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Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDSCs), and periodontal ligament stem cells (PDLSCs) isolation, characterization and the effectiveness of allantoin as bioactive molecule for dental regeneration

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ABSTRACT

Introduction: Dental stem cells are valuable tools in regenerative medicine due to their pluripotency and self-renewal properties. This study aimed to investigate the effects of allantoin (Al) on Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDSCs), and periodontal ligament stem cells (PDLSCs) regarding cytotoxicity, proliferation, wound healing, and osteogenic differentiation.
Methods: Human dental stem cells were isolated from three dental tissues using the explant culture method and cultured in DMEM-F12 medium supplemented with 15 % fetal bovine serum (FBS) and antibiotics. The cytotoxicity and proliferation of allantoin were assessed using the XTT cell viability assay at concentrations ranging from 0.25 to 5 mg/mL. Wound healing was evaluated through a scratch assay at 1 mg/mL, and osteogenic differentiation was assessed using Alizarin Red S staining at 0.5 mg/mL and 1 mg/mL.
Results: Al exhibited no cytotoxic effects across the tested concentrations. It enhanced cell proliferation, particularly in SHEDSCs at 5 mg/mL. DPSCs also showed significant improvement in wound healing in the scratch assay. At 1 mg/mL, Al inhibited osteogenic differentiation in DPSCs and PDLSCs, as indicated by reduced mineralization.
Conclusion: Al shows potential as a non-cytotoxic agent for enhancing the proliferation of dental stem cells, especially SHEDSCs. However, its limited effect on wound healing of SHEDSCs and PDLSCs and inhibition of osteogenic differentiation at higher concentrations suggest that further optimization is required for its application in bone regeneration.
Statement of Clinical Relevance: Evaluation of the effects of plant-based therapeutic compounds on various types of dental stem cells may have the potential to increase the success of stem cell-based therapies in clinical applications in regenerative dentistry.

1. Introduction

Tooth decay and periodontal diseases are among the most common oral diseases worldwide and remain a public health problem for developing countries [1]. Tooth loss due to premature loss of vitality of the dental pulp due to various reasons is still one of the most common health problems in dentistry, and the need for successful pulp regeneration treatments in endodontics is increasing [2]. Regeneration of functional tooth-tissue structures is an important topic that has recently attracted the attention of researchers in dental science. Dental regenerative medicine aims to restore normal pulp function through complex dentin-pulp regeneration using tissue engineering aspects such as stem cells, growth factors, and biomaterials/scaffolds together or in combination [3,4]. In this context, dental stem cells for regenerative endodontic therapy have attracted great attention recently due to their regenerative potential and versatility [5].

Dental stem cells represent a subset of mesenchymal stem cells (MSCs) found in various dental tissues, including dental pulp,

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periodontal ligament, dental follicle, and dental germ [6]. Their ability to differentiate into multiple cell types makes them promising candidates for regenerative medicine applications, as they are accessible through non-invasive procedures, unlike other stem cell sources, and are not subject to ethical and legal boundaries [5,7] Dental stem cell studies are being evaluated for different applications such as the regeneration of dental tissues as well as the regeneration of body tissues with dental stem cells, and it is thought that they will play an important role in regenerative medicine in the future [8,9].

Dental Pulp Stem Cells (DPSCs), the first isolated stem cells of dental origin, are isolated from the dental pulp of permanent teeth [10]. DPSCs exhibit multilineage differentiation potential, with the capacity to form dentin, nerve cells, and other mesodermal derivatives [11]. Stem cells from human exfoliated deciduous teeth (SHEDSCs) are obtained from the dental pulp of primary teeth (baby) and have a high proliferative capacity and the ability to differentiate into various cell lineages, including neurons and adipocytes [12]. Surrounded by fibrous connective tissue, the periodontal ligament is situated between the teeth and the inner wall of the alveolar fossa. Periodontal Ligament Stem Cells (PDLSCs), located in the periodontal ligament, promote the regeneration of periodontal tissues, including cementum, periodontal ligament, and alveolar bone [13]. Dental stem cells have MSC-like properties similar to bone marrow MSCs [14]. Stem cells isolated from all dental sources exhibit fibroblast-like morphology, express a variety of cell surface markers, and can differentiate into mesodermal lineages, namely osteocytes, adipocytes, and chondrocytes [15–17].

Dental stem cells are easily accessible, economical sources of MSCs and are therefore a potential source of stem cells for tissue regeneration [5]. A comprehensive understanding of how to control the expansion and differentiation of these handily accessible stem cells will provide a solid basis for using these cells for regenerative applications. However, increasing the proliferation of these stem cells and preventing their aging is one of the most important stages of cell-based regenerative therapies and modulation of dental stem cells is one of the important issues in this field [18,19]. Recently, researchers have concentrated on investigating the effect of various substances on the survival, proliferation and differentiation of dental stem cells [20]. It has been suggested that adult stem cell proliferation and differentiation, which contribute to the regeneration of damaged tissues, is stimulated by various plant extracts and their phytochemicals.

Allantoin, a naturally occurring compound, has gained significant attention in the fields of medicine and skincare due to its remarkable properties. Allantoin, known chemically as 5-ureidohydantoin, is a nitrogenous compound found in a variety of plants, animals and microorganisms [21]. It was first discovered from *Symphytum officinale*, popularly known as the comfrey plant, and was later identified in various organisms and shown to be widespread [22]. Previous studies have shown that allantoin has numerous pharmacological activities, such as wound healing [23], moisturizing and necrotic tissue removal [24], and stimulation of cell proliferation [25]. It shows anti-inflammatory properties, epithelial stimulation, analgesic effects and also supports keratolytic activity [26]. Due to these various properties of allantoin, it is used by being included in various health and skin care products.

The aim of this study is to evaluate the *in vitro* effects of allantoin on dental stem cells (DPSCs, SHEDSCs, and PDLSCs) in terms of cytotoxicity, cell proliferation, wound healing, and osteogenic differentiation, to determine its potential for enhancing the therapeutic application of these cells in regenerative dentistry

2. Materials and methods

2.1. Materials

In this study, allantoin (Al) was used in powder form. The cell culture materials used were purchased from Pan Biotec. Inc., USA. XTT ((sodium 3′-[1-(phenylaminocarbonyl)−3,4- tetrazolium]-bis(4-methoxy6-nitro) benzene sulfonic acid hydrate) and PMS (N-Methylphenazonium methyl sulfate) using for cytotoxicity assay were purchased from Santa Cruz Biotec. Inc. Dexamethasone, L-ascorbic acid and paraformaldehyde were purchased from Sigma Aldrich-Merck KGaA (Germany). Alizarin Red S Sodium Salt were purchased from Alfa Aesar Thermo Fisher (Kandel)(USA). Sodium b-glycerophosphate were purchased from Cayman Chemical. (USA). All antibodies were obtained from Bio-Legend® Inc (UK).

2.2. Methods

2.2.1. Specimen collection

The protocol was approved by the Istanbul Medipol University Ethics Committee. After obtaining informed consent, two impacted third molars from a healthy young patient were surgically removed at the Istanbul Medipol University Faculty of Dentistry. A healthy human exfoliated primary molar teeth were extracted from a 10-year-old child as a discarded clinical specimen after voluntary informed consent was obtained from both the child and his/her parents. The tooth surfaces were thoroughly cleaned and the pulp tissues were carefully cut and removed. Periodontal tissues were removed along the root surface from third molars of two young healthy patients using a sterile surgical blade. All collected tissues were brought to the laboratory in DMEM-F12 medium containing 3 % penicillin/streptomycin (300 units/mL penicillin, 300 μg/mL streptomycin) in falcon tubes.

2.2.2. Dental stem cell isolation

Samples collected from three different dental tissues were brought to the laboratory in DMEM-F12 basal transport medium supplemented with 15 % fetal bovine serum (FBS) and 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. All subsequent work was carried out in a laminar flow biosafety cabinet under aseptic conditions. Tissue samples were taken from the media and washed 3 times with 1xPBS containing 3 % pen./strep. Cell isolation was performed by explant culture method [27]. For the preparation of explants, samples were transferred to a petri plate and cut into <2 mm^2 pieces using a surgical blade. The resulting explants were then cultured in a T-25 flask containing DMEM-F12 supplemented with 15 % FBS, penicillin 100U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, at 37 °C in a 5 % CO_2 atmosphere. Culture medium changes were made at 3–4 day intervals. Cultures were observed daily under an inverted microscope (BEL, INV-100-FL, Italy) to monitor cell migration and growth from the explant, and possible contamination. Images of the explants were taken at two-day intervals.

2.2.3. Cell culture

It was observed that cell migration started from the explants from the 6th day of the cultures. Passage of cells reaching approximately 70 % to 80 % confluency on day 14 of culture was initiated. For passage, cells were washed twice with PBS to remove cellular debris and waste tissue fragments. Then, 0.25 % trypsin-EDTA solution was added to the flasks and the cells were separated from the flask surface for 3–5 mins. The detached cells were neutralized by adding DMEM-F12 containing 15 % FBS. Then, the cells were transferred to 15 ml tubes by passing through a 70 μm cell strainer to remove tissue fragments and centrifuged at 1500 rpm for 5 mins. The supernatant was carefully removed to obtain a cell pellet. Cell pellet was completed with 1 mL medium and cell suspension was obtained by light pipetting and cell count was performed by staining with 0.4 % Trypan blue solution by means of a hemocytometer. Cells were cultured in basal growth medium at 37 °C in a humidified incubator with 5 % CO_2, by seeding in flasks at certain ratios. Since the use of low passage cells was desired in the intended cellular analyses in the study, cell freezing was performed at each passage after the first passage to five passage numbers. Cells were transferred to cryovials at 1.5×10^6 cells/mL using freezing medium containing 90 % FBS and 10 % DMSO in

liquid nitrogen at −196 °C for long-term storage.

2.2.4. Immunophenotype analysis by fluorescence-activated cell sorting (FACS)

Before performing other experiments, to confirm the in vitro stem cell phenotypic properties of DPSCs, PDLSCs and SHEDSCs, undifferentiated cells in the 4–5 passage number range were subjected to flow cytometry analyses. Cells were characterized for the presence of mesenchymal markers CD44, CD73, CD90 and the absence of the hematopoietic markers CD34, CD11b and CD45. For immunophenotype analysis, stem cells were harvested and resuspended in culture medium at a concentration of 10^6 cells/100 μL. To determine the specific ratios of positive mesenchymal markers, cell types were incubated with antibodies labeled with different fluorescence dyes as follows; CD44-PE/Cyanine7 anti-human (Cat #338,815), CD73-Allophycocyanin (APC)/Fire™ 750 anti-human (Cat #344,035), CD90 (Thy1)-FITC anti-human (Cat #328,107) (BioLegend, San Diego, CA, USA). The following antibodies were used as hematopoietic stem cell markers; PE-conjugated anti-human CD34 (Cat #343,505), anti-human CD45 (Cat #304,007,) and anti-human CD11b (Cat #301,305) (BioLegend, San Diego, CA, USA). Appropriate isotype controls are also included as negative controls for non-specific background staining. Isotype controls as the following antibody; FITC Mouse IgG1 κ Isotype Ctrl Antibody (Cat #400,107), APC Mouse IgG1κ Isotype Ctrl Antibody (Cat #400,119), PE Mouse IgG1 κ Isotype Ctrl Antibody (Cat #400,111), and PE/Cyanine7 Mouse IgG1κ Isotype Ctrl Antibody (Cat #400,125). Cells were then washed three times with PBS, followed by fluorescence-activated cell sorting (FACS) analysis on a CyFlow® Cube 8 flow cytometer equipped with CyFlow® Software (Sysmex).

2.2.5. In vitro cytotoxicity and proliferation

The cytotoxic effects of Al on DPSCs, SHEDSCs and PDLSCs were examined by XTT cell viability assay at varying concentrations (0.25, 0.5, 1, 2, 3, 4 and 5 mg/mL). For cytotoxicity testing, cells in the 3–5 passage range were seeded at 1×10^4 cells/well in 96-well plates and incubated at 37 °C for one day in a 5 % CO_2 humidified incubator to allow the cells to adhere to the surface. Then, the media on the cells were removed and the media containing Al at different concentrations were added to the wells and incubated for 24 h. After the completion of the incubation period, the medium was changed with 0.4 mg/mL XTT and PMS containing medium and incubated for 4 h, and then the absorbance of the wells at 450 nm was read with a microplate reader (Lablineo28,4304, Netherlands) and cell viability was calculated with the formula [1] below. Wells without samples were used as negative control. Experiments were performed with at least 3 replications ($n = 3$) and data were calculated as mean ± standard deviation (SD).

$$\textit{Cell Viability}\ (\%) = \left(\frac{\textit{Optical density (OD) of treated cells}}{\textit{OD of control cells}}\right) \times 100 \quad (1)$$

To examine the effects of longer-term Al treatment on proliferation in dental cells (in a way to examine its effects on proliferation), cell viability tests were performed on the 1st, 3rd and 6th days. For this; DPSCs, SHEDSCs and PDLSCs were seeded at 2×10^4 cells/well in a 24-well plate. After the cells adhered to the surface, the medium in the wells was replaced with a medium containing different concentrations of Al and cultured for a total of 6 days. On the 1st, 3rd and 6th days of culture, absorbance values of the wells were taken at 450 nm by means of a microplate reader and proliferation was evaluated by the increase in absorbance. Experiments were performed with at least 3 replications ($n = 3$) and data were calculated as mean ± standard deviation (SD).

2.2.6. In vitro wound healing activity (Scratch assay)

The effect of allantoin on in vitro cell migration of DPSC, SHEDSC, and PDLSC cells was examined by the scratch closure test. For the scratch assay, cells were seeded in 12-well plates at a density of 4×10^5 cells/well and incubated at 37 °C in an atmosphere of 5 % CO_2 until 95 % confluent. Then, the single cell layer in the wells from which the medium was removed to create the scratches was lightly scratched as a single line with a p200 pipette tip. Cells were washed 2 times with PBS to remove unbound cells. Then, the medium containing Al at a concentration of 1 mg/mL was added to the wells. Medium without Al was used as a control. Scratch closure was followed for 48 h and at different time intervals (0., 12., 24., and 48 h) with a 100X magnification inverted microscope. Percentage closure of the created scratch areas was analyzed using the ImageJ (National Institutes of Health, version 1.520 software, USA) image analysis software.

2.2.7. Osteogenic differentiation

The possible effects of allantoin on the osteogenic differentiation of dental stem cells were examined by Alizarin red staining. Briefly, cells were seeded in 12-well cell culture plates at a density of 3×10^4 cells/well and incubated for 24 h for cell attachment. At the end of 24 h, the medium was replaced with an osteogenic differentiation medium containing different concentrations of Allantoin (0.5 and 1 mg/mL) and cultured for differentiation for 21 days. Osteogenic differentiation medium was prepared using 100 nM dexamethasone, 50 μg/mL ascorbic acid, 10 mM sodium b-glycerophosphate in DMEM-F12 medium containing 10 % FBS. In addition, the direct effects of Allantoin on cell differentiation were studied without differentiation medium. The medium was changed every three days. For Alizarin red staining on day 21 of culture, the medium was removed from the wells and the wells were washed with PBS followed by 10 mM Tris–HCl (pH 7.5) containing 0.9 % NaCl. Then, cells were fixed with 4 % paraformaldehyde and stained with 40 mM (pH 4.18) Alizarin Red S for 20 mins. After staining, the wells were washed four times (5 mins each time with gentle shaking) with deionized water and the stained foci of mineralization were examined with an inverted microscope.

2.3. Statistical analysis

Statistical analyses were performed using GraphPad Prism8 software (La Jolla, USA). Student's t-test was used for two-group comparisons; one-way analysis of variance (ANOVA) was used for multiple-group comparisons. All values are presented as mean ± standard deviation. p-value < 0.05 and < 0.01 were considered significant differences and are represented by the sign "*" and "**" respectively.

3. Results

3.1. Isolation, expansion, and characterisation of stem cells

Human stem cells from three different dental tissue sites were successfully isolated and cultured by the explant culture method. The explant images of different dental tissues between day 6 and day 12 are shown in Fig. 1(a). Cell migration from all explants began visibly on days 5–6 and stem cells were identified with typical fibroblastic-like spindle-like morphologies. After the first passage, cells were observed with a inverted microscope at 200X magnification. Fig. 1(b). In order to preserve the pluripotency of the cells without differentiation, cells that reached 70 % cell density were passaged.

The mesenchymal profile of cells in the study was assessed by flow cytometry using cell surface markers for CD73, CD44, and CD90, and a panel of CD45, CD34 and CD11b negative markers. The results are shown in Fig. 2. Both PDLSCs and SHEDSCs were found to have a similar expression pattern for the analyzed markers. DPSCs showed a generally similar antibody profile to the other two cell types regarding positive markers. In addition, they had a high rate of CD44 marker, which has important roles in processes such as cell-cell and cell-matrix adhesion, and cell migration. In addition, the rate of negative markers was lower than the other two cells. All cell types were strongly positive for the

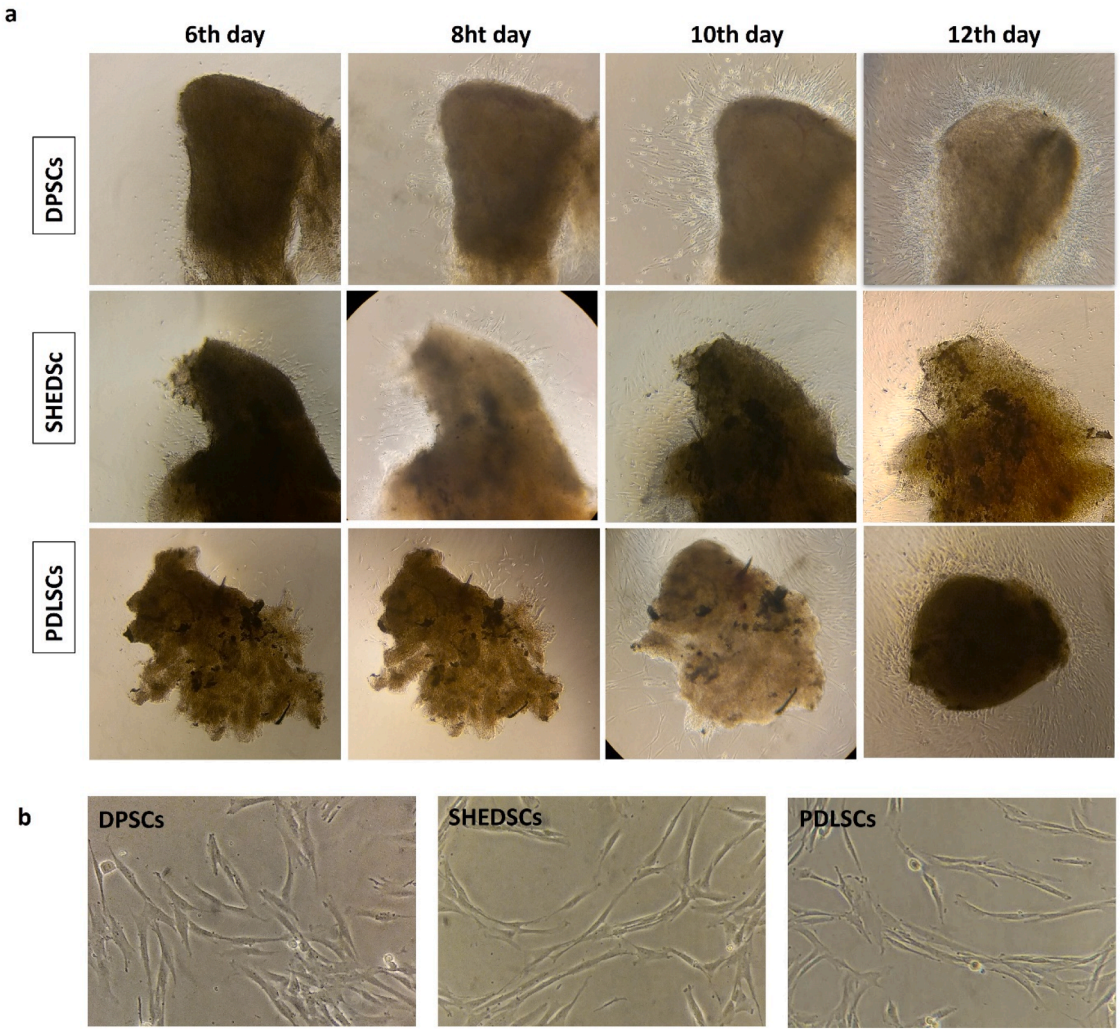


Fig. 1. Different stages of cell isolation (a) Explant microscope images on days 6 and 12 (magnification 40X)(b) Microscope images of first passage cells on days 1 of culture (magnification 200X).

lymphocyte differentiation marker CD73, the early adhesion and hyaluronan marker CD44, and the archetypal membrane marker CD90 also known as Thy-1. For all cell types, no interaction was observed for the leukocyte common antigen CD 45, the endothelial and lymphoma hematopoietic cell marker CD34, and the leukocyte-specific receptor CD 11b. The immunophenotype analysis results confirm that cells from the three populations show expression of generally accepted mesenchymal stem cell markers.

3.2. Effects of allantoin on cell viability and proliferation of dental stem cells

The cytotoxic effects of allantoin on 3 different dental stem cells were examined with the XTT cell viability test at varying concentrations. The cytotoxicity results after 24 h of incubation with different concentrations of allantoin are presented in Fig. 3. In the concentration range of 0.25–5 mg/mL, allantoin did not show cytotoxic activity in all three cell types. However, in Fig. 3(a) for DPSCs, 5 mg/ml concentration caused a significant ($p < 0.05$) decrease in cell viability and decreased by 132.1 % ±6.8. Additionally, allantoin concentrations of 2 mg/mL and above did not create a significant difference in terms of cell viability. According to SHEDSCs cell viability results shown in Fig. 3(b), all studied concentrations had higher cell viability values compared to the control. However, no significant difference was detected in the viability values of cells treated with a range of 0.25–1 mg/mL allantoin. 5 mg/mL allantoin concentration caused a significant ($p < 0.01$) increase in cell viability compared to control and other concentrations and cell viability was 185.8 % + 8.3. As seen in Fig. 3(c), relatively low concentrations of allantoin had no significant effect on cell viability of PDLSCs. Concentrations above 2 mg/mL were significantly effective on cell viability ($p < 0.01$).

Following the observation of the positive effects of allantoin on cell viability, a subsequent investigation was conducted to further explore its impact on cell proliferation. The proliferation of dental stem cells treated with the same allantoin concentrations for six days was examined. Changes in cell proliferation were determined by performing an XTT cell viability test on days 1, 3, and 6, and proliferative effects were expressed as optical densities. Fig. 4 shows the effects of allantoin on the proliferation of dental stem cells. Treatment of different concentrations of Allantoin on DPSCs for 6 days resulted in variable proliferative effects on the cells as shown in the graphs presented in Fig. 4(a). While allantoin concentrations up to 4 mg/mL showed similar cell proliferation to the control up to and including the 3rd day, no significant increase in cell viability was detected on the 6th day. However; cells treated with 0.25 and 0.5 mg/mL allantoin showed lower proliferation than the control on the 6th day. Notably, the 5 mg/ml concentration had a higher proliferation rate than the control group in all three-day periods. The proliferative effects of allantoin on SHEDSCs are shown in Fig. 4(b). Allantoin treatment at different concentrations did not lead to significant differences except 0.5 mg/mL and 3 mg/mL after 24 h incubation.

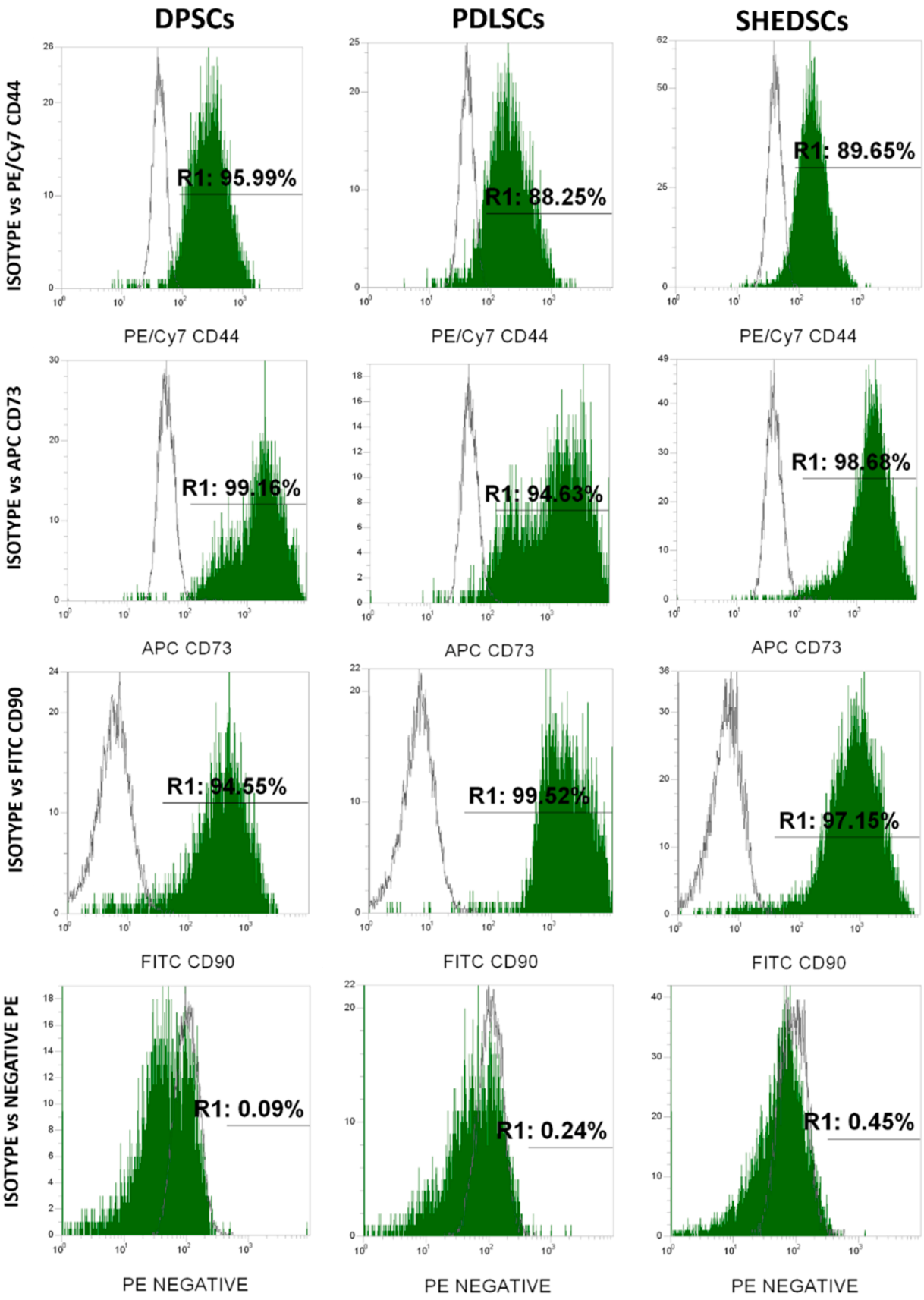


Fig. 2. FACS profiling of common stem cell markers CD73, CD44, and CD90 in DPSCs, SHEDSc, and PDLSCs (Cells derived from dental tissue were stained with the corresponding antibody (green-filled histograms) or mouse IgG1, κ PE isotype control (non-filled histogram)).

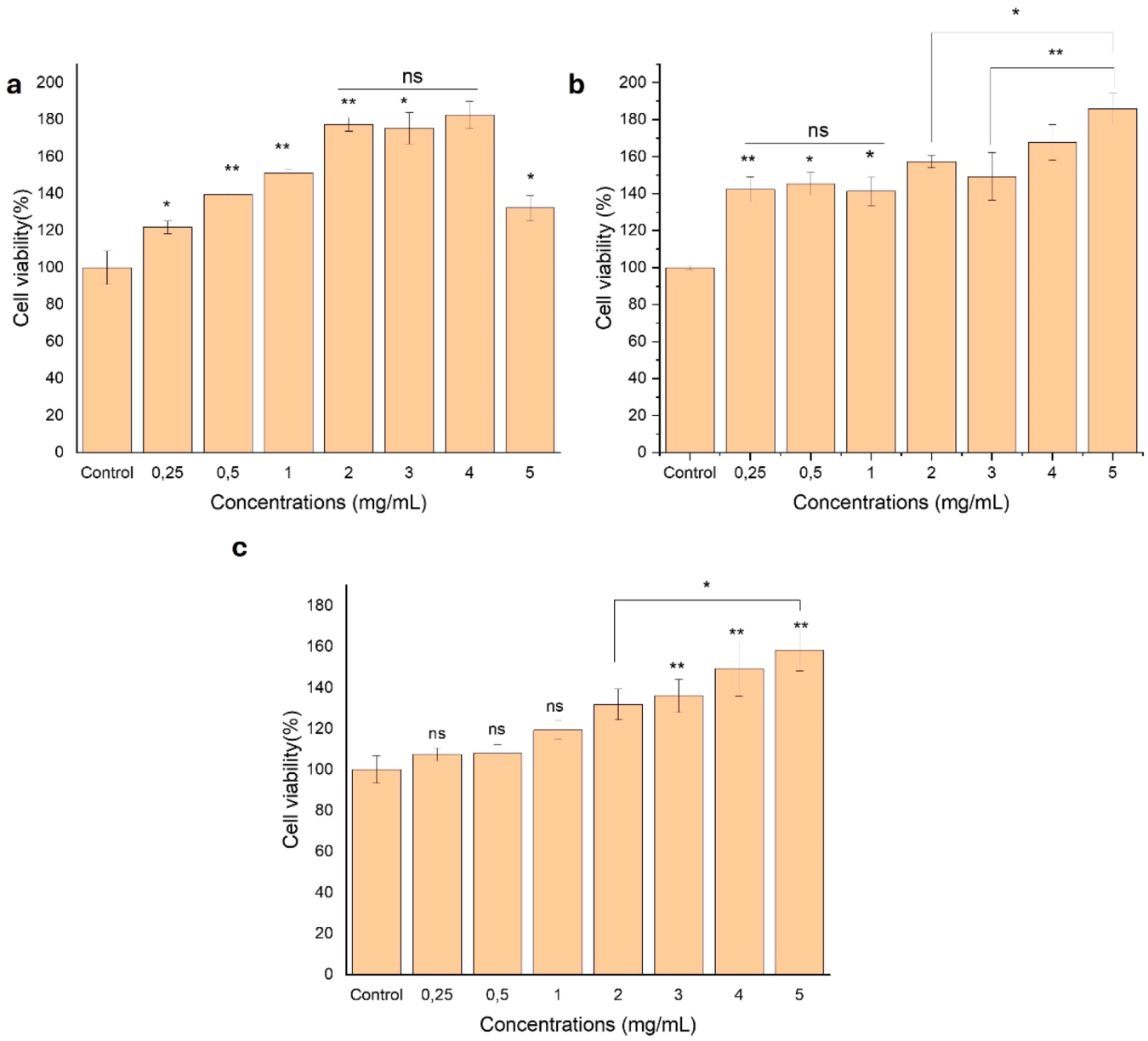


Fig. 3. Cytotoxicity of varying allantoin concentrations on dental stem cells (a) DPSCs (b) SHEDSCs (c) PDLSCs (* $p < 0.05$ and ** $p < 0.01$ indicate significant differences. No significant difference between the control or the specified groups is indicated with "ns").

On the 3rd day of culture, cell viabilities were similar or lower than the control. The proliferative effects of allantoin on SHEDSCs are observed after the 3rd day of culture. On the 6th day, the cell viability of each allantoin group was significantly higher than the control group (except for the 3 mg/mL concentration). Prolonged treatment of allantoin with SHEDSCs shows proliferative effects. The proliferative effects of allantoin on PDLSCs are shown in Fig. 4(c). For PDLSCs, in agreement with the cytotoxicity test result, allantoin treatment at low concentrations (0.25 and 0.5 mg/mL) showed similar cell proliferation with the control (except for a slight decrease on day 3 for 0.5 mg/mL) over a 6-day incubation period, and no significant difference was observed ($p>0.05$). At concentrations of 2 mg/mL and above on the 3rd day of culture, a significant increase in proliferation occurred, consistent with the cytotoxicity results, and on the 6th day, cell viability was significantly higher than the control group ($p < 0.01$).

3.3. *In vitro wound healing activity: Scratch assay*

In this study, the possible effects of allantoin on the migration of dental stem cells were examined with the scratch test for a concentration of 1 mg/ml. Scratch images taken at different time intervals are presented in Fig. 5. There was no significant difference in scratch closure at the end of 48 h for PDLSCs compared to the control group and the results were similar. Similarly, although the average closure percentages were slightly higher for SHEDSCs (there was a significant difference, especially at the end of the 24th hour), there was no significant difference in closure percentages at the end of the 48th hour. The role of allantoin in improving scratch closure was greater for DPSCs. For DPSCs, the closure percentage was higher at the 12th and 24th hour time points compared to the control and at the end of the 48th hour, the scratch closure percentage was determined as 5 % ± 3.89 in the Allantoin group and 15.3 % + 3.12 in the control group.

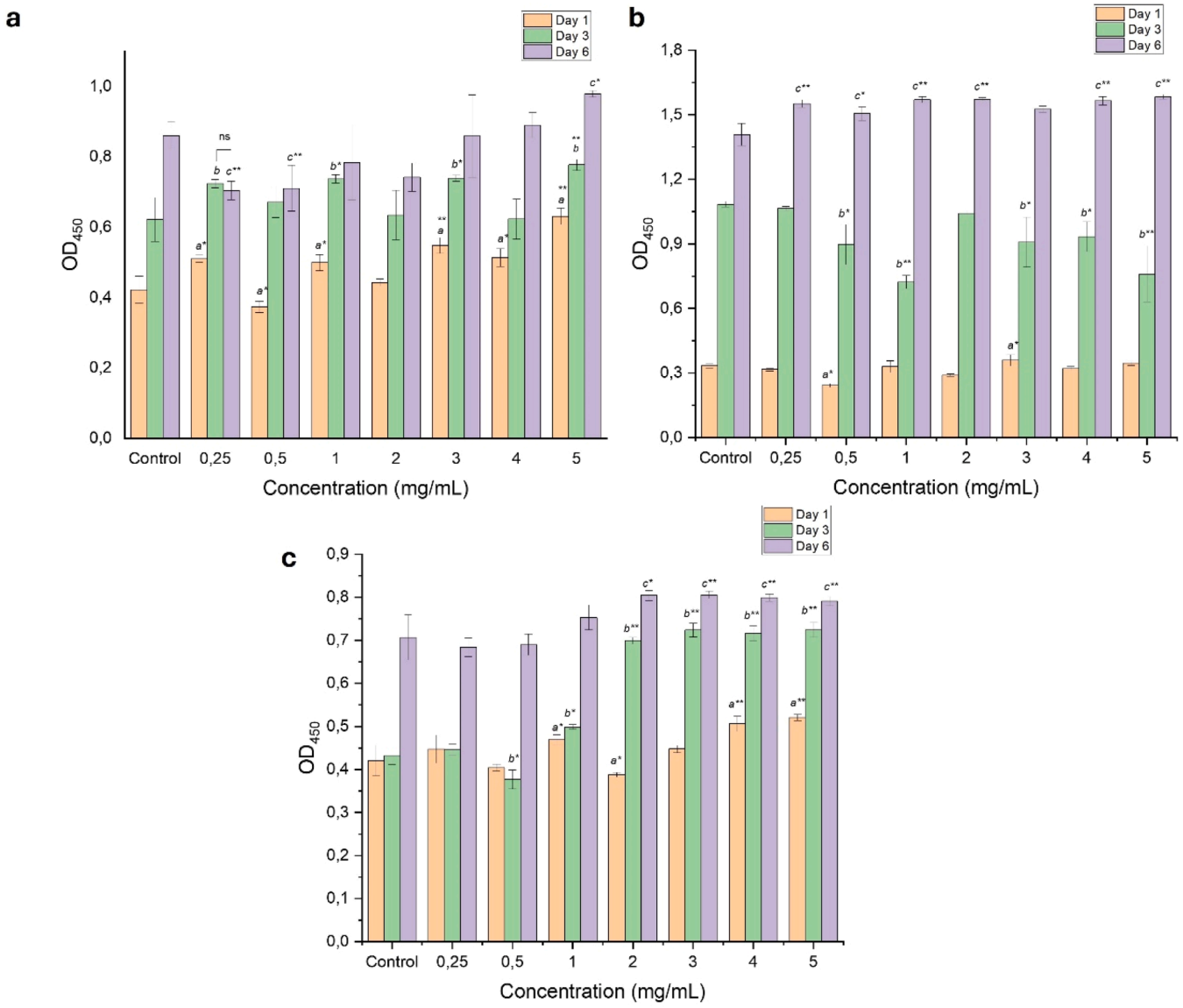


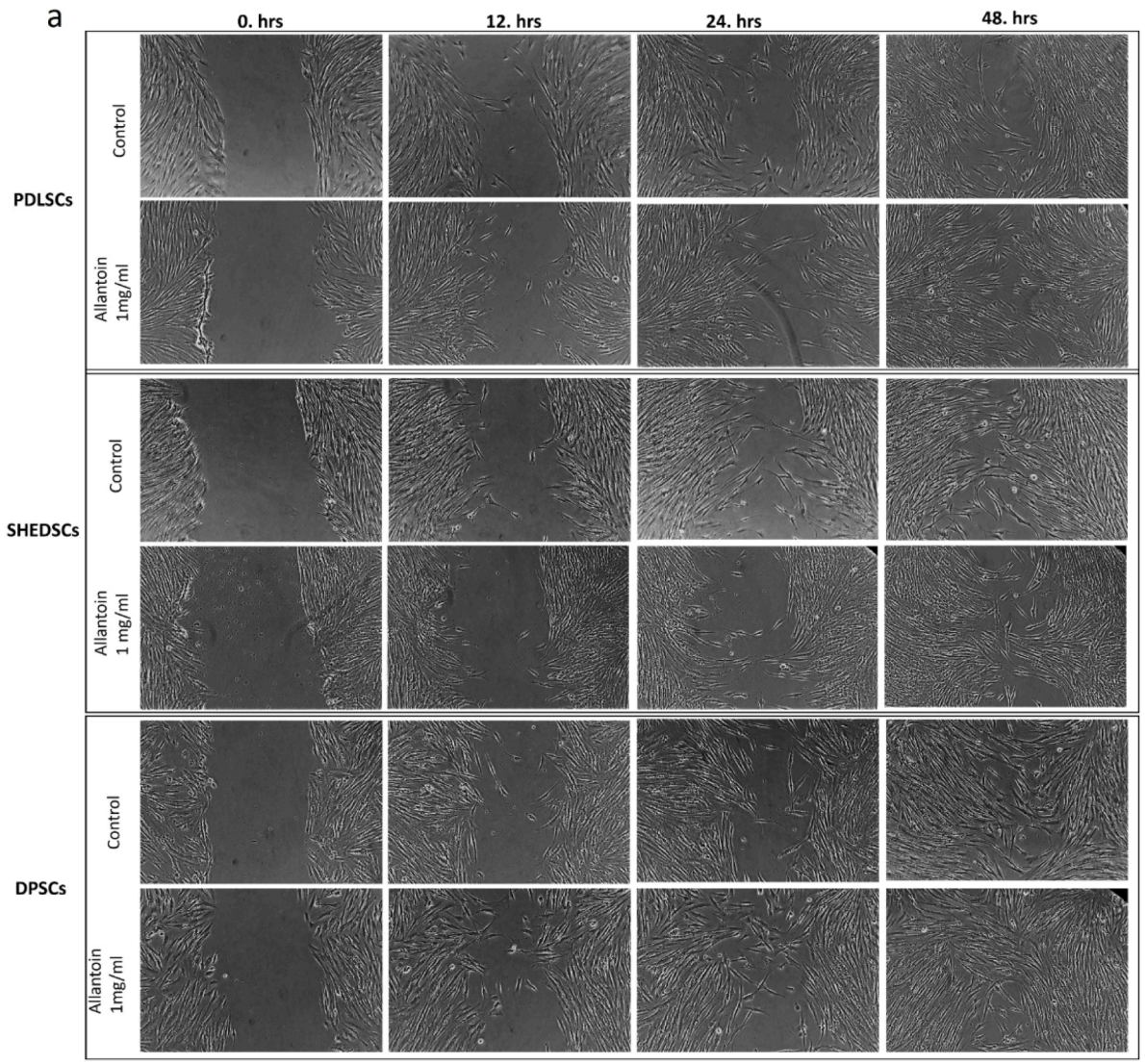
Fig. 4. Effects of allantoin treatment on the proliferation of dental stem cells a) DPSCs b) SHEDSCs c) PDLSCs (Data are presented as mean ± SD. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences compared to the control group. Each concentration was compared for significance to the control group simultaneously; a, b, and c indicate significant differences to the control group at 24, 72, and 114 h, respectively.).

3.4. Effects of allantoin on the differentiation of dental stem cells

The possible effect of allantoin on osteogenic differentiation on dental stem cells was evaluated at two concentrations of 0.5 mg/mL and 1 mg/mL, with osteogenic differentiation medium and without using differentiation medium (control medium). The results of Alizarin Red S staining are presented in Fig. 6. When the staining results were examined, no significant effect of Allantoin on differentiation was observed in the control environment. Only one or two mineralization foci were observed in cells at 0.5 mg/mL llantoin concentrations in SHEDSCs. Under osteogenic differentiation conditions, dental stem cells performed calcium mineralization, which is indicative of osteogenic differentiation. However, there are significant differences between the differentiation potentials of dental stem cells. The formation of mineralization foci was greater in the control DPSCs at the 21-day differentiation period. A similar situation was seen in control SHEDSCs, whereas PDLSCs had dramatically fewer foci of mineralization. Interestingly, the effect of allantoin on differentiation produced concentration-dependent differences on dental stem cells. At 0.5 mg/mL Al concentrations, there was no significant effect on cell differentiation for DPSCs and PDLSCs compared to the control group, while 1 mg/mL Al concentration had a negative effect on differentiation and caused a decrease in the number of mineralization foci.

4. Discussion

MSCs derived from dental tissues play a promising role in future regenerative medicine and tissue engineering due to their ease of assembly and their capacity to undergo self-renewal and polygenic differentiation. However, how to regulate and control the osteogenic differentiation potential of dental stem cells remains an unsolved problem, and the problems such as toxicity and high price of the materials used as osteoinductive factors have led to the need for new bioactive substances for differentiation. Therefore, identification the molecular mechanism of osteogenic differentiation of dental stem cells by various bioactive components is critical for tooth regeneration and oral tissue engineering. This is the first study to examine the in vitro biological activity and osteogenic potential of allantoin on DPSCs, SHEDSCs and PDLSCs dental stem cells. Dental stem cells were successfully isolated and expanded from human dental tissues. Flow



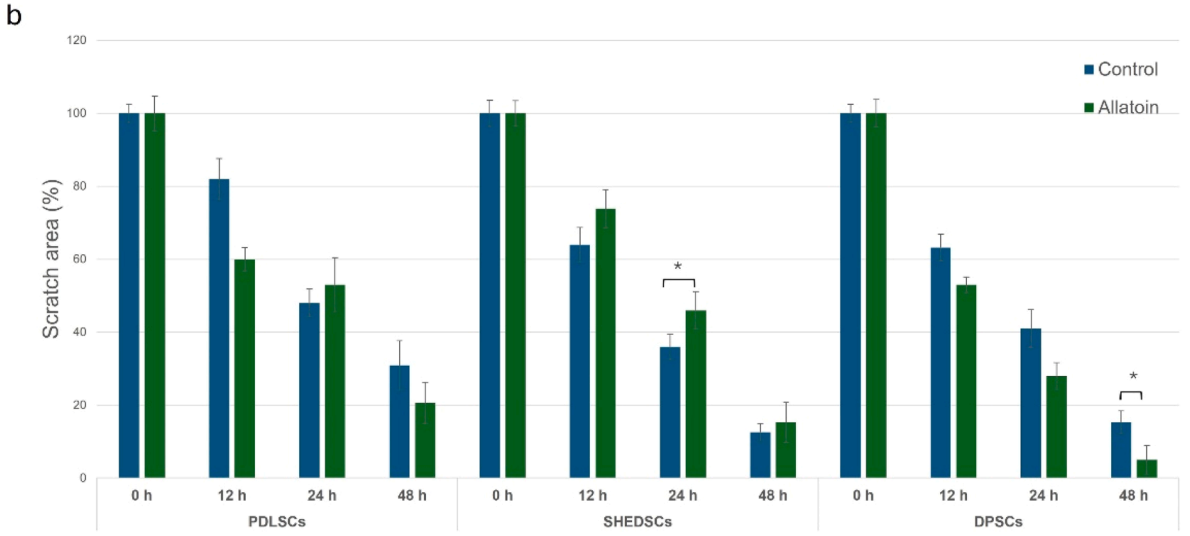


Fig. 5. Effects of allantoin on in vitro wound healing (Scratch Assay) (a) Microscopic monitoring of scratch closure (b) Scratch closure rates at different time points (* $p < 0.05$ indicate significant differences compared to the control group).

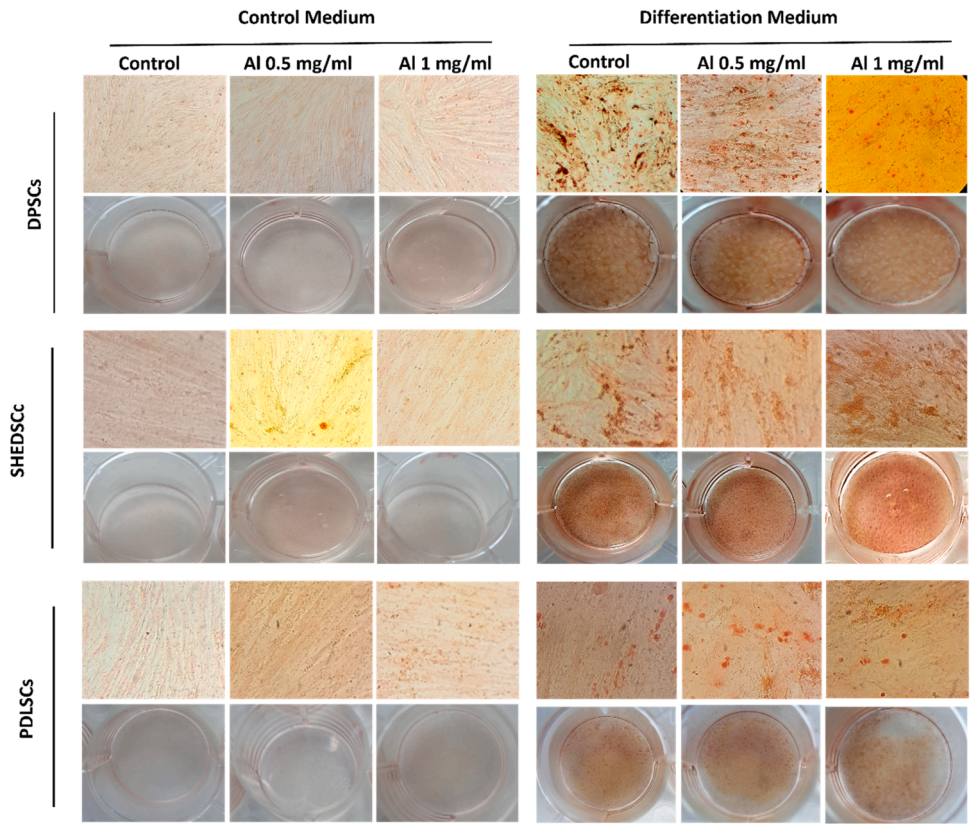


Fig. 6. Effect of 0.5 and 1 mg/mL allantoin treatment on osteogenic differentiation in the presence and absence of differentiation medium. Alizarin red staining.

cytometry results confirmed previous reports that dental stem cells express mesenchymal markers and can be isolated with high purity (88–99 %). Cytotoxicity results confirm that allantoin has no cytotoxic effect on DPSCs, SHEDSCs and PDLSCs and causes a high proliferative effect. Calcium accumulation results evaluated with Alizarin Red S indicate that high doses of allantoin can reduce osteogenic differentiation.

The findings revealed important insights about the suitability of allantoin as a bioactive agent for regenerative dental applications. One of the critical outcomes of this study was the successful isolation and characterization of dental stem cells from three different tissues using the explant culture method. The cells displayed typical fibroblastic spindle-like morphology and were characterized by the presence of mesenchymal markers CD73, CD44, and CD90 and the absence of hematopoietic markers CD45, CD34, and CD11b. These findings are consistent with previous studies, which have shown that dental stem cells, like other MSCs, express a distinct profile of surface markers that confirm their mesenchymal origin [27,28]. The expression of these markers not only ensures the purity of the isolated stem cells but also highlights their potential for therapeutic applications.

The non-cytotoxic nature of allantoin is particularly relevant in the context of stem cell research. As regenerative therapies advance, the biocompatibility of compounds used to enhance tissue regeneration is critical. In this study, allantoin exhibited no cytotoxic effects at concentrations ranging from 0.25 to 5 mg/mL, reinforcing its safety profile as observed in earlier research. Studies involving fibroblasts and MSCs have consistently demonstrated that allantoin, particularly when derived from *Symphytum officinale*, promotes tissue regeneration without inducing cytotoxicity [28,29]. Since there is no previously reported cytotoxic concentration of allantoin on stem cells, a wide concentration range was chosen for cytotoxicity in this study. Results from the *in vitro* cytotoxicity test by parallel analysis of DPSCs, SHEDSCs, and PDLSCs indicate that allantoin does not cause a cytotoxic effect on dental stem cells even at relatively high concentrations. This is an important consideration, as many compounds used in regenerative medicine can exhibit toxic effects at certain concentrations, which limits their clinical applicability. Allantoin's lack of toxicity makes it an attractive candidate for further exploration in stem cell-based therapies.

The proliferative effect of allantoin on dental stem cells is another significant finding. Cell proliferation is a crucial factor in tissue engineering, as the success of regenerative therapies often depends on the ability of stem cells to proliferate and form new tissue. In this study, allantoin stimulated cell proliferation in all three types of dental stem cells, with SHEDSCs showing the most significant increase. This result is in line with previous studies that have demonstrated allantoin's role in promoting cell proliferation in various cell types, including fibroblasts and MSCs [29,30]. The strong proliferative response observed in SHEDSCs may be attributed to their intrinsic ability to proliferate more rapidly than other stem cell types, a property that has been well-documented in the literature [27]. The ability of SHEDSCs to rapidly expand in culture suggests that allantoin could be particularly useful in applications requiring large numbers of cells, such as in tissue regeneration for large defects or complex structures.

The scratch assay is commonly used to evaluate the migratory capacity of cells, which is an essential aspect of wound healing. Although allantoin has been reported to promote wound closure in other contexts, such as in fibroblast cultures [28,29], no significant improvement was observed for SHEDSCs and PDLSCs in this study. However; allantoin significantly improved wound healing in DPSCs, resulting in approximately 10 % greater scratch closure than in the control group. One reason for this effect in DPSCs may be related to the high expression of CD44, which is known to affect cell-cell, cell-matrix adhesion, and migration, as determined by flow cytometry analysis, and is expressed more than in other cell types [30]. It is possible that dental stem cells require different stimuli for migration compared to fibroblasts or that the allantoin concentration used in this study was not optimal to promote cell migration in SHEDSCs and PDLSCs. Previous studies have

shown that the effects of bioactive compounds on stem cell behavior can be highly context-dependent [28,29], and further research is needed to elucidate the conditions under which allantoin can promote wound healing in dental stem cells.

One of the most important aspects of this study was the evaluation of allantoin's effects on osteogenic differentiation, which is critical for bone regeneration. Osteogenic differentiation is a complex process that requires the activation of specific signaling pathways and the expression of key osteogenic markers, such as Runx2, osteocalcin, and alkaline phosphatase. In this study, allantoin did not significantly promote osteogenic differentiation in any of the dental stem cell types. At lower concentrations (0.5 mg/mL), allantoin had no observable effect on mineralization, while at higher concentrations (1 mg/mL), it appeared to inhibit osteogenic differentiation, as indicated by reduced mineralization in DPSCs and PDLSCs. These findings contrast with studies on other natural compounds, such as flavonoids from Drynaria fortunei, which have been shown to enhance osteogenic differentiation by upregulating osteogenic markers [27,31]. The concentration-dependent inhibition of osteogenesis observed in this study suggests that while allantoin may have beneficial effects at lower concentrations, higher concentrations may be detrimental to the differentiation process. Interestingly, the inhibition of osteogenic differentiation was more pronounced in DPSCs and PDLSCs compared to SHEDSCs, which may be related to the inherent differences in the osteogenic potential of these cell types. SHEDSCs have been shown to have a higher capacity for osteogenic differentiation compared to other dental stem cells, likely due to their origin from deciduous teeth, which are more actively involved in tissue remodeling [28,32]. However, even in SHEDSCs, the effect of allantoin on osteogenesis was minimal, indicating that allantoin may not be the ideal bioactive compound for promoting bone regeneration in dental stem cells. This finding underscores the importance of identifying compounds that not only promote cell proliferation but also enhance the differentiation of stem cells into specific lineages required for tissue regeneration.

The role of natural compounds in stem cell differentiation has been widely studied, and there is growing interest in identifying plant-derived bioactive molecules that can enhance the regenerative potential of stem cells. For example, flavonoids, terpenes, and alkaloids have been shown to modulate signaling pathways involved in stem cell differentiation [29,31]. However, the effects of these compounds are often highly specific to the cell type and the concentration used. In the case of allantoin, while it shows promise as a proliferative agent, its inhibitory effect on osteogenic differentiation suggests that it may need to be combined with other bioactive molecules or growth factors to achieve the desired therapeutic outcomes. Future research should focus on investigating the molecular mechanisms underlying allantoin's effects on stem cell differentiation, particularly in the context of bone regeneration. The concentration-dependent effects observed in this study also highlight the importance of optimizing the use of bioactive compounds in regenerative therapies. Many natural compounds, including allantoin, exhibit a dual effect depending on the concentration. At low concentrations, these compounds may promote beneficial processes, such as proliferation or differentiation, while at higher concentrations, they may inhibit these same processes or even induce cytotoxicity [29]. Understanding the concentration-dependent behavior of bioactive compounds is critical for developing effective therapeutic strategies that maximize the benefits while minimizing potential adverse effects.

One of the key limitations of this study is the focus on in vitro conditions, which may not fully replicate the complex environment *in vivo*, where multiple factors influence stem cell behavior, including interaction with surrounding tissues and systemic signals. Additionally, while allantoin demonstrated positive effects on cell proliferation, the lack of significant impact on wound healing on SHEDSCs and PDLSCs and its inhibitory effect on osteogenic differentiation at higher concentrations suggests that its therapeutic application requires further optimization. Another limitation is the narrow concentration range of allantoin tested, which may not have captured its full range of effects. Future studies could explore a broader concentration spectrum and include *in vivo* models to better understand the clinical potential of allantoin in regenerative dentistry. Finally, the study only assessed short-term effects, so the long-term impact of allantoin on stem cell behavior and differentiation remains to be explored.

This study demonstrated that allantoin, a naturally occurring compound, exhibits promising potential as a proliferative agent for dental stem cells, particularly for SHEDSCs, without inducing cytotoxicity. This is a significant finding for regenerative dentistry, as cell proliferation is crucial for tissue regeneration applications. The successful isolation and expansion of DPSCs, SHEDSCs, and PDLSCs further validate their mesenchymal nature and potential use in therapeutic applications. However, the study also highlights the complexity of using bioactive compounds like allantoin, as it did not enhance wound healing for isolated all cell types and showed inhibitory effects on osteogenic differentiation at higher concentrations. These findings suggest that while allantoin has potential as part of regenerative therapies, particularly for enhancing cell proliferation, its application for bone regeneration may be limited unless optimized or combined with other bioactive molecules or growth factors. The results emphasize the importance of further exploring the molecular mechanisms underlying allantoin's effects on dental stem cells, especially in relation to osteogenic pathways. Future research should focus on optimizing the concentrations and exploring potential synergistic combinations with other osteoinductive agents to fully harness the regenerative potential of dental stem cells. Overall, this study contributes valuable insights into the use of natural bioactive compounds in regenerative medicine and underscores the need for continued research to refine therapeutic approaches for clinical applications in tissue engineering.

5. Conclusion

This study demonstrated that allantoin is a non-cytotoxic, bioactive compound that effectively promotes the proliferation of dental stem cells, particularly SHEDSCs, making it a promising candidate for regenerative therapies. However, its inhibitory effect on osteogenic differentiation at higher concentrations suggests limitations in its use for bone regeneration. These findings highlight the need for further optimization of allantoin's application, particularly in combination with other osteoinductive agents, to fully harness its potential in dental regenerative medicine. Further studies are required to better understand the mechanisms underlying these effects and to explore the optimal conditions for its use in clinical applications.

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CRediT authorship contribution statement

Betül Mutlu Özçınar: Writing – original draft, Resources, Methodology, Investigation, Data curation, Conceptualization. **Can Özükoç:** Writing – original draft, Resources, Methodology, Conceptualization. **Emrah Türkmen:** Writing – original draft, Resources, Methodology, Conceptualization. **Rabia Çakır:** Writing – original draft, Resources, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors have declared no conflict of interest

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