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EVALUATION OF THERAPEUTIC BENEFITS OF COLLAGEN BASED BIOMATERIAL FUNCTIONALISED WITH PLATELET RICH PLASMA

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Abstract

Platelet rich plasma (PRP) in combination with different type of biomaterials can promote tissue regeneration. Our study aimed to evaluate the adhesion, proliferation and chondrogenic differentiation potential of canine mesenchymal stem cells on functionalised collagen-based biomaterial. Characterized gingival mesenchymal stem cells ($1x10^5$) were seeded on PRP functionalized synthetic biomaterial and were cultured in DMEM/F12 culture medium supplemented with 10% fetal calf serum and 1% antibiotics-antimycotics. After 24, 72 h and 5 days cellular adhesion, proliferation was assessed using CCK8 assay. The absorbance was recorded using a a BioTek Synergy 2 microplate reader set at 450 nm. The bioactive potential of PRP functionalized biomaterial was evaluated after 21 days. Proteoglycans and sulfated glycosaminoglycan content were assessed. Statistical analysis was performed using GraphPad Prism 5.0 software and statistical significance was considered when $p < 0.05$. Our results indicated the potential of PRP on cells attachment, proliferation and due to the release of growth factors promote the cells differentiation.

Key words*: biomaterials, collagen, platelet rich plasma, mesenchymal stem cells.*

INTRODUCTION

Platelet-rich plasma (PRP), defined as an autologous leukocyte and PRF biomaterial (Tambella et al., 2018), contain various growth factors (platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b) 1, 2, vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) I, IGF-II), extracellular matrix and cell adhesion molecules (fibrin, fibronectin, vitronectin and thrombospondin) (Ramaswamy et al., 2018), proteins, cytokines and other signalling molecules with important function in regulation of regeneration and tissue repair by supplying growth factors, cytokines, chemokines and other bioactive compounds (Boswell et al., 2012; Hsu et al., 2013; Jason et al., 2013; Albanese et al., 2013; Carr et al., 2016; Chang et al., 2018; Wenqiang et al., 2020). PRP are an autologous source of platelets (least 1,000,000/1 L in a small volume of plasma) (Marx, 1999; Saluja et al., 2011; Albanese et al., 2013) it is widely used in different areas of dentistry, orthopedics, reconstructive medicine, among which can be remembered mandibular reconstruction, periodontal defects and periodontal plastic surgery, head and neck surgery, surgical repair of the alveolar cleft, otolaryngology, cardiovascular surgery, maxillofacial surgery (Albanese et al., 2013), soft tissue injuries, osteoarthritis of the knee, ulnar collateral ligament tears, lateral epicondylitis (Mlynarek et al., 2016), hip dysplasia (Yun et al., 2016), hip osteoarthritis in dog (Malek et al., 2012) and muscle damage. Our hypothesis is that PRP in combination with different type of biomaterials can promote tissue regeneration. Therefore, the objective of the current study was to evaluate the adhesion, proliferation and chondrogenic differentiation potential of canine mesenchymal stem cells on PRP functionalised collagen-based biomaterial.

MATERIALS AND METHODS

Canine gingival tissue derived mesenchymal stem cells (MSCs) was used for this study. The gingival tissue and blood sample was harvested from a mixed breed dog, presented at a private

practice with dental disorders. The samples were collected after the informed consent from the owner. The MSCs were isolated using enzymatic dissociation method (Collagenase 0.075%, Sigma-Aldrich), after several passages, the cells were characterized according to the International Society for Cellular Therapy recommendations. For PRP the blood was collected from the external jugular vein under sterile conditions. The samples were centrifuged at 300x g for 7 min to separate the blood phases. The upper phase was harvested (approximately 1.5 ml) and freshly added on collagen-based biomaterial (Evolution, OsteoBiol^{R}) in three different concentrations (5%, 7%, and 10%). Prior the PRP was activated with calcium chloride (Sigma-Aldrich). The canine gingival stem cells at a density of 1 x 10^4 cells were seeded onto the functionalized biomaterial (0.7 cm x 0.7 cm) placed in culture plates. The cell-seeded biomaterials were transferred to a 12 well plates containing normal propagation medium containing DMEM/F12 (Gibco), 1% Antibiotic Antimycotic 100x (Sigma-Aldrich), 5% Horse serum (Gibco Life Technologies), at 37.5°C in a humidified 5% CO atmosphere for the attachment and proliferation tests. The medium was changed every day. Collagen based biomaterials without PRP was considered the negative control. The propagation medium in the control group was supplemented with 10% FCS (fetal calf serum, Sigma-Aldrich). After 24 h cellular adhesion was assessed using cytochemical stains, tetramethylrhodamine isothiocyanate (TRITC) phalloidin (Sigma-Aldrich) 1: 20 in phosphate-buffered saline (PBS; Sigma-Aldrich) for actin filaments and DAPI (4,6-diamidino2-phenylindole) for cell nuclei. The results were evaluated using Axiovision 3.0 image analysis software (Carl Zeiss, Hitech Instruments). Cell proliferation was assessed using CCK-8 assay after 24, 72h and 5 days of culture. CCK-8 solution was added in each well (100 µl/ml) followed by incubation at 37°C for 1 hour. The absorbance was recorded using a BioTek Synergy 2 microplate reader set at 450 nm. For chondrogenic differentiation, pellet cultures method was used. The cells (5 x 10^4/ml) were aggregated using hanging drops method. After 48h, the aggregates were washed and added onto the PRP treated collagen-based biomaterial in normal propagation medium. The control groups were treated with chondrogenic induction medium: DMEM/F-12 (Sigma-Aldrich) supplemented with 1% ITS (Insulin-Transferrin-Selenium, Sigma-Aldrich), 50 nM L ascorbic acid 2-phosphate (Sigma-Aldrich), 100 nM dexamethasone, 10 ng/ml of transforming growth factor (TGF-b; Sigma-Aldrich) and 1% antibiotic/antimycotic (Gibco).

The morphological characteristics of the cells from both cultures were monitored daily. Proteoglycans and sulfated glycosaminoglycan content were evaluated after 21 days. The proteoglycan accumulation was assessed by Alcian blue staining. The cultures were fixed with methanol for 20 minutes and were stained with Alcian blue in 0.1N HCl for 20 minutes, washed with distilled water and treated with 6 M guanidine-HCl (Sigma-Aldrich). The absorbance was measured at 620 nm. The glycolsaminoglycans were assessed after treatment of the paraformaldehyde fixed cultures with 0.02% Safranin-O (Sigma-Aldrich). The Safranin-O dye was extracted using 10% cetylpyridinium chloride (Sigma-Aldrich) for 20 min. The optical density was evaluated at 550 nm. Canine gingival cells cultured in normal propagation medium were represented the control group. The evaluations were performed in triplicate.

Statistical analysis was performed with GraphPad Prism 6 software. A value of $P < 0.05$ was considered to be statistically significant difference for all tests.

RESULTS AND DISCUSSIONS

In order to evaluate the potential of functionalized collagen-based biomaterial, characterized canine gingival stem cells were used. The biomaterial was functionalized with different concentration of PRP. Prior the biomaterial treatment the PRP was activated with calcium chloride.

This treatment is important for releasing the granule's contents (Cuervo et al., 2020). Cellular adhesion was assessed using cytochemical staining and quantitative morphometric analyses were performed.

The number of adherent cells was quantified in control group and in PFR functionalized

biomaterials. Cells were counted in 5 different microscopic fields (Figures 1, 2).

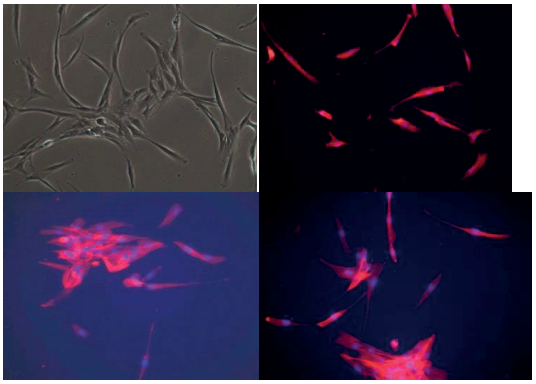

Figure 1. Gingival MSCS attached to the PRP treated biomaterial (1st images: cells cultivated on plastic surface, negative control, 2nd images: cells seeded on biomaterial functionalized with 5% PRP, 3rd images: cells seeded on biomaterial functionalized with 7% PRP, 4th images: cells seeded on biomaterial functionalized with 10% PRP), blue nuclei stained with DAPI, red actin filaments stained with TRITC-phalloidin

Microscopic fields were randomly selected. After 24 h of seeding on collagen-based biomaterial statistically significant results were identified especially for biomaterials treated with 5% PRP (Figure 1).

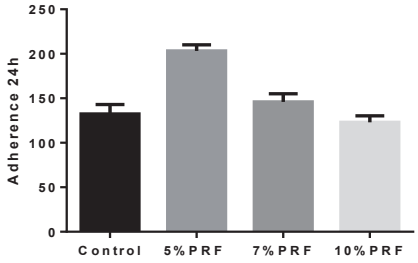

Figure 2. Cell number on functionalized collagen-based biomaterial 24 h after seeding (cells seeded on biomaterial functionalized with 5% PRP, 7% PRP and 10% PRP)

CCK-8 test was performed to examine the effect of different concentrations of PRP on canine gingival stem cells proliferation after 24 h, 72 h and 5 days of exposure (Figure 3). The results of the CCK-8 assay demonstrated that PRP-treated MSCs in different concentration showed improved cell growth compared to control groups.

After 24 hours of seeding the cells on 7% and 10% PRP treated biomaterial, a slight increase in cell proliferation rate can be observed.

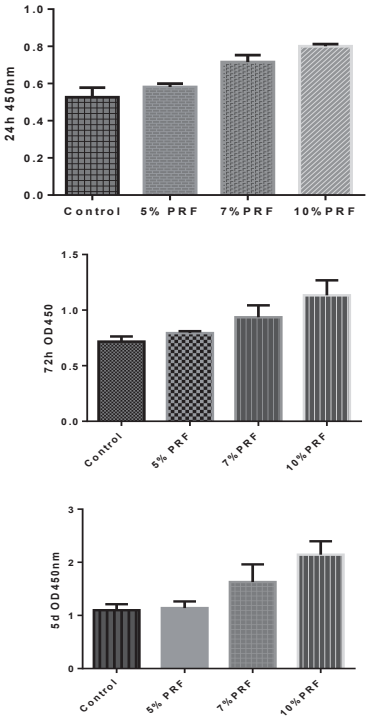

Figure 3. Canine gingival tissue derived MSCs proliferation on PRP functionalized collagen-based biomaterial (Evolution, OsteoBiol[R]). CCK-8 assay was performed to evaluate the effect of three different concentrations of PRP in three different period of time. The absorbance was read at 450 nm

After 5 days statistically significant results were identified in cultures treated with 10% PRP. Our results are in agreement with certain data from the literature. A similar behaviour was observed by Wang et al. (2019) in bone marrow aspirate derived mesenchymal stem cells treated with of the PRP. They study demonstrate that PRP in concentration of 1500 $\times 10^9$ pl/L. exerted different modulatory effects on cell proliferation and the osteogenic, adipogenic and chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. The differentiation capacity is closely related to the increased content of cytokines and growth factors, a group of diffusible polypeptides that control cell growth, proliferation, differentiation, cellular

metabolism (Marx et al., 1999, Cuervo et al., 2020). PRP also promoted the production of the anti-inflammatory cytokines IL-10 by mesenchymal stem cells, but can suppress the IL-1β, IL-6 and TNF-α secretion (Wang et al., 2019).

To explore the bioactive potential of PRP, chondrogenic induction potential of functionnalized biomaterials were assessed. The canine gingival tissue derived stem cells were maintained on functionalized biomaterial for 21 days. The PRP supplementation was performed in every 3 days until the cells were fixed. The positive control group were treated with chondrogenic induction medium without PRP and for negative control the cells were treated with normal propagation medium. After 21 days Alcian Blue and Safranin O staining indicated the presence of glycosaminoglycans and proteoglycan deposition (Figures 4, 5).

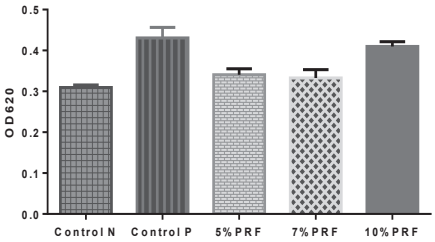

Figure 4. Chondrogenic differentiation - proteoglycan deposition
(The absorbance value of the solubilized Alcian blue at 620 nm. The results are shown as mean ± SD
Control N - negative control: canine gingival stem cells cultured in normal propagation medium; Control P - positive control: canine gingival stem cells treated with chondrogenic induction medium)

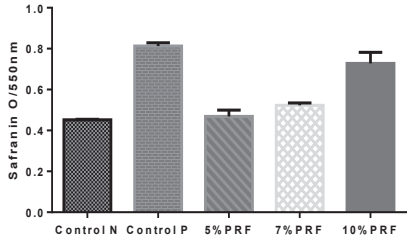

Figure 5. Chondrogenic differentiation-glycosaminoglycans evaluation with Safranin O staining
(Control N - negative control: canine gingival stem cells cultured in normal propagation medium; Control P - positive control: canine gingival stem cells treated with chondrogenic induction medium)

Compared with negative control (0.309 ± 0.006), the average of absorbance for proteoglycan deposition was 0.340 ± 0.01 on biomaterial functionalized with 5% PRP, 0.332 ± 0.02 on 7% PRP and 0.410 ± 0.01 on 10% PRP. The differentiation capacity of cells maintained on 10% PRP treated biomaterial was similar with positive control group where the average of absorbance was 0.430 ± 0.02. These results suggested that normal propagation medium supplemented with 10% PRP may be preferable for the accumulation of proteoglycans (Figure 4). Moreover, similar effects of PRP in glycosaminoglycans production were observed. The average of absorbance for positive control was 0.810 ± 0.01 compared with the negative control where the average of absorbance was 0.452 ± 0.002 (Figure 5).

The potential of the glycosaminoglycans synthesis increased exponentially with increasing PRP concentration. These data demonstrate that 10% PRP is effective and efficient for the chondrogenic differentiation of canine gingival stem cells. Numerous applications based on PRP have been tested for bone and cartilaginous disorders (Marmotti et al., 2015; Wang et al., 2019). All these applications suggested that the concentration of PRP is important for the restoration of the affected tissues. Our data showed that PRP in concentration of 10% would be effective. Our future concerns will be focused on evaluating the tested PRP concentrations on different biological models including organotypic cultures. Wang et al. (2019) data showed that PRP with platelet concentrations of 1000×10^9 pl/L to 3000×10^9 pl/L supported MSC adipogenesis, osteogenesis and chondrogenesis. They also suggest that for cartilage regeneration a concentration of 2000×10^9 pl/L PRP provides a strong proliferation and induce chondrogenesis (Wang et al., 2019). Platelet rich plasma in combination with stem cells may represent a promissory treatment for many diseases, but multiple clinical trials are required to establish the true efficiency of *in vitro* and *in vivo* treatments. PRP-based therapy could be valuable as an alternative therapy alone or in combination with other conventional treatments for different diseases (Ramaswamy et al., 2018).

CONCLUSIONS

Our results indicated the potential of PRP in combination with collagen-based biomaterial on cells attachment, proliferation and due to the release of growth factors promote the cells differentiation. These results may suggest promising clinical strategies for different diseases.

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REFERENCES

Albanese, A., Licata, M.E., Polizzi, B., Campisi, G. (2013). Platelet-rich plasma (PRP) in dental and oral surgery: from the wound healing to bone regeneration. *Immun Ageing*. 10(1): 23.

Boswell, S.G., Cole, B.J., Sundman, E.A., Karas, V., Fortier, L.A. (2012). Platelet-rich plasma: a milieu of bioactive factors. *Arthroscopy*. 28(3): 429–39.

Carr, B.J., Canapp, S.O., Mason, D.R., Cox, C., Hess. T. (2016). Canine Platelet-Rich Plasma Systems: A Prospective Analysis. *Front Vet Sci*. 2: 73.

Chang, N.J., Erdenekhuyag, Y., Chou, P.H., Chu, C.J., Lin, C.C., Shie, M.Y. (2018). Therapeutic Effects of the Addition of Platelet-Rich Plasma to Bioimplants and Early Rehabilitation Exercise on Articular Cartilage Repair. *Am J Sports Med*. 46(9): 2232–2241.

Cuervo, B., Rubio, M., Chicharro, D., et al. (2020). Objective Comparison between Platelet Rich Plasma Alone and in Combination with Physical Therapy in Dogs with Osteoarthritis Caused by Hip Dysplasia. *Animals (Basel)*. 10(2): 175

Hsu, W.K., Mishra, A., Rodeo, S.R., Fu, F., Terry, M.A., Randelli, P., et al. (2013). Platelet-rich plasma in orthopaedic applications: evidence-based recommendations for treatment. *J Am Acad Orthop Surg.* 21: 739–48.

Jason, D., Smith, Lee E., Weiss, James E., Burgess, Alan I. West, Phil, G. (2013). Biologically Active Blood Plasma-Based Biomaterials as a New Paradigm for Tissue Repair Therapies. *Disruptive Science and Technology*, 127–137.

Malek, S., Sample, S.J., Schwartz, Z., Nemke, B.,Jacobson, P.B., Cozzi, E.M., Schaefer, S.L., Bleedorn, J.A., Holzman, G., Muir, P. (2012). Effect of analgesic therapy on clinical outcome measures in a randomized controlled trial using client-owned dogs with hip osteoarthritis. *BMC Vet. Res.,* 8, 185.

Marmotti, A. R., Rossi, F., Castoldi, E., Roveda, G., Michielon, G.M. (2015). Peretti PRP and articular cartilage: a clinical update. *Biomed Res Int*., 542502.

Marx, R.E. (2004). Platelet-rich plasma: evidence to support its use. *J Oral Maxillofac Surg.* 62(4): 489–96.

Marx, R.E., Carlson, E.R., Eichstaedt, R.M., Schimmele, S.R., Strauss, J.E., Georgeff, K.R. (1998). Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod*. 85, 638–646.

Marx,RE. In: Tissue Engineering: Applications in Maxillofacial Surgery and Periodontics. Lynch SE,Genco RJ, Marx RE, editor. Chicago: Quintessence; 1999. Platelet-rich plasma: a source of multiple autologous growth factors for bone grafts; pp. 71–82.

Miron, R.J., Fujioka-Kobayashi, M., Hernandez, M., et al. (2017). Injectable platelet rich fibrin (i-PRP): opportunities in regenerative dentistry? *Clin Oral Investig*. 21(8): 2619–2627.

Mlynarek, R.A., Kuhn, A.W., Bedi, A. (2016). Platelet-Rich Plasma (PRP) in Orthopedic Sports Medicine. *Am J Orthop (Belle Mead NJ)*. 45(5): 290–326.

Ramaswamy Redd, SH., Reddy, R., Babu, N.C., Ashok, G.N. (2018). Stem-cell therapy and platelet-rich plasma in regenerative medicines: A review on pros and cons of the technologies. *J Oral Maxillofac Pathol*., 22(3): 367–374.

Saluja, H., Dehane, V., Mahindra, U. (2011). Platelet-Rich fibrin: A second generation platelet concentrate and a new friend of oral and maxillofacial surgeons. *Ann Maxillofac Surg*. 1(1): 53–57.

Tambella Adolfo, M., Martin, S., Cantalamessa, A., Serri, E., Attili, A.R. (2018). Platelet-rich Plasma and Other Hemocomponents in Veterinary Regenerative Medicine, *Wounds.* 30(11): 329–336.

Wang K, Li Z, Li J, et al. (2019). Optimization of the Platelet-Rich Plasma Concentration for Mesenchymal Stem Cell Applications. *Tissue Eng Part A*., 25(5-6): 333–351.

Wenqiang, Y., Xingquan, X., Qian, X., Ziying, S., Qing, J., Dongquan, S. (2020). Platelet-rich plasma combined with injectable hyaluronic acid hydrogel for porcine cartilage regeneration: a 6-month follow-up. *Regenerative Biomaterials*, 7(1), 77–90.

Yun, S.; Ku, S.K.; Kwon, Y.S. (2016). Adipose-derived mesenchymal stem cells and platelet-rich plasma synergistically ameliorate the surgical-induced osteoarthritis in Beagle dogs. *J. Orthop. Surg. Res*. 11, 9.