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ORIGINAL RESEARCH

Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue, and Dental Pulp as Sources of Cell Therapy for Zone of Stasis Burns

Ozan Luay Abbas, MD, Assistant Professor [1], Orhan Özatik, MD, Assistant Professor,[2] Zeynep Burçin Gönen, DDs, PhD, Assistant Professor,[3] Serdal Öğüt, PhD, Associate Professor,[4] Fikriye Yasemin Özatik, MD, Assistant Professor,[5] Hasan Salkın, MSc,[6] Ahmet Musmul, PhD[7]

[1]*Ahi Evran University, Faculty of Medicine, Department of Plastic, Reconstructive and Aesthetic Surgery, Kırşehir, Turkey,* [2]*Dumlupınar University, Faculty of Medicine, Department of Histology and Embryology, Kütahya, Turkey,* [3]*Erciyes University, Gen Kök Genome and Stem Cell Center, Kayseri, Turkey,* [4]*Adnan Menderes University, Faculty of Health Science, Department of Nutrition and Dietetics, Aydın, Turkey,* [5]*Dumlupınar University, Faculty of Medicine, Department of Pharmacology, Kütahya, Turkey,* [6]*Beykent University, Vocational School, Department of Medical Services and techniques, Istanbul, Turkey,* [7]*Osmangazi University, Faculty of Medicine, Department of Biostatistics, Eskişehir, Turkey*

ABSTRACT

Introduction: The implantation of mesenchymal stem cells (MSCs) has been shown to exert benefits for the survival of the zone-of-stasis. However, the clinical experience indicates the importance of selecting the right source and type of stem cells. Therefore, we planned the current study to perform a quantitative comparison of MSCs isolated from three different sources to provide information useful in selection of the optimal source and to see whether critical mechanisms are conserved between different populations. *Methods*: The protective effects of MSCs derived from bone marrow, adipose tissue and dental pulp were compared in a rat model of thermal trauma. The stasis zones were evaluated 72 h after the burn using histochemistry, immunohistochemistry and biochemistry. *Results:* Gross evaluation of burn wounds revealed that the differences between the mean percentages of the calculated necrotic areas weren't statistically significant. Semi-quantitative grading of the histopathological findings revealed that there were no significant differences between damage scores. Immunohistochemical assessment of apoptotic and necrotic cell deaths revealed that the differences between the mean numbers of apoptotic and necrotic cells weren't statistically significant. Myeloperoxidase activity was found to be significantly lower in the adipose tissue group. Biochemical and immunohistochemical assessment of tissue malondialdehyde revealed that the differences between the groups weren't statistically significant. Finally, the number of neo-vessels in the dental pulp group was found to be significantly higher. *Conclusion:* Our findings suggest that bone marrow, adipose tissue and dental pulp may serve as a universal donor MSC source for the prevention of burn wound progression.

Keywords: Burn; stasis zone; mesenchymal stem cell; bone marrow; dental pulp; adipose tissue

INTRODUCTION

Burn wounds are dynamic injuries that are characterized by a central area of irreversible coagulation necrosis that may progress to compromise the initially viable tissue in the surrounding ischemic zone-of-stasis during the first few days after the burn.[1] This expansion has significant clinical implications resulting in important therapeutic conundrums.[2,3] Hence, several studies have focused on investigating the mechanisms of burn wound progression in order to develop strategies aimed at preventing the conversion of the zone-of-stasis to necrosis. It has been reported that prolonged acute inflammatory reaction, oxidative stress,

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Address correspondence to Ozan Luay Abbas, MD, Assistant Professor, Ahi Evran University, Faculty of Medicine, Department of Plastic, Reconstructive and Aesthetic Surgery Postal code: 40100, Kırşehir, Turkey. E-mail: ozanluay@hotmail.com

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and impaired tissue perfusion are key to this event.[4] Therefore, a logical strategy to limit the progression of burn wound would be to ameliorate these secondary sources of damage. From this point of view, mesenchymal stem cells (MSCs) are an attractive option because of their ability to self-renew, transdifferentiate, modulate innate immune response and provide trophic support.[5]

In a previous study, using a rat model of contact thermal trauma, we demonstrated that MSC transplantation into the zone-of-stasis significantly reduced burn injury progression (unpublished work). We further documented that the protective effects of MSCs were associated with the decreased susceptibility of cells within the stasis zone to apoptosis through; (1) an immunomodulatory effect that involves the reduced neutrophilic infiltration, upregulation of anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines in the local tissue, (2) attenuation of oxidative stress by the inhibition of free radical generation and stimulation of endogenous antioxidant enzymes, (3) and the promotion of functional angiogenesis within the ischemic zone-of-stasis to maintain and restore blood perfusion.

Although our results suggest that MSC therapy has a significant benefit for the survival of the zone-of-stasis, the overall clinical experience indicates that stem cells can be effectively applied only when the results are reproducible, indicating the importance of selecting the right stem cell type in the right clinical case. Therefore, before the translation of MSC application from such preclinical studies to the clinic, one significant challenge is the identification of the optimal source. This is especially important because there is convincing evidence that MSCs from diverse tissue are different. In addition, the characteristics of MSCs can be affected by their preparation.[6] Therefore, in the current study we performed a quantitative comparison of MSCs isolated from three different sources to provide information useful in selection of the optimal source and to see whether critical mechanisms are conserved between different cell populations.

METHODS

Animals

This study was approved by the Ethical Committee for Experimental Research on Animals and supported by Ahi Evran University Research Fund. All rats used in the current study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health and published by the National Ethics Council. Adult male Wistar rats weighing between 300 and 325 grams were caged individually with 12-h light/dark cycle in a humidity- and temperature- controlled environment and free access to water and food.

Tissue Collection and Isolation of MSCs

1. Bone marrow derived mesenchymal stem cells (BMSCs): The femura and tibia were excised and washed by phosphate-buffered saline (PBS) (Biological Industries, Kibbutz Beit Haemek, Israel). After removing the epiphyses, the bone marrow was flushed into a culture dish using complete medium (low glucose Dulbecco's Modified Eagle's Medium (L-DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). The cell suspension was disaggregated by pipetting several times to break the cell clumps and filtered by a 70-mm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove bone particles. After centrifugation, the pellet containing the cells was re-suspended in complete medium. 3 hours later the not attached cells removed and new media added for ongoing culture.
2. Adipose derived stem cells (ASCs): Approximately $1\times\ 1\ cm^3$ periperitoneal adipose tissue was harvested and washed in PBS containing 1% penicillin/streptomycin solution. Harvested tissue was enzymatically digested with 2.5 mg/ml collagenase type 2 *(Sigma–Aldrich, Taufkirchen, Germany)* for 30 minutes at 37°C. The cell suspension was filtered through 70 μm cell strainer *(Becton Dickinson Labware, Franklin Lakes, NJ, USA)* to separate cells from debris and undigested adipose tissue fragments. Next, the cell suspension was centrifuged and the cell pellet was re-suspended in complete medium.
3. Dental pulp derived mesenchymal stem cells (DPSCs): After disinfection of the tooth surface, the teeth were mechanically fractured and the dental pulp was gently isolated. The pulp fragments were washed in PBS containing 1% penicillin/streptomycin solution and enzymatically digested with 3 mg/ml collagenase type 1 *(Serva Electrophoresis GmbH, Germany)* and 4 mg/ml dispase *(Stem Cell Technologies, Canada)* for 30 minutes at 37°C. After centrifugation, the pellet containing the cells was re-suspended in complete medium: alpha-modified Eagle medium *(Biological Industries, Beit-Haemek, Israel)* supplemented with 2 mM L-glutamine *(Gibco, USA)*, 100 U/ml penicillin, 100 μg/ml streptomycin *(Lonza, MD, USA)*, 20% FBS *(Biological Industries, Beit-Haemek, Israel)* and 100 μM L-ascorbic acid 2-phosphate *(Sigma-Aldrich, USA)*.

Culture of MSCs

The three cell populations were seeded separately into culture flasks containing complete medium. The flasks were cultured in incubator at 37°C and 5% CO_2. The medium was refreshed at the fourth day and subsequently replaced every 3 days to discard the non-adherent cells. After cells reached 80% confluence in the primary culture, they were detached by 0.025% trypsin-EDTA and re-plated at the 1:4 ratio for subculture. For subsequent in vivo studies, MSCs from passage 3 were used.

Flow Cytometric Analysis of MSCs

The following antibodies were used for flow cytometric immunophenotyping of MSCs: CD11b, CD44, CD45, CD73, CD90 and CD105. All of them were purchased from BD Biosciences. Flow cytometric analysis was performed using a Navios flow cytometer *(Beckman Coulter, USA)*. The data were analyzed using the Kaluza analysis software *(Beckman Coulter, USA)*. The cell phenotyping was performed just before releasing the cells for transplantation. The duration between the releasing and transplantation was less than 24 hours.

Quantitative Real-time Polymerase Chain Reaction Assay (qRT-PCR)

Whole RNA was extracted from cells using the High Pure RNA Tissue Kit (Roche Diagnostics, GmbH, Mannheim, Germany), quantified using a NanoDrop spectrophotometer. The extracted RNA was reverse transcripted to complementary DNA (cDNA) using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics). qRT-PCR was carried out using the LightCycler® 480 II RT-PCR system (Roche Diagnostics). The housekeeping gene GAPDH served as an internal control. Results are expressed as a percentage relative to $1/\Delta Ct$ of GAPDH gene. Primer sequences of the target genes are shown in Table 1.

Count and Viability Assay

Viable MSCs were detected using the dual fluorescent probes of Muse Count & Viability Assay kit *(Merck Millipore, Germany)* on a flow cytometer.

MSCs in-vitro Cellular Differentiation

The *in-vitro* lineages differentiation was performed and each differentiation experiment was done in triplicate.

- *Adipogenic differentiation*. Rat bone marrow, adipose tissue and dental pulp derived MSCs from passage three (3,000 cells/cm^2) were seeded in 6-well plates to induce adipogenic differentiation. When they reached 90–100% confluency, the growth medium was replaced with adipogenic differentiation medium. To this end, adipogenic induction medium (Lonza, MD, USA) and adipogenic maintain medium (Lonza, MD, USA) were used. MSCs were stimulated for 21 days.
- *Osteogenic differentiation*. Rat bone marrow, adipose tissue and dental pulp derived MSCs in passage three were seeded on 0.1% gelatin coated 12-well plate (3000 cells / cm2). Twenty-four hours later, the growth medium was replaced with osteogenic differentiation medium (Lonza, MD, USA).

Burn Injury Model

All burning procedures were performed under general anesthesia with intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg Xylazine. The entire dorsal skin of each animal was shaved and burn wounds were created according to a previously described contact thermal trauma model.[7] This model uses a specially manufactured brass comb with four rows separated by three notches that produces four burn sites separated by three unburned interspaces that represent the stasis zones. The comb was immersed in boiling water for

TABLE 1 Primers used for real-time polymerase chain reaction analysis

Gene		Sequence
CD11	Foreword	GGGAGCCCCACACTGATA
	Reverse	AGAGGGAGGCCCCAAAATA
GAPDH	Foreword	CATCGTGGAAGGGCTCAT
	Reverse	CGCCACAGCTTTCCAGAG
CD44	Foreword	TTGGGGACTACTTTGCCTCTT
	Reverse	CGGCAGGTTATATTCAAATCG
CD73	Foreword	CTTCTGAACAGCACCATTCG
	Reverse	CCTCCACTGGTTAATGTCTGC
CD90	Foreword	CCACAAGCTCCAATAAAACTATCAA
	Reverse	AGCAGCCAGGAAGTGTTTTG
CD29	Foreword	ATCATGCAGGTTGCAGTTTG
	Reverse	CGTGGAAAACACCAGCAGT

five minutes and then placed on the back of each rat and held for thirty seconds without any pressure.

Experimental Design

Forty rats were randomly divided into four groups as follows:

1. BMSC group (n = 10): Thirty minutes after burn induction, 2^{-7} m^3 of phosphate-buffered saline containing 1×10^6 BMSCs was injected subcutaneously to each zone-of-stasis.
2. ASC group (n = 10): Thirty minutes after burn induction, 2^{-7} m^3 of phosphate-buffered saline containing 1×10^6 ASCs was injected subcutaneously to each zone-of-stasis.
3. DPSC group (n = 10): Thirty minutes after burn induction, 2^{-7} m^3 of phosphate-buffered saline containing 1×10^6 DPSCs was injected subcutaneously to each zone-of-stasis.
4. Control group (n = 10): Thirty minutes after burn induction, 2^{-7} m^3 of PBS was injected into the subcutaneous plane of each interspace.

The animals were returned to their individual cages for a period of 72 hours, after which they were re-anesthetized. After gross evaluation of burn wounds, the exact areas of the three interspaces were biopsied for subsequent histologic, immunohistochemical and biochemical evaluations.

Assessment of Gross Necrosis

The extent of necrosis in the stasis zone was evaluated 72 hours after the burn using the paper template method. In this method, a template was drawn on each interspace with delimitation of the necrotic area. The template was then scanned on a computer and the necrotic area was measured as a percentage of the total interspace using Adobe Photoshop CS5 (*Adobe Systems, Inc., San Jose, CA*).

Assessment of Burn-related Histopathological Alterations

Histologic evaluation was conducted on formalin-fixed, paraffin-embedded, hematoxylin and eosin (H&E) stained 5-micron sections. Sections were first evaluated at 10x magnification to obtain an overview of the morphologic alterations. Thereafter, 10 random fields were examined under 40x magnification and the following histopathological variables were scored semi-quantitatively (0, none; 1, mild; 2, moderate; 3, severe) as previously described[8]: epidermal injury, disorganization of collagen, tissue edema, and damage to the skin appendages.

Assessment of Apoptosis

We examined apoptotic cell death in the zone-of-stasis immunohistochemically using antibodies against cleaved caspase 3a (CC3a) *(Thermo Scientific, Waltham, MA)* according to the manufacturer's recommendations. The stained slices were observed and the number of apoptotic cells was counted in 10 random fields at 40x magnification and the mean number per microscopic field was used.

Assessment of Necrosis

Skin biopsies were stained with antibodies against anti-high mobility group box 1 (HMGB1; *Thermo Scientific, Waltham, MA)* for evidence of necrosis according to the manufacturer's protocol.[9] The number of necrotic cells was determined at 40x magnification and the mean value of 10 random fields per slide for each animal was used.

Assessment of Inflammatory Cell Infiltration

Infiltrating neutrophils in the zone-of-stasis was quantitated by assaying the activity of myeloperoxidase, an enzyme found predominantly in the azurophilic granules of neutrophils. Using a previously described method,[10] myeloperoxidase activity was measured spectrophotometrically using a series of reactions with o-dianisidine, hexadecyltrimethylammonium bromide, and hydrogen peroxide. One unit of myeloperoxidase activity was defined as the amount of enzyme that causes a change in absorbance measured at 460 nm for 3 min.

Assessment of Oxidative Stress

As a marker of lipid peroxidation and oxidative stress, malondialdehyde levels were evaluated in the skin samples by using both biochemical and immunohistochemical methods. For the biochemical evaluation, the thiobarbituric acid reaction method was used to determine malondialdehyde levels in tissue homogenates. In this method, we measured the absorbance of the pink color produced by the interaction of malondialdehyde with thiobarbituric acid at 532 nm.[11] On the other hand, immunohistochemical assessment was performed using anti-malondialdehyde antibodies *(Thermo Scientific, Waltham, MA)* according to the manufacturer's protocol. The numbers of positive cells were determined at 40x magnification and the mean value of 10 random fields per slide for each animal was used.

Assessment of Microvascular Density

Immunohistochemical staining of endothelial cells was performed using antibodies against CD31 *(Thermo Scientific, Waltham, MA)* according to the manufacturer's protocol. Microvascular density was assessed by measuring the number of CD31 positive vessels in 10 random fields under 40x magnification. Results were expressed as the mean number of stained cells per microscopic field.

Statistics

Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Variable distribution was evaluated using the Shapiro-Wilk test. Samples with normal distribution were evaluated by one-way analysis of variance (ANOVA). Non-normally distributed variables were compared across the groups by using Kruskal–Wallis one-way ANOVA on ranks Test. Tukey test was used for the multiple comparisons. A p value < 0.05 was considered statistically significant. The results of the statistical analysis were expressed as mean $\pm$ standard deviation.

RESULTS

BMSCs, ASCs, and DPSCs Exhibited Homogenous Cell Populations with Similar Morphologies

The adherent cells from bone marrow, adipose tissue, and dental pulp exhibited heterogeneous morphologies with different shapes, including elongated, flat and triangular. After the third passage, all populations became relatively homogenous and exhibited similar fibroblast-like morphology. Cell populations were very heterogeneous by cell size (from 10 to 300 μ) (Figure 1).

BMSCs, ASCs, and DPSCs Exhibited Homogenous Cell Populations with Similar Expressions of Surface Markers

At the end of the third passage, the MSCs were analyzed by flow cytometry to document the surface marker profile after their *ex vivo* expansion. The flow cytometry analysis data indicated that all BMSCs, ASCs, and DPSCs did not differ by the expression of the main MSC markers CD44, CD73, CD90, and CD105. Furthermore, all cell populations were negative for the hematopoietic cell markers CD11b and CD45 (Figure 2).

In accordance with the flow cytometry data, qRT-PCR analysis demonstrated high gene expression levels of the markers CD44, CD29, CD73 and CD90. Moreover, low expressions were detected for the markers CD11b and CD45. While the frequency of positivity for CD11b was low in BMSCs, no positivity was detected for CD45. These cells were positive for CD44 and CD73 and strongly positive for CD29 and CD90. On the other hand, no positivity was noted for CD11b and CD45 in DPSCs. However, these cells were positive for CD29 and CD 90 and strongly positive for CD73 (Figure 3).

This is a valid protocol for confirming the characteristics of transplanted cells since cell phenotyping was performed just before releasing the cells for transplantation and the duration between the releasing and transplantation was less than 24 hours.

Finally, viability was quantified by flow cytometer analysis after staining with count and viability kit before application and more than 90% viable cells were detected.

Multi-lineage Differentiation Capacities of MSCs

The presence of intracellular lipid droplets was confirmed by AdipoRed Assay Kit (Lonza MD, USA) in all cell sources (Figure 4). At the end of the third week

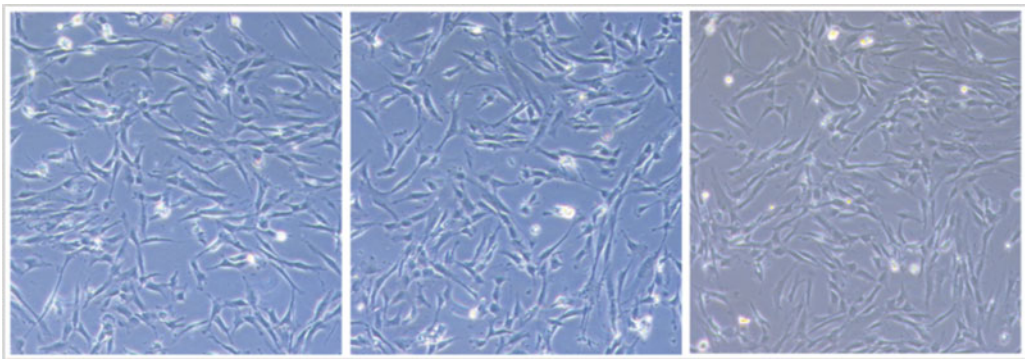

FIGURE 1 Cell morphologies of rat BMSCs *(A)*, ASCs *(B)* and BMSCs *(C)* after the third passage (x20 magnification).

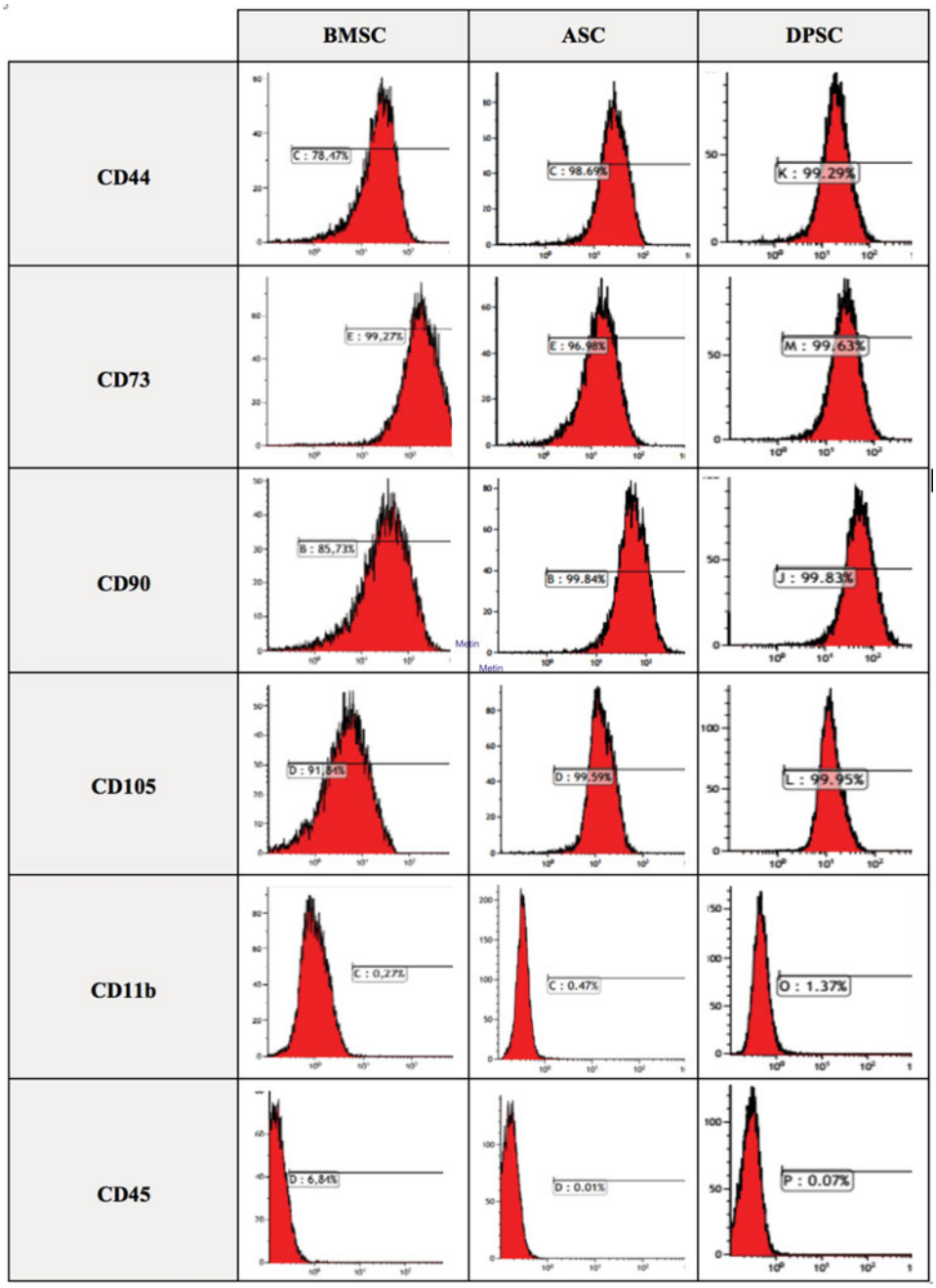

FIGURE 2 Representative flow cytometry analysis of cell-surface markers in BMSCs, ASCs and DPSCs at the third passage. All MSCs expressed mesenchymal stem cell markers including CD29, CD 44, CD73, CD90, and CD105; however negative for CD 11b and CD45.

calcium storage in differentiated cells was demonstrated by staining with 1% alizarin red (Figure 4).

No Differences in The Extent of Gross Necrosis Were Noted Between Stem Cell Groups

The boundaries between necrotic and viable areas in the unburned interspaces were clearly demarcated 72 h after the burn, considering black color and hardness as markers for necrosis. The mean percentage of the calculated necrotic area by the paper template method in the control group was significantly higher than those of the stem cell groups (all $p < 0.05$; Figure 5). However, the differences in the mean percentages of the necrotic areas between the stem cell groups weren't statistically significant (all $p > 0.05$; Figure 5).

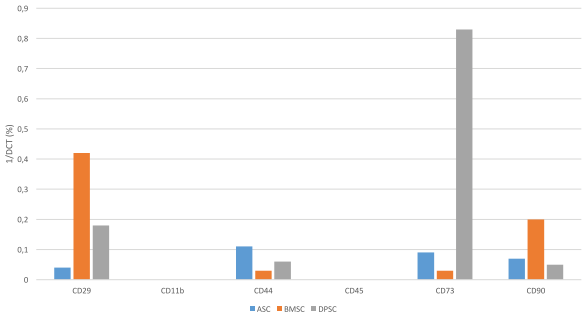

FIGURE 3 qRT-PCR analysis demonstrated high gene expression levels of the markers CD44, CD29, CD73 and CD90. Low expressions were detected for the markers CD11b and CD45.

No Differences in The Degrees of The Histopathological Alterations Were Observed Between Stem Cell Groups

Evaluation of H&E stained sections in the control group revealed dramatic morphological alterations such as epidermal desquamation, loose collagen matrix, profound edema and damage to skin appendages. However, MSCs markedly attenuated these signs of injury. Semi-quantitative grading of the histopathological findings revealed that there were no significant differences between damage scores in BMSC-, ASC- and DPSC-treated groups (all $p > 0.05$; Figure 6).

No Differences in Apoptotic Cell Count Were Noted Between Stem Cell Groups

Immunohistochemical assessment of apoptotic cell death using antibodies against CC3a revealed that the number of apoptotic cells in the control group was significantly higher ($p < 0.001$; Figure 7) than those in the stem cell groups. However, the differences in the mean numbers of apoptotic cells between the stem cell groups weren't statistically significant (all $p > 0.05$; Figure 7).

No Differences in Necrotic Cell Count Were Noted Between Groups

As a marker of necrotic cell death, the translocation of HMGB1 from the nucleus to the cytoplasm was evaluated.[9] In all groups, both nuclear-to-cytoplasmic and cytoplasmic-to-extracellular redistribution of HMGB1 were observed. There were no significant differences between groups with respect to necrotic cell count (all $p > 0.05$; Figure 8).

ASCs Exhibited More Potent Immunomodulatory Effects than BMSCs and DPSCs

Compared with the controls, MSC-treated rats had significantly lower myeloperoxidase activities (all $p < 0.001$; Figure 9). On the other hand, myeloperoxidase activity didn't differ significantly between the BMSC- and DPSC-treated groups ($p = 0.098$). However, myeloperoxidase activity was found to be significantly lower in the ASC-treated group in comparison with the BMSC- and DPSC-treated ones (both $p < 0.05$; Figure 9).

No Differences in Tissue Oxidative Stress Were Noted Between Groups

Tissue malondialdehyde level, as a marker of oxidative stress, was evaluated by thiobarbituric acid reaction. Compared with the tissues of the stem cell groups, burned tissues obtained from control rats presented with significantly higher malondialdehyde levels (all $p < 0.05$; Figure 10). On the other hand, we found that malondialdehyde levels didn't differ significantly between BMSC-, ASC- and DPSC-treated groups (all $p > 0.05$; Figure 10). Correspondingly, immunohistochemical labeling of malondialdehyde revealed similar staining intensities in all stem cell groups (Figure 10).

DPSCs Showed More Potent Angiogenic Effects That ASCs and BMSCs

The capillaries that were counted on H&E stained slices were confirmed by CD31 immunostaining. Compared with the control group, all MSC-treated groups revealed significantly higher numbers of CD31 positive vessels (all $p < 0.001$; Figure 11). The number of CD31 positive vessels in the DPSC-treated group was significantly higher than either the ASC- and BMSC-treated groups (both $p < 0.05$; Figure 11).

DISCUSSION

MSCs are one of the promising cell types for regenerative medicine.[12] The ability to adhere to plastic surfaces, the expression of specific surface markers, the lack of expression of hematopoietic markers and the ability to differentiate along adipocytic, osteoblastic and chondrocytic pathways have been suggested as minimal criteria to characterize MSCs by the International Society for Cellular Therapy.[13] With the ongoing advances made in this field, many alternative MSC sources such as bone marrow,[14] adipose tissue,[15] dental pulp,[16] synovium,[17] skeletal muscle,[18] and

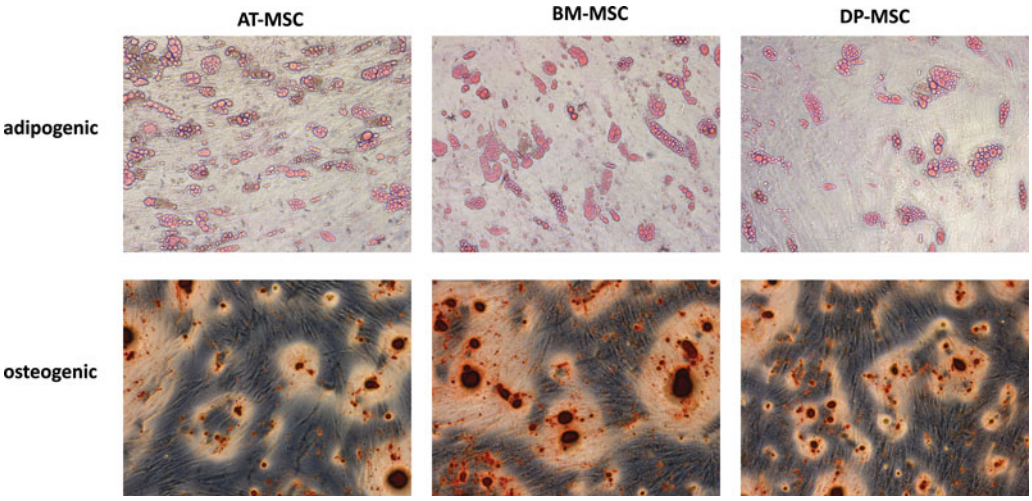

FIGURE 4 Multi-lineage differentiation capacities of MSCs.

periosteum[19] have been described. However, one important question that remains to be answered is which source is more effective and suitable for MSC transplantation, particularly in the treatment of burn wounds. In this study, we compared the benefits and mechanisms of variously sourced MSCs in the prevention of burn wound progression.

Bone marrow was long regarded as the major source of MSCs, however, the collection of bone marrow from the patient requires an invasive and painful procedure that may be accompanied by a risk of infection. In addition, bone marrow suppression has been documented in major burns either as a result silver sulphadiazine toxicity or a result of sepsis.[20,21] Compared with BMSCs, ASCs are superior candidate cells for autologous cell transplantation, as they can be harvested by less invasive liposuction procedures. Moreover, it has been shown that the application of ASCs from the hypodermis of the discarded skins of severely burned patients contributes significantly to the wound healing.[22] Recently, dental pulp has been considered as an interesting source of MSCs due to the high content of cells and to the relatively low invasive procedures required for cell isolation. While the benefits of both BMSCs and ASCs have been demonstrated in burn wounds, this is the first study to evaluate the effects of DPSCs on burn wound healing.[15,23]

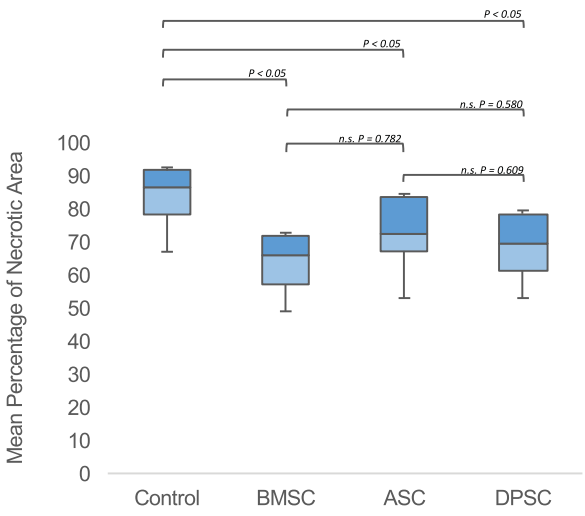

FIGURE 5 Plot showing the mean percentage of necrotic area measured in each group. The sample size was n = 10 for each group. n.s., non-significant.

Our comparison of MSCs derived from bone marrow, adipose tissue, and dental pulp revealed homogenous cell populations with similar morphologies and surface markers in agreement with previous reports.[24,25] MSCs were compared at the same passage because their potential of differentiation has been shown to decrease after each passage.[26] BMSCs, ASCs, and DPSCs exhibited heterogeneous morphology with different shapes at the first culture phase, with the morphology becoming gradually homogenous with subsequent passages. All cells showed a similar fibroblast-like morphology in parallel patterns. All cells in the three groups were positive for commonly accepted MSCs surface markers. Furthermore, they were very low in all hematopoietic markers. Taken together, these findings are in line with the notion that MSCs are similar irrespective of their original tissue source.[27,28]

Despite the aforementioned similarities in morphology and surface markers, many studies have documented significant biological differences concerning differentiation capacities, proliferation rates, and gene expression profiles of variously sourced MSCs.[29–33] Accordingly, conflicting results have been reported regarding the clinical effectiveness of these cells.[34,35] We think that data from different descriptive reports devoted to cells of a certain tissue cannot be correctly compared because of differences in the acquisition,

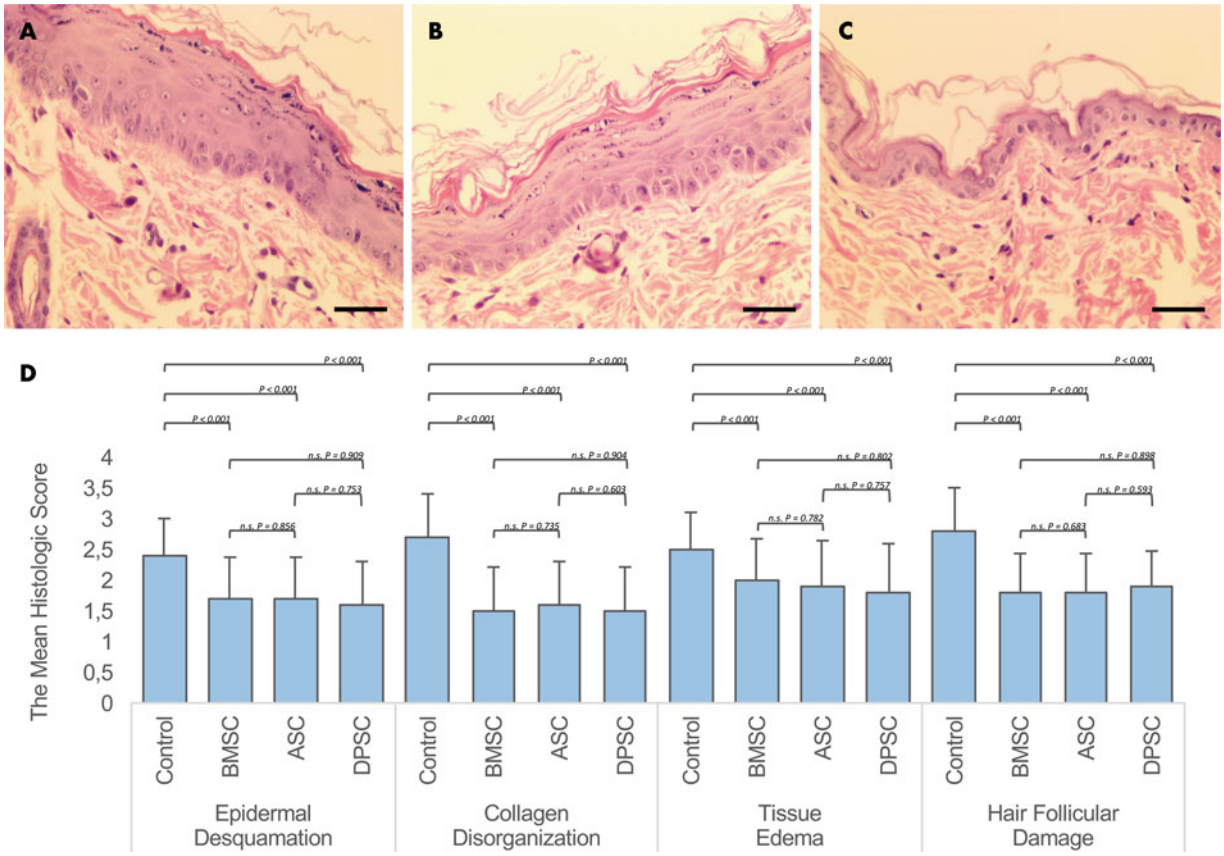

FIGURE 6 Representative images of a hematoxylin and eosin stained section in the BMSC-treated *(A)*, ASC-treated *(B)* and DPSC-treated *(C)* groups 72 hours after burn. The following histologic characteristics were observed: epidermal injury, loose collagen matrix and mild interstitial edema (x40 magnification). Scale = 100 μm. Semi-quantitative assessment of burn-related histopathological alterations in hematoxylin and eosin stained sections *(D)*. The sample size was n = 10 for each group. Data was presented as mean ± SD. n.s., non-significant.

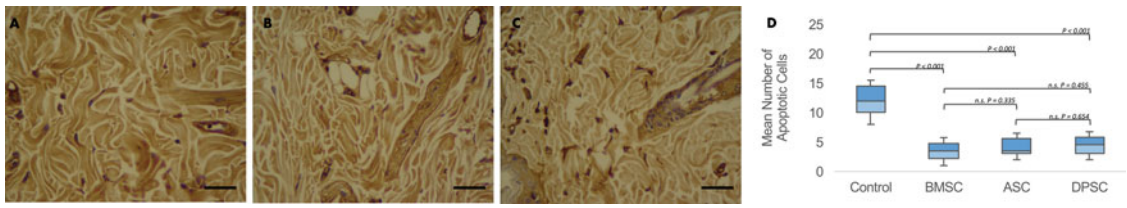

FIGURE 7 Representative images of immunohistochemical staining with antibodies against CC3a in tissues obtained from the BMSC-treated *(A)*, ASC-treated *(B)* and DPSC-treated *(C)* groups (x40 magnification). Scale = 100 μm. Quantitative assessment of apoptotic cell death (*D*). The sample size was n = 10 for each group. n.s., non-significant.

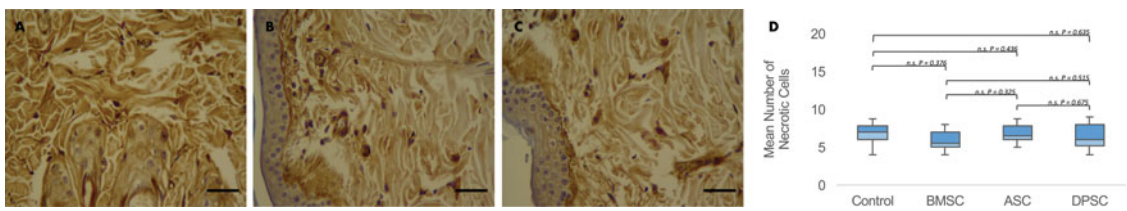

FIGURE 8 Representative images of immunohistochemical staining with antibodies against HMGB1 in tissues obtained from the BMSC-treated *(A)*, ASC-treated *(B)* and DPSC-treated *(C)* groups (x40 magnification). Scale = 100 μm. Quantitative assessment of necrotic cell death (*D*). The sample size was n = 10 for each group. n.s., non-significant.

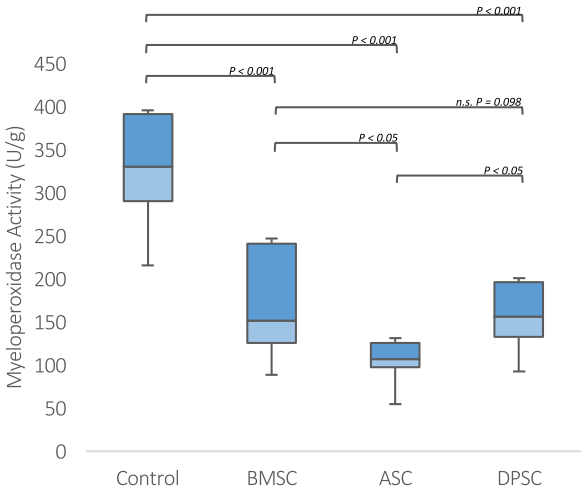

FIGURE 9 Quantitative analysis of myeloperoxidase activity. The sample size was n = 10 for each group. n.s., non-significant.

separation, in-vitro cultivation, and expansion of MSCs.

In our previous study, we investigated the effects of BMSCs on burn wound progression and found that MSC transplantation significantly reduced the area of necrosis in the zone-of-stasis. Moreover, histologic assessment revealed that MSCs effectively ameliorated the histopathological changes associated with burn wound progression such as tissue edema, collagen disorganization, damage to adnexal structures, and epidermal desquamation. In the current study, we showed that injection of equivalent numbers of ASCs and DPSCs resulted in comparable effects without any significant differences between groups. This indicates that the beneficial results of MSCs are irrespective of the source tissue.

It is now clear that tissue loss within the zone-of-stasis is directly associated with apoptotic cell number.[4] Our results and previously reported data indicate similarly marked activation of apoptosis in the zone-of-stasis, and it has been also reported that BMSC transplantation can prevent burn wound progression by decreasing the susceptibility of cells to apoptosis.[23] Consistent with the results of gross evaluation, the current study documented that apoptosis count within the burn stasis zones did not differ significantly between groups, indicating that critical anti-apoptotic mechanisms are conserved between BMSCs, ASCs, and DPSCs.

The role excessive inflammatory response in contributing to burn wound progression has been well documented.[36] In our previous study, we documented that MSC transplantation ameliorates burn wound progression through an immunomodulatory effect that involves reduced neutrophilic infiltration, downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines in the local tissue (unpublished work). In the current study, we found that ASCs have more potent immunomodulatory effects than BMSCs and DPSCs, similar to previous results.[24] This suggests that ASCs might be substituted for BMSCs and DPSCs for the treatment of severe burns. However, further studies in models with more severe degrees of burn-induced inflammation are needed to confirm this prediction since the extent of gross necrosis was found to be similar in all groups.

At the molecular level, the over-production of free radicals resulting from activation of neutrophils may have contributed to progressive tissue destruction in the zone-of-stasis.[37] The underlying mechanisms include lipid peroxidation of cell membranes and disruption of nucleic acids and proteins. Although the number of infiltrating neutrophils in the ASC-treated group was significantly less than either the BMSC- and DPMSC- treated groups, oxidative stress levels were found to be the same. We think that this could be associated with the fact that the cause of oxidative stress is multifactorial. For example, it has been shown that thermal injury is capable of directly producing free radicals through homolytic bond fusion.[4] Additionally, recent studies have focused on increased nitric oxide after the burn as a source of oxidative stress.[38]

We documented that myeloperoxidase activity, indicative of neutrophilic infiltration, was significantly lower in the ASC-treated group in comparison with the BMSC- and DPSC-treated ones. We speculate that strong CD44 positivity in ASCs might play a role in this finding. In different models of acute tissue injuries, the blockade of CD44 led to reduced activation of the anti-inflammatory TGF-β1, impaired removal of apoptotic neutrophils, and accumulation of hyaluronan, suggesting a role for CD44 in limiting the inflammatory response.[39–41]

It is well known that neovascularization is critical for the prevention of burn wound progression.[4] Our previous report demonstrated that BMSCs transplantation significantly enhanced vascular density in the zone-of-stasis. Moreover, we documented higher expression levels for VEGF, PDGF, FGF, and TGF after BMSCs transplantation, indicating that this mix of factors collectively mediates, at least in part, the vasculotropic properties of MSCs. In the present study, we demonstrated that DPSCs have more potent angiogenic effects that ASCs and BMSCs. It has been shown that DPSCs, like BMSCs, are able to secrete various pro-angiogenic factors.[42,43] Moreover, our qRT-PCR analysis revealed that DPSCs were strongly positive for CD73. A growing body of evidence suggests that CD73 can promote new vessel formation, endothelial cell migration and tube-like structure generation.[44,45] These effects can be attributed to both enzymatic and non-enzymatic actions. CD73 is a membrane-bound glycoprotein that mediates the hydrolysis of

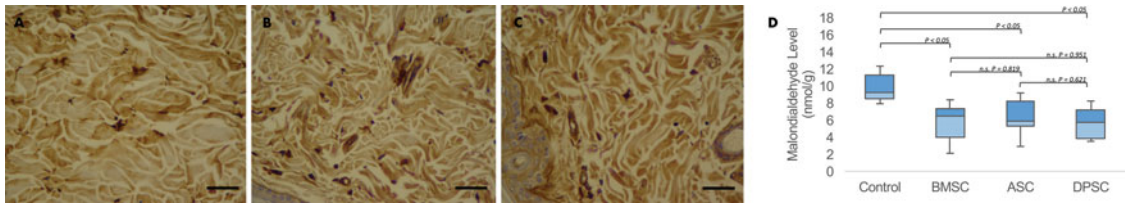

FIGURE 10 Images of immunohistochemical staining with antibodies against malondialdehyde in tissues obtained from the BMSC-treated group (*A*), ASC-treated group (*B*) and DPSC-treated group (*C*) (x40 magnification). Scales = 100 μm. Quantitative assessment of tissue malondialdehyde levels *(D)*. The sample size was n = 10 for each group. n.s., non-significant.

adenosine monophosphate to adenosine and inorganic phosphate.[46,47] CD73-derived adenosine induces the release of VEGF through A2A receptors, thus promoting neo-angiogenesis.[45,48] In addition, it has been shown that CD73 promotes endothelial growth by the upregulation of the pro-proliferative protein cyclin D1.[45,48] Interestingly, gross survival rates were similar between three groups. This situation could be associated with the fact that stasis zone survival may not completely rely on blood supply. Other protective factors of MSCs may also play a role.

The therapeutic effects of transplanted MSCs were initially considered to be mediated by the incorporation of MSCs into the healing tissue and differentiating into appropriate cells. However, a growing body of evidence proposes that their therapeutic effects are attributed to their release of paracrine mediators.[31,49] Correspondingly, in our study benefits occurred at a time point at which it is highly unlikely that MSCs would integrate into the tissue, differentiate and replace damaged cells. This suggests that the paracrine capacity of the transplanted MSCs is the principal mechanism that contributes to tissue repair. Characterization of these mediators would be beneficial for identification of alternative soluble factor-based therapies, the definition of the mechanisms of action of MSCs, and the prediction of potential diseases for the application of MSCs.

MSCs must be expanded in number over several weeks prior to implantation and this time-consuming process precludes autologous use in such emergency situations.[50] However, a growing body of evidence suggests that these cells possess hypo-immunogenic properties, which allows them to be tolerated when transplanted over major histocompatibility complex barriers in humans.[51] Consequently, ex vivo expanded MSCs from HLA-mismatched "third party" donors could be potentially used as a universal donor product. The use of MSCs from healthy donors for severely burned patients in allogenic transplantation is a promising approach to treat such acute cases.

The main limitation of the current study is that all evaluations were made only on the third day after the burn. This day was chosen because the boundaries between necrotic and viable areas in the unburned interspaces usually demarcate before the third post-burn day.[23] Moreover, this timing allows us to evaluate the early changes in apoptotic and necrotic cell deaths, oxidative stress, inflammatory reaction, and neo-angiogenesis. However, there could be distinctions between the aforementioned results in different days after the burn. Therefore, further investigation is required to determine the long-term impact of MSCs.

Another important limitation of this study is that the proteomic comparison of secreted factors between groups was not performed. In the first part of this study, we evaluated the expression of selected inflammatory cytokines after BMSC transplantation into the stasis zone using real-time polymerase chain reaction assay. Compared with the baseline levels of the healthy animals, the relative expression levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β significantly increased after the burn. Additionally, the expression of the anti-inflammatory cytokine IL-10 significantly elevated. After BMSC transplantation, the relative expression levels of the aforementioned

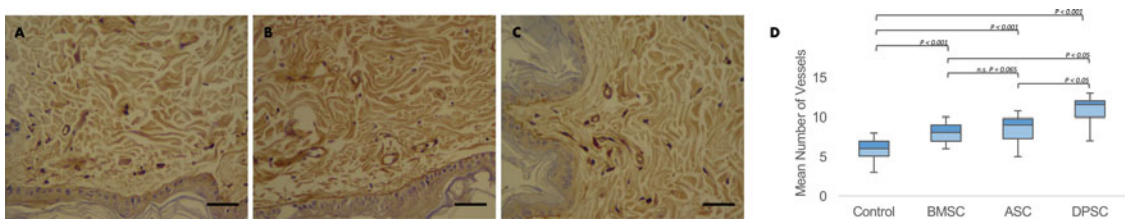

FIGURE 11 Evaluation of microvascular density in the BMSC-treated *(A)*, ASC-treated *(B)* and DPSC-treated *(C)* groups using immunohistochemical labeling of CD31. Scale = 100 μm. Quantitative assessment of vascular density (*D*). The sample size was n = 10 for each group. n.s., non-significant.

pro-inflammatory cytokines displayed significant reductions in comparison with the control group. Furthermore, the elevation of the anti-inflammatory cytokine IL-10 became more significant. Moreover, we evaluated the expression of some pro-angiogenic factors that could mediate vasculotropic effects. Compared with the baseline levels of the healthy animals, the relative expression levels of the pro-angiogenic factors VEGF-A, PDGF, basic FGF and TGF-β significantly increased after the burn. After BMSC transplantation, the upregulation of expressions became more significant. However, these findings couldn't be generalized to include other stem cell groups and more research will in fact be necessary to compare the secretion level of cytokines and growth factors between the cells.

CONCLUSON

Taken together, our findings suggest that bone marrow, adipose tissue, and dental pulp may serve as a universal donor MSC source for the prevention of burn wound progression. However, important questions remain to be addressed before clinical translation: How can we standardize the isolation and culture of MSCs? Is it better to use autologous MSCs, if clinically applicable, over the third party? What are the optimal dose and route of administration? What is the ultimate fate of MSCs after transplantation?

FINANCIAL DISCLOSURE

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AUTHORS' CONTRIBUTIONS

All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and final approval of the version to be submitted. Ozan Luay Abbas Corresponding author, Orhan Özatik Histological and immunohistological analysis, Zeynep Burçin Gönen Stem cell isolation and characterization, Serdal Öğüt Biochemical evaluation of oxidative stress, Fikriye Yasemin Özatik Surgical procedures and data analysis, Hasan Salkın Stem cell isolation and characterization, Ahmet Musmul Biostatistical analysis.

ORCID

Ozan Luay Abbas http://orcid.org/0000-0003-0410-1215

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