# Photobiomodulation Effect on Endothelial Cells in Regenerative Endodontics of Mature Necrotic Dog Teeth

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**Abstract**

**Background:**Photobiomodulation therapy (PBMT) was suggested to support regenerative endodontic treatment by modulating cell growth, protein synthesis and secretion processes of several cell types like endothelial cells. Also, it was suggested to activate angiogenesis and affect the organization of endothelial cytoskeletons and creation of new capillary networks from endothelial progenitor cells.**Aim:** The aim of this study was to evaluate the effect of photobiomodulation therapy on endothelial cells count in regenerative endodontic procedures.**Methods:** 54 mature permanent roots of premolar teeth in dogs aged 10-12 months old were included in the current study and distributed into three equal groups (18 roots / each): Group I; REPs was followed using blood clot as scaffold (positive control group) and Group II, was treated with the same regenerative protocol as group I then subjected to photobiomodulation therapy (study group) and Group III (negative control group), no intervention. Each group was further divided into three subgroups (6 roots per subgroup) according to evaluation periods 3, 10, 15 weeks. In each group endothelial cells count was evaluated according to evaluation periods.**Results:** Data analysis revealed significant differences for endothelial cells count between studied groups at the different intervals (P< 0.001). Photobiomodulation therapy was significantly influence the endothelial cells count in regenerated tissue compared to traditional regeneration protocol that used blood clot as scaffold only.**Conclusion:** The results of this study revealed the positive effect of photobiomodulation on vascularity and characteristics of the regenerated tissue. It also supports the REPs in non-vital mature permanent teeth as another treatment method.

**KeyWords:**Endothelial cells. Mature dog teeth. Non-vital permanent teeth. Photobiomodulation. Regenerative endodontic procedures.

**DOINumber: 10.48047/NQ.2022.20.16.NQ880337** **NeuroQuantology2022;20(16): 3255-3264**

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**Relevant conflicts of interest/financial disclosures:**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Introduction:

Regenerative endodontics is defined as biologically based treatment procedures that aims at the regeneration of the pulp's physiology, and cells of pulp- dentin complex [1]. It has been focused mainly on the treatment of young immature non-vital teeth [2] and revealed promising results that encouraged the researchers to study its application in the treatment of non-vital mature permanent teeth [3]. But the regenerative endodontic procedures (REPs) in mature permanent teeth have encountered many challenges, including decreased availability of stem cells, narrow apical foramina for initiation of blood and migration of stem cells, and difficult disinfection of the root canal system of mature teeth [2].

However, it was found that the apex diameter as small as 0.32 mm didn't prevent the ingrowth of vital tissue in the canal space [4], and regeneration without scaffold is also possible [5,6]. Also, induction of periapical bleeding into the canal space brings the critical factors for creating regeneration, such as fibrin scaffold, stem cells, and blood derived bioactive growth factors, in addition to the growth factors that could be delivered from dentin matrix after antibacterial irrigation by 17% Ethelyne diamine tetraacetic acid (EDTA) [7].

In a study performed by **Chrepa et al., 2015** [8], it was revealed that provoked intra-canal bleeding delivers stem cells in the root canals of mature teeth. In addition, Shah and Logani 2012 [9] and **Saoud et al., 2016** [3] reported successful clinical and radiographic results of non-vital mature permanent teeth with periapical lesions subsequent to cell homing-based REPs. But the histological evaluation revealed that the newly formed soft tissue devoid of the pulp's characteristic organization [10]. Since the dental pulp is a loose connective tissue consisting of ground substance, lymphatics, blood vessels, fibers, nerves, and the cellular structure of the pulp's blood vessels such as endothelial cells, and pericytes which play an important role in angiogenesis, pulp regeneration and dentine repair [11].

So, different approaches have been suggested to overcome the lack of pulp' characteristic features after regeneration process by the usual procedures of regeneration to attain more predictable histological features [12]. One of these approaches is photobiomodulation therapy (PBMT) which was suggested by **Marques et al., 2016** [13] as the fourth component of tissue engineering to support regenerative endodontic treatment by modulating cell growth, differentiation, protein synthesis and secretion processes of several cell types like endothelial cells, and fibroblasts [14-15]. In another study by **Ricci et al. 2009** examined the influence of PBMT on endothelial cell morphology, it was suggested that PBMT affects the organization of endothelial cytoskeletons [16]. Also, it has been revealed in several studies that the creation of new capillary networks from endothelial progenitor cells, as well as angiogenesis can be activated by laser light therapy [17-20].

PBMT could also improve micro-vascularization by its mitogenic effects, including production of singlet oxygen by excitation of endogenous porphyrins, absorption of light by mitochondrial enzymes, photoactivation of calcium channels resulting in elevated cellular proliferation [14].

As it was found that, there is only a small percentage of cells are remaining viable after regeneration procedures, and the recruited stem cells die prematurely, impairing the regeneration progression mostly owing to tissue environmental situations, such as the deficiency of proper blood supply and subsequent nutritional stress [21], so, many studies suggested that PBMT can overcome these drawbacks, as it can prevent cell prematurely death by enhancing the viability, migration, proliferation, and differentiation of newly recruited stem cells, in addition to improving their nutrition by encouraging angiogenesis and synthesis of growth factors, comprising the vascular endothelial growth factor (VEGF) and fibroblastic growth factor (FGF) [22–24].

Although, PBMT has been suggested to positively influence the regenerative endodontic procedures [13], however, to date only a few studies have discussed the direct influence of PBMT on endothelial cells in regeneration process [25-27]. Therefore, the present study was designed to assess the influence of PBMT on the endothelial cells count in REPs in mature permanent teeth. The null hypothesis was that no statistically significant difference would be found between the tested groups in terms of endothelial cells count.

## Materials and Methods

The Committee of Scientific Research Ethics, Faculty of Dentistry, Minia University approved the

protocol for this experimental study for the animal experiments regulations and guidelines (reference number **264/2019**). All procedures were performed in accordance with NC3Rs (National Centre for the Replacement Refinement and Reduction) of Animals in Research guidelines and regulations. All procedures were reported according to ARRIVE guidelines of animal experiments.

## Sample size calculation

Sample size calculation was carried out using power analysis (G*Power version 3.1.9.7) that was planned to have sufficient power of 80%, adopting an alpha level of (0.05) a beta of (0.2) and calculated based on the results of a previous study, [28] the predicted sample size was a total of 54 samples (18/group) to apply a statistical test of the null hypothesis that there is no difference would be found between the tested groups. [29]

## Study setting

• A total 27 premolars (of 54 mature roots) were selected from 3 mixed breed canine male dogs about 10-12 months old with weight between 12-15 kg.

• The study procedures were carried out at Veterinary Unit, General Organization for veterinary services, Egypt.

## Randomization and allocation concealment

Simple randomization was assigned by an independent investigator (NMAK) to divide the samples by using the closed sealed envelope method into three equal groups (n = 18 roots/each) based on the treatment protocol. First, one side in each dog was assigned for photobiomodulation group (either upper or lower), then other side was assigned randomly among the two other groups. In Group I (positive control group), teeth were subjected to regeneration endodontic procedures using blood clot as scaffold, whereas in Group II (study group), the same regenerative endodontic procedures were performed as group I then exposed to PBM, while, Group III (negative control group), no procedures have been done as it used for assessment of normal dog's pulp. Each group was subdivided into three subgroups (n=6 roots per subgroup) based on assessment periods, which were; Subgroup A: 3-weeks, Subgroup B: 10-week, Subgroup C: 15-weeks.

## Animal care:

The animals were treated following the National Centre of the three R (Replacement, Reduction and Refinement) of animals in research and Canadian Council protocol of animal care. Animals were housed in separate cages and kept in ventilated air-dried run away from human habitation, noise, parasites-free, and had standard soft balanced diet, fresh water that were placed in a room provided with artificial light to provide stimulated cycle of day and night [30]. Booster doses for rabies and 5-way vaccine were given [28].

## Animal preparation

All dogs were premedicated with intravenous injection of a mixture of Diazepam in a dose of (1 mg/kg) (Neuril – Memphis Co Egypt) and Atropine sulfate in a dose of (0.05mg/kg) (Atropine sulfate – CID Co Egypt), then anesthesia for dogs was induced immediately by intravenous injection of a mixture of Xylazine in a dose of (1 mg/kg) (Xylaject – Adwia Co Egypt) and Ketamine Hcl in a dose of (10 mg/kg) (Ketamine – Sigmatec Co Egypt). The anesthetic depth was maintained with 2.5 % Thiopentone sodium in a dose of (30 mg/kg) (Thiopental – Sandoz Austria) administered intravenously till dose effect. Then the dogs were restrained in lateral recumbence. Following the procedures, Dexamethasone (0.5 mg/kg), and Metacam 2 (mg/kg) were administered intramuscular every 24 hours for three days [31].

## Infection procedures

Periapical radiograph was used to approve closure of roots apex. In positive control group and study group, access cavities in the chosen teeth were achieved with high speed with water coolant and round burs, then the pulp was interrupted using a size 40 sterilized K-file. Then, plaque suspension was prepared by mixing part of supra-gingival plaque with saline and inserted in pulp champers by a sterile sponge soaked in the plaque suspension [32]. After four weeks, periapical radiograph was taken to confirm the periapical radiolucency.

Procedural steps and treatment of the selected teeth were carried out according to The American Association of Endodontics

## Disinfection procedures

Decontamination of teeth surfaces were performed after four weeks with 0.12% chlorhexidine (Hexitol; Arab Drug Company, Cairo, Egypt) and cotton rolls was used for teeth isolation, then the

disinfection step was performed for previously accessed teeth by determining the working length (Root ZX II; J. Mor- ita, Kyoto, Japan) then penetrating the apical cementum with #15 K-file and instrumented up to #60 K-file. The irrigation was performed using 1.5% sodium hypochlorite (Clorox Co, 10th of Ramadan, Egypt) (20 mL/canal for 5 min), normal saline, and 17% ethylene diamine tetraacetic acid (PREVEST, DenPro. Jammu, India) (20 ml per canal each).

Di-antibiotic paste was prepared by mixing equal amounts of powdered ciprofloxacin (Ciprocin 500 mg; EPICO, Cairo, Egypt) and metronidazole (Flagyl 500 mg; Aventis, Cairo, Egypt) with propylene glycol vehicle to a concentration of 1 mg/mL, then inserted up to the CEJ of root canals of positive control group and study group. Glass ionomer cement (NOVA Glass-F; IMICRYL, Konya/ Turkey) was used for sealing access cavities for four weeks, then periapical healing was followed and confirmed by periapical radiograph [33].

## Regenerative procedures

Medications that were inserted in root canals of positive control group and study group were copiously irrigated with 1.5 % NaOCl, normal saline, and 17% EDTA solution for 5 min per canal based on American Association of Endodontists (AAE) guidelines 2017, then root canals were dried with sterile paper points (Dia Dent, Chungcheongbuk-do, Korea). Over-instrumentation of apices was performed by sterile hand files size #25-#35 to allow bleeding flow in root canals up to CEJ. Mineral Trioxide Aggregates powder (MTA) (Rootdent, TehnoDent, Russia), was applied at the orifices, and then followed by coronal seal with glass ionomer [33].

## Photobiomodulation therapy procedure

The study group was subjected to Photobiomodulation therapy after the regenerative procedures, which was applied to the apical root area at the lingual and buccal sides of the tooth, at two days intervals for two weeks period. Diode semiconductor laser, Gallium- Aluminum –Arsenide laser (eleixxon_Claros_Pico, Germany) was used for photobiomodulation therapy with output power of 300 mW and wavelength of 810 nm.

A biostimulation tip of 0.6 cm diameter in contact to the tissues was used to deliver the laser beam with a continuous emission of laser, (E= 2.7 J) and entire dose of each application was 4 J/cm$^2$ [34]. **(Fig. 1).**

All the teeth were observed following the treatment and the animals were sacrificed at various intervals (3 weeks, 10 weeks and 15weeks) and tissues were gathered for histological evaluation. Euthanization procedures

Overdose of thiopental sodium 2 gm in 10 ml distilled water was injected intravenously to euthanize the animals at each predetermined follow up period. The teeth with their surrounding structures were dissected from the jaws [31].

### Histological evaluation

The samples were fixed in 4% paraformaldehyde (Formical, Decal Chemical Corporation, Congers, NY) for 48 hours, washed under tap water overnight then decalcified for 3 months in 10% EDTA (pH = 7.5) and for subsequent preparation the samples were paraffin-embedded [28].

Each root was considered as an independent specimen for histologic assessment. Hematoxylin and eosin (H&E) stain were used for sections staining, [35] then the image analyzer computer system was used for sections analyses using the software Leica Qwin 500 (Germany) at Center of Research and Dental Requirements, Faculty of Dentistry, Cairo University, Egypt. The image analyzer was calibrated automatically to convert the measurement units (pixels) into real micrometer units. Neo-formed tissues were assessed by two blinded calibrated examiners. The endothelial cells count was measured, where five different fields for each subgroup slides were examined using a magnification (x 400). (Fig. 2).

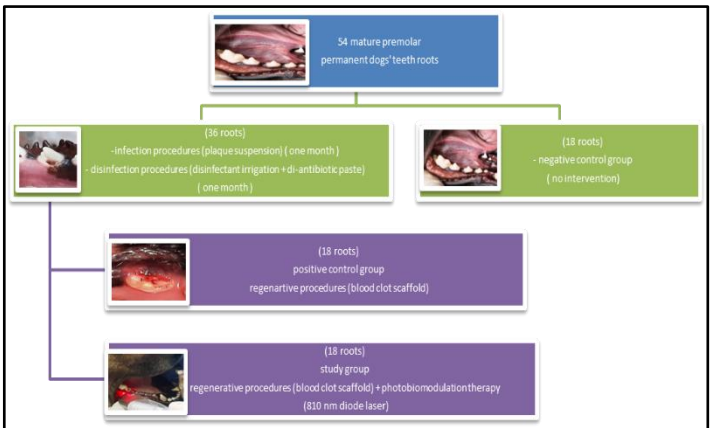


**Figure (1).** Flowchart demonstrating group distribution based on the treatment protocol.

## Statistical analysis

Numerical data were presented as mean and standard deviation (SD) values and were analyzed using independent t-test for intergroup comparisons and repeated measures ANOVA for intragroup comparisons. The significance level was set at $p<0.05$. Statistical analysis was performed with R statistical analysis software version 4.1.3 for Windows [36].

## Results

**Histologic evaluation of the collected samples revealed the following:**

- In negative control group, the photomicrograph of all samples showed normal characteristics of the pulp composed of loose connective tissue with normal appearance of endothelial cells. (H&E X 100um).

- In the samples of positive control group, the formed tissues at different periods of assessment were more similar to granulation tissue lacking the characteristics of organization and structures of normal pulp.

- However, in the samples of study group, the formed tissue showing improvement in constitution, formation, and organization:

At 3 weeks assessment period, the photomicrographs showed formation of tissue start to regenerate with blood vessels, fibroblasts, and less prominent endothelial cells. (H&E X 50 um).

At 10 weeks assessment period, the photomicrographs showed regenerated tissue similar to loose pulp-like tissue formed of blood vessels that were larger and dilated, and prominent endothelial cells, and the formed tissue was connected to each other. (H&E X 100 um).

At 15 weeks assessment period, the photomicrographs showed more organized tissue with numerous dilated blood vessels that were close to each other, newly formed circular blood capillaries, and abundance of endothelial cells. (H&E X 20 um). (Fig. 2)

**Statistical analysis of endothelial cells count revealed:**

- At 3 weeks, there was a significant difference in endothelial cells count between study group and negative control group, where mean values were 31.20±3.27 and 68.53±2.58 respectively, and both values were significantly higher than positive control group (13.40±3.05) ($P<0.001$).

- At 10 weeks, there was insignificant difference in endothelial cells count between study group and negative control group, where mean values were 68.80±10.76 and 69.89±1.65 respectively, however both values were significantly higher than positive control group (31.40±7.50) ($P<0.001$).

- At 15 weeks, there was a significant difference in endothelial cells count for study group and negative control group, where mean values were 95.00±10.61 and 70.33±1.53 respectively, and both values were significantly higher than positive control group (37.60±1.52) ($P<0.001$).

- For positive control group, there was a statistical significant difference between mean values measured at different intervals ($p<0.001$). The highest mean value was measured at 15 weeks (37.60±1.52)

- For study group, there was a significant difference between mean values measured at different intervals ($p<0.001$). The highest mean value was measured at 15 weeks (95.00±10.61)

- For negative control group, there was non-significant difference between the mean values measured at all intervals ($p>0.05$). **Table (1)**

**Table (1):** Mean and standard deviation (SD) values of endothelial cells count among studied groups

| Interval | Number of endothelial cells (mean± SD) | | | p-value |
|---|---|---|---|---|
| | Positive control | Study group | Negative control | |
| 3 weeks | 13.40±3.05$^{Cb}$ | 31.20±3.27$^{Bc}$ | 68.53±2.58$^{Aa}$ | <0.001* |
| 10 weeks | 31.40±7.50$^{Ba}$ | 68.80±10.76$^{Ab}$ | 69.89±1.65$^{Aa}$ | <0.001* |
| 15 weeks | 37.60±1.52$^{Ca}$ | 95.00±10.61$^{Aa}$ | 70.33±1.53$^{Ba}$ | <0.001* |
| p-value | <0.001* | <0.001* | >0.05ns | |

Means with different upper and lowercase superscript letters within the same horizontal row and vertical column respectively are significantly different *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)

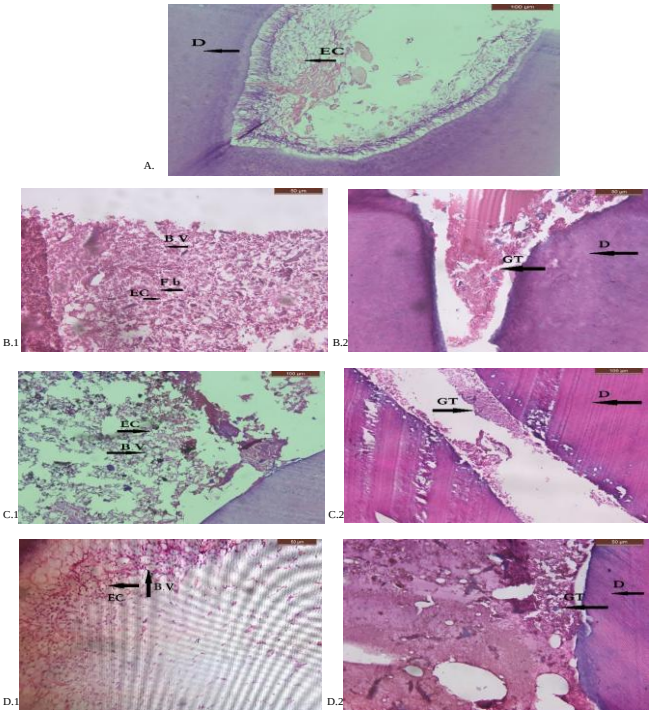


**Figure (1):** Photomicrographs showing: A. the normal characteristics of dog's pulp in negative control group (H&E X 100um), B. At 3 weeks assessment period ( B.1. formation of a tissue starts to regenerate with blood vessels, fibroblasts, and less prominent endothelial cells. (H&E X 50 um). C. At 10 weeks assessment period (C.1. formation of regenerated tissue similar to loose pulp-like tissue formed of blood vessels, and prominent endothelial cells. (H&E X 100 um). D. At 15 weeks assessment period, (D.1. formation of more organized tissue with numerous dilated blood vessels, newly formed circular blood capillaries, and abundance of endothelial cells. (H&E X 20 um). B.2, C.2, D.2, formation of tissue similar to granulation tissue devoid of main characteristics of pulp formation. (B.V: blood vessels, D: dentine, EC: endothelial cells, F.b: fibroblast, GT: granulation tissue). study group (B1, C1, D1), positive control group (B2, C2, D2).

## Discussion

RCT has been used for many years as the conventional treatment protocol of mature permanent teeth with necrotic pulps [37]. But due to its many drawbacks, regenerative endodontics was suggested. Regenerative endodontics was mainly applied in immature permanent teeth, and **Iwaya et al., 2001,** published the first successful revitalisation case in immature permanent teeth [38]. Because of successful results of REP in immature permanent teeth, it was recently proposed to be applied in mature permanent teeth in an attempt to overcome the problems of RCT such as lack of immune mechanisms, elevated susceptibility to root fracture [39]. Furthermore, the use of regenerative endodontics in the treatment of mature permanent teeth may contribute to the re-establishment of the innate immune system [40] and provide a promise for more complicated cases such as perforating root resorption, root fracture, and avulsion [41].

Although, several studies have shown high clinical success rates following REPs [42-44] but their meta-analyses revealed lesser success rates in radiographic analysis [40,43]. Furthermore, the histological analyses showed that the regenerated tissue inside the root canal system was cementoid, osteoid, or periodontal-like tissue, without odontoblasts or the normal characteristics of pulp [45-46]. Moreover, there are some limitations regarding REPs associated with reduced number of stem cells recruited inside the root canal system, their survival capabilities, and proliferation [47].

Some researchers reported that PBMT inhibit cell death [20,48], inducing the release of growth factors [49,50] and could activate TGF- β1 to direct resident stem cell migration and differentiation [22]. Based on these findings, PBMT was selected in the current study as a supportive treatment in the REPs using blood clot scaffold. Dogs were used as the animal model, as they are one of the most commonly used animal species in regenerative endodontics [51] as they have large size teeth making endodontic treatments easier [52,53], also, they have dental tissue structures and apical healing ability similar to human teeth [54]. Moreover, the root apices of the dog's premolar teeth complete at 8 months of age [55], so the chosen dogs were 10-12 months old in the current study.

To consider the newly formed tissue in the root canal as regenerated dental pulp, it must be vascularized and innervated [56]. The endothelial cells considered as a part of the cellular elements of the blood vessels, so it could reflect the vascularity of the regenerated tissue, therefore, it was histologically evaluated at different periods of assessment.

Angiogenesis, which is the growth of new vasculature, defined as a complex process involving EC migration, proliferation, and remodeling of extracellular matrix [57]. EC migration requires

both elevated cell motility and chemotaxis that control the direction of cell movement. VEGF can play a special role on EC to induce vascular permeability, cellular proliferation, and migration [58]. VEGF and other growth factors can be released during the REPs by using EDTA (17%) as disinfectant irrigation, as it is considered a chelating agent that can decalcify the surface of the root canal dentine to reveal its collagen fibers, and it was linked to the release of growth factors implanted in dentine that may promote cellular proliferation [59] and assist recovers of cellular viability [60].

In the present study, PBMT was performed using Ga-Al-As diode laser (810 nm) of 4 J/cm2 energy density as the infrared wavelength 810 nm are preferred to treat deeper tissues as it penetrates 2 mm before losing 37% of its intensity [61], and it can pass the surrounding tissues of the teeth and reach dental pulp cells [47], so could enhance the regenerative endodontic process in view of endothelial cells formation. Moreover, **Góralczyk et al., 2016** described a reduction of inflammation by a decrease in TNF-α and IL-6 after irradiation with infrared wavelength 810 nm diode laser and concluded that the antiinflammation effect of PBMT leads to support of the endothelial function by increasing cell viability [62].

Nitric oxide and ATP are ambiguous molecules acting as anti-inflammatory mediators according to their concentration, target, and time of exposure. Therefore, they are able to downregulate or upregulate some MMPs synthesis and affect TNF and proinflammatory markers [63,64]. In this context, the ability of laser light to induce NO production or release and to change the energetic cell metabolism, such as the ATP production [65,66], can control the proinflammatory molecules. This behavior may lead to a reduction of apoptosis' indices [26], as well as protects against TNF/cycloheximide (CHX)-induced apoptosis pathway by inhibiting p38mitogen-activated proteinkinase (MAPK) and nuclear factor kappa-light chain-enhancer of activated B (NF-κB) signals [67].

The effect of PBMT on the formation process of new blood vessels in the infarcted rat heart monitored by counting the proliferation of EC in blood vessels has been established in a recent study [68]. Also, many studies have demonstrated the direct effect of laser light on endothelial cells, **Schindl et al., 2003**demonstrated positive effects of PBMT with 670 nm on HUVEC proliferation [26], also **Chen et al.** shown the stimulatory influence of PBMT on HUVEC proliferation [14] and concluded that laser irradiation elevate the endothelial cell migration, proliferation, and eNOS gene expression. In addition, **Szymanska et al., 2013** revealed that increased proliferation of HUVEC mediated by light stimulation is mediated by an increase of VEGF and transforming growth factor beta (TGF-β) levels [49].

In addition, the histological findings of the current study demonstrated a prominent increase and abundance in the endothelial cells' formation in the study group that was exposed to PBMT and these finding was supported by the statistical analysis of the collected data that revealed a significant difference in endothelial cells count between the study group and negative control group, however, both values were significantly higher than positive control group ($P<0.001$).

Our finding is comparable to those reported by **Moreira et al., 2017** who reported that Blood clot scaffold with PBM protocol resulted in a dental pulp-like tissue with vessels, nerves, odontoblast-like cell layer, and perivascular SCs [69] and agreed with a previous study which concluded that the direct stimulation by low-intensity laser irradiation could induce the proliferation of EC [70].

This finding may be due to the effect of PBMT that have effective influence on the proliferation and differentiation of Stem Cells (SCs) [71]. Also, the vascular endothelial growth factor, which is expressed by different cell types and acts on regulation of intercellular signals, and the process of angiogenesis, the formation of new blood vessels from pre-existing vessels, and contributing to tissue regeneration [72]. In addition, many growth factors have been reported to modulate endothelial cell proliferation, migration, and angiogenesis [73] including vascular endothelial growth factor (VEGF) [74]. It also seemed that laser therapy stimulated lymphocytes to produce factors that could modulate EC proliferation. In response to the appropriate stimuli, EC ultimately underwent migration and proliferation, forming capillaries of the neovascularization [75].

Therefore, the results of the current study revealed that PBMT enhances the formation of the endothelial cells, so supports the REPs in non-vital permanent mature dog's teeth and rejecting the null hypothesis.

## Conclusions

Within the limitation of the current study and according to their results, the following can be concluded:

1. Revascularization of necrotic mature permanent teeth can be a successful alternative treatment modality for regaining pulp vascularity.
2. Photobiomodulation therapy after blood clot revascularization was found to be an effective way to improve the structure of regenerated tissue.

## Recommendation

Further researches and clinical trials are required to evaluate revascularization procedures in necrotic mature permanent teeth and the effect of photobiomodulation on regeneration process and regenerated tissues.

Abbreviations

Photobiomodulation (PBM), Endothelial cells (EC), mineral trioxide aggregates (MTA), ethylene diamine tetraacetic acid (EDTA), sodium hypochlorite (NaOCl), mesenchymal stem cells (MSC), cementoenamel junction (CEJ), H&E (hematoxylin and eosin), standard deviation (SD), stem cells of apical papilla (SCAPs).

## Declarations

### Ethics approval and consent to participate

The Scientific Research Ethical committee of Faculty of Dentistry, Minia University evaluated and approved the experimental proposal of the current study for the animal experiments guidelines and regulations (reference number 264/2019). All experimental procedures that carried out in the current study were in accordance with NC3Rs (National Centre for the Replacement Refinement and Reduction) of Animals In Research guidelines and regulations. The procedures were reported in accordance with ARRIVE guidelines of animal experiments.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used during the current study available from the corresponding author on reasonable request. All data analyzed during this study are included in this published article in the form of tables and figures.

### Funding

The authors did not receive any funding from any organization.

### Author contributions

**SFK:** study design, conceptualization, data curation, methodology, manuscript preparation; **NMAK:** study design, conceptualization, data curation,methodology, manuscript preparation, data analysis, final approval and agrees to acceptable for all aspects of work ensuring integrity and accuracy; **YFG:** study design, conceptualization, data curation, methodology, manuscript preparation, final approval and agrees to acceptable. **All authors** read and approved the final manuscript.

### Acknowledgement

Not applicable.

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### Competing interests

The authors declare that they have no competing interests.

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