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Collagen I Gel Increases the Osteogenic Potential of Platelet-Rich Plasma in Adipose-Derived Stem Cells

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Abstract

Objectives: Adipose-derived mesenchymal stem cells (ASCs) have osteogenic potential. Platelet-rich plasma (PRP) is an alternative natural replacement for osteogenic growth factors. The present study evaluated the combinatory effect of human PRP (hPRP) and collagen I (Col I) gels on the osteogenic potential of ASCs.

Materials and Methods: In current experimental research, the extracted ASCs from the pararenal fat pad, at passage 3 were used for the experiments. The osteoinductive potential of ASCs was examined by culturing the cells in cell culture media supplemented with 10% hPRP, 10% Col I, and 10% hPRP/Col I. Finally, metabolic activity, osteoblast differentiation, and mineralization were assessed through the MTT method, alkaline phosphatase assay, Von Kossa method, and staining of osteocalcin (OCN) immunocytochemistry, respectively.

Results: Based on the results, 10% hPRP gel, 10% Col I gel, and 10% hPRP/Col I gel increased the metabolic activity and proliferation of ASCs ($P<0.05$). In addition, the activity of alkaline phosphatase in ASCs, supplemented with 10% hPRP/Col I gel was extremely higher compared to the other groups on days 7 and 14 ($P<0.05$). Further, calcified nodules were evident on day 14 after the osteogenic stimulation of ASCs which were cultured in 10% hPRP/Col I gel. Eventually, positive OCN expression was detected in 10% hPRP/Col I gel on days 7 and 14.

Conclusions: These findings indicated that the combination of hPRP and Col I gels provides a natural biomaterial for increasing the proliferation and osteoblast differentiation of ASCs.

Keywords: Osteogenesis, Adipose-derived stem cells, Platelet-rich plasma, Collagen I gel

Introduction

Adult stem cells and growth factors are employed in regenerative medicine. These cells contain bone marrow, dental, periosteal, umbilical, induced pluripotent, synovial membranes, and adipose (1). In addition, bone marrow, periosteal, adipose, and dental stem cells are frequently used in bone engineering (2). Adipose-derived mesenchymal stem cells (ASCs), unlike bone marrow stem cells, are readily and less-invasively extracted and have a considerable proliferative rate in the cell culture media (3). Although ASCs are considered as a suitable option for bone regeneration, their solo usage possesses only partial results in the osteogenesis (4). Therefore, employing efficient osteoinductive factors to enhance the osteogenic potential of ASCs is increasing (5).

According to Marcazzan et al (6), numerous research has focused on the osteoinduction of ASCs resulting in using osteogenic medium and different growth factors like dexamethasone, acid ascorbic, bone morphogenetic proteins, fibroblast growth factor, vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β). However, the appliance of growth factors has some limitations including high cost, short half-life, and high toxicity when used in high-concentrations. Therefore, utilizing a source that contains multiple growth factors to be able to accelerate bone regeneration would be a simple way to overcome the difficulties mentioned earlier. In this regard, human platelet-rich plasma (hPRP) is used as a natural and autologous source of growth factors for clinical applications (6). More precisely, hPRP seems to be a suitable alternative approach for improving osteogenesis because of having growth factors such as insulin growth factor, platelet-derived growth factor, TGF-β, VEGF, and epidermal growth factor (7,8). It has been reported that collagen (Col) could be used as controlled-release systems for PRP (9,10). Considering the above-mentioned

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explanations, this study investigated the impact of the combination of PRP gel and Col I gel on the osteogenic differentiation of ASCs without any supplementation.

Materials and Methods

Human Platelet-Rich Plasma Preparation

hPRP was prepared from the Tabriz Transfusion Organization. The final density of platelets in hPRP was analyzed in an automatic counter and the concentration of used PRP in this research was 10%.

Preparation of Col I From the Tail of the Rats

According to the protocol of previous research (11), type I COL fibers were obtained from the tail tendons of the rats. Tendons were placed in 70% ethanol for 1 hour and then stirred for 7 days in 200 µL/g (5 mmol) glacial acetic acid at 4°C. The resultant suspension was centrifuged at 15 000 rpm at 4°C for 40 minutes. After removing the supernatant, the Col concentration was increased to 10%. To sterilize Col, chloroform steam was used for 24 hours at 4°C. Next, the solution was reached to the pH of 7.3 with the sterile NaOH buffer (Sigma Aldrich, Product No.: 221465) and ice-cold phosphate-buffered saline (PBS, Sigma Aldrich, Product No.: P4417), and then mixed thoroughly in a falcon. For gel formation, it was poured into Petri dishes (SPL, Cat. No.: 20060, Korea) and incubated for 1 hour at 37°C.

ASCs Isolation and Culture

Fresh adipose tissues from the pararenal fat pad were obtained from the rats (12), followed by rinsing the removed adipose tissues in sterile phosphate-buffered saline (PBS), mincing with a sharp scalpel, and incubating in 0.5 mg/mL collagenase I (dissolved in PBS) per gram of tissues with gentle shaking at 37°C for 40 minutes. Furthermore, an equal volume of the cell culture medium (DMEM, Gibco, Product Code: 11594446) containing 10% FBS (Gibco, Product Code: 11573397) and 1% antibiotic penicillin/streptomycin (Gibco, Product Code: 11548876) was added to neutralize the collagenase.

Then, the cellular solution was centrifuged at 1500 rpm for 5 minutes and the cellular pellet was resuspended in the cell culture medium. Moreover, the cells were cultured in 25-cm^2 flasks (SPL, Cat. No.: 70025, Korea) and preserved in the medium at 37°C in 5% CO_2. The medium was replaced every three days with a fresh medium to reach 90% confluence. Next, the confluent culture of ASCs was passaged by 0.25% trypsin- ethylenediaminetetraacetic acid (Gibco, Product Code: 11560626), and ASCs were at passage 3. To investigate osteogenic differentiation, 4 × 10^3 ASCs were cultured in 4 groups of biomaterials in 6-well plates. The groups were control medium consisting of DMEM containing 10% FBS and 1% penicillin/streptomycin (the ASC control group), control medium with 10% hPRP gel (the ASCs/10% hPRP group), control medium with 10% Col I gel (the ASC/10% Col I group), and control medium with %10 hPRP gel and Col I gel (the ASCs/%10 hPRP/Col I group).

Metabolic Activity

The MTT examination reveals cellular metabolic activity and measures the number of viable and dead cells. In this method, 3000 ASCs per 96-well were seeded in 4 groups encompassing the control medium (the ASC control group), 10% hPRP (the ASCs/10% hPRP group), 10% Col I (the ASCs/10% Col I group), and 10% hPRP and Col I (the ASCs/10% hPRP/Col I group). Each well contained 200 µL of cell suspension. After 24 and 48 hours of the cell culture period, the MTT examination was performed to determine the viability of the cells. Briefly, after removing the medium, the 200 uL medium without FBS containing 20 uL of the MTT (5 mg/mL) solution was poured into each well and incubated for 4 hours at 37°C and 5% CO_2. Following the incubation with 100 µL dimethyl sulfoxide (Sigma-Aldrich, Germany), the process of cell lysis was carried out by pipetting up and down. The absorption of the medium was measured by an enzyme-linked immunosorbent assay reader (BioTek Instruments, Inc., VT 05404, USA) at a wavelength of 570 nm. This exam was repeated three times.

Alkaline Phosphatase Analysis

The osteoblast differentiation of ASCs was evaluated using the analysis of ALP activity, bone mineralization, and osteocalcin (OCN) expression. To access ALP activity, 40 ×10^3 ASCs were cultured on 6-well and investigated at 7 and 14 days. The cells were then categorized into 4 groups as the control medium (the ASC control group), 10% hPRP (the ASCs/10% hPRP group), 10% Col I (the ASCs/%10 Col I group), and10% hPRP and Col I (the ASCs/10% hPRP/Col I group). On days 7 and 14, ALP activity was measured by a commercial kit (No.: 97003; Pars Azmoon Company, Iran) based on the manufacturer's protocol. After removing the medium and washing it in PBS, the cells were lysed by 0.5 mL 2% Triton X-100 (Sigma Aldrich, product No.: X 100) and ultra-sonication. Then, the solution was centrifuged at 12 000 rpm for 10 minutes at 4°C. This supernatant was collected to measure ALP. Next, the absorbance was measured at a wavelength of 450 nm (Biolis 24i, Tokyo Boeki Medisys Inc., Japan). Eventually, ALP activity was expressed as unit/ mg protein and the experiment was performed in triplicate.

Von Kossa Staining for Extracellular Matrix Mineralization

To visualize osteogenesis and extracellular matrix (ECM) ECM mineralization, von Kossa staining was performed 7 and 14 days after the culture of 4 × 10^3 ASCs per well in 4 groups as the control medium (the ASC control group), 10% hPRP (the ASCs/10% hPRP group), 10% Col I (the ASCs/10% Col I group), and10% hPRP and Col I (the ASCs/10% hPRP/Col I group) and placed in 6-well plates. It should be noted that ECM mineralization was

evaluated according to the protocol of (13). In brief, ASCs were fixed with 4% parforfaldehyde (Sigma Aldrich, product No.: 158127) for 30 minutes after rinsing twice with PBS. Subsequently, the samples were washed in distilled water and incubated in dark in a 5% silver nitrate solution (Sigma Aldrich, product No.: 209139), followed by its exposure to the UV light for 1 hour. After extensive washing, the cells were fixed by 5% sodium thiosulfate (Sigma Aldrich, product No.: 72049) for 5 minutes and finally stained by 0.1% nuclear fast red (Sigma Aldrich, product No.: 60700) for 5 minutes. The images were obtained by a light microscope (Nikon ECLIPSE E100, Japan) at 40X magnification and the calcified ECM was observed as black deposits.

Immunocytochemistry

The immunocytochemistry (ICC) technique was conducted, according to the protocol of (13), to detect the expression levels of OCN. In this method, after the culture of 4×10^3 ASCs on the above-mentioned 4 biomaterial groups at the end of days 7 and 14, ASCs were fixed with 4% parforfaldehyde about 20 minutes at room temperature after rinsing twice in PBS. The cells were then rinsed in cold PBS and treated with Triton X-100, goat serum, and PBS. In the following steps, the cells were incubated with primary antibodies against OCN (Human/Rat OCN antibody MAB1419, UK) at a dilution of 1:100 at 4 °C overnight. Next, the ASCs were rinsed by PBS 4 times (every 5 minutes) and incubated in a secondary antibody conjugated with a fluorescent probe (goat anti-mouse IgG-PE, sc-3738, USA) at a dilution of 1:200 at 37°C for 2 hours. Eventually, the ASCs were rinsed 4 times with PBS and stained with dye 4'-6-Diamidino-2-phenylindole (Cat. No. 11718096001, Roche, Germany) for 30 seconds. The images were taken by a fluorescence microscope (Carl Zeiss, Germany) at 20X magnification.

Statistical Analysis

The data analysis was done by SPSS, version 22. The difference between the experimental groups was evaluated by ANOVA and Tukey test. The obtained values were indicated as the mean ± standard deviation (SD) and a $P<0.05$ was considered a significant value.

Results

Platelet Counts in Human Platelet-Rich Plasma

The mean platelet number of hPRP samples was 107×10^4 platelets, which were 5-fold higher than the baseline.

Macroscopic and Microscopic Morphology of ASCs in the Gels After 24 Hours

ASCs were observed as floated aggregates in the macroscopic images of the groups that were treated with hPRP and collagen I (Col I) gels. Long protrusions were also observed in the cells which were treated with PRP gels (Figure 1).

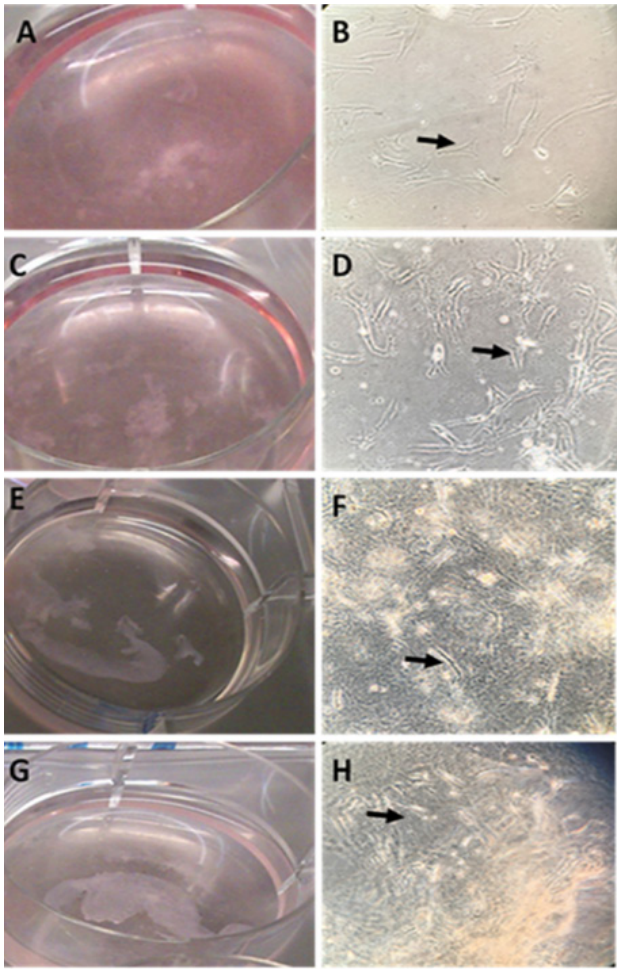

Figure 1. Macroscopic and Microscopic Morphology of ASCs After 24 Hours
Note. ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I. ASC control group (A and B), ASCs/10% hPRP group (C and D), ASCs/10% Col I (E and F), and ASCs/10% hPRP/Col I (G and H). ASCs have fibroblast-like morphology in microscopic images. In addition, the arrow indicates ASCs in B, D, E, and F at ×20 magnification.

High Metabolic Activity and Proliferation of ASCs Cultured in Media Supplemented With PRP and Col Type I

The MTT assay was performed 24 and 48 hours after the ASC culture to examine their viability. As illustrated in Figure 2, the viability and proliferation rate of ASCs significantly increased in all groups from 24 to 48 hours. In addition, the viability of ASCs treated with 10% hPRP, 10% Col I, and 10% hPRP/Col I was greater than that of the ASC control group at 24 and 48 hours ($P<0.05$). However, no significant difference was observed between 10% hPRP, 10% Col I, and 10% hPRP/Col I groups ($P>0.05$). Finally, the MTT assay showed that 10% hPRP, 10% Col I, and 10% hPRP/Col I had no toxicity on ASC viability.

Alkaline Phosphatase Activity Increase After the Culture of ASCs on PRP/Col I

The osteoinductive potential of hPRP and col I was determined through the culture of ASCs in control, 10% hPRP, 10% Col I, and 10% hPRP/Col I groups by Alkaline

Phosphatase (ALP) analysis on days 7 and 14. Based on the results (Figure 3), the ALP activity increased in ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I groups from 7 to 14 days compared to the control group. Further, this activity was significantly higher in the ASCs/10% hPRP/Col I group when compared to the other groups (P<0.05). Although the enzyme activity increased in ASCs/10% hPRP and ASCs/10% Col I groups at days 7 and 14, no significant difference was detected between these groups (P>0.05).

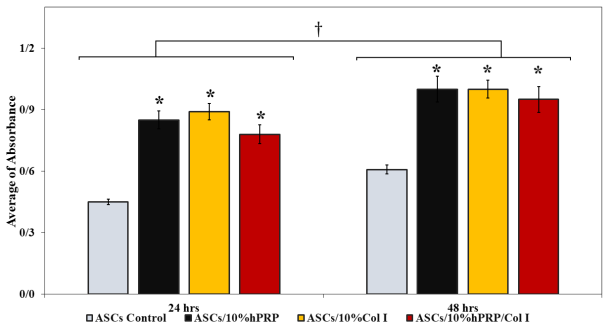

Figure 2. The Viability of ASCs Measured in All Experimental Groups Including ASC Control, ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I by MTT Assay

Note. ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide. A significant increase was observed in the viability and proliferation of ASCs in ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I groups compared to the control group at 24 and 48 hours. † indicates a significant difference in the viability and proliferation of ASCs in all experimental groups at 48 hours compared to 24 hours (P<0.05). In addition, * shows a significant discrepancy when ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I groups were compared to the control group at 24 and 48 hours (P<0.05).

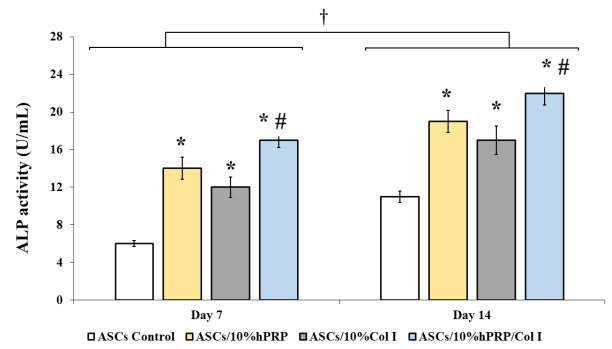

Figure 3. The Activity of ALP in ASCs Cultured in ASC Control, ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I Groups

Note. ALP: Alkaline phosphatase; ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I. The activity of ALP increased in all experimental groups on day 14 compared to day 7. Further, the enzyme activity of the ASCs/10% hPRP/Col I group was considerably higher than that of the other groups on days 7 and 14. † indicates a significant difference in the ALP activity in all groups on day 14 compared to day 7 (P<0.05). * demonstrates a significant difference in the ALP activity of ASCs treated with ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I groups in comparison to the control group at days 7 and 14 (P<0.05). Furthermore, # represents significant differences between the ASCs/10% hPRP/Col I group and the other experimental groups at days 7 and 14 (P<0.05).

Calcified Nodules in PRP/Col I by von Kossa Staining

To identify the presence of ECM mineralization, the Von Kossa method was conducted after 7 and 14 days of ASCs cultured in control, 10% hPRP, 10% Col I, and 10% hPRP/Col I groups (Figure 4 A-D). No calcification was apparent at day 7 in all experimental groups (Figure 4 A-D). Contrarily, specified calcified nodules were visible at day 14 in the ASCs/10% hPRP/Col I group (Figure 4 H) whereas fine calcification was detected in cells in other experimental groups at day 14 (Figure 4 E-F-G).

Osteocalcin Expression in ASCs Supplemented With PRP/Col I

As shown in Figures 5 and 6, the immunocytochemical staining revealed the positive OCN expression in the ASCs/10%hPRP/Col I group at days 7 and 14 although it was not observed in the other groups.

Discussion

In this study, the 10% hPRP/Col I was used to improve the osteoblast differentiation of ASCs. The results represented the positive effects of this mixture on osteogenic differentiation. In bone cell therapy, identifying a special cell with osteogenic ability is important although the limitations of this selection often include their accessibility, invasive procedure, donor site morbidity, biocompatibility, and immunogenicity (14). Common stem cells for bone therapy consists of embryonic, fetal, and adult stem cells. Although embryonic and fetal stem cells possess more osteogenic differentiation potential compared to adult stem cells, their applications are restricted due to ethical issues (1). Among adult stem cells, bone marrow stem cells (BMSCs), osteoprogenitor, ASCs, and dental pulp are mostly used for bone therapy. Furthermore, BMSCs and osteoprogenitors have higher osteogenic capacity, but their extraction is more invasive and their proliferations are lower compared to ASCs (2). ASCs could be considered as a favorable substitute for BMSCs regarding bone therapy. With the similar multilineage potential of BMSCs, ASCs have easy and less invasive extraction procedures and a high proliferation rate (15). Thus, to promote the osteogenic potential of ASCs, the present study used the activated form of the hPRP gel as an osteoinductive factor because the use of ASCs alone has partial osteogenic success (4). Additional effective agents such as osteogenic growth factors, mechanical stimulations, and chemical substances are usually used to increase osteogenicity (8). Moreover, growth factors such as bone morphogenetic proteins, fibroblast growth factor, Notch, and hedgehog increase osteogenesis. On the other hand, the use of growth factors alone displays certain shortcomings including high cost, short half-life, and toxicity in high concentrations (8). Accordingly, identifying an arsenal of growth factors such as hPRP can help to accelerate bone regeneration. In this study, the number of platelet in the hPRP was estimated as 107×10^4 platelet/mL, which was a 5-fold higher than

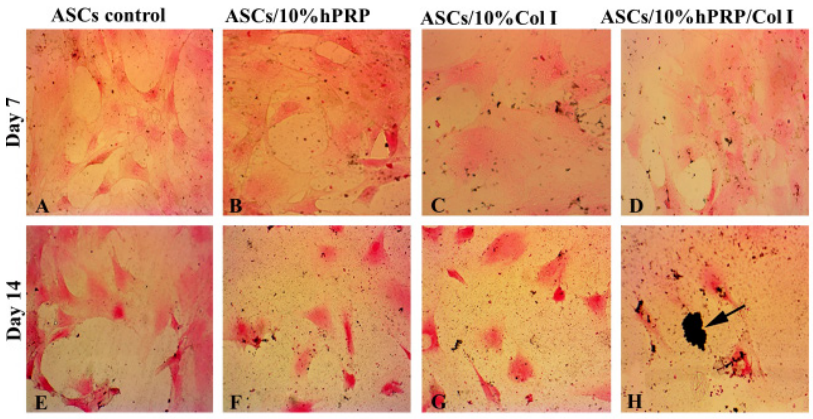

Figure 4. Osteogenic Differentiation of ASCs Visualized After 14 Days in ASCs Control, ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I groups by Von Kossa Staining

Note. ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I. Fine calcification was observed in ASCs on day 7 in all experimental groups (A-D). Moreover, the calcified nodules are evident at day 14 in the ASCs/10% hPRP/Col I group (the arrow in H) whereas the calcification process was shallow in other experimental groups (images E, F, and G at ×40 magnification).

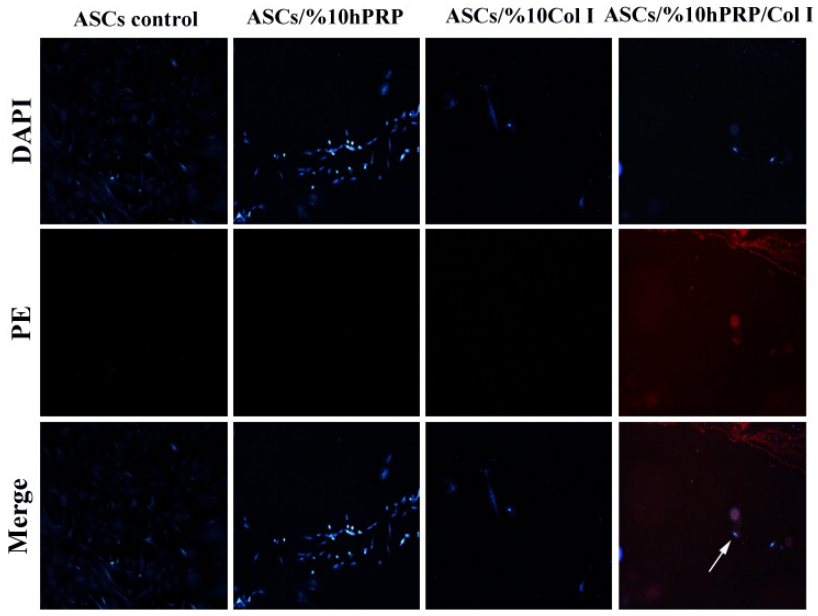

Figure 5. The Expression of OCN Detected by the Immunocytochemical Analysis in ASC Control, ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I Groups

Note. OCN: Osteocalcin; ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I. After 7 days, ASCs were stained with phycoerythrin-conjugated antibodies against OCN and dye 4′-6-Diamidino-2-phenylindole (DAPI). Additionally, positive OCN was detected in the ASCs/10% hPRP/Col I group at day 7. The blue color indicates the cell nucleus stained with DAPI and arrows show positive staining for OCN in the ASCs/10% hPRP/Col I group on day 7 at ×20 magnification.

the baseline value. Marx et al demonstrated the positive effects of hPRP on bone healing when employed at a range of 595 000 to 1 100 000 cells per mL platelet (16). PRP, a part of the plasma, possesses more concentrations of platelet in comparison to the baseline value (7, 17). Next, the current study evaluated the biocompatibility of hPRP and Col I on the proliferation rate of ASCs through the MTT test and demonstrated 10% hPRP/Col I enhancement in cell proliferation and there were no adverse effects in 24 to 48 hours compared to the control group (Figure 2). The results (Figure 3) of this study also showed that the combination of hPRP and Col I is suitable for the osteogenesis of ASCs. To explore the osteogenesis and mineralization of ASCs in *in vitro*, ALP activity, OCN immunohistochemical staining, and von Kossa staining were investigated as well. Some aspects of data suggested that the incorporation of hPRP and Col I is effective in the osteogenesis of ASCs. For instance, the ALP activity increased during hPRP/Col I stimulation from about 7 to 14 days relative to the control group. In addition, the

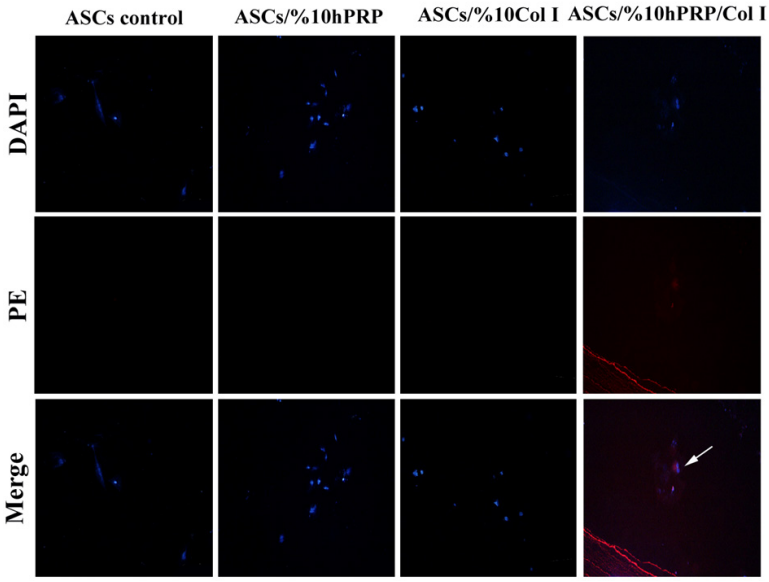

Figure 6. The Expression of OCN Detected by the Immunocytochemical Analysis in ASC Control, ASCs/10% hPRP, ASCs/10% Col I, and ASCs/10% hPRP/Col I Groups

Note. OCN: Osteocalcin; ASCs: Adipose-derived mesenchymal stem cells; hPRP: Human platelet-rich plasma; Col I: Collagen I. After 14 days, ASCs were stained with phycoerythrin-conjugated antibodies against OCN and dye 4′-6-Diamidino-2-phenylindole (DAPI). In addition, positive OCN was detected in the ASCs/10% hPRP/Col I group at day 14. The blue color indicates the cell nucleus stained with DAPI and arrows show positive staining for OCN in the ASCs/10% hPRP/Col I group on day 7 at ×20 magnification.

increase in the hPRP/Col I group was greater on day 14 compared to the other groups (P<0.001).

Additionally, in the von Kossa staining at the ASCs/hPRP/Col I group, positive deposits, and diffused mineralization were found at day 14 after cell culture compared to the other groups (Figure 4). Similarly, OCN protein was detected using immunocytochemistry (positive for OCN) in the ASCs/hPRP/Col I group at day 14 after cell culture while it was not observed in the other groups in 7 to 14 days (Figures 5 and 6). ALP is considered as a routine measurement tool for defining osteogenic differentiation (osteoblastic marker). In addition, the initiation of osteoblast differentiation and bone mineralization can be recognized by the high ALP activity (18, 19). Further, ALP increases the phosphate concentration prerequisite for hydroxyapatite crystallization, nucleation, and ECM mineralization (20-22). Based on our data, hPRP increased ALP activity at days 7 and 14 (~2 time higher) in the ASCs/10% hPRP/Col I group compared to the other groups (Figure 3). These outcomes are like the prior information that indicated increased ALP activity, increased osteogenic differentiation of ASCs in ASCs/HA/PRP (23), or BMSc/10% PRP/calcium phosphate (24, 25).

OCN is a late specific bone matrix marker for osteoblast differentiation and bone mineralization, which significantly increases during ECM mineralization (26). In our study, the positive OCN marker on day 14 indicated that the cultured ASCs in %10 hPRP/Col I differentiated into osteoblasts (Figures 5 and 6). Osteogenesis differentiation was also confirmed by observing mineralized nodule formation in the Von Kossa staining in ASCs /10% hPRP/Col I groups on day 14. These conclusions are comparable to those of some other previous studies (27-29). The Von Kossa method is usually used for identifying mineralized ECM calcium nodules (29). According to the results of this study, the cause of osteogenic differentiation may be contemporary to the cultivation of ASCs in the presence of hPRP/Col I compared to the other groups. hPRP contains more than 300 bioactive molecules which are abandoned by activation (30).

Several of these osteoinductive molecules such as transforming growth factor-β1, platelet-derived growth factor, FG-b, insulin growth factor, and vascular endothelial growth factor may promote the osteogenesis of MSCs (7). Thus, exposure to hPRP stimulates ASCs toward osteogenic differentiation. Furthermore, growth factors and cytokines are released and the PRP gel is formed after the activation of PRP (31). PRP gel can affect bone repair because of the significance of their growth factors on the proliferation and differentiation of the stem cells (32). Moreover, PRP is generally used in the field of orthopedics for healing the defects. Additionally, Li et al reported that hPRP can promote osteogenesis (24). In another study, Yamada et al showed that PRP increased the osteogenesis of MSCs (33). Likewise, Sanchez et al found the significant effect of PRP on bone formation (34). Despite the positive features of PRP, there are some conflictive results in improving the differentiation of mesenchymal stem cells (35-37). Several studies have suggested that PRP has little effect on osteogenesis. For example, Arpornmaeklong et al

exhibited that PRP reduces the osteogenesis and calcium deposition of BMSCs (38). In addition, Plachokova et al found that PRP was not beneficial for bone repairing (39). Further, Choi et al found no effect of PRP on osteogenesis (40). Ranly et al also showed that PRP reduces osteogenesis (41). Different results can depend on soil water, the designed protocols, animals, PRP concentration, stem cell type, and biomaterials. Therefore, further studies are needed to detect the effect of PRP on the osteogenesis of MSCs. In their study, Liu et al used the 10% PRP gel for increasing MSC differentiation and bone regeneration (42). Furthermore, Qi et al confirmed that the 10% PRP gel stimulated the osteoblast differentiation of BMSCs (25). Similarly, Zhou et al stated that 10% PRP increased the osteogenic differentiation of BMSCs (43). In another study, Kazem-Arki et al confirmed that a high concentration of 5% PRP could improve the osteogenesis of ASCs (44). Tavakolinejad et al also used 10%-15% hPRP for increasing the osteogenic differentiation of ASCs (45). The findings of these studies are consistent with those of our study which used 10% hPRP for increasing the osteogenesis of ASCs. The selection of an appropriate hPRP activator before the application is a considerable principle (46). hPRP is often activated by thrombin to eruption growth factors (31). Therefore, the elimination of thrombin is important because of immunological side effects (45). In this study, Col I was used to activate and gelatinize hPRP. A previous study confirmed the absence of significant differences in the density of growth factors between Col I and thrombin as an activator (47). However, using Col I increases the time required for hPRP activation. Moreover, Col I activates hPRP by forming autogenous thrombin to the formation of a gel matrix and thus leading to the release of the growth factors (31). The clotting cascade by Col I is slowly, which prepares the possibility of injecting hPRP before gelation. Col I is considered as an appropriate carrier for the cells and growth factors in the osteoblastic lineage (48). Additionally, Col I is fundamental to bone regeneration because of its high biocompatibility, osteoinductive, osteoconductive, absorbability, and mechanical properties and can activate PRP (49). Some studies suggested that Col combined with PRP is suitable for osteogenesis, growth factor, and stem cell delivery (50). For instance, Scioli et al reported successful osteogenesis of ASCs in PRP/Col combination (51). Nonetheless, some evidence stated contradictory outcomes in this regard. For example, Pryor et al and Goyal et al found no successful bone regeneration with PRP/Col in rat calvaria defects or apicomarginal defects (52, 53). The percentage of the PRP gel and the type of stem cells in our study were different from those of other studies. In addition, the purpose of the application of Col type I was to convert the liquid form of PRP to the gel form and the release of its growth factors. Based on the results of this study, the synergistic application of hPRP and Col type I enhances the osteogenic activity of ASCs, and combining ASCs with hPRP and Col I gels is an efficient approach for clinical bone repair.

Conclusions

The findings of the present study showed that 10% hPRP/Col I gel not only is not toxic to viability and proliferation of ASCs but also enhances the osteogenesis of ASCs. In addition, comparing alkaline phosphatase activity, mineralization, and OCN immunocytochemical staining among the groups demonstrated the synergistic effect of hPRP and Col I gels that could improve the osteogenesis of ASCs. However, further studies are required to investigate the impact of this approach for the *in-vivo* application.

Conflict of Interests

None declared.

Ethical Issues

This research was performed in line with the Ethical Committees of Tabriz University of Medical Sciences, Tabriz, Iran (Ethics No. TBZMED.REC.606175).

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