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The Effect of Light Curing Units on Proliferation and Senescence of Human Dental Pulp Mesenchymal Stem Cells

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Abstract The aim of this study was to evaluate the effect of different dental light curing units on cellular senescence and proliferation rate of human dental pulp mesenchymal stem cells (hDP-MSCs). hDP-MSCs were seeded at a density of $1x10^4$ cells/cm^2 into a 96 well plate. Cells were allowed to attach for 24 h and Halogen curing unit, Light-Emitting-Diode and Plasma arc units (PAC-MOD1, PAC-MOD2, PAC-MOD3) were applied on the cells from 1.2 cm distance (8 mm air + 4 mm growth medium). Media of the wells was refreshed after irradiation and cells cultured at 37 ^oC in a 5% CO_2 incubator for 48 h. Cell growth was determined using the WST-1 cell proliferation assay. The same samples were fixed and evaluated for cellular senescence, the irreversible growth arrest of cells, by staining for SA-β-galactosidase activity. As statistical analyses, Mann-Whitney U test was used for senescence data evaluation and One-way Anova and Tukey HSD tests were used for proliferation data evaluation. Cell proliferation rate was significantly higher under PAC-MOD3 conditions than under Halojen curing unit and PAC-MOD1($p< 0.05$). According to the senescence test results, there was no statistically significant difference between the experimental groups and the control group ($p > 0.05$), but senescence of hDP-MSCs exposed to PAC-MOD2 was greater than the others. When the dental light curing units are used for polymerization of adhesive systems in pulp capping, this procedure may have effects on senescence and proliferation of dental pulp stem cells.

Keywords: *light curing units, dental pulp mesenchymal stem cells, cellular senescence, cellular proliferation*

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1. Introduction

Adhesive systems have been widely used for esthetic procedures due to their superior properties and strength of union to enamel and dentin [1]. However, when they are applied directly or indirectly for pulp capping, adhesive systems may affect pulp tissue [2]. The primary target of restorative dentistry is to restore and maintain tooth health by a suitable restorative treatment.

Dental composites are discussed controversially regarding their content of hazardous substances. In particular, if composites are not cured thoroughly and do not reach a sufficient degree of monomer conversion they can leach toxic and carcinogenic substances into the body of the patient [3].

If a light-activated resin composite is not exposed to sufficient energy at suitable wave-lengths from the light curing units, this can result in wear and greater breakdown of the restoration at the margins, decreases bond strength between the tooth and the restoration, reduces hardness and causes greater cytotoxicity [4].

Various light sources are often applied for polymerization of the dental materials. However, investigators have expressed concerns about the effects of light curing units on vital pulp during the light activated polymerization process. Many different types of light curing units are available for polymerization of dental materials: halogen lamps, plasma arc curing lights, mercury/metal halide lamps, and lasers.

Today, three types of light curing units are widely used to polymerize composites; Halogen curing units, Light-Emitting-Diode (LED) units and Plasma arc (PAC) units.

Light curing units can raise temperature on dental pulp that may lead to irreversible pulp damage [5]. This temperature increase on dental pulp is affected by material shade, thickness, composition, porosity, curing time and residual dentin thickness [6].

If intrapulpal temperature goes above 42.5 ^{o} C, this can cause irreversible damage to the pulp tissue. Polymerization of light-activated adhesives can result in a temperature increase caused by both the exothermic reaction phase and the energy absorbed during curing time [7].

Stem cells can differentiate into many cell types, including odontoblasts, neural progenitors, osteoblasts, chondrocytes, and adipocytes [8]. Odontoblasts generate most of the extracellular matrix (ECM) components in dentin and play a role in dentin mineralization [9]. Yoshiba, et al. [10] reported findings that support the hypothesis that the differenciation of pulp cells into odontoblasts during reparative dentinogenisis is mediated by fibronectin, which is associated with the initially formed calcified layer after pulp capping with calcium hydroxide. Many different types of light curing units are available for polymerization of Some light activated calcium hydroxide cements in deep cavites or expose pulp.

In human postnatal dental tissue, five types of dental mesenchymal stem cells were characterized; dental pulp stem cells from pulp of permanent teeth, stem cells of human exfoliated teeth and immature dental stem cells from deciduous teeth, periodontal ligament stem cells**,** stem cells from apical papilla and dental follicle progenitor cells [11].

The purpose of this study was to evaluate the effect of irradiation time and light intensity of Halogen curing units, LED units, and PAC units on senescence and proliferation of human dental pulp mesenchymal stem cells (hDP-MSCs).

2. Materials and Methods

2.1. hDP-MSCs Isolation, Culture and Characterization

hDP-MSCs were isolated from a deciduous tooth. The tooth was washed in a physiological solution containing antibiotics to eliminate any contamination. Soon after the extraction, pliers (bone forceps) were used to fracture the dental crown into several parts and the dental pulp was uncovered. The pulp tissue was digested using collagenase type I (Sigma- Aldrich, St. Louis MO) to generate a single cell suspension. The cells were cultured in MEM-Earle (Biochrom) containing 15% foetal bovine serum (FBS; Invitrogen/GIBCO, Grand Island, NY, USA) and 100 IU/mL penicilin-100 µg/mL streptomycin (Invitrogen/GIBCO). The cells were seeded into two 25 cm2 plastic tissue culture flasks (BD Biosciences) and incubated at 37°C in a 5% CO_2 incubator for 3 days. The stem cells were isolated based on their plastic adherence ability. On the third day, red blood cells and other nonadherent cells were removed and fresh medium was added to allow further growth. The adherent cells were grown to 70% confluency and were defined as passage zero (P0) cells. Later passages were named accordingly. Immunophenotypic characteristics of hDP-MSCs were determined by flow cytometry prior to use. Cells were labeled with antibodies against hematopoietic antigens (CD34(Hematopoietic Progenitor Cell Antigen; PE), CD45(Protein tyrosine phosphatase, receptor type, C/ PTPRC/ leucocyte common antigen; FITC) and HLA-DR (major histocompatibility complex, MHC class II, cell surface receptor; FITC)) and MSC markers (CD13 (Aminopeptidase N/Vcadherin; PE), CD29 (Integrin β1 chain; PE), CD44 (Hyaluronate/lymphocyte homingassociated cell adhesion molecule-HCAM; PE) CD90 (Thy-1/ Thy-1.1-FITC) and HLA-A,B,C (major histocompatibility class I antigen reseptor; PE)) or their isotype controls. All of the antibodies were supplied by Becton Dickinson. Flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA). The data were analyzed with Cell Quest software (BD Biosciences) and the forward and side scatter profiles were gated out of debris and dead cells. The hDP-MSCs used in this study were subjected to osteogenic differentiation to prove their mesenchymal origin. They were seeded on Type I Collagen coated coverslips at a density of $3x10^3$ cells/cm^2 and incubated in osteogenic media (MEM (Invitrogen/GIBCO) supplemented with 100 nM dexamethasone (Sigma–Aldrich), 0.05mM ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), 10mM β-glycerophosphate (Sigma–Aldrich), 1% antibiotic/antimycotic, and 10% FBS (Invitrogen/ GIBCO)) for 28 days. Osteogenic differentiation was assessed via staining with Alizarin Red (Sigma–Aldich, Fluka Chemie AG, Buchs, Switzerland). Prior to staining, the excess medium was decanted and cells were fixed for 5 min at room temperature in ice-cold 70% ethanol and then allowed to dry completely. The cells on the coverslips were stained with alizarin red solution composed of 2% Alizarin Red S (pH value of the Alizarin Red S solution was adjusted to 4.1–4.3 with ammonium hydroxide) for 30 sec to 1 min, then washed with distilled water (20 dips). The stained cells were dehydrated in acetone (20 dips), fixed in acetone–xylene (1:1) solution (20 dips), cleared with xylene (20 dips), dried completely, and mounted in mounting medium.

2.2. Exposure of hDP-MSCs to Light Curing Units

hDP-MSCs (Figure 1) were seeded at a density of $1x10^4$ cells/cm^2 into a 96 well plate. Cell seeded wells were separate enough from each other and the wells in between were filled with a black dye to prevent any light penetration to other unexposed wells. Cells were allowed to attach for 24 h and halogen units (Megalux CS, MEGADENTA Radeberg, Germany), LED units (Benz, China), PAC units (Plasmastar, Monitex San-chung city, Taipei, Taiwan) lights were applied on the cells from 1.2 cm distance (8 mm air+ 4 mm growth medium). Halogen curing units was applied on hDP-MSCs for 40 s (82 W), LED was applied on hDP-MSCs for 20 s (1200mW/cm^2), PAC-MOD1 (Normal MOD) was applied on hDP-MSCs for 10 s at 100 % light intensity (1800mW/cm^2). PAC-MOD2 (Ramp Curing) that starts with half power and then reaches gradually full power (2 s 50% light intensity, 2 s 80% light intensity, 2 s 100% light intensity) was applied on hDP-MSCs for 6 s. PAC-MOD3 (2 Step Curing) that starts with half power and then works at full power (3 s 50% light intensity, 3 s 100% light intensity) was applied for 6 s, too (Table 1). Media of the wells (Low Glucose DMEM + 10% FBS+ 1% Pen/Strep) was refreshed after irradiation and cells cultured at 37 °C in a 5% CO_2 incubator for 48 hours. Cell growth was evaluated using the WST-1 cell proliferation assay (Roche). The same samples were fixed and evaluated for cellular senescence, the irreversible growth arrest of cells, by staining for SA-*β*-galactosidase activity, which is specific for senescent cells.

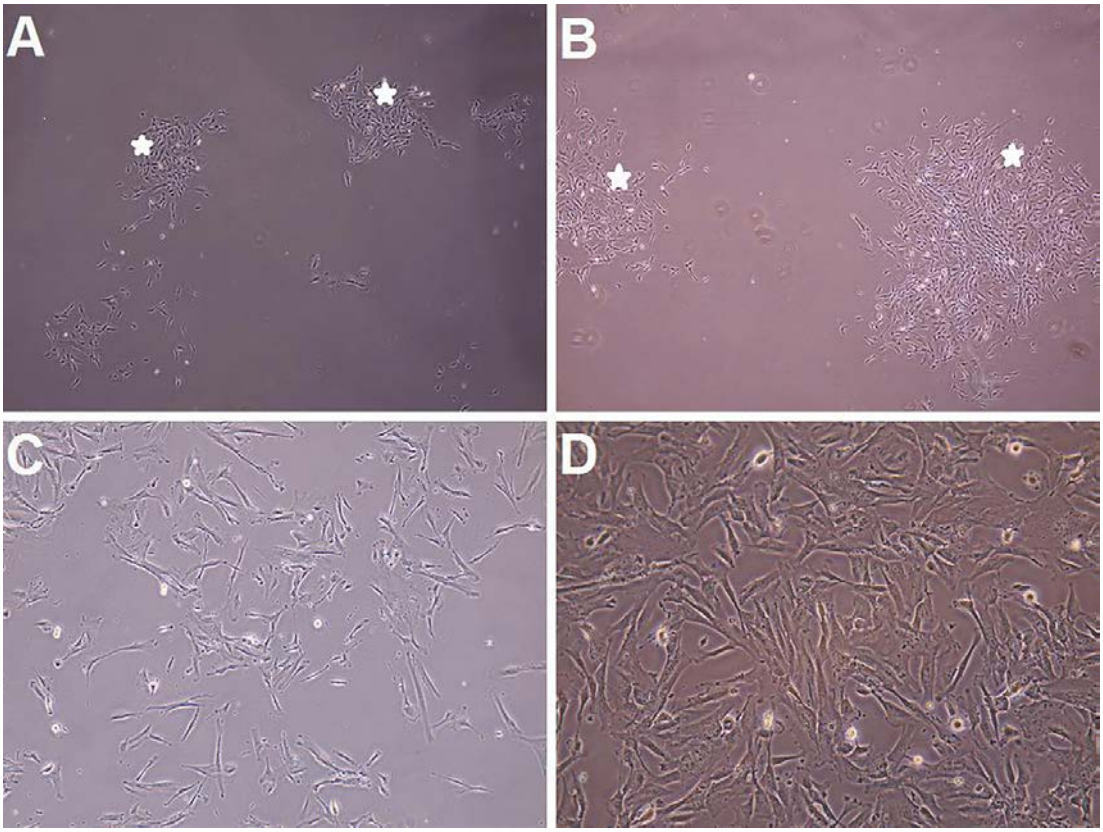

Figure 1. Morphology of hDP-MSCs. Single cell derived colonies (arrows) of isolated cells from dental pulp of wisdom teeth formed (P_0-8thday) **(A)** and spread on the surface of flask (asterisks, P_0-11st day) **(B)**. The colonies represented mesenchymal stem cells like morphology. Later, most of these stem cells exhibited large, flattened or fibroblast-like morphology (**C**, P_1-5thday; **D**, P_2-5thday) (Magnification: **A,B**- X48; **C**- X100; **D**- X200)

Table 1. Light sources used in this study

Light Source	Wave length	Exposure Time	Power	Manufacturer
Halogen unit (Megalux CS)	400-500 nm	40 sec	82 W	Megadenta Radeberg, Germany
LED unit (Benz)	420-480nm	20 sec	1200mW/cm²	Benz, China
PAC unit (Plasmastar) MOD1	410-500nm	10 sec	1800mW/cm²	Monitex San-chungcity, Taipei, Taiwan
PAC unit (Plasmastar) MOD2	410-500nm	6 sec. (2 sec. % 50 light intensity, 2 sec. % 80 light intensity, 2 sec. % 100 light intensity)	1800mW/cm²	Monitex San-chungcity, Taipei, Taiwan
PAC unit (Plasmastar) MOD3	410-500nm	6sec (3 sec. % 50 light intensity, 3 sec. % 100 light intensity)	1800mW/cm²	Monitex San-chungcity, Taipei, Taiwan

2.3. WST-1 Cell Proliferation Assay

The proliferation rate of hDP-MSCs was determined with the WST-1 Test (Roche). Time zero and 48 h culture cell numbers were determined. At each time of analysis, the culture medium was aspirated from the wells and 5% WST-1 solution was added on the cells and incubated at 37^{o}C for 2 h. Absorbance at 480 nm was measured by a UV-visible spectrophotometer (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA). For each group, the assay was performed in triplicate.

2.4. Senescence Test

The same samples were fixed and evaluated for cellular senescence by staining for SA-β-galactosidase -a lysosomal enzyme synthesized by senescent cells - activity. The amount of senescent cells was determined in each well by using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St. Louis, MO) and DAPI as fluorescent counterstain. At the end of the staining procedure, four representative images were taken from diverse areas of each well using phase-contrast microscopy and fluorescence microscopy. For the calculation of the percent senescent cells in the culture the total number of cell nuclei and number of cell nuclei surrounded by cyan dye were enumerated.

2.5. Statistical Analysis

All experiments were performed as triplicates. Data are reported as means ± SD. The results of proliferation experiments were evaluated by One-way Anova and Tukey HSD tests. For statistical analysis of senescence experiment, Mann-Whitney U test was used. Differences were considered statistically significant at $p < 0.05$.

3. Results

In this study we used hDP-MSCs to evaluate effect of different dental light curing units on cell senescence and cell proliferation. Flow cytometric data showed that DP-MSCs used in this study expressed the mesenchymal stem cell markers and were negative for hematopoietic markers like CD34 and CD45 (Figure 2). The cells were able to deposit calcium-phosphate mineral upon osteogenic induction (Figure 2).

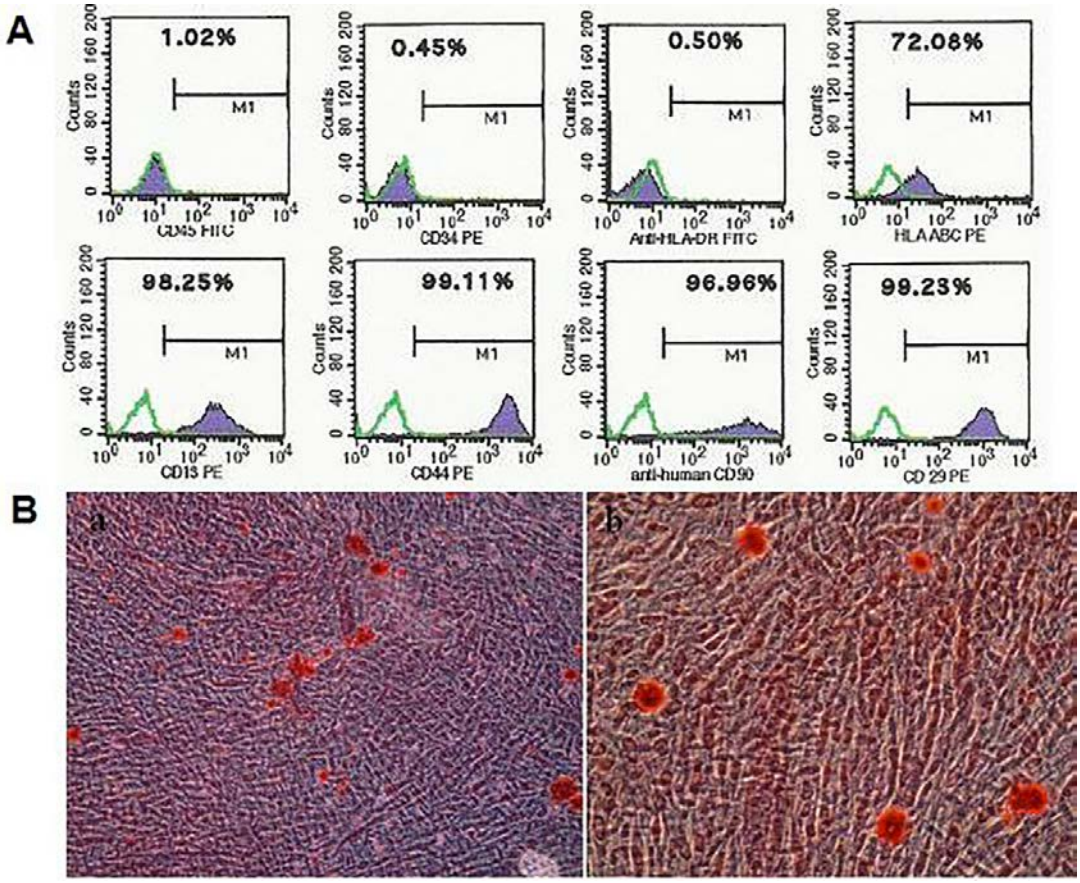

Figure 2. A. Immunophenotypic characteristics of hDP-MSCs obtained by flow cytometry. Cells were labeled with antibodies against hematopoietic antigens (CD34, CD45 and HLA-DR), MSC markers (CD13, CD29, CD44, CD90 and HLA-A,B,C) or immunoglobulin isotype antibodies (Green line: histogram of isotype control immunoglobulin). **B.** Bright field micrographs of the hDP-MSCs differentiated into osteogenic lineage (28 days); arrows point calcified nodules stained with alizarin red S (original magnification: 100X(a) and 200X(b))

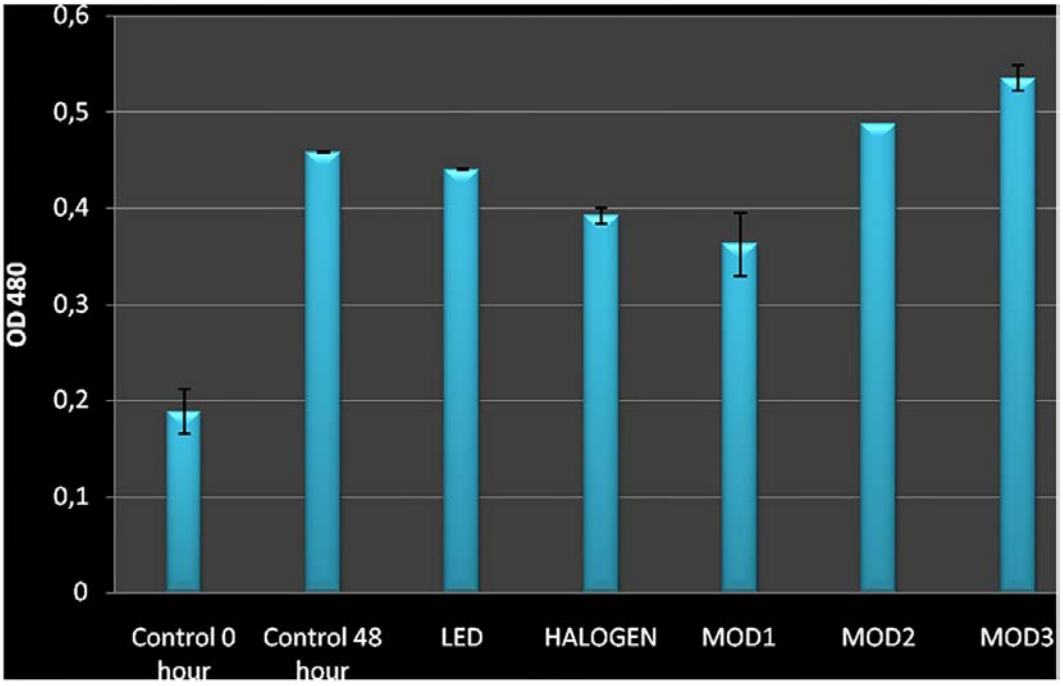

Figure 3. Cell proliferation after light curing (Control represents a well with no light curing applied.)

According to the WST-1 Cell Proliferation Assay, there was no significant difference among cell numbers of the groups when compared to the cell number of the control group ($p > 0.05$), but effect of PAC-MOD3 was significantly different from the effects of Halogen units and PAC-MOD1 (Normal MOD) ($p< 0.05$). The proliferation of hDP-MSCs which were exposed to PAC-MOD3 (2 Step Curing) light for 6 s. was greater than their counterparts' exposed to Halogen units and PAC-MOD1 (Normal MOD) (Figure 3).

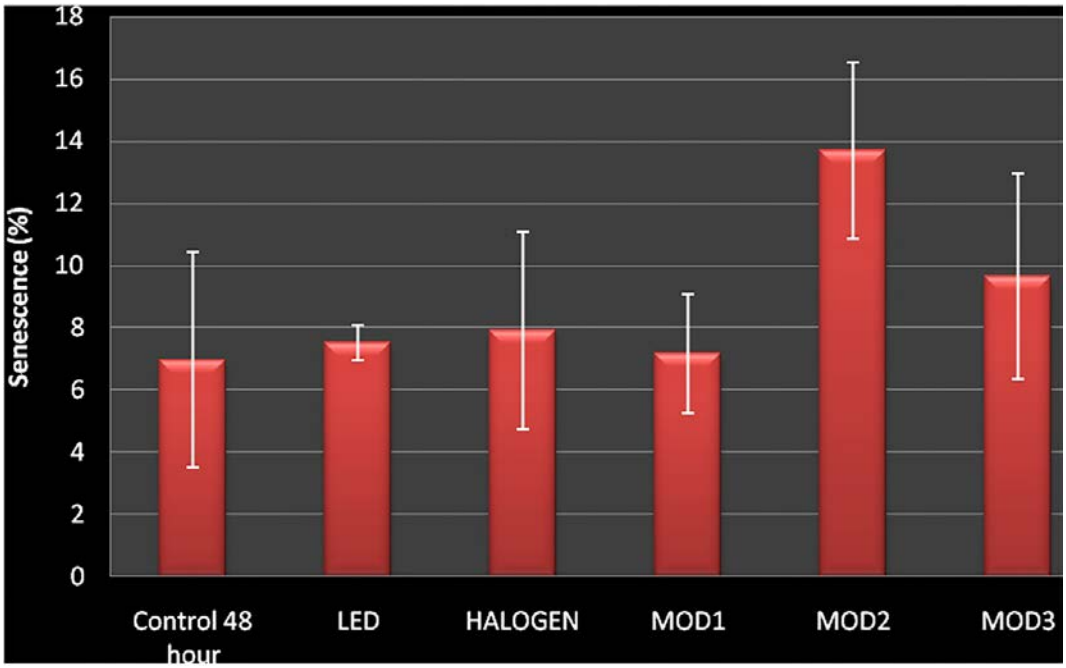

Figure 4. Cell senescence after light curing (Control represents a well with no light curing applied.)

According to the senescence test results, there was no statistically significant difference between the experimental groups and the control group ($p > 0.05$), but SA-β-Galactosidase Activity Test revealed that senescence of hDP-MSCs exposed to PAC-MOD2 (Ramp Curing) light for 6 s. was greater than the others (Figure 4).

4. Discussion

Dental pulp stem cells have become one of the necessary tools for successful tissue repair. Due to their similar characteristics to other mesenchymal stem cells and the relative ease at which they can be obtained and propagated, mesenchymal stem cells derived from dental pulp have become increasingly popular to study. Detailed *in vivo* and *in vitro* studies were previously carried out to examine the proliferation and differentiation capacity of mesenchymal stem cells derived from dental pulp [12]. Because of all these advantages, hDP-MSCs were used in this study. In addition, these cells have proliferative potential and can age by external factors [13].

Different types of light-curing units are used for the photopolymerization of light activated restorative materials including conventional quartz tungsten halogen lights and new photoactivation techniques, such as PAC [14] and, LED [15]. In this study, we compared the effect of Halogen curing units, LED units, PAC units that are widely used in dentistry on dental pulp stem cells.

Conventional halogen light is usually used for curing dental adhesive systems. Halogen units converts only a minor amount of the total energy into light, while it transformes the majority of the energy into heat [16]. The PAC units and LED units are supposed to be safe and useful alternative light curing units to the halogen units, presenting a shorter curing time and lower pulpal temperature rise values to those obtained with the halogen units [17]. These light curing units can be used for polymerization of light activated calcium hydroxide and resin composites in esthetic dental restorative techniques. Light curing units may modify pulp cell metabolism when the materials are used in deep cavities, despite a dentin barrier. In our study, light curing units have affected to proliferation and senence of the dental pulp stem cells.

Cells exposed to hyperthermia (elevated temperature) respond with an increased synthesis of proteins, also called heat shock proteins. The presence of stress proteins has been shown to confer resistance to further stress. Therefore, heat shock proteins might have an impact on temperature studies using cell cultures in a natural or artificial pulp chamber [18].

Studies showed that the highest temperature would result in the lowest rate of surviving cells and there was no statistically significant difference in the number of surviving cells if the different light curing units were used [3]. Most studies agreed that dental temperature rise is significantly higher when resin composites were cured using the Halogen units as compared with the LED and/or PAC curing units [19,20]. Another study showed that LED, standart halogen and high-power halogen light curing units caused a temperature increase within a pulp chamber model but did not have an negative influence on human gingival fibroblasts within the cell medium [3].

The PAC units produced significantly lower temperature changes compared to QTH and LED curing units. The temperature rise must be taken into consideration during polymerization of adhesive resins with LED or QTH in deep cavities when dentin thickness is 0.5 mm or less [20]. In this study, QTH and LED curing units showed less cell

proliferation when compared with the PAC-MOD2 and PAC-MOD3. This result may be due to the high temperature rise in this study. And when direct capping is required, MDPB-containing adhesives like Clearfil Protect Bond can be use, these situation lead to light cure exposure directly to the pulp tissue.

Knezevic et al. [21] studied the effect of curing mode densities on cell culture cytotoxicity/genotoxicity and determined higher DNA damage in cultures cured with low intensity mode when compared to the other modes (high intensity and soft start). Van Buruegel H. et al. [22] have reported that higher power densities and lower exposure times are usually more useful than lower power densities for a longer period of time. In this study, PAC-MOD3 (high power, low exposure time) was more effective in stimulating hDP-MSC proliferation when compared with the other applications.

Less than 400 nm wavelengths of ultraviolet light can have adverse effects on biological cells and is therefore considered as hazard. Accordingly around the 1980s the wavelength of the light for the polymerization of dental composites was shifted from the short wave UV radiation to the longer waves in the visible (VIS) range (400-500 nm). However, recently the negative influence of blue light on mouse fibroblasts has been published stating that blue light suppresses the cellular mitochondrial function [3,23].

The emission of blue LED LCUs is the ideal spectra for composites containing camphorquinon. Studies showed that when the equal light energy was irradiated, the degree of monomer conversion of the composite was not statistically significant if LED, PAC or halogen light curing units was used [3,24].

It has been reported that three common dental photocuring blue light sources significantly and irreversibly suppressed cellular mitochondrial function in a standard cell line when used for clinically relevant durations at clinically relevant distances. The cellular effects did not appear to be caused by increases in temperature alone, and the effects were light-dose-dependent. There was also evidence that the effects were wavelength dependent as well. These results indicate that dental photocuring lights pose at lease some risk to oral cells, and that further study of these effects are warranted [23].

Blue light may not be as innocuous to tissues as has been assumed by the dental community. Reports have documented the effects of blue light on DNA [14], mitosis of cells [25], mitochondria [26] and as generators of intracellular reactive oxygen species via absorption by flavins [27]. Other reports have shown effects on monocytes and monocytic antioxidant pathways [28]. However, in all of these reports, the light sources have not been the same as those used in dentistry, and the doses and durations of light exposure have not been relevant to restorative dentistry (but were usually much higher). The safety of blue light in a dental context has therefore been largely assumed [23]. In our study, PAC-MOD2 have increased to the cell senescence of dental pulp stem cells and PAC-MOD3,PAC-MOD2 have increased to the cell proliferation of dental pulp stem cells.

In summary, dental light curing units may effect of proliferation and senescence of dental pulp stem cells when used deep cavites and expose dental pulp. More studies are needed in this regard.

5. Conclusions

Adhesive systems have been widely used for esthetic procedures. However, when the light curing units are used for polymerization of adhesive systems in direct and indirect pulp capping, this procedure may have effects on senescence and proliferation of dental stem cells.

Acknowledgments

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Competing Interests

There are no conflicts of interest to declare.

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