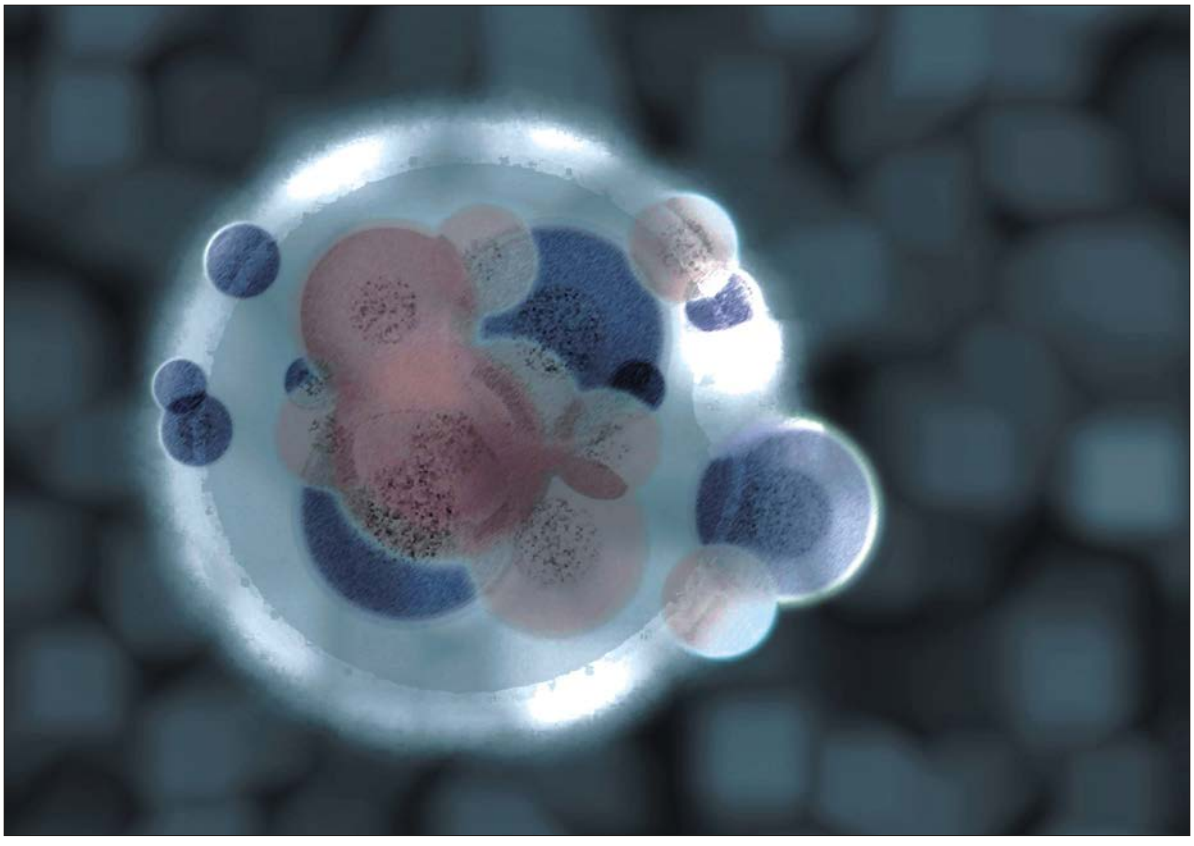

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Research Article
Influence of Vitamin E on Proliferation and Differentiation of Rat's Dental Follicle Stem Cells Treated with Nicotine (An Experimental Study)

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Abstract

Background and Objective: Many lines of evidence suggested that the oxidative stress is important in pathogenesis of diseases. Nicotine inhibited the proliferation and osteoblastic differentiation of mesenchymal stem cells (MSCs) and decrease alkaline phosphatase (ALP) activity. The biological antioxidants, such as vitamin E act in synergy to form an integrated network of antioxidant defense, which is considered to be the first line of defense against any increase in the production of reactive oxygen species (ROS). The objective of the study was to investigate the effect of vitamin E on the proliferation as well as differentiation potentiality of rat's dental follicle stem cells (DFSCs) treated with nicotine. **Materials and Methods:** Rat DFSCs were isolated, cultured and divided into four groups. The 1st group was the control, the 2nd group was cultured with 5 mM of nicotine, the 3rd group was cultured with 2mM of vitamin E (1h) prior to nicotine treatment and the 4th group was cultured with 2 mM of vitamin E (1 h) after nicotine treatment. Methyl thiazol tetrazolium (MTT) assay was used to assess the cell viability among the groups used. Wright staining solution was used to assess the effects of nicotine on cell morphology. The flow cytometric analysis was used to identify the stemness of isolated rat's DFSCs by CD34 and CD29. Alizarin red-sulfate (AR-S) staining and quantitative real time-polymerase chain reaction (qRT-PCR) utilizing alkaline phosphatase (ALP) expression and activity were used to determine the osteoblastic differentiation. **Results:** The results of this study revealed that increase the proliferation rate and the ALP expression and activity in group I followed by group III and IV then group II. The vacuolization and loss of cell membrane of DFSCs appeared to be more extensive in group II compared to group III and group IV. **Conclusion:** These findings indicated that vitamin E could relief the state of oxidative process induced by nicotine. Vitamin E also can increase the capability of DFSCs to proliferate and differentiate into osteogenic lineage detected by AR-S stain and increase the ALP level.

Key words: Vitamin E, nicotine, dental follicle, stem cells, osteogenic differentiation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tissue damage and loss caused by mechanical trauma, degenerative diseases, infections, tumors and other diseases exert a profound negative impact on patient quality of life and impose a heavy social and economic burden. Currently, tissue engineering is believed to be a promising approach to recover the structural integrity and function of the damaged or diseased tissues[1].

Among tissue engineering, the potentially of stem cells (SCs) in regenerative medicine, developmental biology and drug discovery has been well documented. In dentistry, tissue engineering is also considered to be a new frontier in the regeneration of missing oral tissues/organs. These include various degrees of alveolar bone resorption occur after tooth loss/extraction because of periodontal disease, severe caries, root fractures or accidental trauma[2].

Proliferation, differentiation and self-renewal of SCs are strictly regulated and controlled by a number of soluble factors, including cytokines, interleukins and chemokines as well as by extracellular matrix (ECM) and adhesion molecules. Exposure to certain patho-physiologic factors, including cigarette smoke and their byproducts, could lead to an imbalance in SCs properties as well as in the reduction of functionally active immune-competent cells[3].

Among cigarette smoke composition, nicotine is the most prevalent chemical substance, has negatively impact on human embryonic SCs and their development by altering the cell's morphology via a number of free radicals generated that cause oxidation of cells[4].

A protective effect might be possible by means of antioxidants. Vitamin E: α-tocopherol (α TF) is the most important antioxidant and protecting cell membrane from their damage. Vitamin E as a lipid-soluble substance consists of two major groups, which are TFs and tocotrienols (TTs). The distinctive feature between these two groups is the presence of double bonds on the carbon chain of TTs. There are four distinct isomers (α, β, γ and δ) in each group depending on the position of the methyl group on the chromanol ring[5]. Accordingly, it seemed of interest, so the aim of the current study is to investigate the effect of nicotine on mesenchymal stem cell (MSCs) isolated from rat dental follicle (DF) tissue as well as when treated with vitamin E.

MATERIALS AND METHODS

In the present study, 20 neonatal albino rats were selected and sacrificed by decapitation, then DF tissues in the region of mandibular (right and left) 1st and 2nd molars were dissected to be prepared, on the Unit of Biochemistry and Molecular Biology at the Medical Biochemistry Department, Faculty of Medicine, Cairo University (January, 2017), for the following steps:

Isolation of rat's DFSCs: The tissue specimens were minced into 1 mm^3 pieces then collected in eppendorf tubes to which, a digesting solution consisting of (3 mg mL^{-1}) collagenase type II for 30-45 min at 37°C (Sigma-Aldrich, USA), the centrifugation of the tubes was performed for 20 min at room temperature to obtain cell pellet then the culture media was added. The culture medium is consisted of RPMI 1640 (Gibco, Invitrogen Life Technologies, USA) supplemented with 10 % FBS (Gibco, Invitrogen Life Technologies, USA), antibiotics (Penicillin G100 unit mL^{-1} and Streptomycin 100 µg mL^{-1}) and antimycotic agent (Fungizone 0.25 µg mL^{-1}). DFSCs were divided into four groups, group I (control group), group II containing DFSCs cultured with 5 mM of nicotine, group III containing DFSCs cultured with 2 mm of vitamin E (1 h) prior to nicotine treatment and group IV containing DFSCs cultured with 2 mm of vitamin E (1 h) after nicotine treatment. The effect of nicotine on cell survival was determined by methyl thiazol tetrazolium (MTT) assay "3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium". The absorbance of the solution was measured by a microplate reader at 540 nm[6-8] (Spectra MAX 250, Molecular Devices, Inc., Sunnyvale, CA).

Observation of cell morphology: In order to assess the effects of nicotine on cell morphology, cells were seeded at 1×10^5 cells/well and incubated in growth medium in 6-well plates for the indicated durations at the indicated nicotine concentrations. After 5 days, DFSCs were stained with Wright staining solution (Sigma-Aldrich Co.) for 5 min[9].

Identification of DFSCs by the negativity to CD34 and the positivity to CD29 antibodies using flow cytometry: Cell analysis was performed using cytomics FC 500 flow cytometer (Beckman coulter, USA) and CXP software version 2.2. 1×10^5 cells were incubated with 10 µL of monoclonal antibodies against: CD34 to show their negativity and CD29 to show their positivity (Beckman coulter, USA) at 4°C in the dark[10].

Osteogenic differentiation procedures: The 3rd passage culture of the cells was grown to 70% confluence in non-coated 3.5 cm dishes in culture media. Osteogenic differentiation was induced by incubating the confluent monolayers with osteogenic induction in the experimental

group used. Osteogenic medium consisted of osteogenic base media and osteogenic supplement media (ascorbic acid, dexamethasone) (Cat No CCM007, R and D) for 21 days. The medium was changed, twice a week, in all experimental groups used. In order to evaluate the presence calcified deposites, alizarine red sulfate (AR-S) stain was applied and quantitative real time polymerase chain reaction (qRT-PCR) was used to analyze the mRNA level of the osteogenic differentiation for measuring the alkaline phosphatase (ALP) expression and activity.

Statistical analysis: Data were coded and entered using the statistical package for social science version 22 (SPSS). Data were summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (one-way ANOVA test) with multiple comparisons *post hoc* test in normally distributed quantitative variables. Comparisons between the values measured at days 1, 3 and 7 were done using repeated measures ANOVA. When $p<0.05$ were considered as statistically significant.

RESULTS

At day 3, DFSCs culture showed morphological diversity of the cells (spindle and stellate shaped patterns) with varying sizes (Fig. 1). At day 7, group II, III and IV cultured DFSCs showed the same appearance with less distribution compared to group I (Figs. 2a, b, c). DFSCs appeared to proliferate and propagate until 70-80% of the dish area became fully covered with the cells and reached the confluence. This observation was shown in group I at day 10 (Fig. 3), in group II at day 17 (Fig. 4), in group III at day 13 (Fig. 5a) and in group IV at day 13 (Fig. 5b). The cells appeared with morphological diversity: spindle like and stellate shaped patterns. The negativity to CD34 was 1.8% and the positivity to CD29 was 98.8% (Fig. 6). At day 5, the vacuolization of DFSCs appeared to be more extensive in group II compared to group III and group IV. A loss of cell membrane of some cells was noticed (Fig. 7a, b, c). Statistical analysis of MTT of DFSCs revealed that at day 1: group II showed the lowest statistical significance compared to group I, III and IV, respectively ($p<0.001$, <0.005 and <0.001). Similarly, at day 3 ($p<0.001$, <0.002 and <0.002). This observation was also noticed at day 7: ($p<0.001$, <0.003 and <0.001) (Fig. 8).

At day 21, the red calcified deposits were detected with different patterns, small rounded, homogenous and coalescent nodules were seen to be dispersed within the culture cells (Fig. 9a). The calcified deposits appeared to be distinct and surrounded by abundant ECM. In group II, calcified deposits were seen to be aggregating with various

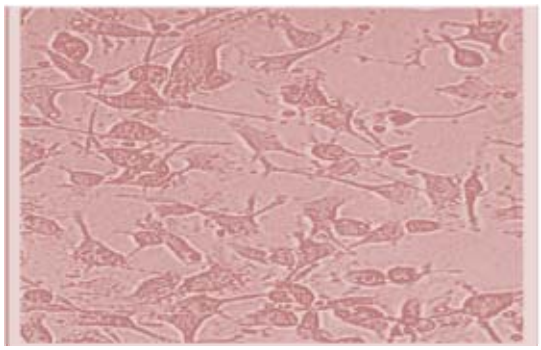

Fig. 1: Group I, at day 3, revealed spindle and stellate shaped cultured rat's DFSCs

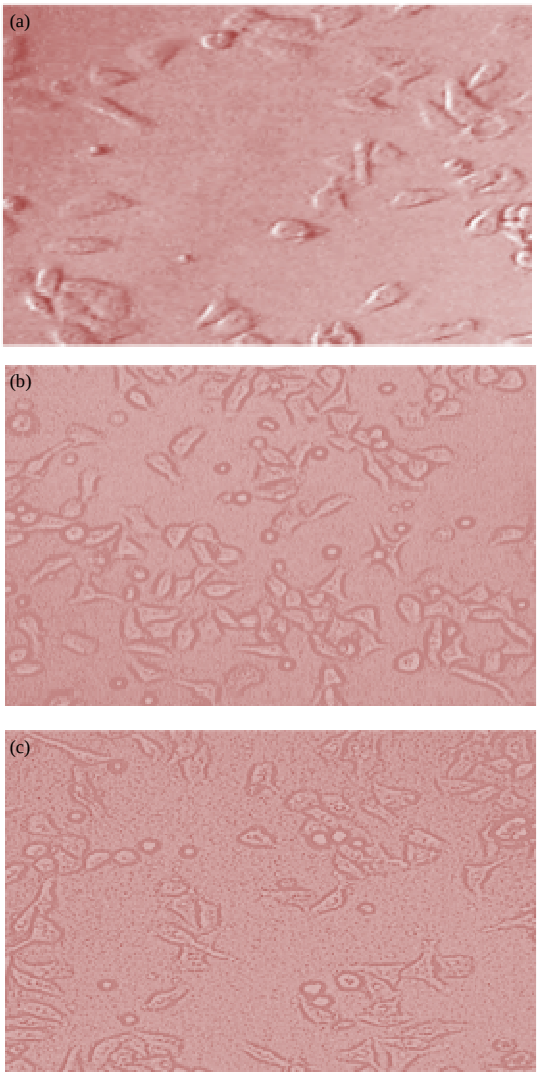

Fig. 2(a-c): (a) Group II, (b) Group III and (c) Group IV, at day 7, revealed spindle and stellate shaped cultured rat's DFSCs

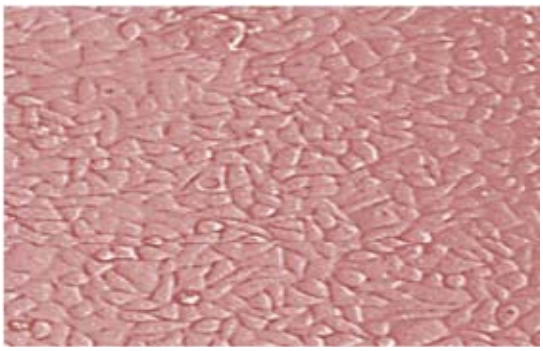

Fig. 3: Group I, at day 10, revealed extensive distribution of cultured rat's DFSCs

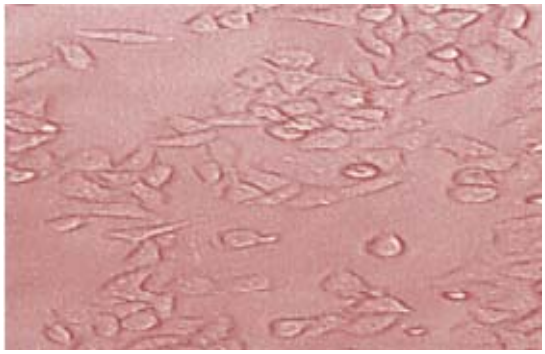

Fig. 4: Group II, after reaching the confluence at day 17

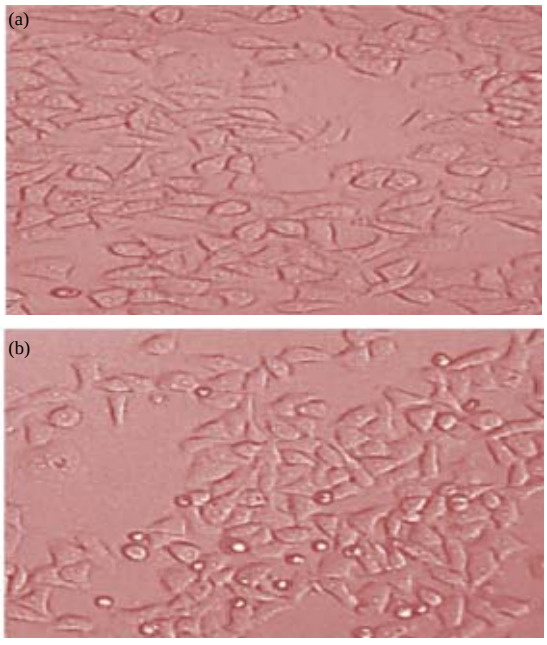

Fig. 5(a-b): (a) Group III and (b) Group IV, after reaching the confluence at day 13 (original magnification 40X)

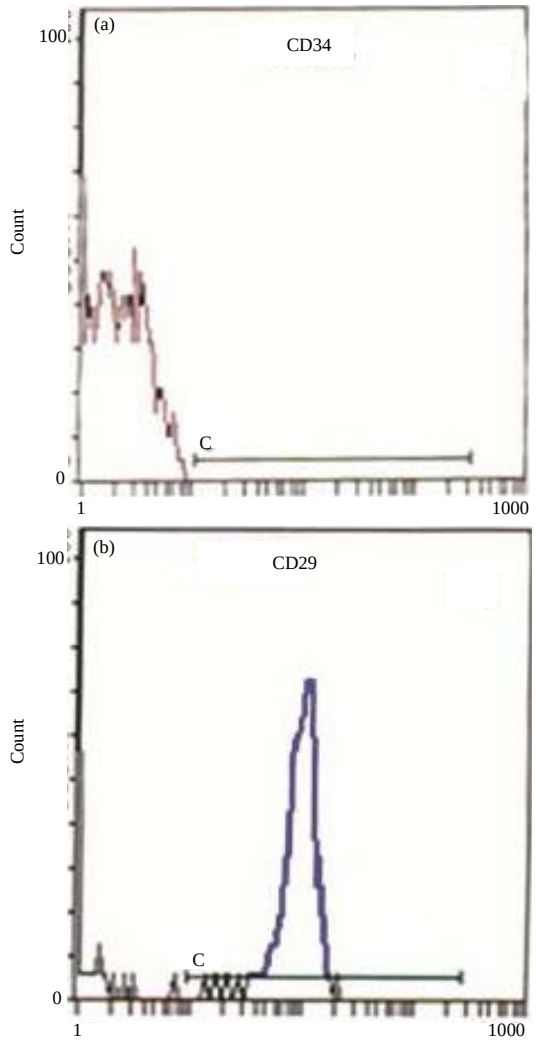

Fig. 6(a-b): A representative histogram of flow cytometric analysis revealed the (a) Negativity of cultured follicle SC to CD34 and (b) The positivity for CD29

morphological patterns: Some scattered and others deposited at the cell wall (Fig. 9b). Group III and group VI, showed scattered calcified deposits like-structures in the form of patches with variable sizes, while others appeared as individual calcified spicules (Fig. 9c, d).

Statistical analysis of ALP expression revealed that the highest statistical significance was shown in group I, followed by groups III, IV and group II ($p<0.001$, <0.001 and <0.002, respectively). There was no significant difference between group III and IV ($p = 1.000$) (Fig. 10).

Statistical analysis of ALP activity revealed that the highest statistical significance was shown in group I followed by groups III, IV and group II ($p<0.001$, <0.047 and <0.047,

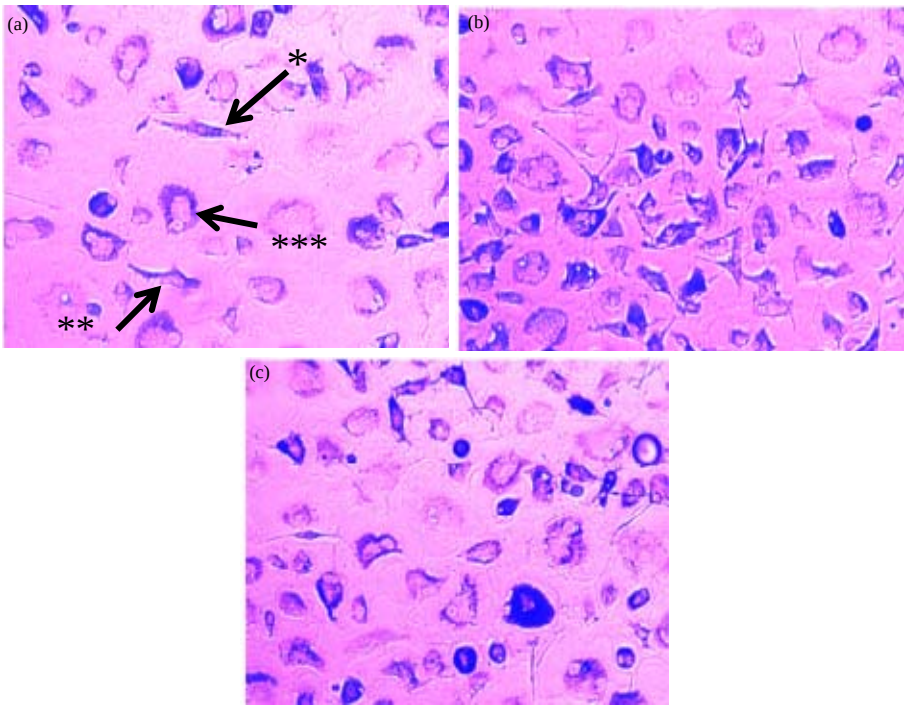

Fig. 7(a-c): Wright stain, in group II (a) Revealed DFSCs with morphological diversity, spindle like (*), stellate shaped patterns with vacuoles (**) and cell membrane loss (***), in comparing with groups those received vitamin E as in (b) Group III and (c) Group IV, Revealed more cell number and less vacuoles
(original magnification 40X)

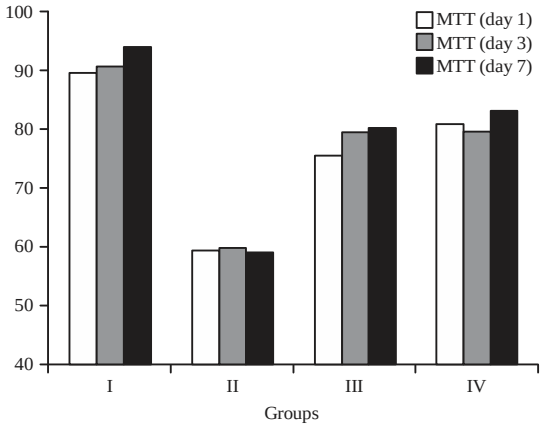

Fig. 8: Bar chart of the groups used, using MTT assay, on day 1, 3 and 7 representing Mean±SD values of DFSCs

respectively). There was no significant difference between group III and group IV (p = 1.000) (Fig. 10).

DISCUSSION

The ability of stem cells (SCs) to regenerate tissues of all germ layers leads to the thought that they may find great use in regenerative therapy of tissues damaged by disease, aging or trauma. For this purpose, DF represents a source of SCs that offers numerous research potentials. Further, their autologous nature will not elicit an undesirable immunological response, when these cells are used in tissue engineering.

The ease of isolation and high expansion potential of rat's DF has made its application as a model system for researches attractive. This was in agreement with that reported by Li *et al.*[11], who found that cells of DF tissue are mesenchymal cells so they were able to survive after several passages and promoting culture media[12]. In the present study, the isolation of SCs from rat's DF tissues through enzymatic digestion was successfully obtained.

In the present study, the effect of concentration of nicotine used (5 mM), on rat's SCs, revealed irreversible changes in the form of vacuolization and cell membrane loss as shown in group II, III and IV. Similarly, several studies revealed that higher nicotine concentration can induce extensive cell death and apoptosis[9,13,14]. This finding can be explained by the hypothesis that nicotine has adverse effect on proliferation and differentiation of DFSCs. Nicotine can

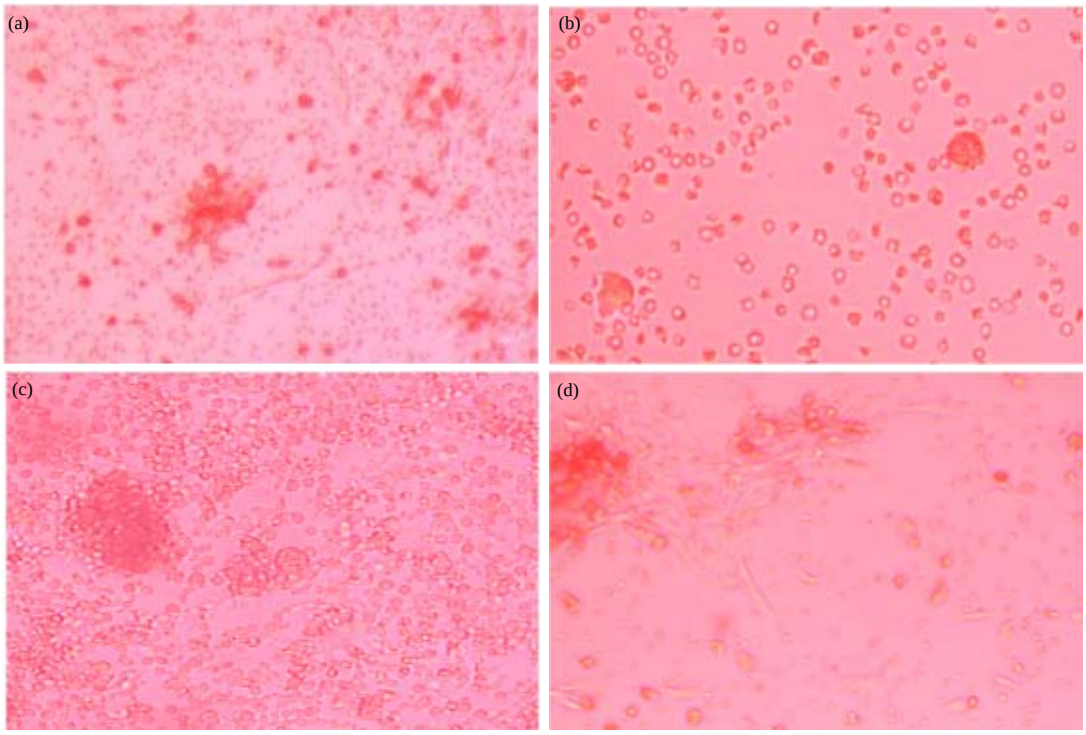

Fig. 9(a-d): AR-S stain, in (a) Group I, at day 21, revealed red-positive calcified deposits with different morphological patterns: small rounded uniform, homogenous nodules and others coalescence of some nodules was noticed, (b) Group II, (c) Group III and (d) Group IV
(Original magnification 20X)

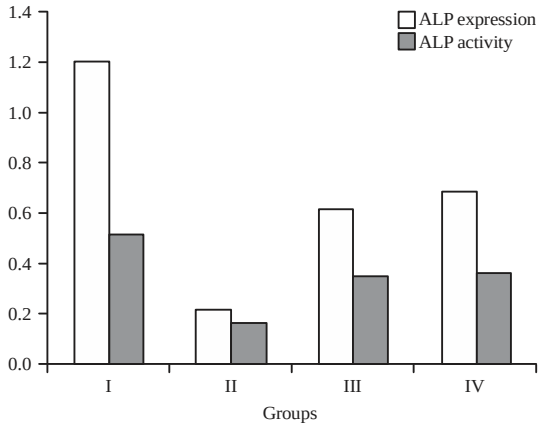

Fig. 10: Bar chart of DFSCs in the groups used on day 21 representing Mean±SD values of ALP expression and activity

increase the generation of free radicals by acting as a pro-oxidant and increase lipid per-oxidation following blocking of antioxidant enzyme. This process leads to the formation of ROS that can cause damage to the cell membrane and DNA fragmentation. MTT assay was used due to its rapidity, precision and the lack of any radioisotope[15]. ANOVA and multiple comparisons *post hoc* test were used to assess the results of MTT assay at day 1, day 3 and 7 among the groups used. Group I (DFSCs only) showed the highest mean value of cell proliferation, this was followed by group III and IV. This was in contrast to group II (DFSCs treated with 5 mM of nicotine) that showed the lowest mean value of cell proliferation. This finding was in accordance to Li *et al.*[11]. A high proliferative capacity is one of the most critical characteristics of MSCs. The cell proliferation rate is of greatest importance especially in cell therapy strategies where the rapid expansion of cells would be desired.

In the present study, for realizations of osteogenesis, the cells were also examined by quantitative RT-PCR for the expression of osteoblast-related gene (ALP). ALP was chosen since its expression marks the early stage of osteoblastic differentiation and considered as one of the first functional genes expressed in the process of calcification. Also, it has been recommended to use of ALP as a marker of hard tissue cell differentiation[16].

In the current study, the expression of ALP activity revealed various finding throughout the groups used. Group I showed the highest mean value followed by group III and IV, while group II showed the lowest mean value. The high

level of ALP activity and expression in group I, groups III and IV (those received vitamin E) provide new and important information into the fundamental mechanisms of hard tissue formation. This provides therapeutic opportunities for treatment of bone diseases and enhances the ability to create useful bone biomaterials. This finding can be explained by the hypothesis that ALP appears to act to increase the local concentration of inorganic phosphate (a mineralization promoter) and to decrease the concentration of extracellular pyrophosphate (an inhibitor of mineral formation)[17].

In the present study, vitamin E groups (III and IV) showed higher mean value compared to group II those having received nicotine only. This result was in agreement with that reported by Ahn *et al.*[18]. The SCs were shown to be proliferating better in αTF media with increase mRNA expressions of RUNX-2 and TGF. This can be explained by the hypothesis that vitamin E is considered as one of the most important naturally occurring antioxidant defense agents. It can act as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation and protecting the cell against oxidative damage through reduces the production of oxygen free radicals[19,20].

CONCLUSION

Nicotine has adverse effect on rats DFSCs proliferation and osteogenic differentiation. Vitamin E significantly reduces the toxic effect of nicotine on DFSCs proliferation as well as osteogenic differentiation as visualized by MTT assay, AR-S stain and ALP activity.

SIGNIFICANCE STATEMENTS

This study discovers that rat DFSCs were able to differentiate into osteogenic cell linage, consequently, those cell may represent a suitable, accessible and potential alternative source that can be beneficial for regenerative medicine and therapeutic application. Moreover vitamin E could relieve the oxidative process induced by nicotine so increase the capability of DFSCs to proliferate and differentiate into osteogenic lineage. This study helps the researchers to cover the area of stem cell proliferation and differentiation mechanism with the effect of nicotine that many researchers were not able to explore. Thus a new theory on role of vitamin E on DFSCs preventing the effect of nicotine may be arrived at.

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