Nicotine Cytotoxicity on the Mesenchymal Stem Cells Derived from Human Periodontium

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Abstract
Smoking is a risk factor for developing severe pathologies, including destructive periodontitis. The study aimed at assessing the cytotoxic effect of nicotine on periodontal mesenchymal stem cells. Four types of cells were isolated from human periodontium: gingival ligament stem cells (GLSCs), periodontal ligament stem cells (PDLSCs), gingival tissue stem cells (GTSCs), and alveolar bone stem cells (ABSCs). Cells were cultured on 96-well plates and exposed to serial concentrations of nicotine, ranging from 0.155µM to 31.125µM for 24h and 48h. The cytotoxic effect was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. Histological examinations were performed, and the optical density was read with a microplate reader. Statistical analysis employed ANOVA and Tukey’s Multiple Comparison Test. The results demonstrated that high nicotine concentrations (3.112µM - 31.125µM) induced marked cell death in all types of cells. Intermediate doses (0.622µM - 3.112µM) induced autophagy in all cells and significantly decreased the number of ABSCs after 24h; contrarily, GLSCs and GTSCs were slightly affected after 48h; PDLSCs were the most resistant. Low nicotine doses (0.155µM - 0.311µM) had no significant effect compared with the control. In conclusion, nicotine exerted dose- and time-dependent cytotoxic effects; however, the periodontal stem cells showed different responses, according to their origin.

Keywords: cytotoxicity, mesenchymal stem cells, nicotine, periodontium

1. Introduction
Smoking significantly reduces life expectancy and is one of the main risk factors for mortality worldwide (CENTERS FOR DISEASE CONTROL AND PREVENTION [1], WOLRD HEALTH ORGANIZATION [2], P. JHA & al. [3]). The role of smoking in developing numerous diseases and disabilities - including cancer, cardiovascular, metabolic and pulmonary diseases - has been well documented (CENTERS FOR DISEASE CONTROL AND PREVENTION [4], U.S. CENTERS FOR DISEASE CONTROL AND PREVENTION [5]).

Moreover, evidence suggests that smoking affects oral health by causing oral mucosal lesions and destructive periodontal disease (SOPORI [6]). Effects such as delayed wound healing, loss of attachment (N.L. BENOWITZ & al. [7], B. HOLTRETER & al [8]), deep periodontal pockets, and reduction of alveolar bone mass have been strongly related to smoking. Additionally, the response to periodontal surgical and non-surgical treatment was less favorable in smokers compared with non-smokers (B.W. BENSON & al. [9], R. KALLALA & al. [10]).

Several clinical and experimental studies proved that nicotine from cigarette smoke...
initiates several pathogenic mechanisms underlying all these conditions (G.R. SOUTO & al. [11]). Systemically, nicotine induces oxidative stress and activates the inflammatory and immune responses (SOPORI [6]); locally, additional effects include vasoconstriction and decreased oxygen concentration in the periodontal tissues (S.C. GOMES & al. [12], H. HERMIZI & al. [13]).

The periodontium is a complex association of specialized tissues which maintain their structural integrity by continuous adaptation, remodeling and regeneration (H. MAEDA & al. [14]). Under normal conditions, homeostasis is accomplished by proliferation and differentiation of mesenchymal stem cells present in the periodontal tissues. Mesenchymal stem cells are multipotent progenitor cells capable of regenerating tissues by differentiating into cells of mesenchymal lineage: fibroblasts, osteoblasts, adipocytes, and chondrocytes (G.T.-J. HUANG & al. [15]). These cells are present in various tissues, including the components of the periodontium, such as the periodontal ligament, gingiva, and the alveolar bone (G.V. THOMAS & al. [16], A.R. NAVABAZAM & al. [17]). However, the regenerative potential of periodontal stem cells may be altered by various factors, including smoking.

Prior in vitro studies demonstrated that nicotine plays a crucial role in regulating cellular processes such as proliferation, differentiation and migration (W. ZHU & al. [18]); thus, it affects the development of stem cells and has a negative biologic effect on a wide range of periodontal cells: periodontal ligament fibroblasts, cementoblasts, periodontal myofibroblasts, gingival fibroblasts and osteoblasts (C.M. CARBALLOSA [19], S. BHAT [20]). However, the mechanisms of tissue damage connected to smoking are not clearly explained.

The purpose of this study was to assess the nicotine cytotoxicity on periodontium derived mesenchymal stem cells regarding cell viability and the mechanisms underlying this effect.

2. Materials and methods

Materials and reagents:
1. Growth media: Dulbecco’s Modified Eagle’s Medium (DMEM) with 4500 mg/l glucose, F-12 HAM, Foetal bovine serum (FBS), Phosphate Buffered Saline (PBS) (Sigma-Aldrich).
2. Chemicals: Antibiotics: Penicillin, Streptomycin, L-Glutamine, Non-essential amino acids, Trypsin, EDTA, beta-mercaptoethanol, natrium pyruvate, DMSO (dimethyl sulfoxide), absolute ethanol and endotoxin free water were purchased from Sigma-Aldrich. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) reagent was purchased from Sigma-Aldrich).
3. Cell lines and cell cultures: we used mesenchymal stem cells (MSCs) derived from the human periodontal tissues. For the cell cultures, we employed 25-cm² culture flasks, 96-well plates purchased from Nunc, and Lab-Tek Permanox chamber slides.

Methods
1. Isolation and culture of human MSCs derived from periodontal tissues
Cells were previously isolated from human periodontal tissues surgically harvested from impacted third molars extracted for orthodontic purposes. The study was conducted on females aged from 18 to 22 years, who showed no comorbidities, and were free of infectious complications, cysts or tumors associated with teeth. All patients confirmed the participation in our study by signing an informed agreement. The clinical protocol was approved by The Ethical Committee of the University of Medicine and Pharmacy “Iuliu Hatieganu”, Cluj-Napoca, registration number: 343/2.10.2014.

The four cell lines were: gingival ligament stem cells (GLSCs) from the cervical zone of
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the teeth, periodontal ligament stem cells (PDLSCs) from the middle third of the root, gingival tissue stem cells (GTSCs) from the gingival lamina propria, and alveolar bone stem cells (ABSCs) from the inter-radicular septum.

Cells were characterized at fifth and sixth passage for expression of stem cells markers by immunocytochemical staining, flowcytometry (CD44, CD49e, CD29, CD73, CD105, CD117, Nanog, OCT ¾, Sox-2, SSEA-4) and RT-PCR (real-time polymerase chain reaction). Additionally, the ability of cells to differentiate into multiple types of cells was tested for osteogenic, chondrogenic and neuronal differentiation (unpublished data).

2. Cell treatments

The nicotine stock solution (20mM) was diluted with endotoxin-free water to obtain the concentrations used for the cell treatments (1:1, 1:10, 1:50, 1:100).

Cells were seeded in 96-well plates (2x10⁴ cells in 200µl complete medium) and incubated for 24h for the cell monolayers to reach 75-80% confluence. Then, cells were treated with serial dilutions of nicotine for 24h and 48h. The final nicotine concentrations in the wells were as follows: N1=31.125µM, N2=15.5625µM, N3=6.225µM, N4=3.1125µM, N5=1.55625µM, N6=1.245µM, N7=0.6225µM, N8=0.31125µM, N9=0.155625µM. All the experiments were performed in triplicate.

3. MTT viability assay

The cytotoxic effects of nicotine were assessed using the MTT assay. After 24h and 48h of exposure to nicotine treatments, the medium from 96-well plates was discarded and 100µl/well MTT solution (1 mg/ml in Hanks solution without phenol red) was added. After one hour incubation at 37°C in the dark, the MTT solution was discarded and replaced with 150 µl of DMSO. The optical density (OD) was measured at a wavelength of 570 nm with a Biotek Synergy 2 microplate reader (Winooski, VT, USA). The results were interpreted by comparing the OD values of untreated controls with nicotine treated samples.

Histological analysis was also performed. Cells in cultures were visualized using a Zeiss Axiovert D1 inverted phase microscope (Carl Zeiss International, GmbH, Germany), equipped with a PlasDIC condenser and a 20X objective. Images were captured with an AxioCam MRC camera.

4. Statistical analysis

- Data were analyzed with the one-way ANOVA, followed by Tukey’s Multiple Comparison Test (p-value <0.05), using the GraphPad Prism 5 software (La Jolla, CA, USA).

In our study, the MTT assay was used as a measurement tool of the mitochondrial activity, and indirectly as a cell viability test. Moreover, MTT staining allowed the histological examination of cells in culture by visualization in inverted phase light microscopy.

MTT is a tetrazolium salt which is transformed by the mitochondrial oxidoreductase that is active in viable cells, into a formazan compound colored in dark blue. Formazan crystals are insoluble in aqueous solution, so DMSO was used as a solvent, and a color reaction was obtained. The OD of the supernatant in each well is correlated with the number of metabolically active cells; therefore, it could be used as an indicator of the cell viability and proliferation. The histological examination provided additional information on the mechanisms underlying the cytotoxic effect of nicotine regarding cellular death and changes in cell morphology: the shape, aspect of the cytoplasmic processes, and signs of autophagy.
3. Results and discussion

The four periodontal stem cell lines (GLSCs, PDLSCs, GTSCs, and ABSCs) were exposed to nicotine and their responses were compared, taking into consideration that the various periodontal tissues are not uniformly affected by nicotine. The gingiva is directly exposed to the cigarette smoke during inhalation, but the profound tissues, such as the periodontal ligament and the alveolar bone are influenced by the nicotine that has been absorbed into the blood and transported to the periodontium.

In order to examine the dose-dependent cytotoxic effect of nicotine, all four types of cells were treated with nine different concentrations of nicotine. Moreover, the time-dependent toxicity was evaluated by comparing the responses after continuous exposure to nicotine for 24h and 48h.

Results of the MTT assay

High nicotine doses (N1-N3) induced a significant cell death in all four cell lines after 24h and 48h. However, there were differences in the effect of intermediate (N4-N7) and low doses (N8, N9) of nicotine at the two-time points.

GLSCs showed no significant response to intermediate and low nicotine concentrations (N4-N9) after 24h. However, some decline in the number of viable cells could be observed at N4-N7 doses after 48h, suggesting a delayed toxicity. (Fig. 1)

Similar results were observed for PDLSCs, with some differences: a stronger cytotoxic effect for N4 nicotine concentration at 24h, and for N5 at 48h. PDLSCs seemed not to be affected by the lowest nicotine concentrations (N8 and N9) with cell numbers similar or even slightly increased compared with the control. PDLSCs appeared to be the most resistant to low doses of nicotine. (Fig.2)
The behavior of GTSCs was quite similar to GLSCs; the main difference was a more pronounced decrease in cell viability after 48h, even for cells treated with the lowest concentrations (N8 and N9). (Fig.3)

![Figure 3. GTSCs response to nicotine after 24 and 48h (***p < 0.0001; **p<0.001; *p<0.05).](image)

**ABSCs** showed an increased sensitivity to nicotine even at the lowest doses (N6- N9) after 24h, compared with the other three cell types. Surprisingly, bone stem cells treated with intermediate and low nicotine concentrations (N5-N8) restored their functions after 48h and reached cell numbers comparable to the control. (Fig.4)

![Figure 4. ABSCs response to nicotine after 24 and 48 h (*** p < 0.0001; **p<0.001; *p<0.005).](image)

Nicotine in doses ranging from 3.112µM to 31.125µM exerted a significant cytotoxicity on all periodontal stem cells after 24h and 48h, whereas intermediate and low doses induced different effects, depending on the type of cells. Nicotine in doses ranging from 0.622µM to 1.556µM slightly reduced the cell number after 24h and showed a more severe effect after 48h on GLSCs, PDLSCs, and GTSCs. Lower doses (0.155µM and 0.311µM) did not significantly affect cell viability but stimulated cell proliferation. By contrast, ABSCs were more sensitive: intermediate and low doses of nicotine (0.3112µM to 1.556µM) significantly reduced cell numbers after 24h; interestingly, cells treated with doses lower than 1.556µM proliferated and reached a comparable number to the control.

These results suggest that the cytotoxic effect of nicotine is both dose-dependent – high doses induced significant death in all types of cells – and time-dependent – the reduction in cell number was more important after a longer exposure when intermediate doses were used. Particularly, the ABSCs were the exception: they were dramatically affected by intermediate and even low doses of nicotine after 24h, but their number was restored after 48h. Therefore,
there was a minimum time-dependent toxicity level.

The intense cell death after incubation with high doses of nicotine for 24h suggests fast transport of the drug through the plasma membrane and its rapid association with intracellular components. This is consistent with published data reporting that nicotine from cigarette smoke was rapidly absorbed in the oral cavity during inhalation due to its ability to bind to cell membranes (TANG [21]).

A number of studies support our findings referring to the in vitro cytotoxicity, demonstrating that nicotine-induced dose-dependent negative effects on various cells (S. BHAT [20], F.L. ULMER & al. [22]). However, the nicotine doses and the time of exposure were different than ours. Fang et al. reported that nicotine in doses ranging from 0.01mM to 1mM decreased gingival fibroblasts proliferation after 30h and inhibited their differentiation into myofibroblasts; they also demonstrated that nicotine inhibited attachment and proliferation of human periodontal ligament fibroblasts in a dose-dependent manner (Y. FANG & al. [23]). In a study conducted by Gullihorn et al., low doses of nicotine (12.5-250ng/mL) induced bone cells proliferation and activated cell metabolism (L. GULLIHAM & al. [24]).

Published data reported that nicotine may also induce various biological effects, including generation of inflammatory cytokines and reactive oxygen species in cementoblasts (C.S. CHEN & al. [25]), inhibition of protein synthesis in periodontal ligament fibroblasts (R. KALLALA & al. [10]), increased alkaline phosphatase activity and stimulation of protein and collagen synthesis in bone cells (H. HERMIZI & al. [13], Y. FANG & al. [23]).

When comparing the responses of the four types of cells, our results suggested that ABSCs were the most sensitive to nicotine after 24h, whereas GTSCs appeared to be the most affected by intermediate and low doses of nicotine after 48h. There was no significant difference between GLSCs and PDLSCs. These observations are not consistent with data reported by Chen et al., who observed that human periodontal ligament fibroblasts were more sensitive to nicotine treatment compared with human gingival fibroblasts (C.S. CHEN & al. [25]).

Interestingly, by taking together our results and data reported by other studies, it can be observed that periodontal stem cells were more sensitive to nicotine compared with adult cells. Several papers demonstrated that nicotine was cytotoxic to cementoblasts in doses higher than 1.5mM (C.S. CHEN & al. [25]), and to periodontal ligament fibroblasts in concentrations of 2.5mM (Y.C. CHANG & al. [26]) gingival fibroblasts proliferation was inhibited by doses higher than 7.8mM, and 25µM nicotine solution had the same inhibitory effect on periodontal ligament fibroblasts (C.S. CHEN & al. [25]).

**Results of the histological examination**

Phase contrast microscopy images confirmed the MTT results.

**GLSCs** treated with high nicotine concentrations (N1 and N2) were unstained suggesting that they were no longer viable, although cell bodies showed no major morphological changes. A marked decrease in cell numbers was observed for N3 and N4 concentrations at both time points, whereas N5 and N6 induced a reduction in cell metabolism after 48h. The lowest concentrations seemed not to affect the viability and cell numbers even after 48h. A characteristic aspect of autophagy has been found when using N4-N7 concentrations, with the presence of autophagosomes in metabolic active cells. The autophagic activity decreased in cells treated with N7 concentration and at N8 and N9 no autophagy could be observed. (Fig.5)
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The highest nicotine concentrations induced a violent PDLSCs death, suggested by the unstained cells that still preserved their morphology. Cells treated with N3 and N4 became round-shaped and presented a strong decrease in cell numbers. Autophagy was observed starting with the N5 concentration. The lowest doses (N7-N9) increased the proliferation rate. (Fig.6)

The morphological aspects of GTSCs were identical to GLSCs and PDLSCs, regarding the presence of autophagy starting with the N5 dose, and an increased proliferation rate in cells treated with N6-N9 concentrations. (Fig.7)
ABSCs showed the same aspects: cell death at the highest concentrations (N1-N3), autophagy or cells treated with N4-N6 concentrations and a slight proliferation for N7-N9 doses. (Fig.8)

The four types of periodontal stem cells exhibited similar histological features when treated with identical nicotine doses. Cells treated with high doses (15.562µM – 31.125µM) appeared unstained, proving that the mitochondrial oxidoreductases were no longer active when the MTT assay was performed; moreover, cells preserved their shape, suggesting an instantaneous cell death.

Doses ranging from 3.112µM to 6.225µM caused the death of the majority of cells in each well after 24h; cells that were still viable showed significant morphological changes and a delayed cell death occurred after 48h. Intermediate doses (0.622µM – 1.556µM) induced autophagy in all cells after 24h and 48h. The lowest doses (0.155µM – 0.311µM) did not affect the cell morphology, and autophagy vesicles were not present; contrarily, these doses even induced an increase in cell number and density.

Our histological findings suggest, like in other studies (S.Y. KIM & al. [27]), that autophagy initiated in viable cells by the intermediate doses may be one of the pathogenic mechanisms underlying the nicotine cytotoxic effect.

Autophagy is an alternative cellular catabolic process that allows the orderly degradation and recycling of the cellular components; it is also one of the mechanisms associated with programmed cell death (BAEHRECKE [28]). Autophagy is characterized by the formation of autophagosomes, which are double membrane vesicles delimiting the cytoplasmic contents marked for destruction, such as abnormal protein aggregates and excessive or altered organelles (Y. TSUJIMOTO & al. [29]); subsequently, the autophagosome fuses with a lysosome and the contents are degraded by the hydrolases (BAEHRECKE [28]).

In pathological conditions, the autophagosomes-lysosomal degradation of cytoplasmic contents leads to morbidity and cell death. However, recent research sustains the argument that autophagy in injured cells is an adaptive response to stress in order to promote survival (BAEHRECKE [28]). In our study, the autophagic activity in periodontal stem cells could be regarded as a repair mechanism and an attempt to maintain cell viability.

A number of studies demonstrated several other mechanisms underlying nicotine cytotoxicity: intracellular thiol depletion (C.S. CHEN & al. [25]), DNA fragmentation (S.Y. KIM & al. [27]), generation of reactive oxygen species (Y. TSUJIMOTO & al. [29]) and activation of the nicotinic acetylcholine receptor in periodontal ligament fibroblasts (S.Y. KIM & al. [27]).
In the present study, nicotine-induced dose- and time-dependent cell death and inhibited periodontal stem cell proliferation. These observations provide an important clinical significance since stem cells have a crucial contribution to periodontal regeneration by their proliferation, differentiation, and migration. Therefore, factors that alter stem cells functions would also impair tissue repair and regeneration. This suggests that nicotine might be one of the factors responsible for rendering cigarette smokers more susceptible to the destruction of the periodontium and less responsive to regenerative procedures during periodontal therapy.

To our knowledge, the present study was the first to assess and compare the cytotoxicity induced by nicotine on different types of periodontal stem cells. Until now, various adult cells, such as: periodontal ligament fibroblasts and myofibroblasts, gingival fibroblasts, cortical neurons, osteoblasts, and cementoblasts have been examined for their in vitro response to nicotine (G.T.-J. HUANG & al. [15], F.L. ULMER & al. [22]).

Our further studies will focus on the effect of nicotine on the ability of periodontal stem cells to differentiate into chondrogenic, osteogenic and neurogenic lineages.

4. Conclusions
Nicotine exerted dose- and time-dependent cytotoxic effects on mesenchymal stem cells derived from the human periodontium. The periodontal stem cells exhibited different responses to nicotine according to their origin.

One of the mechanisms associated with nicotine cytotoxicity was the induction of autophagic activity in the periodontal stem cells.

Nicotine decreases viability of periodontal stem cells and alters the regenerative potential of the periodontium. Thus, it might promote severe periodontal destructions and impair the periodontal treatment outcome.

Taken together, the results of the present study provide significant evidence that support the implementation of individualized therapeutic approaches for a more efficient management of the cigarette smoking-related periodontal diseases.

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