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EFFECT OF DIODE LASER WITH AND WITHOUT ENAMEL MATRIX DERIVATIVE ON PERIODONTAL HEALING OF REPLANTED TEETH AFTER EXTENDED EXTRA-ORAL DRY TIME (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

The aim of the study was to evaluate the effect of enamel matrix derivative and high power diode laser irradiation on healing of periodontal ligament (PDL) of experimentally avulsed and replanted upper incisors teeth of albino rats after extended extra oral dry time through histological examination and immunohistochemical detection of fibronectin. **Materials and methods:** 30 adult male Albino rats divided into three groups, 10 rats each; **group I** served as controls were no treatment for root surface of extracted incisors before their replantation, **group II** were root surface treated with enamel matrix derivative (Emdogain) gel, **group III** were root surface treated with diode laser and Emdogain gel**.** Five rats of each group were sacrificed four weeks from the beginning of the experiment while the other five rats were sacrificed after eight weeks. The jaws of the upper incisors regions of the sacrificed animals were dissected out for histological examination and immunohistochemical analysis. **Results: The histological examination** of group I rats showed massive degeneration and loss of orientation in the collagen fibers of PDL of replanted teeth, group II rats revealed signs of PDL regeneration in the form well-defined but unorganized fibers of PDL, while group III rats showed the best regenerative features in the form of well oriented and organized fibers of PDL. **Immunohistochemical** detection of fibronectin of group I showed negatively to weakly positive reaction. PDL of group II presented moderately positive reaction. While, group III presented strongly positive reaction. **Conclusions**: Application of Emdogain only to the root surface of replanted teeth has limited inductive effects on the regeneration of PDL cells and fibers while using of Emdogain in combination with diode laser on the root surface leads to better periodontal healing.

KEYWORDS: Replantation, Emdogain, Diode laser, Fibronectin.

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INTRODUCTION

Avulsion is one of the most severe and complicated forms of dental injuries, in which the total displacement of the tooth from its alveolar socket affects the alveolar bone, gingival tissues, periodontal ligament, pulp and cementum layer[1]. Avulsed tooth can usually be retained by replantation however, the prognosis depends on the extra alveolar duration, the condition of the avulsed tooth, the patient's age and root development. Vitality of the periodontal membrane attached to the root is of great importance to the success of a replanted tooth[2].

The ideal treatment for an avulsed tooth is its immediate replantation into the socket, which significantly improves the prognosis. The prognosis of the treatment as well as the survival of an avulsed tooth in the mouth depends on intrinsic and extrinsic factors, such as the duration of the tooth's extra-alveolar period, its storage medium, replantation time, PDL status and duration of splinting. Sixty minutes of extra-oral dry time is considered to be critical. In order to protect PDL cells and provide optimum healing, immediate replantation is the most ideal treatment among other options[3].

Enamel matrix derivative (Emdogain) is an extract of porcine fetal tooth material used to stimulate the soft and hard tissues surrounding teeth to regrow[4] . Emdogain (EMD) was shown to promote periodontal healing during replantation in some studies, whereas in other studies Emdogain did not prevent root resorption[5].

Emdogain has been successfully used in periodontal regenerative procedures as reported by several investigations. Both animal and human studies have shown that Emdogain may lead to the formation of periodontium. The use of Emdogain in periodontal regeneration has been suggested because of its biological origin and properties[6].

Diode laser is able to accelerate wound healing, to promote angiogenesis, to augment growth factor release and to prevent root surface ablation[7]. Furthermore, when used on the root surface, lasers can promote fusion and melting of the dental structure, thus making it more homogeneous and favoring the adhesion of connective tissue fibers and cells, and new cementum formation. This renders the root surface more resistant to microbial and clastic cell action[8]. Root surface treatment with high-power diode laser irradiation prior to delayed replantation has shown potential to reduce the occurrence of external root resorption and to inhibit ankylosis[9].

The periodontal ligament is fetal-like connective tissue that is the tissue of attatchment of the tooth to the alveolar bone. It consists of fibers, ground substance (fibronectin, tenascin), Cells, blood supply, nerve fibers and lymphatic vessels [10].

Fibronectin is a glycoprotein that is thought to promote attachment of cells to the extra cellular matrix, especially to collagen fibrils. Furthermore, cells also preferentially adhere to fibronectin and it may be involved in cell migration and orientation. Fibronectin is uniformly distributed throughout the periodontal ligament and is localized over collagen fibers and at certain sites on the cell collagen interface[11].

Recent guidelines from the International Association of Dental Traumatology refer to 60 minutes as the extraoral dry time threshold and outline different treatment approaches for avulsion cases with an extraoral dry time of less than 60 min[12]. The healing potential of the PDL tends to decrease exponentially as the dry time increases. Some literature even suggests that if replantation does not occur almost immediately, the healing of PDL is likely to be compromised and low healing rates should be expected, even after only 20 min of extraoral dry time [13].

The present study was designed to evaluate the effect of Emdogain in combination with high power diode laser irradiation on periodontal healing of replanted teeth after extended extra oral dry time.

MATERIAL AND METHODS

The study was done by extraction of maxillary incisors of 30 adult male Albino rats weighing 180–200 g and divided into three groups. Rats were caged in wire top cages seven per cage in the animal house of Faculty of Dentistry, Suez Canal University. They were supplied natural diet and tap water throughout the experiment. This study was carried out in accordance with the Guidelines of Research ethical committee (REC) at Faculty of Dentistry, Suez Canal University (Approval number 27, 2017).

Surgical procedure

Rats were anesthetized under intramuscular general anesthesia, using ketamine (Rotexmed, Germany) and xylazine (Adwia, Egypt) at 0.7:0.5 ml ratio,and in dose of 0.1 ml per100g of weight[14] **(Figure 1).**

Fig. (1) Photographs showing the ketamine and the way of intramuscular injection.

After that, the extraction was done by laxation of the tooth by using amalgam carver as an elevator to break down the periodontal ligament. Followed by extraction using dental adam plier. After extraction, the tooth was left on the napkin for 60 min[15]. **(Figure 2)**.

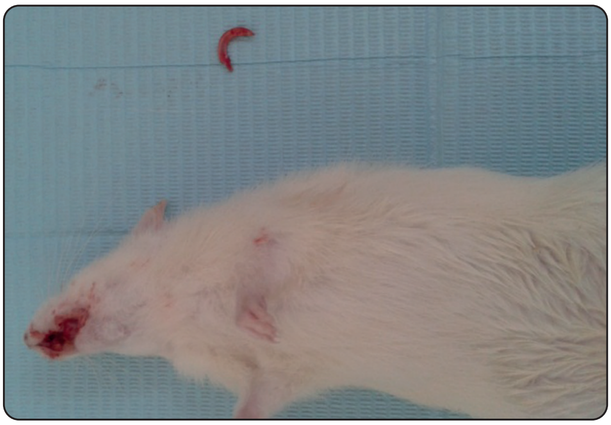

Fig. (2) A Photograph showing extracted tooth left for 60 min. on napkin.

Then removal of the pulp was done using endodontic files after the time previously established, the pulp was removed via the apical foramen with a precurved 25-mm length #20 K files. The root canals were irrigated with 5 ml of saline solution dried with absorbent paper points and filled with calcium hydroxide paste with idoform Metapex (Meta biomed Korean)**.**

Then rats were classified into three groups ,10 rats in each group:

-Group I (Control group) no treatment for the root surface and alveolar wound was performed for the extracted incisors before their replantation.

-Group II (EMD) application of Emdogain gel (Emdogain, Straumann, Sweden) to root surface and alveolar wound was performed for the root surface of extracted incisors before their replantation **Figure (3)**.

- **Group III** (Dioade laser & EMD) root surface of extracted incisors treated with Zolar dental diode laser (810nm, contionous mode, 1.0w, 30s) and topical application of emdogain gel performed for the extracted incisors before their replantation[16] **Figure (4)**.

Fig. (3) A photograph showing Emdogain used in the experiment.

Fig. (4) A photograph showing Zolar dental Diode laser device used in the experiment.

Replantation was done then splinting using flowable composite (composan bio-esthetic flow; Promedica, Germany) to the neighboring central maxillary inscisor prior to tooth replantation. Where acid etch was applied to the crown the washed away after 15 sec., then the bond was applied and light cured for 20 sec., then the flowable composite and light cured for 40sec[17]. Administration of intramuscular dose of antibiotic (amoxicillin 1gm) in dose of 0.005ml per 100g of body weight[18].

Half of each group were euthanized and sacrificed after 4 weeks, while the other half will be after 8 weeks. The rats were sacrificed by cervical dislocation. The jaws of the upper incisors regions of the sacrificed animals were dissected out for histological examination and immunohistochemical analysis of fibronectin.

The jaw specimens were decalcified in 10% ethylene diamine tetra acetic acid (EDTA) PH 7-7.4. After complete decalcification, jaw specimens will be prepared for light microscopic examination. They will be dehydrated in alcohol, cleared in xylene and embedded in paraffin. Serial sections were cut in a mesio-distal plane and parallel to the long axis of the root and subjected to:

a) **Hematoxylin and eosin stain:** for histological examination of the periodontal ligament and alveolar bone.

b) **Masson's trichrome stain:** for collagen fibers demonstration[19].

c) **Immunohistochemical localization** of fibronectin in PDL & alveolar bone.

Immunohistochemical detection of fibronectin

Rabbit polyclonal anti-fibronectin (diluted 1:200) was from LSL (Tokyo, Japan). To confirm antigen masking by other molecules, we tested trypsin digestion (0.1 % w/v, porcine pancreas, Wako Chemicals, Tokyo, Japan) for 30 min at room temperature, or testicular hyaluronidase digestion (25 mg/ml, Sigma, St. Louis, MO) for 2 h at 37^{o}C, or 0.1 M acetic acid treatments for 10 min at room temperature. The Histofine SAB kit (Nichirei, Tokyo, Japan) was used to perform streptavidin–biotin labeling, Sections were treated with diaminobenzidine (DAB, brown) to visualize protein localization. Negative control sections were incubated with normal rabbit IgG (10 lg/ml) instead of primary antibodies. Sections were observed after counterstaining with hematoxylin. The images were captured under light microscope [20].

Statistical analysis

The immunostained sections were examined under the light microscope. The positive staining reaction appeared in the form of brown staining. Data were collected and expressed as mean ±

S.E.M. For statistical analysis, t-test for multiple comparisons was applied. Data analysis was performed employing the statistical package for social sciences, version 21 (SPSS Software, SPSS Inc., Chicago, USA). The mean values are considered significant when the P value ≤ 0.05.

RESULTS

a) Hematoxylin and Eosin stain

Histological examination of the periodontal ligament specimens of group I rats revealed apparent degeneration and dissociation of the collagen fibers of PDL after four weeks from the beginning of the experiment associated with marked degeneration of the fibroblasts. Frequent areas of loss of attachment of the PDL fibers to the bone and cementum surfaces were encountered together with surface bone resorption and thinning of bone trabeculae**.** While PDL specimens that were taken after eight weeks showed massive degeneration, dissociation and loss of orientation in the collagen fibers. Massive alveolar bone resorption with osteoclastic activity and cementum resorption, extreme widening of bone marrow cavities associated with thinning of bone trabeculae were observred (Figure 5 a&b). The periodontal ligament of groups II rats treated with EMD revealed signs of PDL regeneration in the form well-defined but unorganized fibers found close to cementum after 4 weeks**.** Reversal lines of bone which represent past osteoclastic activity were present indicating that teeth treated with EMD exhibited signs of osseous replacement. While specimens taken after 8 weeks characterized by

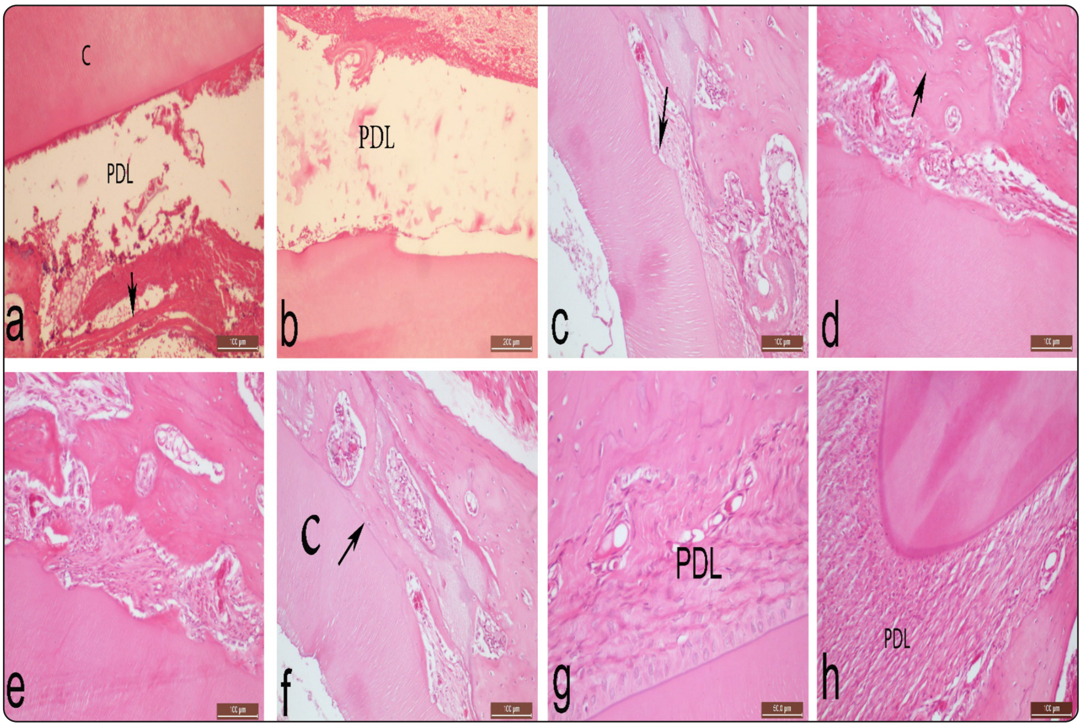

Fig. (5): Histological sections (hematoxylin and eosin staining) of group I a: showing degeneration of cells and fibers with loss of attachment of PDL associated with alveolar bone resorption and thinning of bone trabeculae (arrow), b: showing massive degeneration of the collagen fibers of PDL, group II c: showing well-defined but unorganized fibers of PDL with focal areas of cementum resorption (arrow), d: formation of bundles of PDL fibers with reversal lines of bone (arrow), e: showing great number of viable and undamaged PDL cells and fibers,f: showing focal areas of ankylosis between cementum (C) and alveolar bone (arrow), group III g: showing the least degenerative changes in PDL fibers and cells, h: showing well oriented and organized fibers of PDL.

the presence of well vascularized connective tissue having apparent number of viable and undamaged periodontal cells and oriented fibers. Also there were focal areas of ankylosis between cementum and alveolar bone and other areas of cementum resorption (Figure 5 c, d, e & f). The periodontal ligament of groups III rats treated with diode laser & EMD and replanted for 4 weeks showed the least degenerative changes in the periodontal ligament fibers and cells when compared to the extracted teeth which treated with EMD only**.** The best regenerative features represented by well oriented and organized fibers of PDL were apparent in the specimens taken after 8 weeks with higher rate of osseous replacement resorption when compared to groups II rats**.** The areas of alveolar bone and cementum resorption were reduced (Figure 5 g&h).

b) Masson's trichrome stain

Jaw specimens of **control** rats revealed localized degeneration of the collagen fibers of PDL with weakly positive reaction to Masson's trichrome stain after four weeks (Fig 6 a). Moreover, the specimens taken after eight weeks showed generalized massive degeneration of the collagen fibers which stained negatively to weakly positive reaction to Masson's trichrome stain (Fig 6 b). While, PDL fibers of EMD treated rats presented the number of fibers was increased and denser collagen fibers formed with weak to moderate positive stain after 4 weeks (Fig 6 c) and moderate positive stain after 8 weeks (Fig 6 d). However, Jaw specimens of rats treated with diode laser & EMD presented further increase of collagen fibers with moderately to strongly positive stain after 4 weeks (Fig 6 e) and well organized bundles of collagen fibers with strongly positive stain after 8 weeks (Fig 6 f).

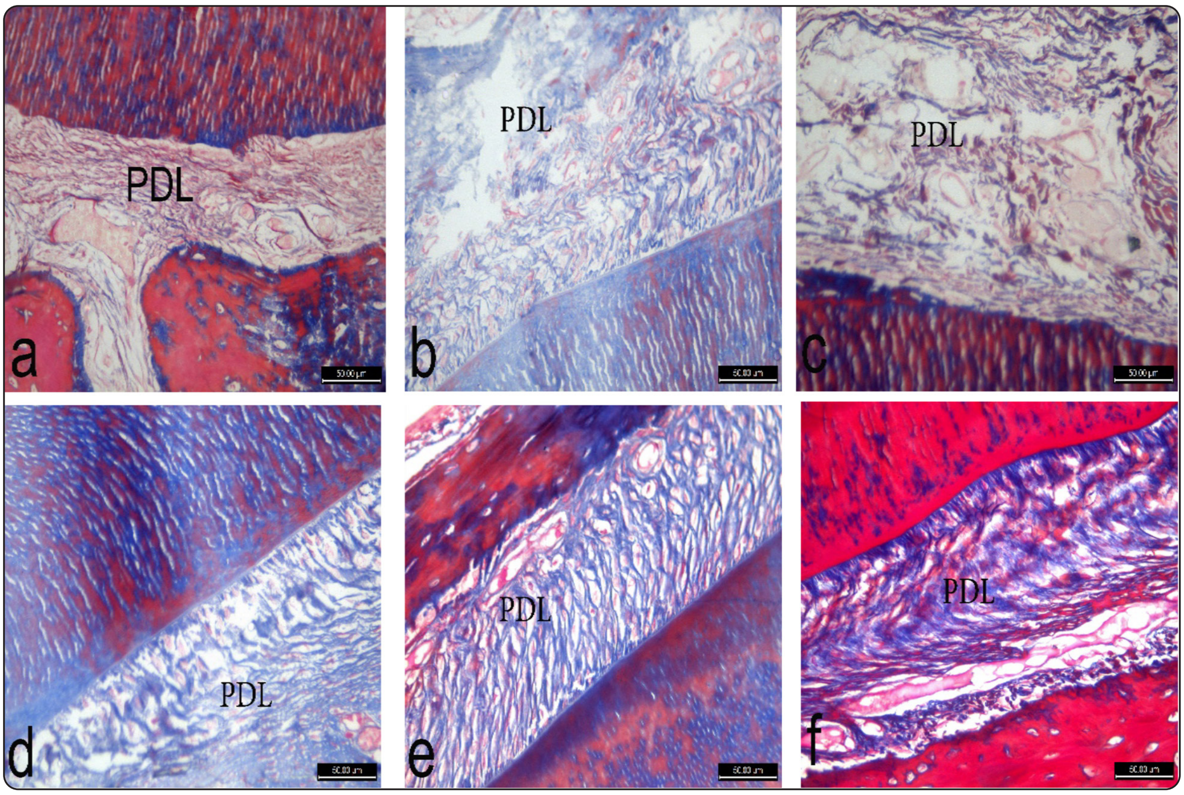

Fig. (6): Histological sections showing collagen fibers of the periodontal ligament stained with Masson's trichrome stain of group I control rats (a&b), group II EMD treated rats (c&d), group III Diode laser & EMD treated rats (e&f).

c) Immunohistochemical detection of Fibronectin

Jaw specimens of control rats showed weakly positive fibronectin reaction in the PDL fibroblasts, extracellular matrix &bone marrow cavities after 4 weeks (Fig 7, a) and negatively to weakly positive reaction after 8 weeks (Fig 7,b).While, PDL fibers of EMD treated rats presented mild to moderate positive fibronectin reaction after 4 weeks (Fig 7, c) and moderate positive reaction after 8 weeks (Fig 7, d). However, Jaw specimens of rats treated with diode laser & EMD presented moderately to strongly positive reaction after 4 weeks (Fig 7,e) and strongly positive reaction after 8 weeks (Fig 7,f).

Evaluation of fibronectin immunostaining by image analysis

A statistically significant difference between the rats of treated groups II and III when compared with those of group I using t-test. Also, the analysis of variance clarified a high significant difference (p<0.01) between group III when compared with those of group II **Table (1) & Table (2)**.

The results showed that the immunostaining of fibronectin in PDL of groups II and III rats gradually increased throughout the treatment periods when compared with group I rats. Also, there was a marked increase of fibronectin immunostaining in groups III (Diode laser & EMD) when compared with those of group II (EMD) with significant difference.

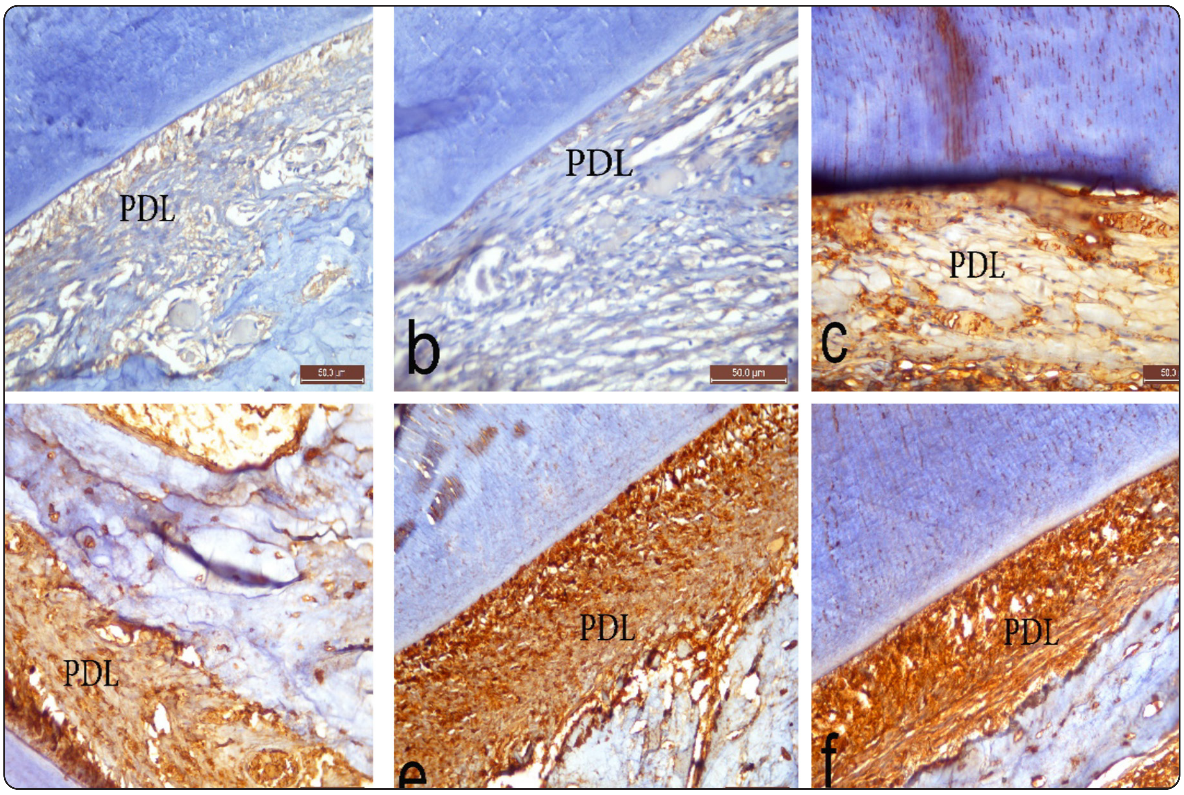

Fig. (7): Photomicrographs showing immunohistochemical localization of fibronectin in PDL & alveolar bone of group I control rats (a&b), group II EMD treated rats (c&d), group III Diode laser & EMD treated rats (e&f).

TABLE (1): Illustrates the mean labelling index of fibronectin in PDL at the different groups at 4 weeks:

		Positive Control (group I)	**Emdogain (group II)**	**t. test**	**p. value**
4 weeks	Range	121.65 – 130.66	139.31 – 151.44	9.248	0.001*
	Mean ± S. D	126.90 ± 3.50	146.39 ± 4.34		

		Positive Control (group I)	**Diode laser + Emdogain (group III)**	**t. test**	**p. value**
4 weeks	Range	121.65 – 130.66	160.54 – 176.91	17.834	0.001*
	Mean ± S. D	126.90 ± 3.50	170.10 ± 5.37		

		Emdogain (group II)	**Diode laser + Emdogain (group III)**	**t. test**	**p. value**
4 weeks	Range	139.31 – 151.44	160.54 – 176.91	9.091	0.001*
	Mean ± S. D	146.39 ± 4.34	170.10 ± 5.37		

Significant if P ≤ 0.05** * Highly significant if p ≤ 0.01***

TABLE (2): Illustrates the mean labelling index of fibronectin in PDL at the different groups at 8 weeks:

		Positive Control (group I)	**Emdogain (group II)**	**t. test**	**p. value**
8 weeks	Range	95.21 – 105.32	152.8 – 164.91	26.523	0.001*
	Mean ± S. D	101.36 ± 3.65	158.55 ± 4.39		

		Postive Control (group I)	**Diode laser + Emdogain (group III)**	**t. test**	**p. value**
8 weeks	Range	95.21 – 105.32	170.21 – 189.20	27.625	0.001*
	Mean ± S. D	101.36 ± 3.65	180.39 ± 6.63		

		Emdogain (group II)	**Diode laser + Emdogain (group III)**	**t. test**	**p. value**
8 weeks	Range	152.8 – 164.91	170.21 – 189.20	7.263	0.001*
	Mean ± S. D	158.55 ± 4.39	180.39 ± 6.63		

**** Significant if P ≤ 0.05*** **** Highly significant if p ≤ 0.01***

DISCUSSION

Delayed treatment of the avulsed tooth gives very poor prognosis due to dehydration of the tooth and loss of vitality of the periodontal ligament. So the current study aimed to find way to improve prognosis of delayed replantation by evaluating the effect of both Emdogain with and without high power diode laser irradiation on periodontal healing of replanted teeth after extended extra oral dry time.

Histologically, the application of Emdogain to the root surface and alveolar wound of replanted tooth resulted in regeneration of PDL characterized by presence well vascularized connective tissue having apparent number of viable periodontal cells and oriented collagen fibers associated with focal areas of ankylosis and others with root resorption.

The results histologically were in agreement with **Gestrelius et al** (21) who found that EMD increased the proliferation of PDL cells and protein synthesis The same was found with **Kim et al.** (5) who showed that application of enamel matrix derivative for replanted teeth can promote periodontal healing during replantation but did not prevent root resorption.

Whereas **Manuel et al.** (22) showed the effect of Emdogain gel on periodontal regeneration in autogenous transplanted dog's teeth that Emdogain gel used on the surface of transplanted teeth improves the occurrence of complete healing and reduces root resorption.

Similar pattern of results was obtained by **Schjøtt et al.** (23) who concluded that the regenerative ability of Emdogain on the periodontal ligament also worked in a trauma situation where a significant number of PDL cells have been eliminated because of unphysiologic storage or actual damage during avulsion or replantation, Furthermore if ankylosis sites already established because of earlier replantation after avulsion could be surgical removed and application of Emdogain could revert the ankylosis stage to a normal PDL situation.

In agreement with **Iqbal et al.** (24) who assessed the effect of Emdogain on periodontal healing in teeth replanted after different dry storage time and showed that lower incidence of replacement resorption in dog teeth when EMD was applied to the root surface prior to replantation ,compared with teeth not pretreated before replantation . Similar results were obtained by **Sculean et al** (25) who performed surgical periodontal treatment of deep intrabony defects with EMD to promote periodontal regeneration in vivo study. Treatment of recession-type defects with coronally repositioned flaps and EMD may promote formation of cementum, periodontal ligament and bone.

On other hand, the best regenerative features represented by well oriented and organized fibers of PDL were apparent in group III specimens with higher rate of osseous replacement when compared to groups II rats. The areas of alveolar bone and cementum resorption were reduced associated with reduction of both ankylosis and root resorption when the root surface treated with combined diode laser and EMD. These results were in accordance with **Biloklytska et al.**(26) who studied the combined usage of Diode Laser and Emdogain during the surgical phase in treatment of generalized periodontitis, they found that significant inflammatory reduction in the periodontal tissues on pre-surgical stage. The diode laser may be beneficial due to its bactericidal simultaneous effects in the preservation of marginal periodontal tissues, promotion of progressive regeneration process and long-term of generalized periodontitis .

Similar results were obtained in **Felipe et al.** (27) study who found that laser phototherapy (LPT) could be used as a new therapeutic strategy to accelerate and improve the periodontal repair process of replanted teeth when evaluated histologically the effect of LPT with different wave lengths on angiogenesis in the periodontal tissue of replanted teeth in rats. Also, the use of laser phototherapy is an important tool in controlling external inflammatory root resorption, ankylosis, osetoclasts and inflammation after replantation of teeth rats increasing the success rate of the replantation procedure[28].

Another study by **Balsam et al.** [29] who used diode laser to restore tooth supporting structure and its functional attachment through regeneration of cementum, periodontal ligament and surrounding alveolar bone. In agreement with **Mahmood et al**.[30] who reported that 940 nm of diode laser stimulate bone regeneration in dental socket after human tooth extraction using 11.9 W/cm2 power density. The same was found with **Donald et al**.[31] who revealed that clinical use of Emdogain for regeneration of periodontal defects were preferable to both patients and clinicians because of the opportunity of Emdogain to restore lost hard and soft tissues and decrease probing depth.

The results are supported by Masson's trichrome staining of the Jaw specimens of the different experimental groups which revealed that PDL fibers of EMD treated rats presented the number of fibers was increased and denser collagen fibers formed with moderate positive staining reaction. However, Jaw specimens of rats treated with diode laser & EMD presented further increase and well organized bundles of collagen fibers with strongly positive staining reaction.

These results were validated by **Felipe et al.**[32] who declared the effect of laser photobiomodulation (LPBM) on the periodontal repair process of replanted teeth, they found reduction of root resorption and increase the perimeter of periodontal repair and type I and III collagen deposition.

Also, Araujo et al. [33] who concluded that application of EMD to root surface of replanted teeth can enhance the formation of cementum with large number of collagen fibers of PDL.

The results of the current study are confirmed by the immunohistochemical detection of fibronectin of jaw specimens of EMD treated rats presented mild to moderate positive fibronectin reaction after 4 weeks and moderate positive reaction after 8 weeks. However, Jaw specimens of rats treated with diode laser & EMD presented moderately to strongly positive reaction after 4 weeks and strongly positive reaction after 8 weeks.

The same results were obtained by **Zhou et al.** [34] who reported minimum signs of inflammation were observed and periodontal ligament and cementum tissue regeneration were observed with high collagen type III, typeI and fibronectin expression.

These were in accordance with **Amorim et al**. [35] who showed that lasers therapy promote fibroblast and keratinocyte motility, collagen synthesis, angiogenesis and growth factors release, so facilitating the healing process.

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