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The Role of Graphene and Biodentine™ on Proliferation and Odontoblastic Differentiation of Dental Pulp Stem Cells

Grafen ve Biodentine™'in Dental Pulpa Kök Hücrelerinin Proliferasyonu ve Odontoblastik Farklılaşması Üzerindeki Rolü

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ABSTRACT

Objective: The need for new biomaterials with biocompatibility, mechanical, and antimicrobial properties continues in regenerative endodontic clinical applications in dentistry. Biodentine™ is successfully used in vital pulp therapies and has regenerative effects. Additionally, graphene, which possesses good physicochemical, mechanical, and biological properties, promotes cellular biomineralization and osteogenic differentiation in dentistry, along with its neuroregenerative effect. This study aims to investigate the effects of graphene and Biodentine™ on cell proliferation, oxidative stress, and odontogenic differentiation in human dental pulp stem cells (hDPSCs).

Methods: Cryopreserved hDPSCs purchased from American Type Culture Collection (ATCC) were used in our study (Cat No: PT-5025). hDPSCs were seeded into the E-plate wells and subsequently four different doses of graphene (12.5, 25, 50 and 100 µg/ml) and Biodentine™ (2, 4, 8 and 16 µg/ml) were added. Results of MTT, total antioxidant capacity (TAC), total oxidant status (TOS) and alkaline phosphatase (ALP) tests were obtained at the end of the 24th hr. 96 hr-real time cell index data were collected with xCELLigence® system. Resulting data were compared using *one-way* analysis of variance (*ANOVA*).

Results: 12.5 µg/ml graphene and 2 µg/ml Biodentine™ were found to be the subgroups with the highest levels of cell proliferation and the lowest oxidative stress. Antioxidative effect was determined in all Biodentine™ doses but only in 12.5 µg/ml graphene. Odontogenic differentiation was observed in all doses of graphene and Biodentine™.

Conclusion: 12.5 µg/ml graphene and 2 µg/ml Biodentine™ were observed to have positive impacts on the proliferation, oxidative stress and odontogenic differentiations of hDPSCs.

Keywords: Cell proliferation, Graphene, Oxidative stress, Odontogenic differentiation, Tricalcium silicate

ÖZ

Amaç: Diş hekimliğinin rejeneratif endodontik klinik uygulamalarında biyouyumluluk, mekanik ve antimikrobiyal özelliklerine sahip yeni biyomateryal ihtiyacı devam etmektedir. Biodentine™ vital pulpa tedavilerinde başarıyla kullanılmakta ve rejeneratif etkisi bulunmaktadır. Ayrıca, iyi fizikokimyasal, mekanik, biyolojik özelliklere sahip grafenin diş hekimliğinde hücresel biyomineralizasyonu ve osteojenik farklılaşmayı sağlayan, nörorejeneratif etkisi bulunmaktadır. Bu çalışmada grafen ve Biodentine™ 'in insan dental pulpa kök hücreleri (hDPSC) üzerindeki hücre proliferasyonu, oksidatif stres ve odontojenik farklılaşma etkilerinin incelenmesi amaçlanmıştır.

Metot: Çalışmamızda Amerikan Tipi Kültür Koleksiyonu (ATCC) insan DPSC'si (Cat No: PT-5025) kullanılmıştır. DPSC'ler E-platelere ekildikten sonra dörder farklı dozda grafen (12.5, 25, 50 ve 100 µg/ml) ve Biodentine™ (2, 4, 8 ve 16 µg/ml) eklenmiştir. Deney gruplarının 24 saat sonunda MTT, total antioksidan seviye (TAS), total oksidan seviye (TOS) ve alkalen fosfataz (ALP) analiz sonuçları elde edilmiştir. Ayrıca 96 saatlik gerçek zamanlı hücre indeks verileri xCELLigence® cihazı kullanılarak elde edilmiştir. Verilerin karşılaştırılmasında tek yönlü varyans analizi kullanılmıştır.

Bulgular: Çalışmada proliferasyonu en yüksek ve oksidatif stres düzeyi en düşük gruplar grafen 12.5 µg/ml ve Biodentine™ 2 µg/ml olarak belirlenmiştir. Antioksidan etki grafenin sadece 12.5 µg/ml grubunda Biodentine™' in ise bütün dozlarında tespit edilmiştir. Grafen ve Biodentine™' in bütün dozları için odontojenik farklılaşma gözlenmiştir.

Sonuç: İnsan DPSC'si üzerinde grafen 12.5 µg/ml ve Biodentine ™ 2 µg/ml gruplarının hücre proliferasyonu, oksidatif stres ve odontojenik farklılaşma bakımından olumlu etkileri bulunmuştur.

Anahtar kelimeler: Hücre proliferasyonu, Grafen, Odontojenik farklılaşma, Oksidatif stres, Trikalsiyum silikat

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INTRODUCTION

In dentistry, vital pulp therapy (VPT) performed to maintain the pulp vitality is an important treatment approach preserving the teeth for longer periods of time.[1] VPTs aim to preserve the vitality of dentin-pulp-complex, which contains a pulpal tissue of mesenchymal origin and involves the specialized cells known as odontoblasts. The materials used in VPTs need to be non-cytotoxic and bioactive to preserve the vitality of the dentin-pulp complex and stimulate odontogenic differentiations. Although several new materials with different ingredients have been recently introduced into dental clinical applications, there is still an ongoing need for novel biomaterials with superior biocompatibility, mechanical and antimicrobial properties.[2,3]

Vast majority of the materials used in dentistry induce oxidative stress by producing free radicals. Excessive generation of reactive oxygen species greater than the antioxidant capacity of the cells for many reasons, cause damage to cellular macromolecules, such as lipids, proteins and DNA and lead to cell injury.[4] Impact of overwhelming production of cellular oxidative stress on both recovery time and cell viability necessitates the assessment of different dental materials' effects on cells.[5]

In addition to cell viability and proliferation, odontogenic cell differentiation is essential for pulp vitality. And alkaline phosphatase (ALP), secreted by the osteoblasts during bone formation is a marker of odontogenic differentiation regarding the hard tissue mineralization process.[6]

Biodentine™, used in the VPTs stimulating odontogenic differentiation is a tricalcium silicate (Ca_3SiO_5) based commercially available inorganic restorative cement also known as "bioactive dentin". Biodentine™ was reported to have better physical and biological properties than other tricalcium silicate cements, such as mineral trioxide aggregate (MTA) and BioAgregate™.[7,8] Although not widely used in clinical applications, MTA has been reported to have some drawbacks, including discoloration, prolonged setting time and worse dentinal tubule penetration. When compared to MTA, Biodentine™ has superior physical and biological properties concerning easier handling, shorter setting time, higher compressive strength and faster dentin bridge formation. The mechanism of these superior features can be explained by Biodentine's™ different particle size and induction of odontoblastic differentiation following to its application and thus initiation of a mineralization appeared similar to osteodentin. The therapeutic mechanism of Biodentine™ is that after application of this material, mineralization induces and occurs in the form of osteodentin, which makes dentin.[9,10]

Among the mostly researched nanocarbon materials in the recent years, graphene, is remarkable for being the thinnest, strongest, and the hardest material tested so far due to the flexible covalent bonds between its carbon atoms.[11,12] It is used for improving the physical, mechanical and biological properties of the biomaterials. Graphene has several types of applications in biomedicine, such as biosensors, and nano-carrier systems for gene and drug delivery, cell imaging, and phototherapy equipment due to not only its ease of handling but also its antimicrobial, antiviral, antitumoral effects.[13]

There is an increasing number of researches assessing the biological properties of graphene, which gradually find applications in the field of biomedicine, including dentistry. The aim of the present study is to evaluate the impacts of graphene and Biodentine™ on human dental pulp stem cells (hDPSCs) regarding cell proliferation, oxidative stress and odontogenic differentiation. In this study, graphene was compared with Biodentine™, which is a bioceramic based material, as it is a successful material in endodontic treatments.[14] Our research hypothesizes that Graphene and Biodentine™ do not have an effect on cell proliferation, oxidative stress, and odontogenic differentiation in hDPSCs.

METHOD

Ethical approval

Ethics committee approval was obtained from the Ataturk University, Faculty of Medicine Clinical Research Ethics Committee (dated as 30 September 2021 and Approval #64).

Cell Culturing

hDPSCs used in the present study (Pioteticsᵀᴹ #PT-5025, Lonza Bioscience, MD, USA) was procured from American Type Culture Collection (ATCC). Cryopreserved hDPSCs in the ampules were immediately thawed, centrifuged at 1200 rpm for 5 min. (Beckman Coulter, Allegra X-30-R Centrifuge) and the supernatant discarded. Pelleted cells were resuspended in a fresh medium containing 10% FBS (Fetal Bovine Solution, Gibco, USA), 1% antibiotic (Penicillin, Streptomycin, Amphotericin B (Thermo Fisher, Germany), 89% DPSC medium (DPSC Basal Medium; catalog #PT-3927 Lonza, USA), 1% L-glutamine and then transferred to a 25cm^2 flask. hDPSCs were subcultured in a humidified incubator (Esco CelCulture® CO_2 Incubator, England) at 37°C and under a 5% CO_2 atmosphere. It was determined with an inverted microscope (Inverted Fluorescent Microscope, Leica, Germany) whether the wells completely filled the bottom of the prepared well plate. The sample preparation phase began when the cells reached a density of 80% (Figure 1).

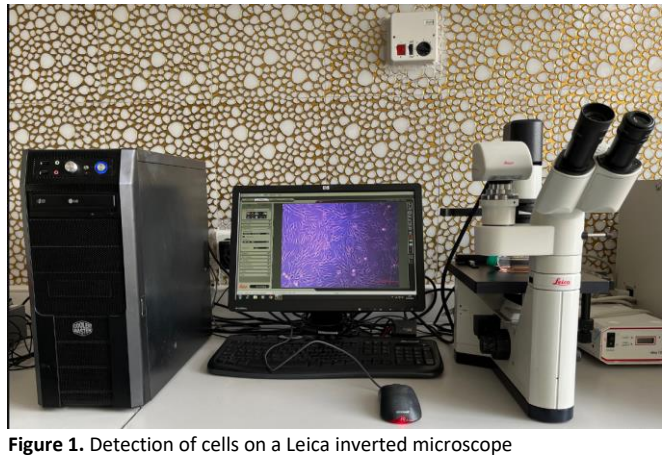

Figure 1. Detection of cells on a Leica inverted microscope

Study Groups

Solid form graphene and Biodentine™ (Septodont, Saint-Maurdes-Fosses, France) were tested in our study. The present study consisted of a negative control group consisting of only hDPSCs, a positive control group composed of hDPSCs+dimethyl sulfoxide (DMSO- C2H6OS) and two study groups, in which Biodentine™ was administrated in the doses of 2, 4, 8, and 16 µg/ml and graphene in the doses of 12.5, 25, 50, and 100 µg/ml. The sample size in each group (n=10) was determined using power analysis (effect size f = 1.4, $1 - \beta = 0.80$, $\alpha = 0.05$). Tests were repeated 10 times for each dose and different doses of both agents were analyzed with MTT (thiazolyl blue tetrazolium bromide), xCELLigence®, total antioxidant capacity (TAC), total oxidant status (TOS) and alkaline phosphatase (ALP).

MTT Assay

hDSPCs were transferred to 48 well flat bottom- plates at a density of apx. 0.25 x 10^4 cell/cm^2 and subcultured, then incubated until reaching a cell density of 80%. Positive control group consisted of DMSO-treated cells whereas negative control group composed of untreated cells. Impacts of graphene and Biodentine™ doses on the viability of hDPSCs were assessed by MTT test. At the end of a 24 hr incubation period, cell media were dispensed into Eppendorf Tubes® (Eppendorf Limited, UK) for bioanalysis. In order to volume-up the wells to 100 µL, a 10 µL of MTT solution (5mg/ml) was added to each plate well and incubated at 37 °C. 100 µL DMSO was added to dissolve formazan crystals after removing the medium in the wells and absorbance was measured at wavelength of 570 nm using a microplate (ELISA) reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Finland).[15]

Biochemical Analyses

TAC and TOS levels were measured by using Rel Assay Diagnostics® TAC and TOS assay kits (Rel Assay Diagnostics, Gaziantep, Türkiye) in the cell medium for 24 hr. ALP levels were assessed with ready-to-use ELISA test kits (Sunlog Biotech Co, China).[16,17]

xCELLigence® Analysis

50 µl of agar was added to E-plate wells, in which 10^4 hDPSCs were seeded and incubated at room temperature for 30 min. Then the E-plate was inserted in the cradle of real time analyzer (xCELLigence RTCA, ACEA Biosciences Inc.-Agilent, California, USA) and real time logarithmic proliferation of the cells was monitored at the scheduled temporal resolutions. Media in the wells were removed following to a 24 hr of incubation time and 100 µl agar solution with the different doses of the agents was added to each well, only medium and medium+DMSO were added into the control wells. Tests had been continued for 72 hr after the addition of the agents and meanwhile, cell index (CI) values were measured. In order to monitor the short-term cellular responses to the agents tested, CI values were measured in every 2 min during the post-addendum first hour then every 30 min for following-up the long-term responses.[18]

Statistical Analysis

GraphPad Prism 8.0a for Mac OS X (GraphPad Software, Inc., La Jolia California, USA) was use for assessing the study data at a 0.05 level of significance. One-way analysis of variance (One Way ANOVA) was used to evaluate the results of MTT, TAC, TOS, LDH and ALP assays. Fisher Least Significant Difference (LSD) test was used for determining the differences between groups' means.

RESULTS

MTT Assay

Cytotoxic effects of graphene and Biodentine™, administrated in different doses on hDPSCs were determined with MTT assay. Dose-depended changes were observed in cell viability whereas a statistically significant difference was found between negative control and DMSO groups (*P*<.05) (Figure 2).

The highest cell viability was seen in 12.5 µg/ml graphene and 2 µg/ml Biodentine™ subgroups. No significant difference was observed between the cell proliferation rates in these subgroups. Higher cell viability was observed in 25 µg/ml graphene, and 4 and 8 µg/ml Biodentine™ groups compared to DMSO group (*p*=0.59).

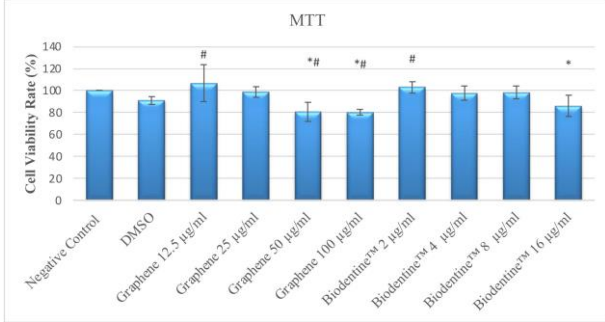

Figure 2. 24 hr-MTT assay results of control, graphene and Biodentine™ groups
** P<.05 significant difference in comparison with the control group # P<.05 significant difference in comparison with DMSO group*

xCELLigence® Analysis

For determining the influence of different Biodentine™ doses on of hDPSC proliferation, each dose was monitored in 3 wells in xCELLigence® system (RTCA) for 72 hr. Following to the 2 µg/ml, 4 µg/ml and 8 µg/ml Biodentine™ administration at the 24th hour, hDPSC viability was observed to increase at the end of the 72nd hour whereas cell viability significantly decreased in the 16 µg/ml Biodentine™ subgroup (Figure 3).

After dosages of 12.5 µg/ml and 25 µg/ml graphene were added into the media, an increase was observed in hDPSC viability. The highest CI value was detected in the 12.5 µg/ml graphene subgroup (Figure 4).

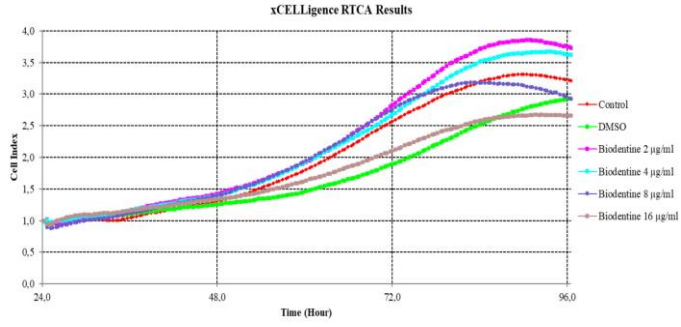

Figure 3. Distribution of Biodentine™ subgroups' hourly cell index values compared to DMSO group

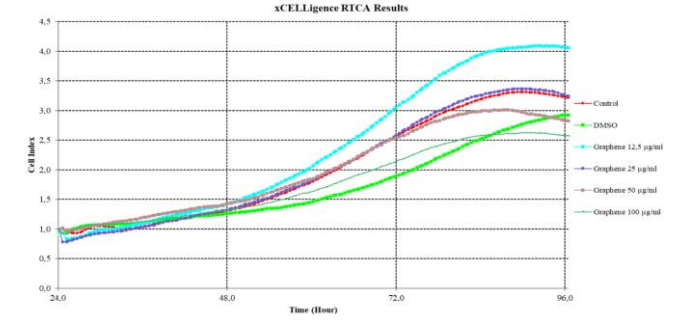

Figure 4. Distribution of graphene subgroups' hourly cell index values compared to DMSO group

TAC and TOS Assays

TAC and TOS assay kits were used to measure hDPSC oxidant and antioxidant levels 24 hr after administrating different doses of graphene and Biodentine™. Among all graphene subgroups, higher levels of antioxidant than DMSO (positive control) group seen only in 12.5 µg/ml dosage and presence of greater antioxidant capacity in all doses of Biodentine™ than both control groups were found to be statistically significant (*P*<.05) (Figure 5).

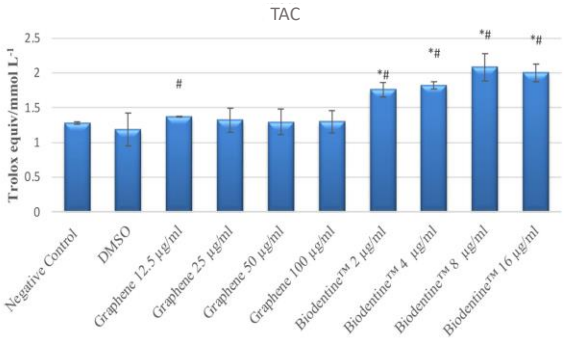

Figure 5. 24 hr-TAC assay results of control, graphene and Biodentine™ groups
** P<.05 significant difference in comparison with the control group # P<.05 significant difference in comparison with DMSO group*

12.5 µg/ml graphene and 2 µg/ml Biodentine™ subgroups had lower oxidant levels than DMSO group (*P*<.05) (Figure 6). Moreover, no statistically significant difference was observed between the median oxidant levels of these groups (*P*=0.34). 100 µg/ml graphene and 16 µg/ml Biodentine™ groups were observed to have greater oxidant levels than DMSO group (*P<.05*).

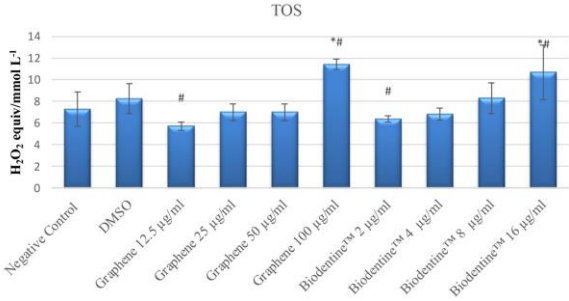

Figure 6. 24 hr-TOS assay results of control, graphene and Biodentine™ groups
** P<.05 significant difference in comparison with the control group # P<.05 significant difference in comparison with DMSO group*

ALP Assay

Effects of different Biodentine™ and graphene doses on the odontogenic differentiation of hDPSCs were detected using ALP activity assay. Significantly higher levels of ALP enzymes were seen in all study groups than DMSO group (*P*<.05) (Figure 7). ALP levels were observed to decrease in the doses more than 4 µg/ml Biodentine™ and 12.5 µg/ml graphene. The groups of Biodentine™ 4 µg/ml and graphene 12.5 µg/ml exhibit the highest ALP enzyme activities, and there is no statistically significant difference between the means of these groups (*P*=0.16).

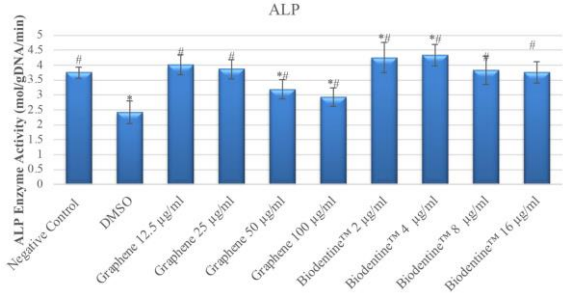

Figure 7. ALP enzymatic activity values of study and control groups
** P<.05 significant difference in comparison with the control group # P<.05 significant difference in comparison with DMSO group*

DISCUSSION

This study explored the proliferative, oxidative and odontogenic effects of graphene on hDSPCs. Biodentine™, a bioceramic-based material was used in the control group because it was accepted as the golden standard in post-endodontic restorations. The doses of 12.5 µg/ml for graphene and 2 µg/ml for Biodentine™ exhibited the lowest cytotoxicity based on the MTT and xCELLigence® tests, lower oxidative stress according to the TAC and TOS tests, and the highest odontogenic differentiation based on the ALP test. Therefore, the hypothesis stating that graphene and Biodentine™ have no effect on cell proliferation, oxidative stress, and odontogenic differentiation in human DPSCs is rejected.

Many studies have been conducted for investigating the cytotoxic and proliferative effects of Biodentine™ on DPSCs when used in VPTs.[19-21] Biodentine™ was reported to be bio-compatible since it was not cytotoxic and genotoxic for hDPSCs.[22,23] However, some researches performed in different cell lines remarked that Biodentine™ doses up to 2 µg/ml was bio-safe and its elevated doses led to a decrease in cell proliferation.[19,24] In this study, cell viability level in 2 µg/ml Biodentine™ administrated subgroup was found to be higher than the control groups. A lower viability was observed in 4 µg/ml Biodentine™ subgroup than the negative control group while a greater cell viability was determined when compared to DMSO group. Levels of cell viability in the subgroups treated with 8 and 16 µg/ml Biodentine™ were lower than the control groups. The cell viability of the subgroup treated with 25 µg/ml graphene was found to be higher compared to DMSO group and equivalent to negative control group. There were lower levels of cell viability in 50 and 100 µg/ml graphene-administrated subgroups than the control groups. The greatest cell viability was determined in 12.5 µg/ml graphene subgroup. Both findings of MTT assay matching with the literature and outcomes of xCELLigence® analysis confirmed our results.[19,24] In the 12.5 µg/ml graphene-administrated subgroup, cell viability was observed to be statistically different from all other groups during the 96 hr-RTCA.

Oxidative stress is regarded as an underlying factor for toxicity and ranked among the determinants in cytotoxicity tests. Subsequent to a change in the oxidant/antioxidant balance in favor of antioxidant system, TAC values measuring the oxidative stress increase whereas there is a decrease in the TOS values. However, some agents increase TAC levels although they do not have antioxidant capability. Similarly, Aksu et al.[25] also reported that at the 72nd hr post-administration, Biodentine™ was observed to demonstrate a strong defensive action on hDPSCs as a result of elevated TAC levels with a decline in TOS levels.

In our study, we also found that different doses of Biodentine™ had increased the TAC levels inversely proportional to TOS. Although 12.5 µg/ml graphene subgroup had higher levels of TAC than DMSO group, other graphene doses exhibited no antioxidant effect. Being independent of antioxidant effect, lower TOS levels in the graphene subgroups than the control groups were deemed compatible with the elevated cell viability. In their study assessing the biocompatibility of resin-based dental composites+graphene in in-vivo mandibular bone defect, Dreaneca et al.[26] remarked that following graphene administration TOS levels in the control and study groups were found to be lower than the sham-surgery group with no change in TAC levels. A study assessing the cytotoxicity of graphene-based nanomaterials on human dental follicle stem cells by Olteanu et al.[27] revealed that graphene oxide caused a decrease in superoxide dismutase (SOD) activity, used for measuring the antioxidant balance.

Several in-vitro and in-vivo studies revealed that Biodentine™ had a positive impact on pulp healing when the odontoblastic layer was partially injured.[8,28] In addition to other teccniques, ALP test is also used for assesing odontogenic and osteogenic differentiations.[29,30] ALP is relatively an early diferantiation marker that increases during the proliferation and matrix syntheses stage.[6] ALP activity was also evaluated in the present study since ALP was accepted as the primary marker of osteogenic/odontogenic differentiation.[29,30] 24hr-results of our study also showed higher levels of ALP in all Biodentine™ and graphene doses than DMSO group. Therefore, induction of ALP expression clearly demonstrate the significant role of graphene and Biodentine™ in accelerating osteogenesis/odontogenesis of hDPSC.

Numerous researches indicated that ALP inducted odontogenetic differentiation in DPSCs.[31,32] Jang et al.[33] determined that graphene oxide coated zirconia applications promoted the osteoblastic proliferation and differentiation. And Xie et al.[34] observed that graphene provoked osteogenic rather than odontogenic differentiation. Different from these researchers, we determined that like Biodentine™, all doses of graphene had also stimulated ALP enzymatic activity, which was used as a marker of hDPSC odontogenic differentiation.

However, our study reached consistent results with the other researches in the literature, inability to examine the effects of Biodentine™ +graphene combinations on hDPSCs constituted its limitation.

CONCLUSION

In the present study, at the end of the 24th hr, the strongest proliferative effect was observed in the subgroups of 12.5 µg/ml graphene and 2 µg/ml Biodentine™ whereas 100 µg/ml graphene and 16 µg/ml Biodentine™ exhibited the weakest cell viability. All administrated doses of graphene and Biodentine™ induced odontogenic differentiation. Graphene, which is biocompatible with hDPSCs may be added to endodontic agents or cements for improving their biological and mechanical properties. Nevertheless, comprehensive long-term studies are needed to achieve these goals.

Etik Komite Onayı: Atatürk Üniversitesi Tıp Fakültesi Klinik Araştırmalar Etik Kurulu'nun 30.09.2021 tarihli 6. toplantısında 64 karar numarası ile etik kurul onayı alındı.
Hakem Değerlendirmesi: Dış bağımsız.
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