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THE EFFICACY OF ADIPOSE STEM CELL-DERIVED EXTRACELLULAR VESICLES ON THE STRUCTURE OF ALVEOLAR BONE IN RATS WITH INDUCED PERIODONTITIS (EXPERIMENTAL STUDY)

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ABSTRACT

INTRODUCTION: A plethora of therapeutic interventions are available to treat periodontitis. Mesenchymal stem cell (MSCs) therapy is among the treatment options currently under investigation. Regenerative potential of MSC can be attributed to its paracrine effect carried out by extracellular vesicles (EVs). EVs are nano- to micro-sized particles with great potential in tissue engineering due to their safety, ethical acceptability, and ease of use.

AIM: This study aimed to investigate the effect of local injection of 40µgs of ADSC-EVs on periodontal healing in rats with induced periodontitis.

MATERIALS AND METHODS: Twelve male Albino Wistar rats were randomly divided into two groups: Ligature Group (Lig-G) and EV-treated (EV-G). Adipose derived mesenchymal stem cells were isolated and extracellular vesicles were extracted and characterized. Sutures were secured at the gingival margin of rats' mandibular molars for 2 weeks. Lig-G was left untreated while EV-G, received a single injection of 40µgs of EVs injected into the induced defect at the time of ligature removal. Rats in both groups were euthanized after 4 weeks of ligature removal for histological and histomorphometric analysis.

RESULTS: Histological analysis of LigG revealed signs of bone resorption as for EV-G, EV-treated specimens showed preserved alveolar bone architecture with minimal resorption. Histomorphometric analysis revealed significant increase in bone surface area (BSA) percentage in EV treated group (31.333 ± 11.16) compared to Lig-G (9.444 ± 3.04) as p <0.0001.

CONCLUSION: Local injection of 40 µgADSC-EVs significantly enhanced alveolar bone regeneration in periodontitis-induced defects, suggesting a potential therapeutic strategy for periodontal tissue engineering.

KEYWORDS: Periodontitis, mesenchymal stem cells, extracellular vesicles, bone regeneration.

RUNNING TITTLE: Effect of extracellular vesicles on periodontitis.

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INTRODUCTION

Periodontitis, a chronic multifactorial inflammatory condition, is linked to dysbiotic plaque biofilm and results in the gradual destruction of the tooth's supporting structures.(1) Conventional treatment approaches per se are unable to restore and regenerate damaged periodontal tissues. (2) Subgingival instrumentation and conservative flap therapy aim to only prevent disease progression by eliminating pathogenic factors without restoring damaged periodontium. (3,4) Moreover, results of guided tissue regeneration (GTR) are unpredictable since the prognosis depends on different factors such as patients' compliance, presence of risk factors, choice of material used and surgical skill of operator. (4)

Tissue engineering is a valid treatment modality. Langer and Vacanti identified undifferentiated cells, scaffolds and signaling molecules as integral components of tissue engineering.(5). Mesenchymal stem cells (MSCs) are rapidly proliferating, self-renewing, non-

hematopoietic progenitor cells that can develop into numerous cell types such as cementoblasts and osteoblasts. (6)

Although MSCs from various sources possess similar capabilities, adipose-derived stem cells (ADSCs) are favored due to its availability, easy harvesting and abundance.(7) Nowadays, MSCs are believed to function through paracrine action, as the factors released by MSCs play a crucial role in promoting tissue regeneration. (8)

Owing to its endogenous origin, extracellular vesicles (EVs) are a safer alternative to cell based therapies. (9) EVs are non-replicable bilayer lipid membrane vesicles that comprise multiple signaling chemicals, proteins, nucleic acids and lipids. (10) They are released by different cell types. EVs are classified based on their size, mechanism of production, and cellular origin into three major groups which are: group 1: apoptotic bodies from dying cells, group 2: microvesicles formed by plasma membrane budding, and group 3: exosomes which are endocytic in origin. (11) Additionally, EVs can be supplied in various forms . (12)

The regenerative capacity of EVs can be associated with its ability to control angiogenesis, cell differentiation, proliferation, and apoptosis since EVs can release their cargo or activate specific receptors which can influence a multitude of signaling pathways. The delivery of proteins, messenger RNAs (mRNA), and microRNAs (miRNA) via (EVs) has emerged as a promising mechanism for mediating their regenerative effects. (13)

Investigations in animal models show that EVs have a positive effect on periodontal regeneration. (14–16) It is believed that local administration of EVs is favorable as it has fewer adverse effects and show better control of EV treatment.(17) Given the growing interest in minimally invasive approaches and the regenerative potential of EVs in periodontal therapy, it is important to formulate a minimally invasive treatment protocol that incorporates EVs in the management of periodontitis. However, the optimal dose of EVs in periodontal therapy remains undetermined. (18) Consequently, the current study was conducted to demonstrate the impact of locally injecting 40 µg of ADSC EVs on periodontal healing in a periodontitis rat model. This study's null hypothesis assumes local administration of 40 µg of ADSCS EVs has no influence on periodontal healing in a rat model with induced periodontitis.

METHODS

Prior to conducting the study, approval was granted by the ethical review board at the Faculty of Dentistry, Alexandria University (Alex-Uni) (0734–7/2023).

Collection of ADSCs: (19)

A single 6 months old male Wistar rat weighing 300 grams was used in this study to harvest adipose tissue from which adipose stem cells (ADSCs) and adipose derived extra cellular vesicles (ADSC EVs) were collected.

An adequate amount of adipose tissue was collected through abdominal incisions exposing the subcutaneous adipose layer. The tissue was then repeatedly rinsed with phosphate buffer saline (PBS), mechanically cut, added to collagenase type I solution, and incubated for 30 minutes to 1 hour at 37°C. To halt enzymatic activity, equal amount of Dulbecco's modified Eagle's medium-F12 (DMEM-F12) which is a cell culture medium was added. To pellet the cells, digested tissue was spun at 300 xg for five minutes. Then, it was resuspended in DMEM, 10 % fetal bovine serum, 100IU ml^{-1} penicillin and 100mg ml^{-1} streptomycin. The cells were seeded and cultured in T-25 flasks at thirty-seven °C in a humidified five % CO2 environment. The culture medium was renewed every other day. Upon reaching eighty% confluence, cells were detached using trypsin-EDTA and transferred to T-75 flasks, ultimately yielding conditioned culture media. (fig 1)

Adipose stem cell characterization (20)

MSCs were characterized through immunophenotyping via fluorescence-labeled monoclonal antibodies targeting CD90 and CD45. Following adherence, the cells were separated using trypsin, washed with PBS, and exposed to allophycocyanin (APC)-conjugated antibody for CD90, and fluorescein isothiocyanate (FITC)-conjugated antibody for CD45 (Abcam, Cambridge, UK) in darkness at room temperature for a period of 30 minutes . After incubation, PBS was used to wash the cells thrice then cells were resuspended in 500 µl of FACS buffer. Flow cytometric analysis was conducted on viable cells using a BD FACSCalibur flow cytometer operated via CellQuest software (Becton Dickinson, New Jersey, USA).

Extracellular vesicles (EVs) separation (21)

At p3, ADSCs were grown in serum-free media for forty-eight hours, then conditioned media was gathered for EV isolation via differential centrifugation. To remove cell debris and concentrate the media, it was spun at 300 xg for 10 minutes at room temperature. The resulting supernatant was saved, and the debris pellet was discarded. The following step was high-speed centrifugation (16,500 xg for 40 minutes at 4°C) and removal of larger microvesicles through an 0.2 µm filter. Final EV isolation involved ultracentrifugation of the supernatant (Thermo Fisher Micro UltracentrifugeMX 120 with a fixed angle rotor) at 110,000 xg for 70 minutes at 4°C. The supernatant was removed, and the EV pellet was resuspended in PBS.

Extracellular Vesicles (EVs) characterization (21)

Transmission electron microscopic (TEM) analysis of EVs

In order to investigate particle size and shape, EVs were dissolved, placed on copper grids, and stained at the Electron microscope unit, Faculty of Science, Alex-Uni.

Quantification of EVs using protein assay

The amount of EVs was determined by measuring total protein in an isolated sample via BCA protein assay kit.

Zeta-sizer

Diluted EVs were sonicated then size measurement was done using a zeta sizer. Malvern Panalytical software (Worcestershire, UK) was used to calculate the polydispersity index (PDI)

Experimental Design

12 adult male Albino Wistar rats weighing 210-300 gm were collected from the Experimental Animal Facility at the Department of Medical Physiology, Faculty of Medicine, Alex-Uni. Rats had unrestricted access to food and water and were maintained under a 12 hour light/dark cycle. They were divided to ligature induced periodontitis group (Lig-G) or EV treated group (EV-G). This sample size was based on a power analysis that would be required to achieve eighty percent power and significance level of five percent according to Mohammed et al(16) (alpha error accepted 0.05).

Injections of xylazine (10 mg/kg) and ketamine (50 mg/kg) were used to anesthetize the rats. To induce periodontitis, 4-0 sterile silk ligatures were applied at the marginal level of the mandibular first molar.(22) Ligature was removed after 2 weeks, and the EV-G received a single injection of 40 µg of EVs suspended in 0.2 ml PBS. It was injected to the interdental papilla down to the bone level opposite to the mandibular first molar via buccal approach using 26 gauge syringe. (23) Rats were euthanized after 4 weeks by cervical dislocation and mandibles were collected for histological and histomorphometric analysis.

Light microscope examination (24)

The tissue samples were prepared for microscopic examination by first preserving them in a 10% formalin solution. After rinsing, the samples were subjected to a series of steps: decalcification in 8% trichloroacetic acid, ethanol dehydration, xylene clearing, and paraffin wax embedding. Thin slices, 4 micrometers thick, were cut from the wax blocks then treated with Hematoxylin & Eosin. Histological analysis was conducted in a blinded manner, with the examiner unaware of the group assignments, and all findings were cross-verified by a second blinded examiner.

Histomorphometric assessment (25)

For every specimen, ten images at ×100 magnification were taken by light microscope. The average percentage of newly formed bone surface area (BSA) was compared to the total surface area of bone (TSA) and analyzed using ImageJ 1.46 software (LOCI, University of Wisconsin, Wisconsin, USA). All bone histomorphometric analyses were conducted without prior knowledge of the rats' group assignments, and a second examiner verified the measurements.

2.8 Statistical analysis

The mean ± standard deviation (SD) were used to present the quantitative data. Data normality was assessed by Shapiro-Wilk test. Independent T-test was used to determine statistical significance between groups, with results considered significant if the p-value <0.05 using GraphPad Prizm 9.0 (GraphPad Software, Inc., California, USA).

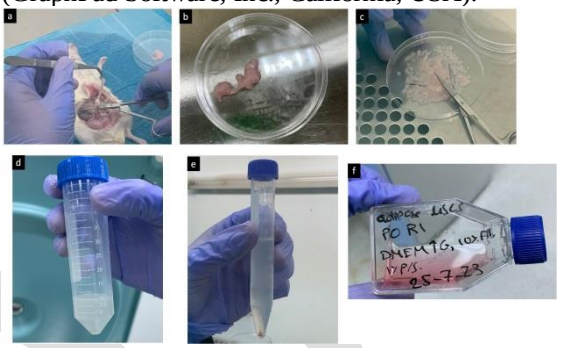

Fig 1 showing the process of ADSC collection: harvesting of adipose tissue (AT) (a) harvested AT (b) AT finely cut (c) collagenase type I solution placed on minced AT (d) cell pellet formed following the spinning of digested tissue at 300 xg for 5 minutes (e) cell pellet placed in DMEM, FBS, penicillin and streptomycin then seeded in T-25 flask (f)

RESULTS

Characterization of ADSCs

Morphological characterization of ADSCs

Daily monitoring of cell culture was performed using Daily monitoring of cell cultures was performed using a phase-contrast inverted microscope. In the primary culture stage, cells initially exhibited rounded morphology, which transitioned to a spindle-like shape after approximately 72 hours. The cultures reached 70–80% confluence within 10 to 12 days. With subsequent passaging, cell proliferation rates increased, and morphological changes included a predominance of flattened, spindle-shaped cells. By the third passage (P3), the cell population formed a uniform monolayer with fibroblast-like characteristics. (fig 2 a)

Immunophenotyping of ADSCs

Flow cytometry analysis for ADSC at P3 revealed: 95.62% of cultured cells expressed MSC surface marker CD90 and 0.03% were negative for hematopoietic marker CD45. (fig 2 c)

Characterization of EVs

Transmission electron microscopic (TEM) analysis of EVs

TEM analysis revealed the presence of round structures of different sizes (56 nm to 309 nm). It also showed an apparent lipid bilayer. (fig 3 a,b)

Zeta sizing

Nanoparticles were detected at peak size of 208 nm and 100 percent intensity (fig 3 c)

EV protein content

BCA assay indicated that the isolated EV solution contained 1200 µg/ml of protein. The amount of protein within (EVs) was representative to estimate the quantity of EVs present.

Histological Analysis

Ligature group (Lig-G)

The alveolar bone exhibited irregular bone margins, with apical shift in the bone crest, and a "punched-out" appearance as well as stained reversal lines. Osteoblasts were discontinuous along the bone surface, and osteocytes showed pyknotic nuclei. Widening of periodontal ligament space was observed. (fig 4 a,b)

EV treated group (EV-G)

EV treated group showed restored alveolar bone crest with a smooth outline. The bone surface was lined by plump osteoblasts and dense bone trabeculae surrounded marrow spaces. Normal thickness and orientation of periodontal ligament were noted. (fig 4 c,d)

Histomorphometric Analysis

Quantitative assessment of the percentage of BSA obtained from the histologic light micrographs showed that the percentage of BSA in Lig-G (9.444 ± 3.04) was significantly less than that of EV-G (31.333 ± 11.16) where p <0.0001. (fig 5)

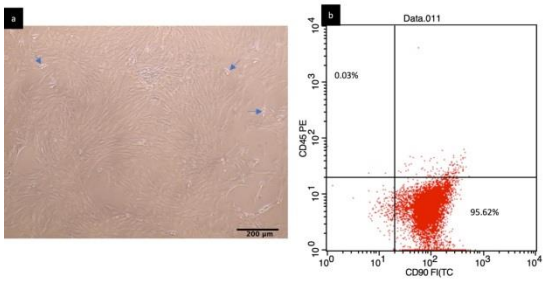

Fig. 2 Phenotypic characterization of ADSC showing spindle shape, fibroblast-like cells at P3. MSCs cells 70 % confluent (100x), scale bar 200 µm using inverted phase microscope. Arrow: Proliferating twin cells (a), Flow cytometric analysis showing the percentage CD90, as 95.62 and negative for CD 45 (0.03%) (c)

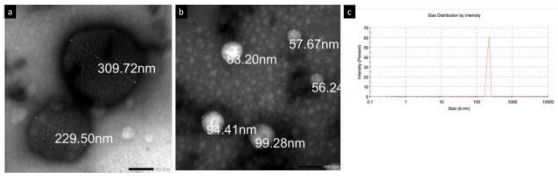

Fig 3 Characterization of EVs: TEM analysis revealed the presence of rounded structures of different sizes (56 nm to 309 nm) with an apparent lipid bilayer. (a,b) graph showing size and peak intensity of isolated EVs(c)

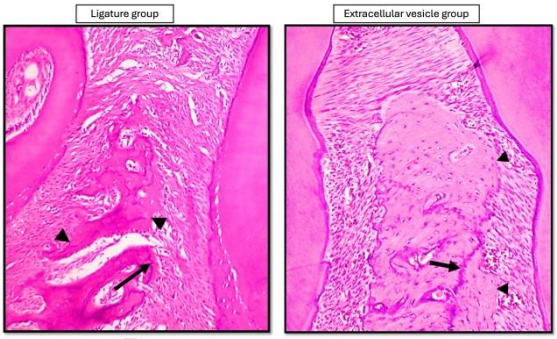

Fig 4 Lig-G showing apical shift in crest of alveolar bone and marked bone resorption with discontinuity of osteoblast and punched out appearance (arrow head) incremental lines indicating bone remodeling (arrow); EV-G group sacrificed after 4 weeks showing restored alveolar bone crest with its smooth outline and presence of osteoblasts along bone surface (arrow head) with incremental lines suggestive of bone remodeling (arrow).

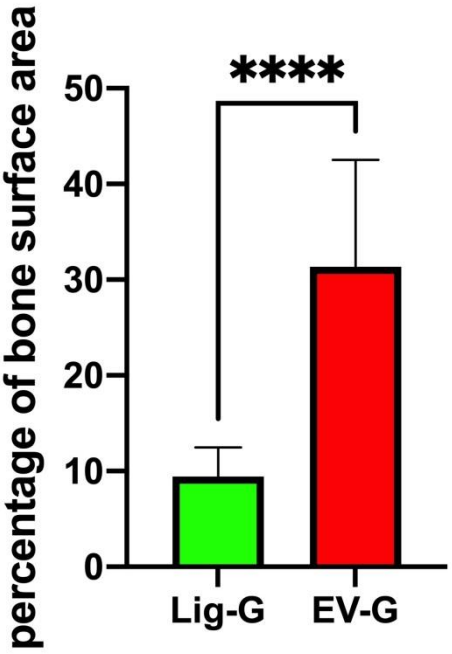

Fig 5 column bar graph was used illustrating the mean percentage of BSA, error bars= SD, n=6 per group, **** p <0.05

DISCUSSION

Complete and functional restoration of periodontal tissues is challenging. (4) EVs can be considered as a reliable treatment modality as they can change the recipient cells' function by supplying genetic information that alter their properties and paracrine factors resulting in tissue regeneration. (26) This study was designed to shed light on the impact of locally injecting ADSC EVs on alveolar bone in ligature induced model and revealed that EVs did

improve the structure of alveolar bone in relation to ligature untreated group.

Zhou et al (27) reviewed the effect of MSC derived EVs in the management of periodontitis induced bone loss and revealed broad range of EV concentrations used (20 µgs to 500 µgs). Therefore, 40 µgs were selected representing a relatively low concentration within the used range.

Isolation of adipose derived stem cells was successful as the isolated cells were flat spindle shaped fibroblast like cells and demonstrated adhesive and proliferative properties. Moreover, isolated cells were positive for CD90 which is a MSC surface marker and were negative for the hematopoietic marker CD 45. The current findings align with Bunnel's (28) findings who reviewed the characteristics of ADSCs.

This study employed the ultracentrifugation method to successfully obtain EVs. TEM was used to characterize EVs where it revealed the presence of round shaped structures of different sizes with an apparent lipid bilayer which are consistent with the appropriate shape and size of EVs confirming the successful mining of EVs as per Pol et al.(29) In addition, nanoparticles were detected at peak size of 208 and 100 percent intensity by zeta sizing. TEM and zeta sizing results are in agreement with Ren et al (21) who confirmed obtaining ADSC-EVs using similar characterization methods. The protein content of EVs was 1200 µg/ml, which reflects both the efficiency of isolating EVs and the high protein content of EVs. The aforementioned results align with guidelines established by the Society for Extracellular Vesicles (ISEV) for EV characterization. (10,30,31)

Rats were used in this study because of the structural similarity of their periodontium and molars to those of humans.. (32) In addition, they are readily available and the housing and maintenance of rats are easier and cost-efficient compared to other animal models. (33)

Tomina el al (34) tested 2 different methods of inducing periodontitis and reported that ligating rats' molars proved to be an effective way in replicating periodontitis in humans. They also stated that the periodontal destruction is due to the increase in inflammatory cytokines TNF-α and IL1-α.

The effectiveness of ligature induced periodontitis method was supported histologically by the apical shift in the alveolar bone crest and bone loss which is in line with Tomina et al (34) findings. Histomorphometry showed noticeable improvement in alveolar bone structure between Lig-G and EV-G. Our results are in accordance with Mohammed et al (16) who investigated the effect of different treatment modalities such as non-surgical instrumentation, injection of ADSCs and injection of its exosomes on ligature induced defect and concluded that the exosome treated group had superior results to ADSC and non-surgical instrumentation as there was evidence of new periodontal tissue formation as early as 2 days and by the 4th week interval, the structure of periodontal tissues was comparable to healthy tissues.

Moreover, our findings align with those of Yue et al (23), who utilized non-dental bone marrow MSC-derived EVs in managing induced periodontitis defects and showed their role in modulating cell metabolism, promoting differentiation, and facilitating the resolution of inflammation. In addition, the present findings are consistent with observations by Wei et al(15)., who demonstrated that EVs obtained from human exfoliated deciduous teeth mitigated bone resorption through the increase in RUNx2 expression and downregulation of IL-6 and TNF-α expression. In contrast to the findings of Nakao et al.(35), who employed 40 µg of pretreated human gingiva-derived MSC-EVs, the current study shows that the same dose of untreated ADSC-EVs was sufficient to enhance BSA.

The above-mentioned results can be attributed to the ability of EVs to modulate inflammatory response. In a mouse model, Parvanian and colleagues (36) demonstrated that ADSV EVs accelerated wound healing by enhancing the production of anti-inflammatory IL-10 as well as suppressing IL-6 and TNF-α. It is worthy to mention that TNF-α is one of the key mediators of inflammation and alveolar bone loss. (37) In addition, Nolde et al (38) presented genetic evidence that suppressing IL-6 is linked to lower chances of developing periodontitis. The attenuation of TNF-α and IL-6 and has great impact on periodontal healing. (37,38)

Furthermore, the rich cargo carried by EVs contributes to the observed enhancement of BSA. This is supported by the findings of Ma et al (14), who reported that EVs induce periodontal ligament stem cell differentiation into osteoblasts by expressing osteogenic genes such as BSP, COL1, RUNX2 and their associated proteins. The group found evidence of bone and periodontal like tissues within the defect site where labelled EVs were found.

Additional in vitro investigations are needed to fully comprehend the mechanism of action of ADSC EVs, which remains a limitation in this study.

Comprehending the functional mechanisms of EVs is fundamental as not all EVs exhibit therapeutic properties. In fact, EVs have been implicated in promoting tumorigenesis and tumor progression via the transfer of specific microRNAs (miRNAs) to neoplastic cells or activating signaling pathways.(39) Further investigations employing a wider range of

concentrations of (EVs) would be crucial for determining the optimal concentration for periodontal regenerative therapies. Prior to initiating clinical trials, comprehensive in vivo evaluation of the dose-dependent and frequency-dependent effects of EV administration on toxicity and immunogenicity is essential. (17)

The null hypothesis was disproven as the EV-treated group (EV-G) exhibited significant periodontal healing, showing improved alveolar bone structure compared to (Lig-G).

CONCLUSION

EVs show promise for regenerating periodontal tissues. They are safe, easy to handle and have strong potential to repair damaged tissues.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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