values followed by the same lower case letters are statistically similar ($p > 0.05$). The higher the number, the better the cell viability achieved. Source: Authors.

3.2 Cell Cytotoxicity – MTT

The MTT results for 24 h indicate no toxicity, in which the Group B had 100% viable cells; however, the Group BP showed a small decrease at this MTT assay. In 48 hours, none of the groups presented toxicity, with 100% viable cells; a similar result was observed in the 72-hour period. The results are shown in Figure 2 (2A, 2B, 2C).

Therefore, the results indicated statistically significant differences for the biomaterial factor ($p = 0.032$) and time ($p < 0.001$). The double interaction was not statistically significant ($p = 0.480$), as shown in Table 2.

Table 2 - Means and standard deviations for cell cytotoxicity values using the MTT method for different biomaterials and times.

Group	Time (h)			
	24	48	72	Total
C	0.1483 ± 0.0142	0.2138 ± 0.0252	0.2600 ± 0.0059	0.2074 ± 0.0498 A
B	0.1592 ± 0,0172	0.1888 ± 0.0047	0.2702 ± 0.1086	0.2061 ± 0.0768 A
BP	0.1420 ± 0.0084	0.1708 ± 0.0098	0.2162 ± 0.0160	0.1763 ± 0.0333 B
Total	0.1498 ± 0,0148 c	0.1912 ± 0.0235 b	0.2448 ± 0.0643 a	

Group C = Control (SHED only). Group B = SHED + Biomaterial. Group BP = SHED + Biomaterial + PRF. In the line, values followed by the same capital letters are statistically similar ($p > 0.05$). In the column, values followed by the same lower case letters are statistically similar ($p > 0.05$). Source: Authors.

3.3 Cell proliferation – Crystal Violet Assay

It can be seen in 24 h that Group B has an anti-proliferative effect with a decrease in cell density. However, a slight stimulus was observed in the Group BP. In 48h, Group B had a more pronounced anti-proliferative effect in comparison to the same group in 24h. Meanwhile, the Group BP showed a greater stimulus in proliferation, like that of the control. Finally, at 72 h, the Group B had a greater anti-proliferative effect in comparison to the 24 and 48 h. Notably, the Group BP had a pronounced proliferation stimulating effect, with results greater than the control. The results are shown in Figure 2 (3A, 3B, 3C).

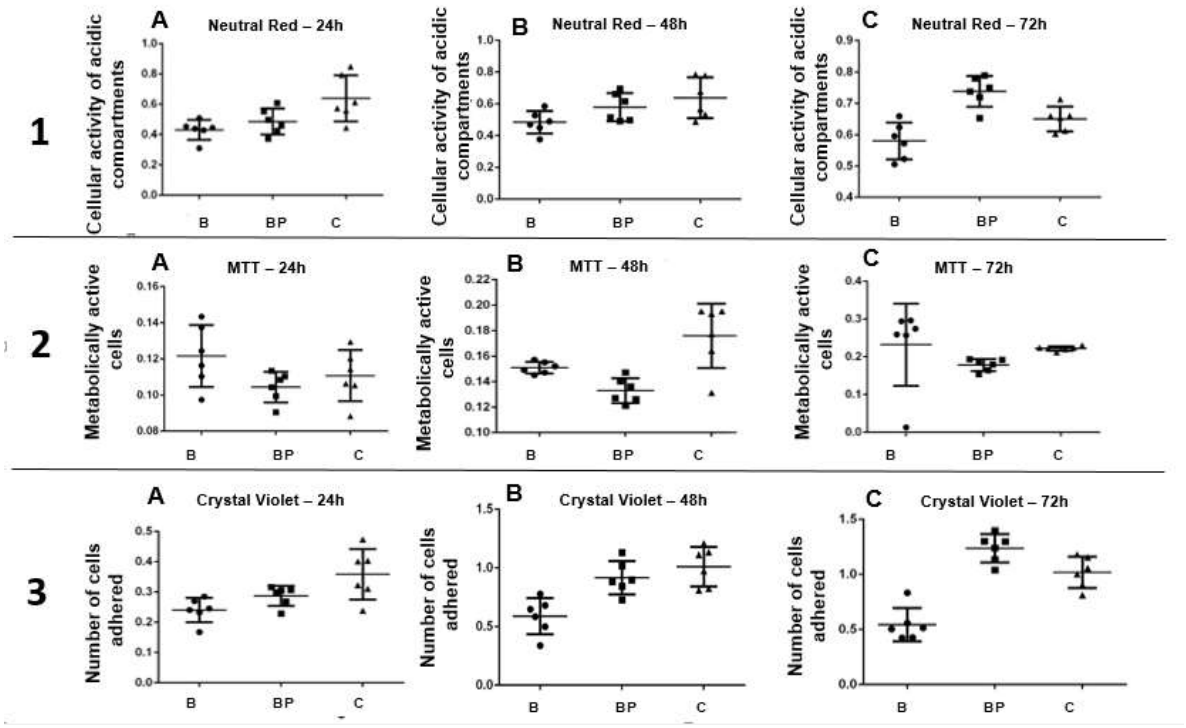
The results indicated statistically significant differences for the biomaterial factor ($p = 0.001$) and time ($p < 0.001$). Double interaction was not statistically significant ($p = 0.001$), as shown in Table 3.

Table 3 - Means and standard deviations for cell proliferation values using the violet crystal method for different biomaterials and times.

Group	Time (h)		
	24	48	72
C	0.3977 ± 0.0837 Ba	1.0582 ± 0.1679 Aa	1.0572 ± 0.1428 Ab
B	0.2795 ± 0.0405 Ba	0.6350 ± 0.1540 Ab	0.5827 ± 0.1510 Ac
BP	0.3265 ± 0.0328 Ca	0.9637 ± 0.1408 Ba	1.2742 ± 0.1286 Aa

Group C = Control (SHED only). Group B = SHED + Biomaterial. Group BP = SHED + Biomaterial + PRF. In the line, values followed by the same capital letters are statistically similar ($p > 0.05$). In the column, values followed by the same lowercase letters are statistically similar ($p > 0.05$) The higher the number, the more cell proliferation was observed. Source: Authors.

Figure 2 - Results of cell viability, cell cytotoxicity and cell proliferation tests for different groups and times.

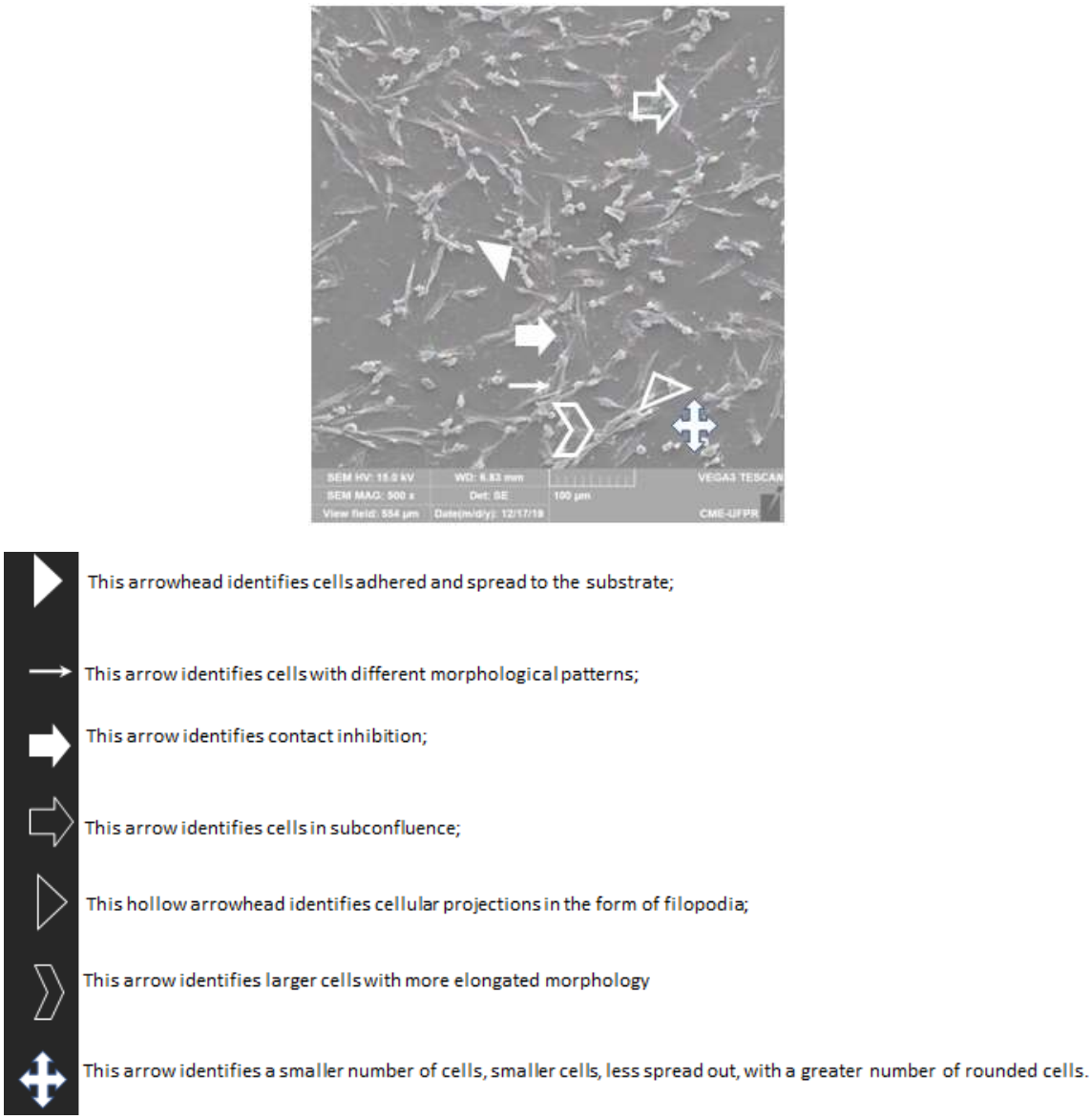


(Letters on the x coordinate in the graphics) B = Group B = SHED + Biomaterial. BP = Group BP = SHED + Biomaterial + PRF. C = Group C = Control group (SHED only). Results of the cell viability by Neutral Red test are shown in line 1, in 24 h (1A), 48 h (1B), 72 h (1C); line 2 shows the cell cytotoxicity through MTT test, in 24 h (2A), 48 h (2B) and 72 h (2C). Line 3 shows the results of cell proliferation by Crystal Violet assay in 24 h (3A), 48 h (3B) and 72 h (3C). Source: Authors.

3.4 Scanning Electron Microscopy - SEM

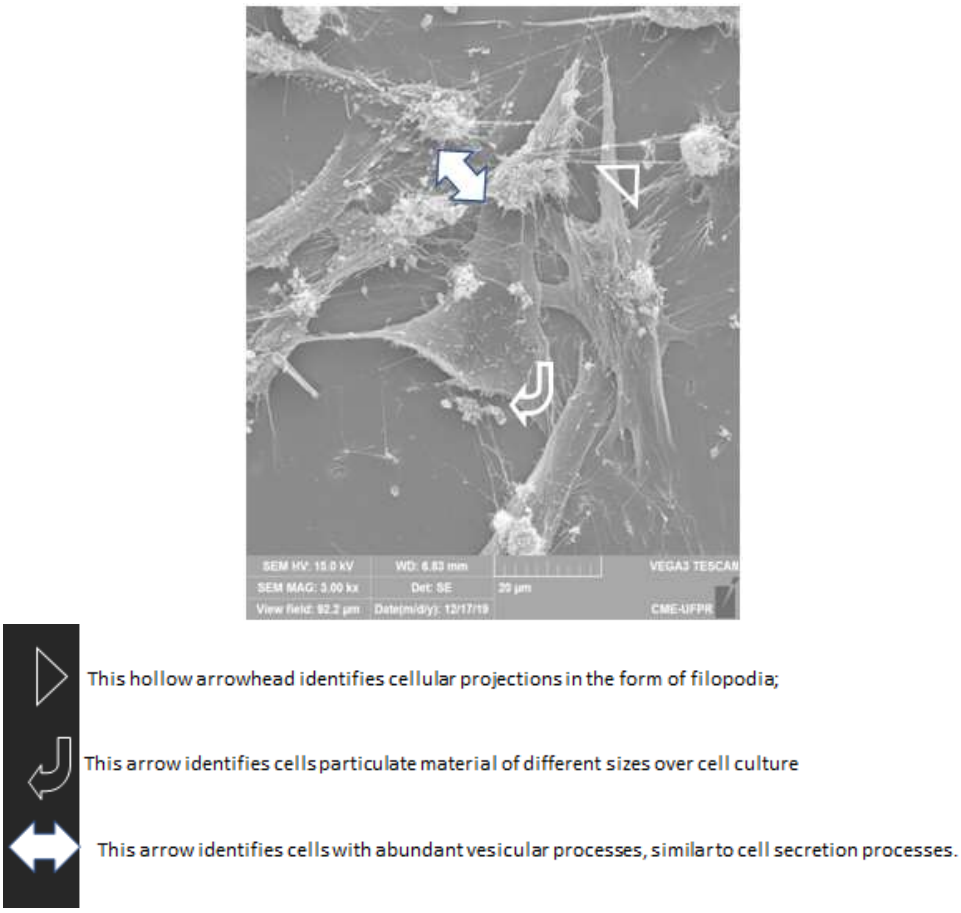
By SEM, it was possible to verify the adhesion to the surface at the Group BP after 72 h of treatment, which presents fusiform morphology (Figures 3 and 4). The SEM refers to sparse cells still with a fibroblastic characteristic after 72 hours of culture with stabilization of cell morphology and increased proliferation.

Figure 3 – Group BP = SHED + Biomaterial + PRF.



Group BP with 72 h. SEM, 500 ×. Source: Authors.

Figure 4 - Group BP = SHED + Biomaterial + PRF.



Group BP with 72 h. SEM, 3000 ×. Source: Authors.

4. Discussion

Isolated from sources such as those of primary tooth pulp, human MSC appears as a model closer to the reality found in the clinic for testing new techniques and products, prior to animal tests or clinical trials. As well as stem cells, additional elements have shown valuable properties for application in the clinical and surgical context, such as PRF, which has great biological potential in wound healing (Massuda *et al.*, 2020). Considering these facts, the objective of this study was the analysis of the viability / cytotoxicity and proliferation of human mesenchymal stem cells, obtained from the collection of pulp from the primary tooth in exfoliation, in the presence of bovine biomaterial associated or not with PRF. Dohan *et al.* (2014) mention an increasing amount of data about the clinical success of PRF use, which corroborates with our results. In the analysis carried out for cell viability (Neutral Red), the group B presented the lower viability within groups. By adding the PRF (Group BP) viability was restored, indicating that PRF might have protective and non-apoptotic actions for MSC.

The standard cytotoxicity MTT assay is important for determining the safety of the analyzed compound. Similarly, to our study, Abuarqoub *et al.* (2020) used MTT in different concentrations of biomaterial in cultures of MSC from the periodontal ligament. Dahake et al (2019) also used MTT to assess the cytotoxicity of three biomaterials with osteoinductive potential but performed the analysis in just 1 time (7 days) in contact with SHED. In this present work, 24, 48 and 72 hours were chosen to evaluate whether or not there would be a toxic action of the studied biomaterials and if this cytotoxicity could change over the hours. The results presented in this work point to a low toxicity of the bovine biomaterial and PRF for the MSC in the analyzed times. Notably, the BP group showed the lowest cytotoxicity compared to the other groups.

In the evaluation of proliferation by means of the crystal violet assay, it was observed that after 72h that Group B had an anti-proliferative effect; in an opposite way, a stimulating effect of proliferation was found in Group BP, suggesting that the association of SHED + biomaterial + PRF influences SHED to multiply with greater intensity. Studies point to such an association as a strong adjunct in the treatment of various diseases (Orlic *et al.*, 2002; Oliveira *et al.*, 2017).

The SEM images of the SHED were obtained from samples of Group BP, at 72 hours, because in this group and at this time they were more proliferative. Sparse cells, with fusiform aspect and characteristics of stabilization of cell morphology and increased proliferation were observed. These results are in line with different studies, such as those described by Yamada and Watari (2003), Lendeckel *et al.* (2004), Ratnayake and Currie (2017), as well as in the study proposed by Amaral (2006), who investigated the morphological characteristics of mesenchymal stem cells and their differentiation capacity.

Naz *et al.* (2019) and Nakajima *et al.* (2019) emphasize the need for using standardized and adequate protocols for this type of assessments to comply with the necessary requirements for the biocompatibility process (Sanada *et al.*, 2003; Rosa *et al.*, 2000). In this study, the followed methodology has proven to be viable and reproducible for future research, suggesting future studies with longer periods and immune characterization, which can further disclose more cell behavior towards biomaterial.

5. Conclusion

Although the results from viability, cytotoxicity, and proliferation among all groups seemed to converge, while exposed to the bovine biomaterial used in this study, SHED decreased their performance. However, when the biomaterial was associated with PRF, a proliferative and stimulatory effect was observed on SHED, indicating that such association can bring an effective benefit in clinical outcomes.

Acknowledgments

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