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ORIGINAL ARTICLE

Does the application of GaAlAs laser and platelet-rich plasma induce cell proliferation and increase alkaline phosphatase activity in human dental pulp stem cells?

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Abstract

Blood extracts containing platelet products are gaining popularity in promoting healing and pulp regeneration. This study was designed to evaluate the effect of platelet-rich plasma (PRP) and gallium–aluminum–arsenide (GaAlAs) laser on proliferation and differentiation of human dental pulp stem cells (hDPSCs). In this ex vivo study, hDPSCs isolated from impacted mandibular third molars were cultured in *Dulbecco's Modified Eagle's medium*)DMEM(with 10% fetal bovine serum (FBS). After reaching the desired confluence, the cells were distributed into 4 groups, namely, control, PRP, laser, and PRP+laser for MTT assay and alkaline phosphatase (ALP) test. In the PRP and PRP+laser groups, 10% PRP was added to each well on the plate. In the laser and PRP+laser groups, as for the proliferation test, laser irradiation was carried out for 45 s, while 135 s was designated for ALP test. After 1, 3, and 5 days, cell proliferation and ALP activity were assessed using MTT and ALP colorimetric assay, respectively. Two-way ANOVA was utilized to analyze data. In PRP and PRP+laser groups, cell proliferation and viability increased until day 3 but began to decline afterwards until the 5th day. In the laser group, the increase in proliferation and viability was observed till day 5 which was less than the control group. Laser and control groups exhibited significantly higher cell viability and proliferation than both PRP and PRP+laser groups. ALP activity was more pronounced in PRP+laser, PRP, and laser in descending order; however, all were less than that of the control group. Only in the control group did the ALP activity augment during the 5-day period. Laser irradiation could induce pulp cell proliferation and demonstrated a better performance than PRP in this regard.

Keywords Platelet-rich plasma · GaAlAs laser · Proliferation · Dental pulp

Introduction

Wound healing and regeneration are composed of a series of complex biologic cascades including cell proliferation and differentiation. These phenomena are governed by the so-called growth factors [1, 2]. At the site of injury or infection, activated platelets are capable of accelerating the healing process by release of a great deal of endogenous bioactive materials or growth factors such as transforming growth factor beta (TGFB), epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [3]. Therefore, the application of products containing these growth factors such as platelet-rich plasma (PRP) to sites in need of repair could expedite wound maturation [4].

PRP is derived from the centrifugation of autologous blood and is converted to a gel formulation by adding thrombin and calcium chloride [5]. It has been used in various medical fields

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including dentistry in order to boost the healing process [4]. However, there are still concerns regarding its application including the short-time nature of its content release as well as limited regenerative potential [6].

Recently, successful application of PRP has been demonstrated in revascularization of immature permanent teeth and regeneration of pulp–dentin complex [7]. PRP is rather easy to prepare, could be obtained from the patient's own blood, and could generate a 3D fibrin scaffold [8]. In an animal study, it has been shown that a PRP scaffold improves dental pulp stem cell (DPSC) migration and proliferation [9, 10]. Additionally, dose-dependent increase in alkaline phosphatase (ALP) expression and osteoblastic activity under the influence of PRP has been demonstrated [11]. PRP in certain concentrations induces calcification of rat dental pulp cells as a result of osteo/odontogenic differentiation of mesenchymal stem cells [12].

Low-level laser (LLL) has been shown to be effective on promoting wound healing [13]. Relieving effect of LLL on oral ulcers and dentin hypersensitivity has been proven in multiple studies [14–16]. Gallium–aluminum–arsenide (GaAlAs) laser as an instance is widely used in pain reduction, tissue healing, and hard tissue formation [17]. Moreover, it might play a role in periodontal attachment gain, $TGFB_1$ expression, angiogenesis, and dentinogenesis of pulp–dentin complex [18, 19]. Increased ALP activity and calcified nodule formation in human dental pulp cells have been demonstrated in response to laser irradiation [20].

In view of the various effects of PRP and LLL on human dental pulp stem cells (hDPSCs), their application might be beneficial in endodontic scenarios involving tissue regeneration or dentinogenesis. Therefore, this study aimed at investigating the impact of PRP with or without GaAlAs laser treatment on proliferation and differentiation of hDPSCs.

Materials and methods

This study obtained approval from the Medical Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran (approval number: 910176).

hDPSCs were purchased from Dr. Torabinejad Research Center, Isfahan University of Medical Sciences, Isfahan, Iran. These cells have been isolated from impacted mandibular third molars and have been kept refrigerated in the cell bank. Cytotoxicity tests were performed according to the ISO 10993-12:2012 (E).

Cell passage

Under sterile condition, culture medium was discarded and the cells were washed with pre-warmed phosphate-buffered saline (PBS). Thereafter, 10 mL 0.25% trypsin/0.05% ethylenediaminetetraacetic acid (EDTA) was added to the cells. After shaking the flask, the cells were incubated for 2 min at 37 °C. Following cell detachment, additional PBS was added to stop the reaction. The cell suspension was transferred into an appropriate tube and centrifuged (5 min, 900 rpm). After removing the supernatant, the cell pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM), split into new flasks according to the rate of cell expansion, and incubated. Upon reaching 90% confluence, the cells were deemed ready for MTT assay which is a colorimetric assay for assessment of the cell metabolic activity.

PRP preparation

In view of the excessive amount of PRP needed, we decided to provide it from Mashhad Blood Transfusion Organization, Mashhad, Iran. To eliminate antigen cross-reactivity, 60 cc of "AB^+" blood was prepared and freeze/defreeze six times to activate the platelets and help release of cytokines. A total of 10 μL of calcium gluconate was added to each 1 mL of PRP and mixed under a laminar flow hood. The suspension was then incubated for 2 h, after which a fibrin clot was formed. The clot was gently detached from the Eppendorf tube which was then centrifuged (15 min, 3000*g*). Afterwards, the supernatant was removed and kept refrigerated in falcon tubes. The part containing fibrin precipitate was discarded.

A 132-well plate was implemented for proliferation test and cell viability (96 wells) along with ALP assessment (36 wells). To perform proliferation and cell viability tests, 8 wells were allocated to each experimental group (control, PRP, laser, and PRP+laser) and each time frame (1, 3, and 5 days). For ALP test, 3 wells were allocated to each experimental group (as above) and each time frame (as above).

Laser parameters

The laser device used in this study was a commercial low-level GaAlAs diode laser (Thor DD2 Control Unit, Thor, London, UK), emitting a wavelength of 810 nm at a maximum power of 200 mW and in a continuous wave (CW) mode. The apparatus radiated a coherent elliptical beam with a Gaussian profile and a spectral width of 1.2 nm FWHM. For the MTT assay, a 96-well plate was used and the laser was held at an approximate distance of 1 cm from the cells and scanned each well for 45 s. To avoid overlapping radiation, every other well was left vacant. The amount of energy transferred to each well was 9 J, and the received dose was estimated to be 22.5 J/cm^2, considering the surface area of ~ 0.40 cm^2 for the well.

In the ALP test, a 24-well plate was employed and the laser scanned each well for 135 s at an approximate distance of 20 mm. The energy transferred to each well was 27 J, and the energy density was calculated to be approximately

10.5 J/cm^2 (surface area of each well ~ 2.55 cm^2). Laser treatment was performed only once in this study.

Quantitative analysis of cell proliferation by MTT assay

Cell suspension was prepared by adding 200-μL complete cell culture medium containing 20,000 cells to each well of 96-well plate. Culture medium contained 10% PRP and 10% fetal bovine serum (FBS) for PRP and PRP+laser groups but possessed 10% FBS and lacked 10% PRP for control and laser groups. Samples were incubated for 24, 72, and 120 h in a CO_2 incubator with 100% humidity. MTT with a final concentration of 0.5 mg/mL was added (equal to 0.1 volume of each well) and incubated for 4 h. Afterwards, dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Ultimately, ELISA plate reader was utilized to assess optical density (OD) of each sample at the wavelength of 550 nm. The percentage of cell viability was calculated with the following formula:

$$\text{cell viability} = \frac{\text{OD test}}{\text{OD control}} \times 100$$

ALP test colorimetric assay

To measure intracellular ALP, washed cells (5 × 105) were homogenized in assay buffer and then centrifuged for 3 min at 13,000*g* in order to remove insoluble material. Various volumes of samples were added into 96-well plate to bring the total volume of each well to 80 μL with assay buffer.

Colored samples may interfere with OD reading at 405 nm; therefore, a control sample was designated. In the control group, the same amount of sample was added to well plate to bring the volume to 80 μL. A total of 20 μL stop solution was incorporated to terminate ALP activity. A total of 50 μL of 5 mM p-NPP was added to each well in test and control samples. After thorough mixing, samples were kept at 25 °C for 60 min away from light. A total of 40 μL of 5 mM p-NPP was diluted with 160 μL of assay buffer to generate 1 mM of p-NPP standard. Afterwards, 0.4, 8, 12, 16, and 20 μL of solution were poured to generate 0.4, 8, 12, 16, and 20 nmol/well p-NPP standard. The final volume was set at 120 μL with the addition of assay buffer. Thereafter, 10 μL of ALP enzyme was added to each well of 24-well plate and mixed well. The suspension was kept at 25 °C for 60 min away from light. A total of 20 μL of stop solution was added to test samples and shook gently. Ultimately, OD reading was carried out by a micro-plate reader at 405 nm.

Data analysis

Statistical data were analyzed using the Statistical Program for Social Sciences (SPSS) 19.0 software (SPSS, Chicago, IL, USA). To examine whether the distribution is normal, the Kolmogorov–Smirnov normality test was used. Standard deviation and mean were implemented to describe data. Two-way ANOVA was utilized to analyze data. The level of significance was set at 0.05.

Results

Based on the Kolmogorov–Smirnov test, the distribution of data was found to be normal. All three variables (proliferation, cell viability, and activity) were found to be meaningful in relation to time, group, and time/group; in other words, these 3 variables were statistically different when comparing between the 4 groups as well as the 3 intervals (Table 1). The combined effect of group and time is also meaningful on proliferation, cell viability, and activity.

In the PRP group, proliferation was initially increased but then decreased; these variations were statistically significant. Tukey's test revealed that the increase between the 1st and 3rd day (as well as the 1st & 5th) was statistically significant. The percentage of cell viability and ALP activity had a significant decline as the time passed; Tukey's test demonstrated statistically significant decrease between the 1st and 3rd, the 1st and 5th, and the 3rd and 5th day.

In the PRP+laser group, proliferation was initially increased but then decreased; these variations were statistically

Table 1 The effects of group, time, and group/time based on ANOVA test results

Source of variation	Variable	Variance	*F* value	*P* value
Group	Proliferation	0.082	38.848	<0.01
	Cell viability	671.846	54.644	<0.01
	Activity	703.571	1596.997	<0.01
Time	Proliferation	0.546	268.673	<0.01
	Cell viability	199.984	16.265	<0.01
	Activity	45.111	102.394	<0.01
Group*time	Proliferation	0.013	6.315	<0.01
	Cell viability	80.457	6.544	<0.01
	Activity	70.330	159.638	<0.01
Error	Proliferation	0.002		
	Cell viability	12.295		
	Activity			
Total	Proliferation			
	Cell viability			
	Activity			

significant. The Tamhane T_2 post hoc test revealed that the increase between the 1st and 3rd day—as well as the 1st and 5th—was statistically significant. The percentage of cell viability and ALP activity had a significant decline as time passed; the Tamhane T_2 post hoc test demonstrated statistically significant decrease in cell viability percentage between the 1st and the 5th day. Also, Tukey's test showed that there was a significant pairwise decrease in ALP activity between various time frames.

In the laser group, proliferation increased as the time passed, and it was statistically significant. A statistically meaningful increase was observed between the 1st and 3rd and the 1st and 5th day, while the increase between the 3rd and the 5th was not significant. Cell viability percentage increased with time albeit not statistically significant. ALP activity perpetually decreased with time; Tukey's test manifested a significant pairwise reduction between various time frames.

The mean proliferation value was statistically different between the four groups in days 1, 2, and 3 (Fig. 1). The Tamhane T_2 post hoc test showed that the value was statistically higher in control and laser groups as compared to PRP and PRP+laser. Cell viability percentage differed statistically between the four groups (Fig. 2); the Tamhane T_2 post hoc test revealed that it was statistically higher in control and laser groups compared with PRP and PRP+laser. The average ALP activity value was statistically different between the four groups (Fig. 3); Tukey's test revealed statistically significant pairwise difference between all four groups except for PRP and laser.

Discussion

Taking biologic aspects into account, the application of PRP seems to be well-justified [21]; however, the most appropriate methodology to yield predictable results is yet to be determined. Based on the current study, proliferation and cell viability in the PRP group were lower than those of the control group at all times. Between days 1 and 3, proliferation boosted significantly with time albeit less than that of the control group, whereas it decreased from day 3 to day 5. The percentage of cell viability had a significant decline between days 1, 3, and 5. ALP activity in the PRP group was significantly lower than that of the control group at all times and decreased constantly from day 1 to day 3 and then to day 5. Contrary to our findings, one study showed that maximum proliferation was observed at day 7 in the PRP group as compared to the addition of $rhTGFB_1$ and rhPDGF-AB [22]. ALP expression was also higher in the PRP group in the 2nd and 3rd days. This

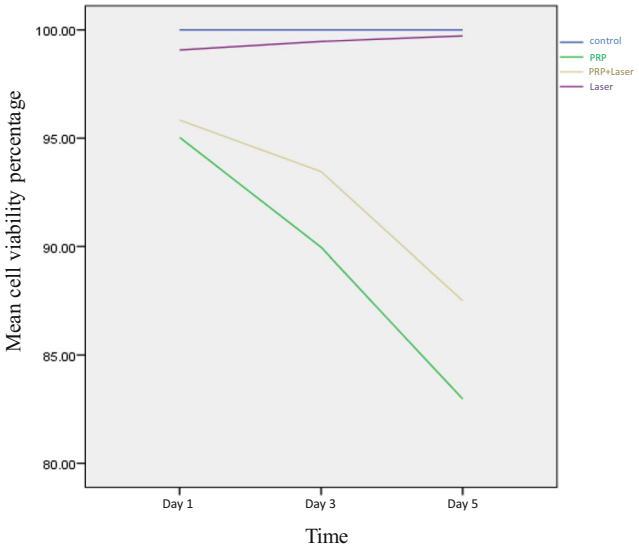

Fig. 2 Mean cell viability percentage of each group in relation to time elapsed

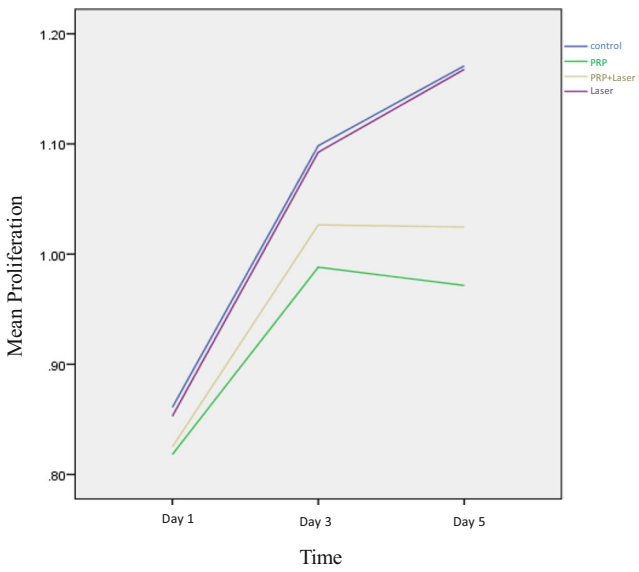

Fig. 1 Mean proliferation of each group in relation to time elapsed

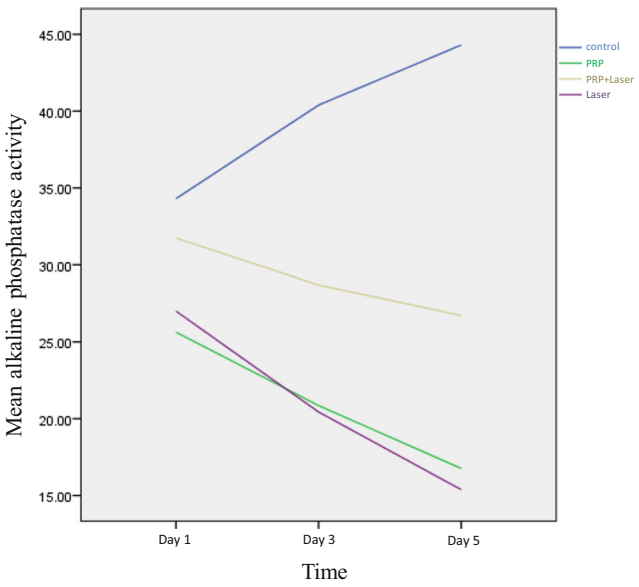

Fig. 3 Mean ALP activity of each group in relation to time elapsed

discrepancy may be justified by the difference in cell types used (periodontal ligament cells vs. pulp cells in the present study). Additionally, platelet activation was induced by 10% calcium chloride and thrombin (as opposed to calcium gluconate in the present study).

There might be a dose-dependent relationship between PRP concentration and cell proliferation [23–25]; however, the presence of such correlation has not been established [26–28]. Higher concentrations of PRP induced insignificant increase in ALP activity which may implicate the use of 10% product [11]. Collectively, the application of 1 to 10% PRP has been recommended, with a significant effect on osteogenic differentiation of hDPSCs [21]. Taking all the evidence into account, 10% PRP was used in this study.

Based on the findings of the present study, in the laser group, cell proliferation and viability increased with time, whereas ALP activity constantly decreased. In accordance with these results, recent evidence from a systematic review assessing stem cells derived from human deciduous teeth (SHEDs) and dental pulp (hDPSCs) suggests that photobiomodulation has the potential to improve cell proliferation and viability in both groups [29]. In another study [30], LLL irradiation has been found to induce dental pulp cell bio-mineralization [30]. However, contrary to our results, multiple studies have reported increased ALP activity in response to laser irradiation [30–33]. Not only different cell lines utilized in the studies may affect the results but various irradiation parameters could also be influential. Theocharidou et al. [30] applied LLL irradiation at 660 nm with a maximum output power of 140 mW which was lower than that of the present study. In spite of the fact that high energy density might be attributed to attenuated photobiomodulatory effects [29], one study [34] did not find a meaningful correlation between such effects and various output power.

One of the applications of platelet products in endodontics is in revascularization/regeneration of immature teeth [35]. The application of PRP and platelet-rich fibrin (PRF) more frequently induced apical closure as compared to blood clot in another study [7]. Considerable root length increase has also been observed in relation to the use of PRP in revascularization [36]. In the present study, the control group was found to be more successful in cell proliferation than the PRP group. Some researchers stated that concurrent application of PRP and hDPSCs was no different from the conventional method of inducing blood clot inside the root canal [37]. This is to some extent consistent with the results of the present study which might imply a probable cytotoxic effect on hDPSCs.

There are certain drawbacks to PRP usage including the application of anticoagulants which reduces regenerative potential, and short-term release of cytokine content [38]. In an in vitro study, 20% human platelet lysate was more effective than various PRP concentrations in terms of proliferation and angiogenic potential [39]. PRF as the next-generation material could be generated without the use of anticoagulants and needs considerably shorter centrifugation time [38]. In comparison to PRP, liquid PRF exhibits higher inductive potential on the regeneration of hDPSCs and reduces the inflammatory state caused by lipopolysaccharides [6]. Moreover, PRF showed a better performance than PRP in terms of mineralization and cell proliferation [40].

One of the major limitations of this study was the substantial cost incurred in the provision of equipment which made us restrict the assessment time to 5 days. Longer durations in future studies might yield more reliable results. A thorough search was conducted to set the assessment intervals. Finally, the assessments were done at days 1, 3, and 5, which is comparable to other studies [11, 41, 42].

In the present study, "AB^{+}" PRP (60 CC) was used because of the obligation for donor/receiver subtype compatibility. AB plasma is regarded as universal donor since it lacks both anti-A and anti-B immunoglobulin [42]. The application of this product does not necessitate Rh antigen compatibility; therefore, Rh-positive plasma could be used for an Rh-negative individual [43]. Considering these facts and unknown blood subtype of pulp cell donor, AB^{+} PRP was used. Consistent with similar studies, 10% calcium gluconate was utilized to activate platelets [44–46].

It could be inferred that PRP is not well-suited for vital pulp therapy since it decreases proliferation of pulp cells (day 5) as well as ALP activity with time. Though, the effect of PRP on different cell lines is variable. The appropriate concentration of PRP for fibroblasts has been found to be 8.5–17.5% [28]. Another study recommended concentration of 16.5% for fibroblasts and osteoblasts. Others [47] suggested 10 and 20% PRP for bone marrow stem cells, while more recently, a concentration of 2–5% was proved to be amenable to osteogenic differentiation. However, based on the findings of this study, a concentration of 10% did not turn out to be suitable for pulp cell proliferation and ALP activity. Consequently, further studies are required to focus on the optimal concentration of PRP for maintaining DPSC proliferation and viability.

Conclusion

GaAlAs laser irradiation increased hDPSC proliferation and viability while decreasing ALP activity. Nevertheless, the application of PRP was not as effective as laser radiation in terms of inducing cell proliferation. In view of the positive impact of laser on pulp cell proliferation in the present study, it is regarded as a promising non-toxic means to vital pulp treatment. Yet, complementary studies are still warranted to determine the most effective dose of irradiation and power output.

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Data availability All data are available and ready to release to the editorial board.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval (include appropriate approvals or waivers) The ethical approval number in Ethics Committee of Mashhad University of Medical Sciences is 910176.

Consent to participate Not applicable.

Consent for publication The publisher has our consent to publish this article.

Code availability Not applicable.

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