EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 71, 1293:1304, APRIL, 2025
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 16-12-2024 • Accept Date : 08-01-2025 • Available online: 20-04-2025 • DOI : 10.21608/edj.2025.343111.3300

HISTOPATHOLOGICAL EVALUATION OF THE EFFECT OF MESENCHYMAL STEM CELLS ON PERIODONTAL DISEASE: IN-VIVO STUDY

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ABSTRACT

Background: In the oral cavity, the potential application of stem cell therapy has been implied in different conditions including periodontal disease. The increased incidence rate of periodontitis, especially in developing countries, with lack of novel treatment modalities introduced that can help speed-up the healing process can imply the need for new approaches such as using stem cells.

Aim of the study: To histologically evaluate the effect of mesenchymal stem cell therapy on the healing of periodontal fibers, in albino rats suffering from periodontal disease.

Material and Methods: Periodontitis was induced in a total number of 36 albino rats, divided into 4 groups evenly. 2 control group, and 2 study groups based on the date of sacrifice (2 and 6 weeks). Evaluation of the periodontal condition was performed using ordinary Hematoxylin and eosin staining, Masson's trichrome special stain, and β-catenin immunohistochemical staining to evaluate number of proliferating periodontal fibroblasts. To investigate the effects of the use of stem cells, time, and their interactions on the mean cell count, two-way ANOVA was conducted. If the ANOVA test revealed a significant outcome, Bonferroni post-hoc correction was used to identify which specific groups differed from each other.

Results: Highest area percentage of Masson's trichrome stain, and β-catenin immuno-positive cell count was found in the group treated with stem cells for 6 weeks.

Conclusion: Stem cells administration improved collagen quality and encouraged proliferation of periodontal cells in a time-dependent manner, when compared to groups not treated with stem cells.

KEYWORDS: Stem cells, Periodontal disease, Albino rats, Masson's trichrome, β-catenin.

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INTRODUCTION

Stem cells possess an incredible capacity to grow into various cell types within the body. This unique characteristic enables them to regenerate damaged tissues and offer potential cures for a wide spectrum of medical disorders, including hearts disease, Parkinson's disease, and malignant neoplasms[1–3]. Importantly, there are diverse categories of stem cells, namely embryonic, adult, and induced pluripotent stem cells. Each type demonstrate distinct capabilities as well as associated advantages and disadvantages[4].

Stem cell research possesses the potential to revolutionize the field of medicine, as we currently understand it. This groundbreaking field could be utilized for cultivating new organs, thus revoking the necessity for organ donors and diminishing the risk of rejection. Despite existing challenges yet to be defeated, the possibilities it presents are undeniably promising[5].

The adult stem cells, that can be present in several tissues of the body, for instance, the brain, skin, and bone marrow, offer promising potential for treating several diseases. Unlike embryonic stem cells, these cells are multipotent and can differentiate into certain types of cells. Research has shown their effectiveness in addressing conditions such as diabetes, heart disease, and Parkinson's disease[5–7]. It has also shown promising results with different blood disorders including leukemia and lymphoma, as well as bone and cartilage defects[1,2,8]. However, the use of adult stem cells is challenging, especially in terms of their availability, which makes isolation difficult. Additionally, their limited differentiation abilities mean they cannot form all types of cells within the body[9].

Among the various kinds of adult stem cells, stem cells from the bone marrow play a significant role. These multipotent cells are in the bone marrow and can transform to different sorts of blood cells. These proteins play crucial roles in the production of different components of the blood, such as platelets, as well as red and white blood cells. For decades, bone marrow stem cells have been utilized to treat several blood disorders and cancers such as leukemia, lymphoma, and multiple myeloma. The results have been astonishing, as they effectively restore patient blood cell production and enhance overall health[10]. However, beyond that, there is still investigation for their potential in treating various diseases. Researchers are particularly intrigued by their potential application in regenerative medicine, where they could aid in repairing damaged tissues and organs, such as the brain, after radiation injuries[11].

The oral cavity offers various potential applications for stem cells in different procedures. One such example is the use of stem cells to re-form tooth-supporting tissues, which can be beneficial in implant dentistry. Additionally, research has explored the possibility of using stem cells to repair damage to the oral mucosa, gums, and other soft tissues. Restoring salivary gland function with the help of stem cells has also been proposed as a future application within intraoral sectors[12].

Among the most common conditions affecting the oral cavity is periodontal disease, which occurs mainly in the form of inflammation. Some studies have reported that the incidence of periodontal disease is high, especially in older age groups. Huang and Dong[13] reported that more than 92% of patients above 35 years of age suffer from periodontal disease, which manifests as gingival bleeding and accumulation of calculus. In addition, they reported that 38% of 35-44-year-old patients experienced periodontal attachment loss, whereas more than 61% of older patients experienced this loss. The possible effect of mesenchymal stem cells in the healing of periodontal inflammation has not been thoroughly investigated. Thus, the main goal is to assess, histologically and immunohistochemically, the effect of bone marrow derived stem cells on the quality of collagen fibers, in albino rats suffering from periodontitis.

MATERIALS AND METHODS

This work was performed in the animal facility of the Ain shams university faculty of medicine. Ethical approval obtained from the committee of the Ain Shams University ethical Committee (FDASU-Rec IR012447), and in line with the national research council's guide for the care and use of laboratory animals. Sample size estimation was performed via G*power software, version 3.1.2, and the amount of collagen fibers in the periodontal ligaments was used as the primary outcome. The effect size (d) was (1.53), using alpha level = 0.05 and 80% power. The degree of the effect to be identified was anticipated as mean and standard deviation (M ±SD) for the variable of interest, and Hasegawa et al.[14] was the referrence used for sample size determination. The minimum estimated sample size is a total of 28 rats, this was increased to 36 (9 rats per group) to compensate for the possible deaths of the rats during the study duration. All the rats were acquired from the Faculty of Medicine, Ain shams university animal house and selected according to the inclusion criteria (race, gender, weight, age). The rats were males, of Rattus norvegicus race, approximately weighed 150–200 g, and their age was 6 weeks. The exclusion criteria for this study were the presence of any visible signs of disease, or the involvement of any rat in other studies. The animals were maintained at a controlled temperature (12-hour dark/light, cycle at 23 ±5°C), with simple gain to a basic pellet diet and tap water. Every 4 rats were placed in a separate, well-aerated, stainless-steel cage. The animals were divided at random into groups via a program called "the random sequence generator" (https://www.random.org/).

The rats were randomly distributed into 4 groups at which periodontitis was induced: 2 untreated control groups, and 2 study groups which bone marrow mesenchymal stem cells (BMMSCs) were used to treat periodontitis. Periodontitis was induced by using ligatures. A sterile 4/0 non-resorbable sterile silk thread was placed subgingivally at the cervical region of the rat's first molar. The ligature was placed for 14 days and was checked daily to ensure proper placement. After the fourth day of ligature placement, notable changes in the gingiva were apparent. Changes included color alteration from pink to red, plaque accumulation around the silk and gingival tissue bleeding occurred three days before removal of the ligature, denoting the development of periodontal disease. The rats in the study group received a single injection, intravenously, of 50 million cultured BMMSCs in phosphate buffered saline (PBS) through the tail vein[15].

Bone marrow stem cells were retrieved from the tibia of the rats using PBS (Invitrogen, Grand Island, USA), which was then centrifuged for 5 minutes at 1,000 rpm. 35 mL of flushed BM cells, then put in layers over 15 mL Ficoll-Paque™ (Invitrogen) for 35 minutes, where they were centrifuged at 400 × g. The topmost layer was removed, leaving behind a mononuclear cell (MNC) layer at the interface, which was collected, rinsed twice with PBS, and centrifuged at 200 ×g at 10°C for 10 minutes. Bone marrow stem cells were cultured using RPMI-1640 medium (Merck, Darmstadt, Germany) complemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, USA), 0.5% penicillin, and streptomycin (Thermo Fisher Scientific). The cells were then grown in 25-ml culture flasks and incubated at 37°C with 5% CO2 until they reached 80–90% confluence after culturing for 14 days[16].

Bone marrow MSCs were identified following the guidelines established by the International Society for Cellular Therapy [17]. This involved assessing their shape, adherence properties, and fluorescence-activated cell sorting (FACS). The cells were confirmed positive for markers CD90+, CD105+, and CD73+, and negative for CD14−, CD34−, and CD45−. Additionally, their capacity to differentiate into various cell types, including osteoblasts, adipocytes, and chondroblasts, was evaluated

The rats in both groups of the study were sacrificed equally after 2 and 6 weeks, after BMMSCs injection, using an anesthetic solution[18]. No animals were lost before the sacrifice point. Scarification was performed by intraperitoneal overdose injection of a mixture of ketamine/xylazine (Sigma-Aldrich)[19]. Every mandible was cut-up, processed, and then assessed histologically to evaluate bone quality and periodontal condition.

The samples were decalcified for 4 weeks, dried up in rising alcohol concentrations, cleared in xylol, and fixed in paraffin blocks. Serial sections of 5–6 μm thickness were cut, mounted on glass slides, and stained with hematoxylin and eosin (H&E) for routine histopathological examination. Masson's trichrome stain was utilized to assess periodontal ligaments, while β-catenin immunohistochemical staining (Mybiosource, USA) was employed to evaluate periodontal cell proliferation levels. The collagen fiber area percentage beneath the first molar in each sample was measured using an objective lens with ×20 magnification (resulting in a total magnification of ×200). Data collection was performed with a Leica Qwin 500 image analysis system (Wetzlar, Germany), and five regions were analyzed per sample. The collagen area percentage was calculated relative to a standard measuring frame with an area of 118,476.6 μm^2. Image analysis and cell counts for all samples were conducted by a blinded examiner.

To analyze the data, we first checked whether the data followed a normal distribution or not. This was done by looking at how the data were presented and using statistical tests called the Kolmogorov-Smirnov and Shapiro-Wilk tests. All the datasets were found to be normally distributed. The data were then summarized using the mean (average) and standard deviation (SD) values. To investigate the effects of the use of stem cells, time, and their interactions on the mean cell count, two-way analysis of variance (ANOVA) was conducted. If the ANOVA test showed a significant effect, Bonferroni post-hoc correction was used to identify which specific groups differed from each other. The statistical analysis was executed via IBM SPSS Statistics for Windows, version 23.0.

RESULTS

H&E staining of both the treated and untreated groups at the two sacrifice points revealed different results (Fig. 1). The first untreated group showed disorganization of the periodontal ligament (PDL) fibers and a scalloped bone surface, indicating damage to the periodontium, but this damage was not as severe as that in the other untreated group, which was sacrificed after 6 weeks. The latter group showed complete disorganization and tear of the periodontal fibers with large interstitial areas filled with extravasated red blood cells (RBCs), in addition to much more intense infiltration of the inflammatory cells. For the groups treated with stem cells, better arrangement of collagen fibers and less inflammatory cell infiltration were noted after the 1st sacrifice, whereas more signs of healing occurred after the 2nd sacrifice, where better attachment to the adjacent bone could be seen, normal organization of the collagen fibers, fewer interstitial spaces and fewer inflammatory cells, and a more even PDL bone interface were noted.

To evaluate the condition of the periodontal fibers, Masson's trichrome stain was used (Fig.2), and the findings from the different study groups were statistically analyzed. Jaw samples from control rats revealed degeneration and mal arrangement of the collagen fibers of the PDL, with a weakly positive reaction to Masson's trichrome stain after the 1st sacrifice. Moreover, the samples taken after the 2nd sacrifice exhibited great degeneration and disorganization of the collagen fibers, with multiple interstitial spaces containing extravasated RBCs and weakly positive Masson's trichrome staining. In the groups treated with BMMSCs, PDL

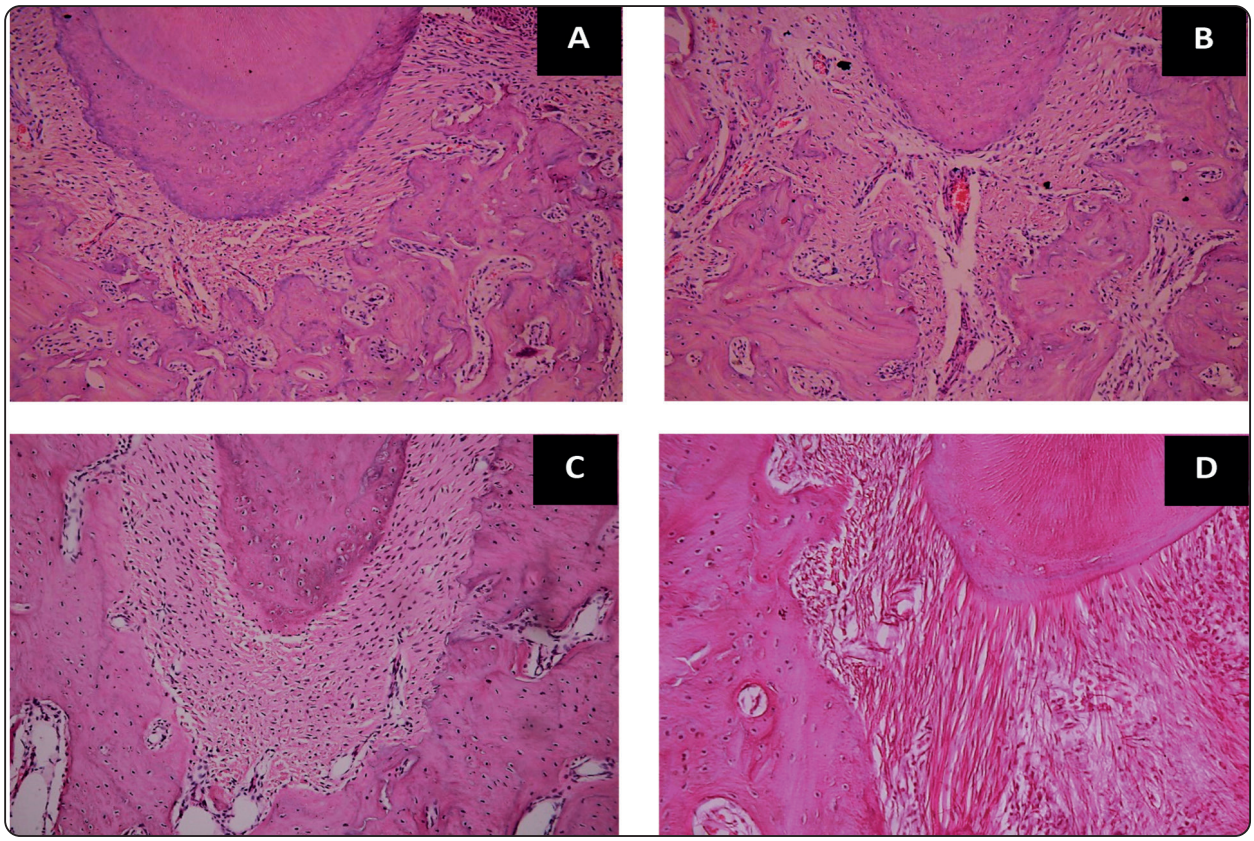

Fig. (1) Photomicrographs of the PDL fibers and adjacent bone of all groups stained with H&E, control groups sacrificed after 2 and 6 weeks (A, B), and the groups treated with BMMSCs after 2 and 6 weeks (C, D) (original magnification X100).

fibers increased, and more dense collagen fibers formed with moderate positive staining after the 1st sacrifice. After the 2nd sacrifice, the stem cell-treated rats presented a further increase in collagen fibers with strongly positive staining and well-organized bundles of collagen fibers. To statistically assess the results of Masson's trichrome staining, two-way ANOVA test was used, which revealed that the use of stem cells, regardless of the time, significantly increased the average area percentage. Additionally, the time factor in the control groups, when stem cells were not used, also had a significant effect on the average area percentage. Moreover, the interaction between stem cell administration and time had a significant effect on the average area percentage. These findings suggest that the effects of stem cells and time are not independent of each other and are influenced by their combined effects (Tables 1-a, b). When the different groups compared at the different sacrifice dates, the BMMSCs-treated groups at both sacrifice dates presented significantly higher mean area percentages than did the control groups. With respect to the stem cell group, there was a statistically significant increase in the mean area percentage in the group treated for 6 weeks. For the control group, there was a statistically significant decrease in the mean area percentage in the group treated for the same duration (Table 1-c).

Immunohistochemical staining was performed with a β-catenin antibody. The first sacrifice sample from the control group revealed moderate nuclear β-catenin-immunoreactivity in periodontal fibroblasts, whereas the second sacrifice sample

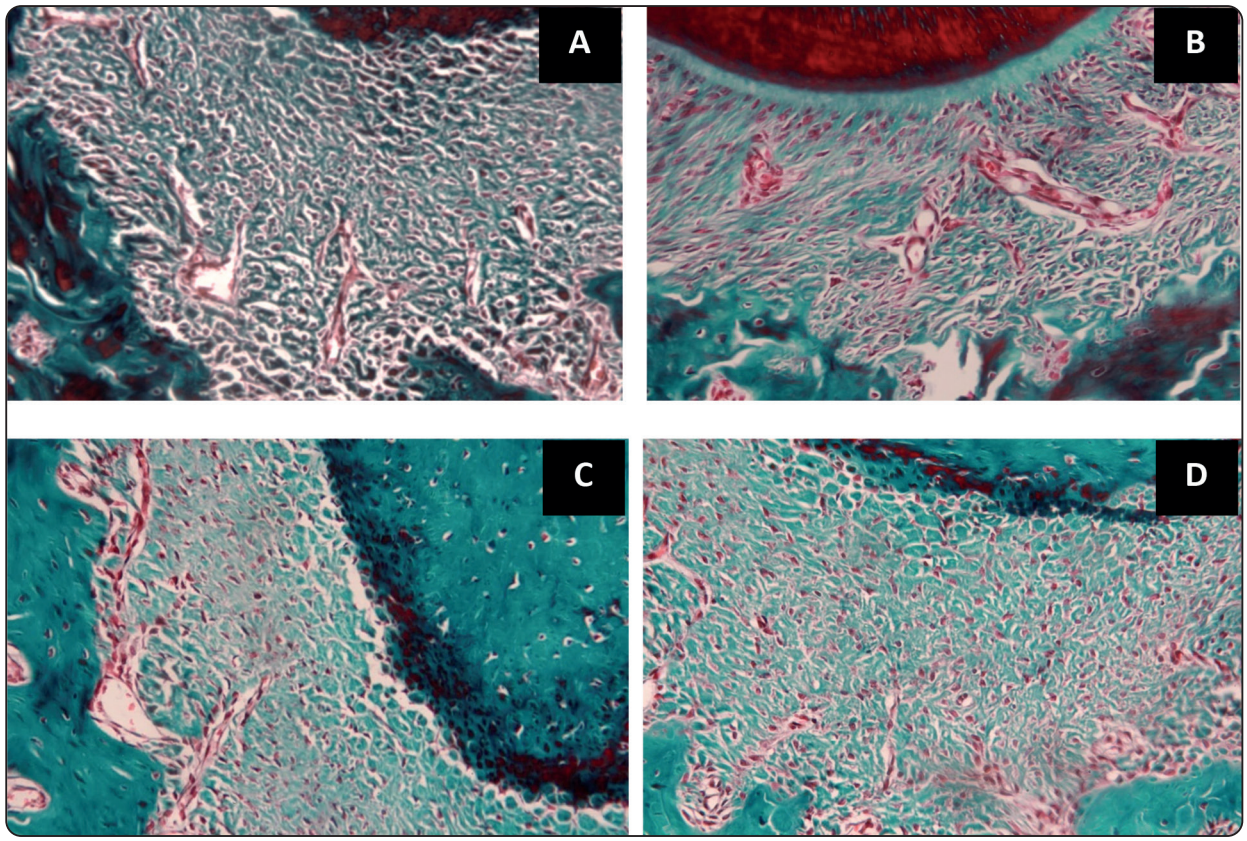

Fig. (2) Photomicrographs of the PDL fibers showing collagen fibers in the periodontal ligaments of the control and stem cell-treated groups (A, B), control groups (C, D), and BMSCs-treated groups. (Masson's trichrome, original magnification X100).

TABLE (1-a) Two-way ANOVA results for the effect of different variables on mean area %.

Source of variation	Type III Sum of Squares	df	Mean Square	F-value	P-value	Effect size (Partial eta squared)
Using stem cells	441	1	441	93.38	<0.001*	0.745
Time	29.16	1	29.16	6.174	0.018*	0.162
Using stem cells x Time interaction	198.81	1	198.81	42.097	<0.001*	0.568

*df: degrees of freedom = (n-1), *: Significant at P ≤ 0.05*

TABLE (1-b) The mean, standard deviation (SD) values and results of two-way ANOVA test for main effects of the two variables on area %.

Variables		Mean	SD	P-value	Effect size (Partial Eta squared)
Using stem cells	Stem cells	19.8	2.3	<0.001*	0.745
	Control	12.8	4.1		
Time	T2	17.2	2.6	0.018*	0.162
	T6	15.4	6.3		

**: Significant at P ≤ 0.05*

TABLE (1-c). The mean, standard deviation (SD) values and results of two-way ANOVA test for comparison between area % with different interactions of variables.

Time	Stem cells		Control		*P*-value	Effect size *(Partial eta squared)*
	Mean	SD	Mean	SD		
T2	18.3	1.8	16	2.9	0.032*	0.136
T6	21.2	1.9	9.5	2	<0.001*	0.803
P-value	0.008*		<0.001*			
Effect size *(Partial eta squared)*	0.2		0.557			

**: Significant at P ≤ 0.05*

showed weak nuclear immunoreactivity in periodontal tissue fibroblasts. For the groups treated with stem cells, the group treated for 2 weeks presented moderately strong nuclear β-catenin immunoreactivity. On the other hand, the group treated for 6 weeks presented the strongest nuclear β-catenin-immunoreactivity in periodontal tissue fibroblasts (Fig. 3). The study revealed that the use of stem cells, regardless of time, significantly increased the average area percentage. Furthermore, the time factor, even without the use of stem cells, also had a significant effect on the average area percentage. Moreover, the interaction between stem cell use and time had a significant influence on the

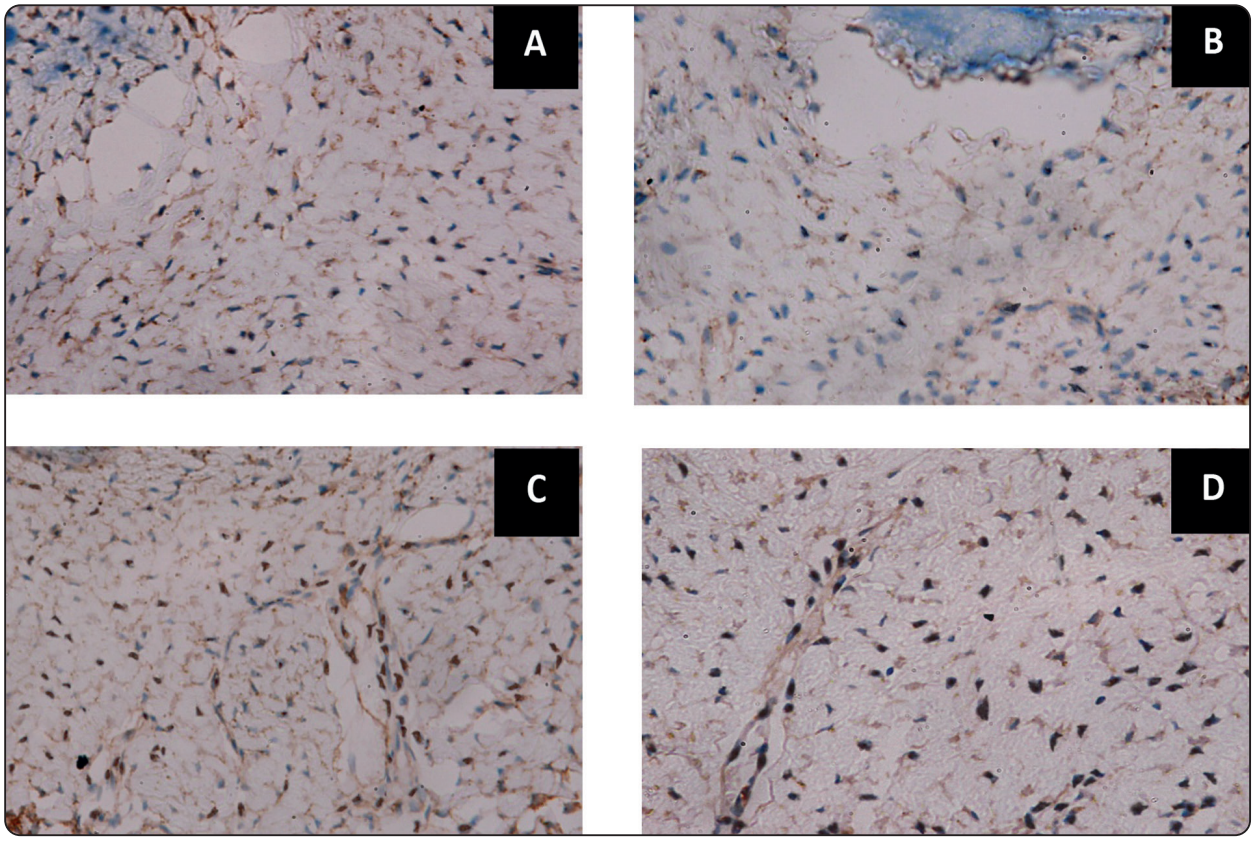

Fig. (3) Photomicrographs of all the studied groups, showing different patterns of nuclear immunoreactivity in periodontal tissue fibroblasts. (A,B) Control groups, (C,D) BMSCs-treated groups (original magnification X200).

average area percentage. These findings suggest that the effects of stem cell use and time are not independent of each other and are influenced by their combined effects (Tables 2-a, b). Comparison among the groups at different sacrifice dates revealed that the stem cell-treated groups exhibited significantly higher mean cell counts compared to the control group. Furthermore, the group treated for six weeks showed a statistically significant increase in mean cell count compared to the group treated for two weeks. In contrast, the control groups demonstrated a significant decrease in mean cell count at the second sacrifice date (Table 2-c).

TABLE (2-a). Two-way ANOVA results for the effect of different variables on mean cell count.

Source of variation	Type III Sum of Squares	df	Mean Square	*F*-value	*P*-value	Effect size *(Partial eta squared)*
Using stem cells	447561	1	447561	1653.423	<0.001*	0.981
Time	12321	1	12321	45.517	<0.001*	0.587
Using stem cells x Time interaction	103041	1	103041	380.664	<0.001*	0.922

*df: degrees of freedom = (n-1), *: Significant at P ≤ 0.05*

TABLE (2-b). The mean, standard deviation (SD) values and results of two-way ANOVA test for main effects of the two variables on cell count.

Variables		Mean	SD	*P*-value	*Effect size (Partial Eta squared)*
Using stem cells	Stem cells	277	77.1	<0.001*	0.981
	Control	54	36.7		
Time	T2	147	60.2	<0.001*	0.587
	T6	184	171.1		

**: Significant at P ≤ 0.05*

TABLE (2-c). The mean, standard deviation (SD) values and results of two-way ANOVA test for comparison between cell count with different interactions of variables.

Time	Stem cells		Control		*P*-value	Effect size *(Partial eta squared)*
	Mean	SD	Mean	SD		
T2	205	6.2	89	9.9	<0.001*	0.875
T6	349	30.7	19	2.4	<0.001*	0.983
P-value	<0.001*		<0.001*			
Effect size *(Partial eta squared)*	0.915		0.718			

**: Significant at P ≤ 0.05*

DISCUSSION

The introduction of stem cell-based therapies has recently been an encouraging field for research. Several studies have shown promising results regarding the possibility of using BMMSCs in the treatment and management of several conditions such as diabetic kidney disease[20], immune mediated inflammatory diseases[21], Alzheimer's disease[22], and ischemic heart disease and in improving stroke recovery[23,24]. With respect to the oral cavity, stem cells hold positive potential in the management of different situations, including maxillofacial bone repair, and dental pulp regeneration. Although their ability to differentiate into different types of periodontal cells, such as cementoblasts and osteoblasts, a definite role in the healing of periodontal ligaments in cases of inflammation has not yet been fully established[25].

To evaluate the effect of BMMSCs on the healing of periodontal fibers periodontitis, Masson's trichrome special stain was used to assess the condition of collagen fibers in albino rats treated with stem cells, and the results were compared with those of the untreated groups, at different durations. Our findings revealed that the fibers in the treated group sacrificed after 6 weeks were more intensely stained and had a normal arrangement than those in the group sacrificed after 2 weeks. This means that the longer the duration is, the more stem cells have time to exert their effects on diseased tissues and induce healing by promoting collagen production. Compared with the treated groups, the untreated groups had less positive reactions. In addition, the group that was left untreated for a longer duration presented more interstitial spaces and disorganized collagen fibers, which is normal considering that inflammation is left untreated for a longer duration. These findings coincide with the findings of Wu et al.[26], who studied the effect of stem cells on skin repair and regeneration, and reported that one of the mechanisms by which stem cells promote healing involves increasing levels of collagen production. Sharun et al.[27] also used Masson's trichrome and reported increased collagen production in response to stem cell therapy. Notably, research on the effect of BMMSCs on patients with myocardial infarction revealed that a longer duration of treatment leads to better vascularization[28]. The increased blood supply also contributes to elevated levels of collagen production[29], resulting in better expected outcomes from stem cell therapy, which matches our findings.

To confirm the previous findings, immunohistochemical staining was used to measure the levels of β-catenin in periodontal fibroblasts. The reaction to the antibody was apparent in the nucleus of the periodontal fibroblasts, matching the findings of Lim et al.[30]. Similar to the findings from Masson's trichrome staining, the group that presented the highest level of nuclear immunoreactivity was the group treated with BMMSCs for 6 weeks, followed by the group treated for 2 weeks. Moreover, the lowest levels were found in the control group, which was sacrificed after 6 weeks. Since β-catenin plays a major role in the Wnt/β-catenin signaling pathway, which, when upregulated, promotes cellular proliferation[31], increased expression indicates fibroblastic proliferation as a result of effects of BMMSCs. This finding is similar to the findings of Voza et al.[32] reported that increased levels of β-catenin expression are associated with an increased number of fibroblasts. For BMMSCs, the β-catenin-dependent Wnt pathway, known as the canonical pathway, promotes self-renewal and differentiation of stem cells into periodontal cells[33,34], which can explain the increase in the number of fibroblasts reflected by the elevated levels of immunoreactivity.

CONCLUSION

Histological and immunohistochemical investigations confirmed that the bone marrow-derived stem cells improved the quality of the collagen fibers in albino rats suffering from

periodontitis in a time dependent manner. This led to the restoration of healthy functional periodontal ligaments. Despite our findings, further research should be conducted to explore all the possible benefits of using BMMSCs for improving the periodontal condition in diseased patients.

Abbreviations:

- BMD: Bone mineral density.
- BMMSCs: Bone marrow mesenchymal stem cells.
- PBS: Phosphate buffered saline.
- SD: Standard deviation.
- ANOVA: Analysis of variance.
- PDL: Periodontal ligament.
- MNC: Mononuclear cell layer.
- FACS: Fluorescence-activated cell sorting.
- RBCs: Red blood cells.

Ethics approval:

Ethical approval was granted by the Ain Shams University Ethical Committee (FDASU-Rec IR012447).

Consent for publication: Not applicable

Availability of data and materials:

The datasets utilized and/or analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests.

Funding: This research received no specific grant from any funding agency in the public, commercial, or non-profit sectors.

Authors' contributions:

- TS have made substantial contribution to the conception and design of the work. IS has written the original draft, worked as project administrator, and submitted the manuscript.DM made the acquisition, analysis, and interpretation of data. AT contributed to the practical work (staining procedures) and revised the written manuscript. All authors have approved the submitted version, and any substantially modified version that involves the author's contribution to the study.

Acknowledgements:

Would like to acknowledge and thank Dr.Khaled Mohamed Keraa, Biostatistician at Misr International University, for his contribution in the statistical analysis of this study.

Clinical trial number: not applicable.

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