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Research Paper

Cytotoxic and Genotoxic Effects of Lambda-Cyhalothrin Insecticide on Human Dental Pulp Stem Cells

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ABSTRACT

Background: Lambda-cyhalothrin (LCT) belongs to pyrethroid insecticides, the use of which has increased for pest control. It is essential to study the effects of LCT on the DNA of living organisms to prevent its mutagenic and carcinogenic properties. Currently, there is a lack of information on the effects of LCT on humans. This study examined the cytotoxicity and genotoxicity of LCT insecticides on human dental pulp stem cells (DPSCs).

Methods: We examined the cytotoxicity of LCT at serial concentrations of 0.5, 1, 2.5, 5, 10, 25, and 50 µM using an MTT assay. Four concentrations of LCT at 0.5, 1, 25, and 50 µM were selected from the cytotoxicity curve and subjected to a comet assay to assess genotoxicity.

Results: The results of the MTT assay showed that LCT inhibited cell proliferation at 1 µM concentration of the 5% formulation, while the other concentrations of LCT at 0.5, 2.5, 5, 10, 25, and 50 µM increased cell proliferation rates by 10, 1, 4, 20, 59, and 76%, respectively. The results of the comet assay provided evidence that the LCT insecticide induced a statistically significant increase in DNA damage in DPSCs at all tested concentrations compared to those of the negative controls (P>0.05).

Conclusion: The LCT insecticide was genotoxic to DPSCs but was not cytotoxic at the tested concentrations, except at 1 µM. Instead, it increased cell proliferation. This suggests that LCT may function through an additional mechanism that mimics that of estrogen and may potentially become a candidate as a xenoestrogen.

Keywords: Comet assay, Cytotoxicity, Dental pulp stem cells, Genotoxicity, Lambda-cyhalothrin, LCT compound

Introduction

Due to the harmful effects of pesticides on the genetic material of living organisms, it is essential to study and analyze their hazards to human health, including their cytotoxic and genotoxic properties [1]. Such investigations are crucial to preventing the mutagenic and carcinogenic effects of pesticides [2]. Genetic damages can occur in human DNA, such as strand breaks and DNA adducts, or at the level of chromosomes, such as a change in the chromosome number (aneuploidy), deletion, and/or breaks (clastogenicity) [3, 4]. Chromosomal aberrations and DNA damage are considered the primary events that lead to the carcinogenicity and/or mutagenicity of numerous chemicals [5]. The genotoxicity of many pesticides has been studied through *in vitro* and *in vivo* experiments. It has been found that most organophosphates, organochlorine, and synthetic pyrethroid pesticides have caused concerns in the development of human cancer. In recent years, 56 pesticides from various groups have been classified as carcinogens [6], with 29 other pesticides being genetically toxic [5].

Because of the restrictions and warnings on the use of organophosphate insecticides, pyrethroid pesticides, such as Lambda-cyhalothrin (LCT), have been widely used by farmers for pest control [7]. This compound belongs to synthetic pyrethroid groups, which are analogues to natural pyrethrins and are extracted from dried flowers of *Chrysanthemum cinerariaefolium* plants [8]. However, the latter agents are more toxic to insects and mammals than the synthetic pyrethrins [9]. Synthetic pyrethroid insecticides, including LCT, are widely used for pest control in agriculture and public health. This compound is a synthetic pyrethroid type-II insecticide (Figure 1) containing an alpha-cyano group [10], which is categorized as a class D carcinogen by the U.S. Environmental Protection Agency [11].

Based on *in vitro* and *in vivo* experiments, previous studies have shown that LCT is highly toxic to aquatic organisms, such as fish and arthropods [12, 13]. Other studies have found that LCT affects the total protein and

albumin contents in the fish kidneys and liver and causes endocrine disruptions [14, 15]. Genotoxic studies on LCT have also shown increases in micronucleus formation in bone marrow cells, the intestinal epithelia of rats [16-18], and the blood cells of *Gambusia affinis* fish [19]. Moreover, LCT is thought to induce DNA damage in murine macrophage cell lines [7], chromosomal aberrations in rabbits' lymphocytes [20], and the gill cells of *Mystusgulio* fish [21]. In an earlier study [22], LCT was also found to be genotoxic to an insect cell line (Sf-9).

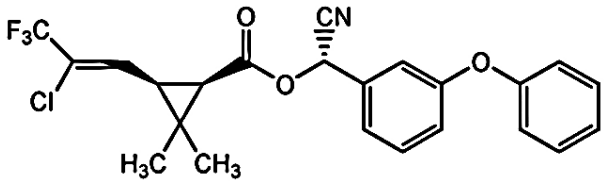

Figure 1. Chemical structure of lambda-cyhalothrin.

Most previous studies have focused on the genotoxicity of LCT in animal models. However, few investigations have been conducted *in vitro* on the cytotoxicity and genotoxicity of LCT in human models. These studies have shown that LCT causes cell death, inhibits cell division, forms micronuclei, and leads to DNA damage in human lymphocytes. This agent also induces oxidative stress in human erythrocytes [23]. In this context, scientific insight is lacking on the effects of LCT on humans, and the available *in vitro* data are limited to peripheral blood lymphocytes. Therefore, it is necessary to conduct further studies to assess the impact of LCT on cells derived from other human tissues.

Aim of the Study: The aim of this study was to examine the *in vitro* cytotoxic and genotoxic effects of LCT insecticide on human cells. To this end, dental pulp stem cells (DPSCs) were chosen as the *in vitro* model for human cells because they are undifferentiated natural cells that are capable of renewing themselves and can be continuously cultured in their undifferentiated state [24].

Materials and Methods

Chemicals: LCT (RS)-α-cyano-3-phenoxybenzyl (1R)-cis-3-(Z)-(2-chloro-3, 3, 3-trifluorop1-enyl)-2,2-dimethylcyclopropanecarboxylate, with a purity of 98.7%, was obtained from Syngenta©. A stock solution of 10 mM LCT was prepared using dimethyl sulfoxide (DMSO) freshly made before cell treatment.

Cell Culture: In the current study, DPSCs represent healthy and normal cells with a typical fibroblast-like morphology (Figure 2). They express several biomarkers, including the mesenchymal and bone-marrow stem cell markers, STRO-1, CD146, and the embryonic stem cell marker, OCT4 [25]. The DPSCs were isolated from the tooth pulp of a 17-year-old patient [26] and cultured in T25 culture flacks (TPP). These cells were grown in low-glucose Dulbecco's modified eagle medium (DMEM). The culture medium was supplemented with 10% fetal bovine serum (FBS, 200 mM), L-glutamine (100 U/10 mg), antibiotics (Streptomycin and Penicillin), and 0.25 µg/ml antifungals (Amphotericin B). The culture dishes were kept in a CO_2 incubator (Lab Tech-Lco-065 AI) at 37ºC with 5% CO_2. The chemicals used for cell culture were purchased from Euroclone©, except for the DMEM medium, which was purchased from Sigma-Aldrich© (St. Gallen, Switzerland). The first and third subculture passages of DPSCs were used for the purpose of cytotoxicity and genotoxicity tests.

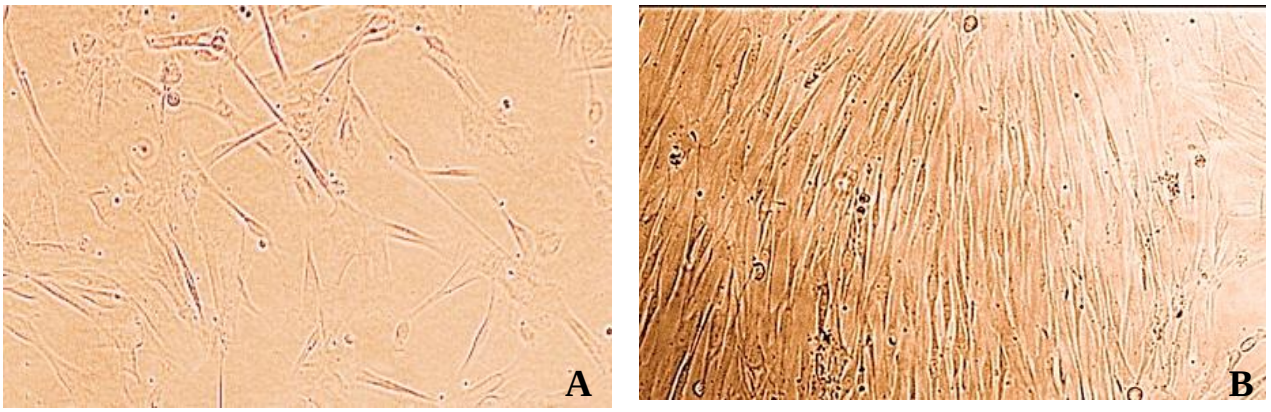

Figure 2. (A) Primary culture of DPSCs during the first week, and (B) Culture of DPSCs at 75% confluence.

Cytotoxicity Tests: The cytotoxicity of LCT was assessed by an MTT assay, as described by an earlier study [27]. Cells were seeded at a concentration of 1×10^5 cells/ml in the DMEM culture medium without phenol red or FBS. A total of 100 µl of the cell suspension was added to each well on 96-well culture plates (TPP, Switzerland). The culture plates were then incubated overnight in a CO_2 incubator at 37ºC. The medium was replaced the next day with the test medium at a concentration of 0.5, 1, 2.5,5, 10, 25, or 50 µM of LCT for 24 h. Cell suspensions with 1% DMSO were used as the negative control. Each of the concentrations tested consisted of four replicates, and these tests were repeated in duplicate.

Following the exposure period, the test medium was

replaced with 20 µl MTT (2 mg/ml) in PBS and incubated overnight. Next, an aliquot of DMSO (150 µl) was added to each well to solubilize purple formazan crystals. The absorbance of the solution in each well was measured at 540 nm using a microplate reader (SCO, Germany). Regression analyses were also carried out to determine the concentration-response relationships. The inhibition rate (IR) was calculated by the following formula [28]: **(**1-At/Ac)×100=IR, where At=Absorbance value of the tested wells and Ac=Absorbance value of the control wells.

Alkaline Comet Assay: This assay was performed using the Comet Assay® silver staining kit catalog #4251-050-K (Trevigen, Minneapolis, USA) and Alkaline Comet Assay® following the manufacturer's instructions with minimal modifications. Briefly, the DPSCs were treated in a 6-well culture plate, then harvested and embedded in low-melting agarose (at 37°C) at a ratio of 1:10. After mixing the sample, a 50 µL aliquot was pipetted onto the Comet Slide™ area immediately. The cells exposed to ultraviolet-C (UVC, 257.3 nm) for 45 min were used as positive controls. The cells treated with DMSO alone were considered the negative controls. To prevent additional damage, all of the steps described above were carried out under dim light. Slides were then transferred to 40 ml pre-chilled lysis solution (cat #: 4250-050-01) containing 10% DMSO and incubated overnight at 4°C. The comet slides™ were immersed in an alkali unwinding solution at pH 13, 300 mM NaOH, and 1 mM EDTA in the dark at room temperature for 30 min.

Electrophoresis was performed at 1 Volt/cm and 300 mA for 30 min. The slides were then immersed twice in distilled water (dH_2O) for 5 min and then in 70% ethanol for 5 min. These samples were dried at 37°C for 10-15 min and stained using the silver staining method. The stain intensity was observed under light microscopy at 100× magnification. The reaction was terminated when the comets were easily visible by covering samples with 100 µl 5% acetic acid for 15 min and rinsed in dH_2O. The comets were analyzed by visually scoring them, as described in a previous study [29]. The DNA damage was calculated by arbitrary units (AU) according to an earlier method [30], based on the following formula:

$$AU = \frac{(0 \times N0 + 1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4)}{N^{\circ}\ \text{comets analyzed}} \times 100$$

Where N0, N1, etc. are the numbers of comets in the respective categories. For each treatment, two slides were prepared from two independent experiments.

Statistical Analyses: The statistical analyses were performed using the Mann-Whitney U test in SPSS software (version 17). Error bars represent standard deviations. The results were considered statistically significant at $P<0.05$.

Results

Cytotoxicity: Figure 3 demonstrates that only the 1 µM concentration of LCT induced a slight inhibition of cell proliferation (about 5%), while at other concentrations (0.5, 2.5, 5, 10, 25, and 50 µM), this compound was non-toxic to cells. These concentrations increased cell proliferation rates by 10, 1, 4, 20, 59, and 76%, respectively. The correlation coefficient was strong at R=0.983. Four concentrations were selected from the cytotoxicity curve of LCT for the genotoxicity study. These were the lowest (0.5 µM) and the two highest concentrations (25 and 50 µM) that increased cell proliferation rates, and the 1 µM concentration that inhibited cell viability.

Genotoxicity: Based on the visual scoring method [29], the comets were classified into five different categories, from zero (no tail) to four (almost all DNA in the tail), depending on the tail length and the amount of DNA present in it (Figure 4). Varying degrees of DNA damages were observed by the comet assay (0 to 4) for human stem cells isolated from the tooth pulp. These cells had been exposed to varying concentrations of LCT at 0.5, 1, 25, or 50 µM, in addition to those observed in the negative and positive controls (see Figure 5). As represented by Figure 6, LCT caused DNA damage in DPSCs at all tested concentrations. Based on these tests, DNA damage indices were 78, 160, 131, and 162 AU, corresponding to the LCT concentrations at 0.5, 1, 25, and 50 µM, respectively, as derived from the cytotoxicity curves. The DNA damage indices were significant at $P<0.05$ for all concentrations compared to those of the negative controls (17 AU). However, they were not significant compared to the positive controls (145 AU). The highest value representing DNA damage was 162 AU at 50 µM LCT.

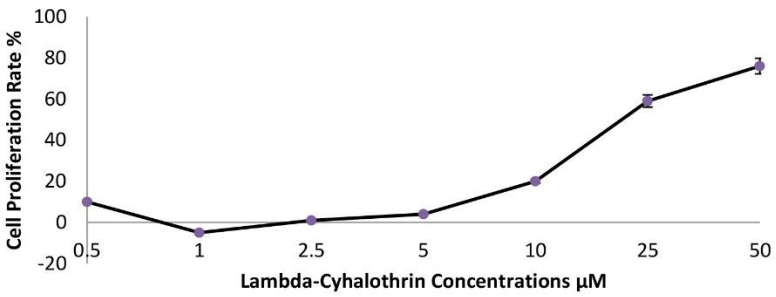

Figure 3. Cytotoxicity of lambda-cyhalothrin on human DPSCs.

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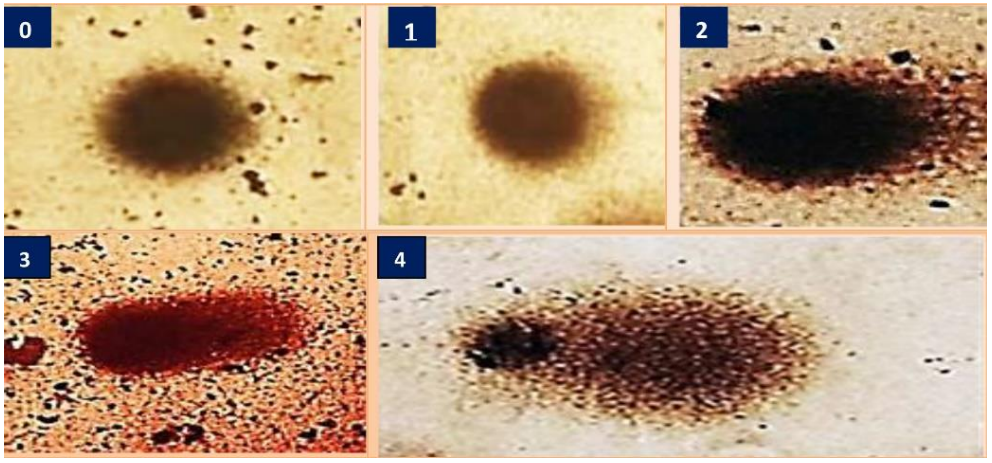

Figure 4. Silver-stained comet images of human DPSCs classified into five categories as: Class 0 (undamaged cells) and classes 1, 2, 3, and 4 (damaged cells).

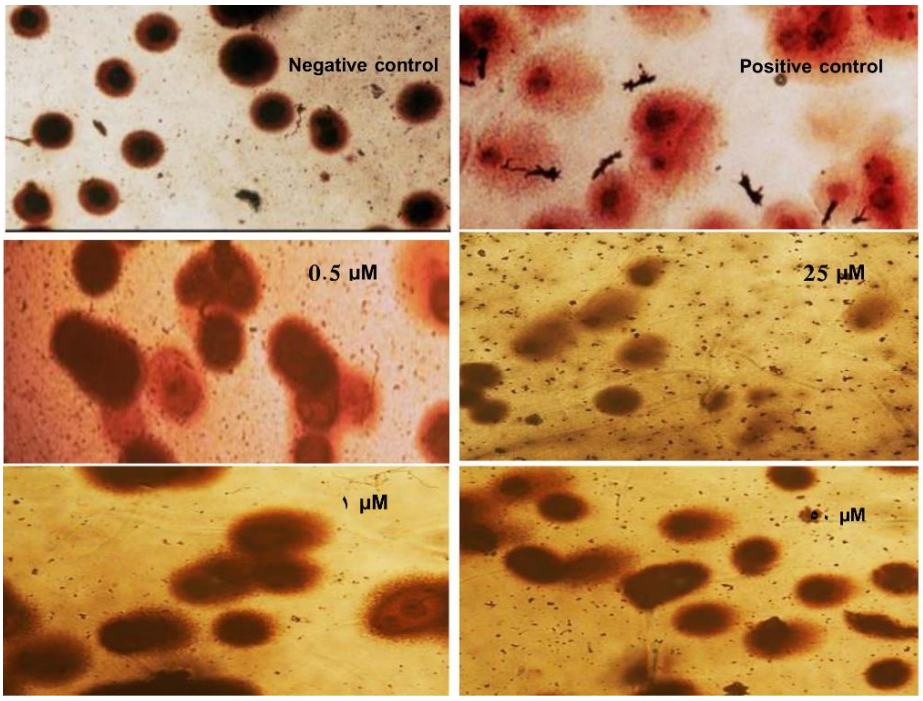

Figure 5. Different degrees of DNA damage from 0 to 4 in human tooth pulp cells treated with different concentrations of lambda-cyhalothrin at 200× magnification

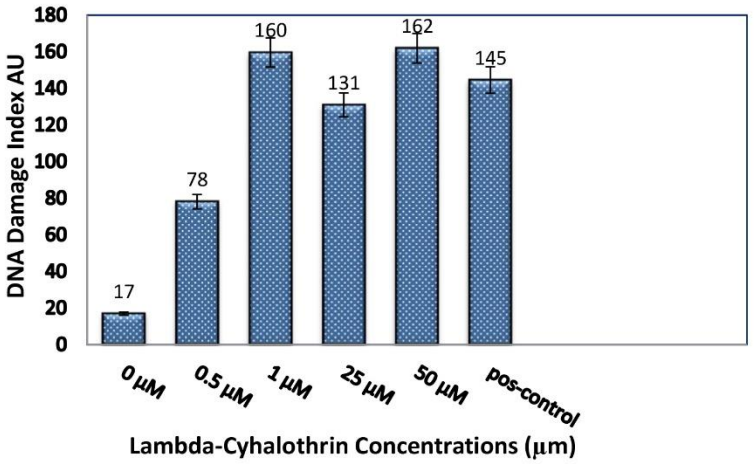

Figure 6. DNA damage induced by Lambda-cyhalothrin in DPSCs expressed in arbitrary units (AU) in the comet assay. Data are means of values for repeated experiments±standard deviations. A statistically significant increase ($P<0.05$) was determined by comparing the values of DNA damage induced by various concentrations of Lambda-cyhalothrin with those of the negative controls (with 10% DMSO).

Discussion

Pyrethroid insecticides target the central nervous system in insects and other non-target organisms. The principal mechanism of action of these compounds is to interact with voltage-gated sodium channels in the neurons' cell membrane. They cause neuronal hyper-excitability in the insects' central nervous system [31]. In addition to their neurotoxicity, these insecticides also affect the endocrine system, exhibiting estrogenic potentials [15], genotoxic effects [31], and oxidative stress [32].

In this study, the cytotoxicity and genotoxicity of LCT insecticides were examined on human DPSCs using MTT and comet assays. The MTT assay results demonstrated that LCT inhibited cell proliferation at 1 µM concentration (~ 5%), while at other concentrations (0.5, 2.5, 5, 10, 25, and 50 µM), this agent increased cell proliferation rate by 10, 1, 4, 20, 59, and 76%, respectively.

Previous studies [13, 33] have found that LCT has been highly toxic to lymphocytes in culture, completely inhibiting cell division and inducing cell death at significantly higher concentrations. However, *in vitro* results on cells may vary due to differences in the cell types used, the regulation of cell growth, and different cell responses to the toxic effects of various insecticides. The LCT's stimulating effect on DPSCs' proliferation may be due to its estrogenic property. Several studies have suggested that the estrogenic potential of LCT [15, 34-37] is due to its stimulating effects on the proliferation of such cells as BG-1 ovarian cancer cells [15] and MCF-7 human breast carcinoma cell line [35]. These studies indicate that LCT possesses estrogenic properties, which may be the reason behind its xenoestrogenic function based on a mechanism similar to that of estradiol [35]. Estrogens are hormones that are important in sexual and reproductive development and bone formation [38, 39]. Estrogens and estrogen-like molecules can modify the biology of several cell types, including MSCs [40]. They regulate the proliferation, differentiation [41], apoptosis, and metabolism of various cell types [42].

In the present study, we used DPSCs as an *in vitro* human cell model. These cells, which are similar to adult stem cells, have self-renewing potential and multiple differentiation functions and play important roles in the regeneration of dental tissue [38]. Dental cells express estrogen receptors [43], and LCT, by binding to them, increases their proliferation rate, except at a 1 µM concentration. This observation explains the fact that LCT did not affect estrogen receptors at a 1 µM concentration. Furthermore, it is likely that the response of DPSCs to LCT is achieved by cell cycle arrest or cell death via apoptosis, thereby not allowing their continuous proliferation.

In the current study, the genotoxicity of LCT was evident by the alkaline comet assay on DPSCs. The results demonstrated that this insecticide was toxic to the DNA in DPSCs at all concentrations used. This finding was consistent with those reported previously by numerous *in vitro* and *in vivo* studies that applied this compound to other organisms [7, 13, 17-19, 22, 33]. The DNA damage indices were evaluated at LCT concentrations of 0.5, 1, 25, and 50 µM using the cytotoxicity curves, which resulted in the AU values of 78, 160, 131, and 162, respectively. Among these concentrations, the 1 µM concentration inhibited cell proliferation, while other concentrations promoted proliferation. In this study, the cells responded to genetic damage by activating specific pathways, resulting in the repair of damaged DNA, activation of checkpoints, and inhibition of the cell cycle. If this repair process does not succeed, the cells are likely to go through programmed cell death, that is, apoptosis, thereby preventing possible mutations [44, 45].

The exact genotoxic mechanism of LCT is currently unknown. However, according to previous studies [7, 16, 23], the possible mechanism could be the induction of oxidative stress by raising the formation of reactive oxygen species (ROS), thereby disrupting the balance between ROS generation and antioxidant defense capability. Further, LCT may also cause damage to cell membrane lipids and proteins [32]. Based on the results of this study, we observed that LCT caused DNA damage in DPSCs while still continuing to proliferate at the tested concentrations. This suggests that LCT may have another operational mechanism that mimics the effects of estrogen.

According to other studies [46, 47], one mechanism by which estrogens promote the proliferation of breast cancer is through inducing DNA damage. Additionally, estrogen alters the DNA damage response (DDR) and repair by regulating proteins that are key effectors. It has been hypothesized that estrogen receptor signaling converges to suppress effective DNA repair and apoptosis in favor of proliferation [47]. Further studies are warranted to discover if the estrogen-mimicking effect of LCT can cause DNA damage and the repair pathways similarly to those induced by estrogen.

Conclusions

Based on the findings of this study, LCT caused DNA damage in human DPSCs but did not induce cytotoxic effects on the cells at the examined concentrations, except for the 1 µM one. Instead, LCT caused increases in cell proliferation. This suggests that LCT may have an additional mechanism of action that mimics the effects of estrogen, and thus it may function as a potential xenoestrogen.

Conflict of Interests

The authors declare no conflict of interest.

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Compliance with Ethical Guidelines

Human stem cells protocol was reviewed and approved by the Ethics Committee of the Dental Collage of Damascus University (Approval #: 8; 30/01/2011).

Authors' Contributions

Manal Saleh performed the experiments, designed the study, analyzed the data, wrote the manuscript, and reviewed and approved the manuscript.

Aroub Al-Masri supervised and analyzed the data and reviewed and approved the manuscript. Daas Ezzedin supervised, reviewed, and approved the manuscript.

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