EFFECTS OF EXOSOMES DERIVED FROM MSC-iPSC ON FIBROBLASTS *IN VITRO* IN THE PRESENCE OF PLATELET-RICH PLASMA

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EFFECTS OF EXOSOMES DERIVED FROM MSC-iPSC ON FIBROBLASTS *IN VITRO* IN THE PRESENCE OF PLATELET-RICH PLASMA (Abstract): Mesenchymal stem cells, as well as mesenchymal stem cells-induced pluripotent stem cells, can definitely induce alterations of fibroblasts and osteoblasts micro medium, stimulating the development of bone around the dental implants. **Aim**: The purpose of the current study was represented by the following of the effects of stimulation and inhibition of multiple intracellular functional pathways of senescent gingival fibroblasts in culture when exosomes released from pluripotent stem cells reprogrammed from mesenchymal stem cells and platelet-rich plasma were used. Materials and m**ethods**: We used flow cytometry to measure the production of the beta-galactosidase level by gingival fibroblasts in culture after 30 passages and ultraviolet treatment. **Results**: Reduction of β-galactosidase concentrations in senescent gingival fibroblasts was evident upon administration of platelet-rich plasma, exosomes and exosomes co-administered the same time with: LY-294002, nutlin-3, berberine, resveratrol, collagen, thrombin, and fibronectin. On the other side, lipopolysaccharide (which might induce a pro-inflammatory profile on iPSC-MSC) enhanced the production of β-galactosidase. The most intense effects were associated with collagen, thrombin, fibronectin, resveratrol, and berberine. **Conclusions**: Exosomes isolated from pluripotent stem cells derived from mesenchymal stem cells, administered in the culture medium of senescent gingival fibroblasts, can modify the degree and evolution of the senescence of these last cells by potentiating, activating, or stimulating some intracellular pathways of biological signal transduction. **Keywords:** MSC, MSC-iPSC, GINGIVAL FIBROBLASTS, EXOSOMES, LY294002, NUTLIN-3, BERBERINE RESVERATROL, LPS, COLLAGEN, THROMBIN, FIBRINOGEN, PLATELET-RICH PLASMA.

INTRODUCTION

Large vertical and horizontal alveolar ridge deficits in mandibular and maxillary bone remain a barrier for doctors practicing implant dentistry. One of the disadvantages of porous blocks for bone repair in big lesions in the oral cavity and in the musculoskeletal system is that the fibrin clot does not adequately cover the interior pores and, the same time, does not persist long enough

to accommodate cell migration into the center of the block (1).

Platelet concentrates, namely platelet-rich plasma, fibrin, or concentrated growth factors, represent cost-effective autologous preparations comprising platelet-derived growth factor, transforming growth factor β, insulin-like growth factor 1, as well as vascular endothelial growth factor. As a result, they are commonly employed in regenerative medicine to repair wounds, nerve damage, cartilage, and bone abnormalities. Unfortunately, following administration, these preparations rapidly release growth factors, which quickly lose their effectiveness. As a result, the therapy must be repeated, which causes the patient more pain and anguish. Recent studies indicate that mixing platelet concentrates with biomaterials solves this problem by releasing growth factors in a more sustainable manner. Furthermore, this notion fits with the most recent advancements in tissue engineering, including biomaterials, bioactive agents, and cells (2).

The progress made in tissue engineering is the result of a thorough investigation of cell-tissue interactions. The selection of a specific biomaterial in Tissue Engineering is crucial because it serves as an interface for adherent cells in the establishment of a microenvironment suited for cell proliferation and differentiation (3).

Lesions linked with medication-related osteonecrosis of the jaws (MRONJ) are resistant to many therapeutic options. As a result, alternative treatments capable of enhancing patient outcomes should be investigated. Leukocyte- and platelet-rich fibrin (LPRF) is a second-generation platelet concentrate composed of natural autologous fibrin matrix. It exhibits anti-infective activity via immunological modulation and accelerates the angiogenesis and multiplication of fibroblasts and osteoblasts; as a result, it promotes soft tissue repair and reduces alveolar bone exposure in the oral cavity. As a result, this could be a noninvasive, rapid, and novel technique to managing bone exposure. The LPRF membrane contributes to a favorable outcome by acting as a physical barrier against microorganisms, preventing secondary infections (4).

Cellular therapy is a new treatment option in regenerative endodontics. Allogeneic mesenchymal stromal cells were tested for their ability to stimulate dental pulp and apical bone regeneration in an endodontically treated tooth. After 14 months after MSC transplantation, the tooth being studied demonstrated sensitivity to cold and electric pulp testing. Radiographic and cone-beam computed tomography images revealed improved periapical bone density, healing of the periapical lesion, and nearly complete apical remodeling. Thus, allogeneic MSCs could be a first-line therapy in regenerative endodontics (5).

The purpose of our research was to investigate the effects of stimulating and inhibiting multiple intracellular functional pathways of senescent gingival fibroblasts in culture, in the presence of microvesicles released by pluripotent stem cells derived from mesenchymal stem cells and platelet-rich plasma.

MATERIALS AND METHODS

The laboratory research aimed to quantify the evolution of the degree of senescence of gingival fibroblasts (senescent cells) by evaluating β-galactosidase through flow cytometry (6, 7) in a biculture, in the presence of platelet-rich plasma and pluripotent stem cells, repro-

grammed from mesenchymal stem cells (in culture), or under the action of exosomes/micro vesicles released from MSC-iPSCs, in the form of concentrated supernatant, administered in the culture medium.

Storage rat gingival fibroblasts (cell line no. 1) were cultivated under conventional laboratory conditions and analyzed for β-galactosidase using flow cytometry after passages 1, 10, 20, and 30. To obtain senescent gingival fibroblasts, they were treated with ultraviolet (λ=315 nm, 80 mJ/cm^2) and multiplied through 30 consecutive passes.

To produce MSC-iPSCs, we used the episomal technique with iPSC reprogramming vectors and the appropriate Stemline culture media (Invitrogen), in accordance with the manufacturer's instructions. We picked this method since the usage and manipulation of potentially genetic material, which is mentioned in the "dangerous" chapter, is completely prohibited. The growth and multiplication of pluripotent stem cells reprogrammed from mesenchymal stem cells were carried out utilizing an Alvetex 3D bio support -a gift from Reinnervate-, as previous investigations revealed the highest efficiency for this form of support (8).

The platelet-rich plasma (PRP) gel was prepared as previously described (9) and delivered at a concentration of 1%.

For exosome separation, the culture medium of pluripotent stem cells produced from rat aortic smooth muscle cells was centrifuged at 100,000 x g (10).

The following compounds were employed in tests and protocols with senescent gingival fibroblasts: 1 µM LY294002, 1 µM nutlin-3, 1 µM berberine, 1 µM resveratrol, 1 µM lipopolysaccharide (LPS), 1 µg/ml collagen, 1 µM thrombin, and 1 µg/ml fibrinogen.

All experimental results obtained during the current study were processed entirely by statistical methodologies, using a number of well-known and optimized tests over time and for medical research, among which the One-Way ANOVA test should be mentioned, which was also supplemented, on occasion, with the Student-Newman-Keuls test.

All the experiments were approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy from Iaşi.

RESULTS

The best qualitative and quantitative development in the case of pluripotent stem cells experimentally reprogrammed from mesenchymal stem cells, beginning with the second passage in culture, was obtained when we multiplied them in a preconditioning medium containing only 1% O2 (8), as previously demonstrated. We always assumed a 100% increase (n=5 experiments per set, data not shown, personal observation).

In addition, the most important β-galactosidase reduction effects in senescent gingival fibroblasts in monoculture were observed in the presence of exosomes/micro- vesicles at a concentration of 0.01%, obtained in concentrated form by ultracentrifugation of the MSC-iPSC supernatant. These latter cell types were also developed in a medium preconditioned with 1% O2. We observed a 20% drop in β-galactosidase levels in senescent gingival fibroblasts under these circumstances, consistent with earlier studies.

In the first series of tests, senescent gingival fibroblasts were treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet-rich plasma.

Previously, MSC-iPSCs were treated with 1 μM LY294002, a highly selective PI3K kinase inhibitor. In senescent gingival fibroblasts, we observed an average 15% drop in β-galactosidase activity (fig. 1).

In the second series of tests, a separate batch of senescent gingival fibroblasts was treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet rich plasma. Previously, MSC-iPSCs were treated with 1 μM nutlin-3, which effectively stabilized the non-genomic activities of p53 (MDM2/p53 pathway). Senescent gingival fibroblasts showed an average 13% drop in β-galactosidase levels (fig. 1).

In the third series of tests, a large number of senescent gingival fibroblasts were treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet rich plasma. MSC-iPSCs were treated with 1 μM berberine, which inhibits ROS-dependent and JNK-driven cell death via PI3K/Akt. We observed a 23% drop in β-galactosidase levels in senescent gingival fibroblasts (fig. 1).

In the fourth series of studies, a completely separate batch of senescent gingival fibroblasts were treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet-rich plasma. MSC-iPSCs were treated with 1 μM resveratrol, potentially improving their survival, self-renewal, lineage commitment, and anti-aging benefits. In senescent gingival fibroblasts, we observed an average 52% drop in β-galactosidase levels (fig. 1).

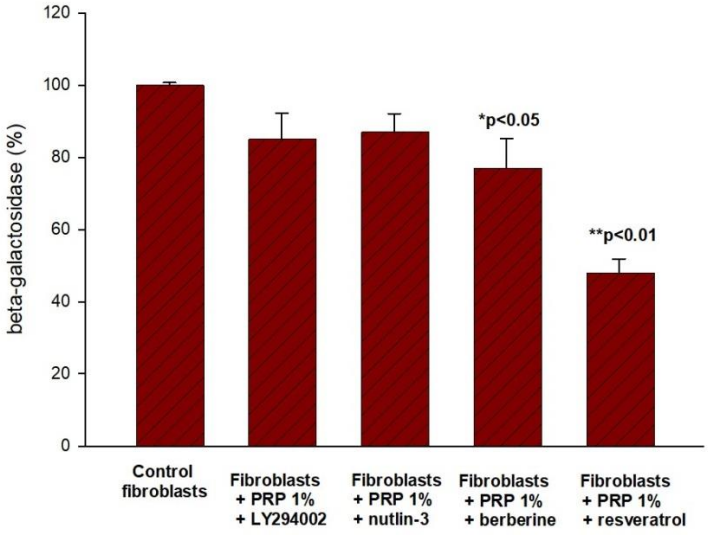

Fig. 1. Determination of β-galactosidase in senescent gingival fibroblasts, when cultured with 0.01% exosomes obtained from the supernatant of MSC-iPSC and 1% platelet-rich plasma. Previously, MSC-iPSCs have been treated with 1 μM LY294002, nutlin-3, berberine, and resveratrol. (*p<0.05 as compared to the normal culture medium. **p<0.01 as compared to the normal culture medium)

In the fifth series of tests, senescent gingival fibroblasts were treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet-rich plasma. Previously,

MSC-iPSCs were treated with 1 μM lipopolysaccharide (LPS), which induces a proinflammatory profile on iPSC-MSC. We observed a 37% drop in β-galactosidase levels in senescent gingival fibroblasts (fig. 2).

In the sixth series of studies, a separate batch of senescent gingival fibroblasts was treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet rich plasma. Previously, MSC-iPSCs were treated with 1 μM collagen, which increased chemokine and growth factor production by MSCs. We observed a 61% drop in β-galactosidase levels in senescent gingival fibroblasts (fig. 2).

In the seventh series of tests, a large number of senescent gingival fibroblasts were treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet rich plasma. MSC-iPSCs were treated with 1 μM thrombin, which increases fibronectin production through protease-activated receptor-mediated signaling pathways. In senescent gingival fibroblasts, we observed an average 58% drop in β-galactosidase levels (fig. 2).

In the eighth series of trials, a completely separate batch of senescent gingival fibroblasts was treated with 0.01% exosomes derived from MSC-iPSC supernatant and 1% platelet rich plasma. Previously, MSC-iPSCs were treated with 1 μM fibrinogen, which promotes self-renewal by stabilizing bFGF and activating autophagy. We observed a 48% drop in β-galactosidase levels in senescent gingival fibroblasts (fig. 2).

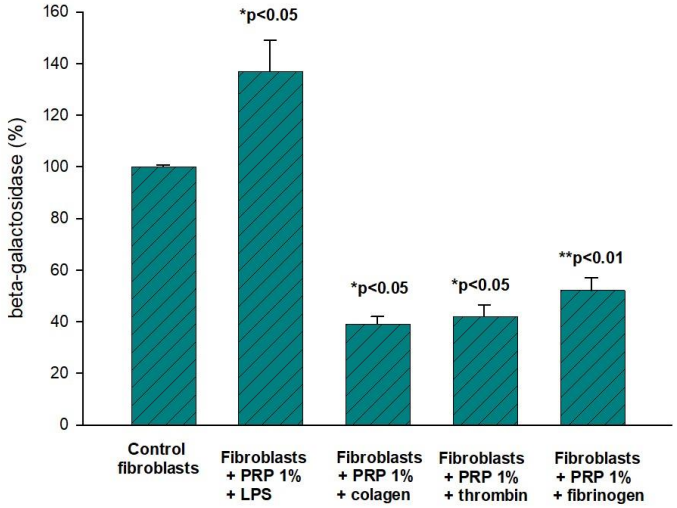

Fig. 2. Measurement of β-galactosidase in senescent gingival fibroblasts, when developed in the presence of 0.01% exosomes obtained from the supernatant of MSC-iPSC and 1% platelet-rich plasma. Foregoing, MSC-iPSCs have been treated with 1 μM LPS, collagen, thrombin, and fibrinogen. (*p<0.05 as compared to the normal culture medium. **p<0.01 as compared to the normal culture medium)

DISCUSSION

There are studies underway to investigate platelet-rich plasma (PRP) effects in human mesenchymal stem cells, which may support or limit the clinical use of MSCs in conjunction with PRP. The effects of PRP

on proliferation, migration, stemness, the preservation of MSC immune-modulatory capabilities, and the emergence of the senescence phenotype were investigated. Overall, PRP promotes MSC proliferation, maintains multipotency, and does not impede with lineage differentiation. PRP (platelet lysate or releasate) protects MSCs' immune-privileged potential and may postpone the onset of the senescent phenotype. Currently, there is little evidence tying specific chemicals to biological mechanisms. Several knowledge gaps must be addressed in order to gather enough usable information for translation (11).

Platelet-rich plasma is defined as an enriched platelet suspension in plasma. In addition to its clinical applications in numerous orthopedic illnesses and beyond, PRP and platelet lysate (PL) have received attention in the field of tissue engineering. PRP has been used for a variety of tissue engineering applications, including bone, cartilage, skin, and soft tissue repair, with the majority of studies focused on bone tissue engineering. These methods take advantage of either the release of chemo attractive, angiogenic, proliferative, and potentially pro-regenerative growth factors from PRP, or the hydrogel properties of activated PRP, which make it suited as a cell delivery vehicle. Many of these researches combine PRP with biomaterials, cells, and, in some cases, recombinant growth factors. Although the experimental design frequently does not allow for judgments on the pro-regenerative effect of PRP, the majority of articles show positive results when PRP is introduced to the tissue-engineered construct. Furthermore, it was established that the release of growth factors from PRP can be customized and regulated by combining PRP with growth factor-capturing materials. Platelet-derived preparations (PRP and PL) are a promising source of autologous growth factors that can be used as a cell culture supplement or to induce regeneration in tissue-engineered constructions. Furthermore, activated PRP shows promise as an autologous cell carrier. However, research exploring PRP in these circumstances frequently provide inconsistent results, which are most likely due to a lack of standardized preparation methods, notably in terms of platelet composition and donor variance. Finally, the use of PRP must be customized for each application (12).

Soft tissue abnormalities can be treated using autologous fat grafts or adipose-derived stem cells (ASCs). However, the results are inconsistent and can include tissue resorption and necrosis. This could be owing to poor vascularization. Platelet-rich plasma (PRP) contains concentrated autologous platelets. Platelet-derived growth factors and cytokines can promote angiogenesis. The concomitant use of PRP may boost the regeneration potential of fat grafts. The ideal ratio has yet to be identified. PRP preparation produces platelet-poor plasma (PPP). PRP and PPP can both be utilized to increase ASCs in cell culture without the use of xenogeneic chemicals. A PRP concentration greater than 20% exerts inhibitory effects (13).

Platelet-rich plasma (PRP) is a biological therapy that stimulates biological processes like cell growth. The extent of PRP's effect is determined by a variety of parameters, the most important of which is the composition of PRP. The study aimed to investigate the correlation between cell proliferation and growth factors (namely IGF-1, HGF, PDGF, TGF-β, and VEG) in platelet-rich plasma. First, PRP's composi-

tion and effect on cell proliferation were compared to platelet-poor plasma (PPP). The association between each PRP growth factor and cell proliferation was then investigated. Cell proliferation was greater in cells treated with PRP lysates than in cells treated with PPP lysates. PRP showed considerably greater levels of PDGF, TGF-β, and VEGF. When the PRP growth factors were examined, only IGF-1 showed a significant correlation with cell proliferation. IGF-1 levels were the only ones that did not link with platelet levels. The extent of PRP's effect depends not only on platelet count, but also on platelet-independent molecules (14).

Platelets produce platelet growth factors (as PDGF, IGF-1, EGF-, HGF, TGFβ, bFGF, and VEGF) that regulate wound healing at all stages. These chemicals are derived from platelet-rich plasma. Over the last five decades, there has been a substantial growth in interest and utilization of platelets' regenerative qualities in a variety of medical sectors around the world. PRP and PRF derived preparations are used in dentistry, sports medicine, as well as dermatology, and cosmetology to treat alopecia, further hair reconstruction (FGF-7, HGF), acne scars, skin so in rejuvenation and regeneration, chronic wounds, burns, and acquired vitiligo. Another important domain is represented by gynecology and reproductive medicine (as treatment of infertility, but also erectile dysfunction - using PDGF-β, TGF-β, IGF-1, sexual dysfunction - PDGF, vaginal atrophy). Ophthalmology is becoming more and more important from the point of view of using platelet-rich plasma derivatives (as healing of corneal epithelial wounds, dormant corneal ulcers, dry eye syndrome and, last but not least, corneal surface reconstruction). Treatments in neurology are involving also substances obtained from platelet-rich plasma (speaking about regeneration of neurons, pain relief, and clinical symptoms -through the use of TGF-β 1, IGF-1, PDGF, VEGF, and FGF). So, platelet-rich plasma therapy is a very interesting alternative and supplement to established treatment procedures. However, the potential use of platelets is not entirely known. Many factors influence the composition of platelet-rich plasma, which can have an impact on its efficacy and clinical advantages. Additional study is required to standardize PRP delivery preparation protocols and strategies for a specific disease entity or clinical instance (15).

Mesenchymal stem cells (MSC) added to bone replacement materials can aid in bone repair. Bone development in bio composites is highly reliant on the type of biomaterial, its pre-treatment, and the cells used. Supplements that may be immunogenic or infectious, such as fetal calf serum (FCS), should not be used in cell expansion media. Cells cultured in two different mediums were examined on several biomaterials for bone forming capacity following ectopic implantation in vivo, as well as growth rate and differentiation capacity in vitro. The replacement of FCS with PRP removed the hazards associated with the use of xenogeneic supplements. It increased the expansion of MSC and retained their differentiation and in vivo bone forming potential in an environment suitable for autogenous usage (16).

Implant therapy is a popular therapeutic option in dentistry and orthopedics, however it is frequently accompanied with an increased risk of microbial contamination of the implant sites, which can cause bone tissue damage. This work intends to create two silver-enriched platelet-rich plasma (PRP) multifunctional scaffolds that can

prevent implant-associated infections while also boosting bone repair. In vitro tests were conducted on both commercial silver lactate (L) and freshly synthesized silver deoxycholate: β-Cyclodextrin (B). Initially, the antibacterial activity of the two silver soluble forms and PRP supplemented with the two silver forms was investigated on microbial planktonic cells. At the same time, the biocompatibility of silver-enriched PRPs was evaluated using an MTT test on human primary osteoblasts (hOBs). Following that, a study was done to assess the efficacy of various concentrations and types of silver-enriched PRPs in suppressing microbial biofilm formation and boosting hOB differentiation. PRP-L (0.3 μg/mm2) and PRP-B (0.2 μg/mm2) inhibit planktonic cell proliferation and biofilm formation in Staphylococcus aureus, Staphylococcus epidermidis, and Candida albicans, while retaining hOB viability and differentiation capability. As a partial conclusion, the results obtained suggest that L- and B-enriched PRPs could constitute a promising preventive strategy against biofilm-related implant infections and can be used as a new silver formula that, in addition to increasing fibrin binding protecting silver into the truncated cone-shaped cyclic oligosaccharides, to be able to achieve comparable inhibitory results on prokaryotic cells, but with a lower concentration (17).

Regulatory authorities oppose the use of fetal bovine serum (FBS) as a cell culture supplement in order to reduce the danger of zoonotic and xenogeneic immunological reactions in the transplanted host. Furthermore, FBS production has come under fire because to animal welfare problems. Platelet derivatives have been proposed as FBS alternatives for the ex-vivo expansion of mesenchymal stem/stromal cells (MSCs), as platelet-derived growth factors can enhance MSC multiplication. Platelet-derived growth factors can be found in platelet lysate (PL), which is generated by freezing and thawing platelet-rich plasma repeatedly or by adding physiological stimuli such as thrombin or CaCl2.PL-expanded MSCs have already been used in clinical trials, taking advantage of their quicker proliferation compared to FBS-expanded preparations. If PL is used to other biopharmaceutical drugs, demand is anticipated to skyrocket. The use of fresh platelet units in the manufacturing of PL poses problems because to the scarcity of platelet donors. Expired units could be an option, however further information is needed to determine safety, including pathogen decrease, and usefulness of the obtained PL. Furthermore, significant problems about the establishment of PL release criteria, such as the concentration ranges of certain growth factors in PL batches for different therapeutic reasons, must be addressed. We are still distant from a uniform definition of PL and standardized PL production due to our poor knowledge of the processes that drive PL-promoting cell proliferation (18).

Dental pulp stem cells (DPSCs) are intriguing candidates for therapeutic use due to their high proliferative capability, multipotent differentiation, immunomodulatory properties, and lack of ethical problems. Clinical research on DPSCs is still in its early phases. The failure to obtain clinically effective results may be due to issues with the DPSC production process. Cell properties may be impacted by differences in DPSC preparation procedures and reagent formulas, resulting in conflicting experimental outcomes. The preparation of clinical-grade DPSCs is far from complete. To reach therapeutic use, it is required to transition the manufacture of stem cells

from laboratory to clinical grade (19).

Human platelet lysate (PL) as well as human platelet lysate serum (PLS) are used as cell culture supplements instead of fetal bovine serum (FBS) due to ethical concerns, batch variability, and the possibility of introducing xenogenic pollutants. Some studies examined the content and efficiency of PL, PLS, as well as FBS as supplements during the culture and cryopreservation of human gingival fibroblasts, also Wharton's jelly-derived mesenchymal stem cells (WJ-MCS), and finally adipose tissue (AdMSC). Biochemical components, certain growth factors, and cytokines were examined in each of them; additionally, the cells were cultivated in media supplemented with 5% PL, 5% PLS, and 10% FBS and exposed to various freezing and thawing solutions containing the supplements under investigation. Biochemical parameters in PL and PLS were found to be similar to those in FBS, with some variations in fibrinogen and calcium concentrations. PL and PLS contained higher levels of growth factors and cytokines than FBS. Cell proliferation and morphology did not differ significantly between the three culture mediums. PLS and FBS provided better results for cell cryopreservation and thawing. To summarize, PL and PLS are an ideal choice to substitute the conventional supplement of animal origin (FBS) in the media necessary for the culture and cryopreservation of fibroblasts, WJ-MSC, and AdMSC (20).

CONCLUSIONS

Exosomes were found to reduce β-galactosidase concentrations in senescent gingival fibroblasts by around 20% on average. Exosomes were also delivered in combination with: LY-294002 (a non-selective inhibitor of phosphatidylinositol 3-Kinase (PI3K) pathway, α/δ/β, 15% on average); nutlin-3 (a potent and selective small-molecule MDM2 antagonist that activates p53, 13% on average); berberine (which protects against MSC apoptosis by preventing ROS-dependent, as well as JNK-driven cell apoptosis, in a PI3K/Akt-dependent manner, 23% on average); resveratrol (may improve the therapeutic effects of MSCs by enhancing their survival, as well as self-renewal, lineage commitment, and anti-aging effects, 52% on average); collagen (promotes the secretion of the chemokines and growth factors by MSCs, 61% on average); thrombin (promotes fibronectin secretion by MSCs via the protease-activated receptor mediated signaling pathways, 58% on average); and fibronectin (supports self-renewal of MSCs through stabilization of bFGF and activation of autophagy, 48% on average. On the other side, lipopolysaccharide (which might induce a proinflammatory profile on iPSC-MSC) enhanced the production of β-galactosidase (37% on average).

All of these findings suggest that exosomes derived from pluripotent stem cells induced from mesenchymal stem cells and administered in the culture medium of senescent gingival fibroblasts can influence the degree and evolution of senescence in these cells by potentiating, activating, or stimulating some intracellular biological signal transduction pathways, particularly those involving resveratrol, collagen, thrombin, and fibronectin.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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