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RESEARCH ARTICLE

EFFICACY OF PLATELET RICH FIBRIN IN COMBINATION WITH ENAMEL MATRIX DERIVATIVE ON PERIODONTAL TISSUES REGENERATION IN RATS.

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Abstract

Objectives: Evaluating the efficacy of enamel matrix derivative in combination with platelet rich fibrin on periodontal tissues regeneration in rats.

Material and methods: This experimental trail that was conducted on twenty-four adult healthy male Wistsr rats, animals were housed in separated cages as two rats per cage. Rats were kept in suitable conditions like temperature, humidity, standard food for rodents. Animals randomly allocated into 3 groups (8 rats each); G1: control group, left to heal without intervention, G2: treated by enamel matrix derivatives (EMD), G3: treated by enamel matrix derivatives plus platelet rich fibrin (EMD+PRF). Induced periodontal fenestration defects were created at buccal side of the distal root of the 1st mandibular molar and the mesial root of the 2ed mandibular molar. After surgery rats housed in a clean sterile cage and received special care. After induction of the fenestration defect by one month, four rats from each group were sacrificed. While, the remaining four rats of each group were sacrificed after another one month.

Results: Histological analysis showed that periodontal tissues had best regeneration of periodontal fibers with more mature bone formation in the EMD+PRF group (G3) comparing to other groups. In addition, other groups showed favorable regeneration of periodontal tissues.

Conclusions: Application platelet rich fibrin in combination with biological mediators such as enamel matrix protein in periodontal treatment improves regeneration of periodontal tissues.

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Introduction:-

Periodontitis is an inflammatory disease of the periodontal tissues which caused by bacterial biofilm resulting in destructions of the supporting structures of the tooth. It is considered as one of the major causes for early tooth loss.[(1)]

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It is present in all age ranges of population from children to the elderly. The traditional methods of treatment for periodontitis include education of the patient about the disease nature, oral hygiene instructions, scaling and root planning (SRP), chemical treatment and periodontal surgery if indicated. However, the final success rate of the treatment depends upon the status and maintenance of oral hygiene.[2]

The goal from periodontal treatment is to regenerate the tissues lost by infection or trauma. One of the most complicating issues of this variety of treatment has been the nature of periodontal tissues. These include both hard tissues such as the cementum, bone, and dentin, as well as soft tissues likes gingival tissue and periodontal ligament.[3]

Enamel matrix derivatives (EMD) protein plays an important role in cementum differentiation and periodontal tissues formation. According to previous vivo and vitro studies where EMD protein has been shown to be a group of proteins produced by the epithelial root sheath of Hertwig's and are able to promote periodontal regeneration.[4, 5]

EMD has been shown to have an important role in influencing the cell activities of many cells by facilitating cell association, proliferation, diffusion, survival and differentiation, as well as expression of transcript factors, cytokines, growth factors, extracellular matrix (ECM)components and other particles involved in regulating bone formation.[6] In addition, it has confirmed that EMD plays an important role in wound healing for the benefit of soft tissue regeneration and vascular activity.[7]

Platelet-rich fibrin (PRF) was developed as an improved formulation of the previously utilized platelet-rich plasma (PRP).[8] Unlike PRP, which requires the addition of anticoagulants such as bovine thrombin during initial blood collection, PRF is obtained simply by centrifugation without anticoagulants and is therefore strictly autologous. This fibrin matrix contains platelets and leukocytes as well as a variety of growth factors and cytokines including transforming growth factor beta1(TGF-β1), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), interleukin - 1 beta (IL-1β), interleukin-4 (IL-4) and interleukin-6 (IL-6).[9]

Furthermore, fibrin that forms during the final stages of the coagulation cascade, combined with cytokines secreted by platelets, makes PRF a highly biocompatible matrix especially in damaged sites where the fibrin network acts as a reservoir of tissue growth factors.[10] These factors act directly on promoting the proliferation and differentiation of osteoblasts, endothelial cells, chondrocytes, and fibroblasts .[11]

Clinical applications of PRF in dentistry are numerous, for example, the development of soft tissue root coverage [12, 13], socket preservation, bone graft protection and remodeling.[14, 15] It is also useful for membrane protection or as an osteo-conductive restorative material during a sinus-lift surgery,[16] and also useful in plastic surgery.[17] In addition, PRF used to seal cavities of defects either directly or mixed with another graft or regenerative material. [18] In implant surgical procedure, the use of PRF improves the healing quality and quantity of the hard tissue.[19] Also, PRF decrease pain, edema, accelerate healing and reduce the postoperative discomfort. Using of PRF in periodontal therapy for bone affected area of chronic periodontitis showed a better decrease in pocket depth, and increase in clinical attachment level (CAL) gain and better bone fill of the affected area. [20]

Material and methods:-

Animals:

Twenty four adult healthy male Wistar rats ranging in weight from (300–400 g) were included in this study. Rats were housed in Medical Experimental Research Center (MERC) Faculty of Medicine, Mansoura University. According to the ethical committee of the national research center-Egypt with registration number (09/189), all animals were housed in separated cages as two rats per cage. Rats were housed under ambient temperature of 25^{o}C with 50% relative humidity and a12 hour's light-dark cycle. The cages were allowed free access to water and fed on standard diet for rodents and then were allocated randomly into groups.

Groups:

The animals were divided into 3 groups; 8 rats each, all groups were subjected to the aim of the surgery and then divided into: G1: control group, left to heal without additional procedures, G2: Enamel matrix derivative group (EMD) Emdogain® *,G3:Enamel matrix derivative group plus Platelet rich fibrin (EMD+PRF). First 4 animals in

* Straumann, Basel, Switzerland.

each group were euthanized after one month of treatment and the remaining 4 animals in each group were euthanized after two months of treatment.

Surgical technique:
According to **Padial et al 2015** [21], all animals were exposed to surgery in right side of mandible and anesthetized by intra-peritoneal injection (IP) of xylazine∂ 0.1mg/kg body weight and ketamine$ 75mg/kg body weight. Animal's hair was shaved in the area of surgery and disinfected with povidine-iodine. The animal's eyes were protected by application of eye ointment.

Landmarks for the initial incision are identified by masseter muscle, parotid gland and inferior border of the mandible and labial angle. Magnifying stereoscope was used during surgery. Clean cut longitudinal incision was created (only dermal layers) by scalpel number 15, along the surface of the base of the mandible. Masseter muscle was exposed to see anatomical landmarks (parotid duct and facial nerve). The area of interest was dissected through the masseter muscle slightly under the lower ligamentous line by a second incision until the buccal plate of the body of the mandible is reached.

Once the bone was exposed and the first molar region has been accessed, a more opaque and bulbous bone region with a tear-like shape (buccal plate) became visible. According to **King et al 1997** bone fenestration defects (4 mm in width, 3 mm in length and 1 mm deep) created in the tear-like shape area by using 2 mm diameter round bur at low speed under saline solution (NACL 0, 9%) irrigation.[22] A chisel was used to denuded the periodontal ligaments, cementum and superficial layer of dentin of the distal root of the first mandibular molar and the mesial root of the second mandibular molar. The surgical area was irrigated with saline solution (NACL 0, 9%).

The experimental study groups are; G1: left to heal without additional procedures, G2: Emdogain (EMD) was applied into the created defect area with a syringe, G3: Emdogain gel (EMD) then Platelet rich fibrin (PRF) was applied into the created defect area. Finally, absorbable sutures were used to close both the muscle flap and the skin flap by using a simple interrupted suture method.

Post-operative care:
After surgery, monitoring of the rats for at least 30 minutes in a cage covered with electrical heating pads to preserve body temperature. Then, they were housed in a clean sterile cage and fed soft diet and regular food. Pain killer **§** 0.2ml intramuscular (IM) and antibioticsΔ 0.5ml/200gm (IM) were administrated by veterinarist for three days after surgery, some animals were taken additional antibiotic dose for another two days determined by veterinarist.

Preparation of Platelet rich fibrin (PRF) :[23]
Blood samples were collected direct from heart during the surgery 5ml of blood was collected and added in sterile vacutioner tube of 5ml capacity without anticoagulant. The samples were centrifuged for 10 minutes at 3000rpm. After which it settles into the following layers: lower red fraction containing red blood cells, upper straw colored cellular plasma and the middle fraction containing the fibrin clot. Then the upper straw colored layer is removed and the middle fraction which is the PRF is collected 2 mm below the lower dividing line.

Euthanasia for animals:
Four animals from each group at two different sacrifaction times (after 1st and 2nd months induction of the defect and there treatment according to their groups) were anesthetized then exposed to halothane by placing fully saturated cotton piece with halothane in locked container containing the animals. Then, the whole mandibles were collected by cutting through the cheek, dividing the temporo-mandibular joint and divided the two mandibles at the midline. The right side of mandible was fixed in 10% natural buffered formalin and decalcified in 10% natural EDTA. Then the right side of mandible is processed for: Haematoxyline & Eosin stain (H&E) and Masson's Trichrom stain.

∂ Xyla-Ject® ADWIA Co, Cairo, Egypt.
$KETAMAX-50 ® TROIKAA pharmaceutical ltd, India.
§Ketoprofen® 100mg AMRIYA pharmaceutical co, Alexandria, Egypt.
ΔAmoxicillin 500® E.I.P.I.CO. 10th of Ramadan city, Cairo, Egypt.

Computer Assisted digital image analysis (Digital morphometric study):
Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 10 X objective. The result images were calculated on Intel® Core I5® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for area, percent area measurement and object counting.

Statistical analysis:-

Data was analyzed using Statistical Package for Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation. One way analysis of variance (ANOVA) and tukey were used for comparing more than two different groups. P value less than 0.05 was considered statistically significant.

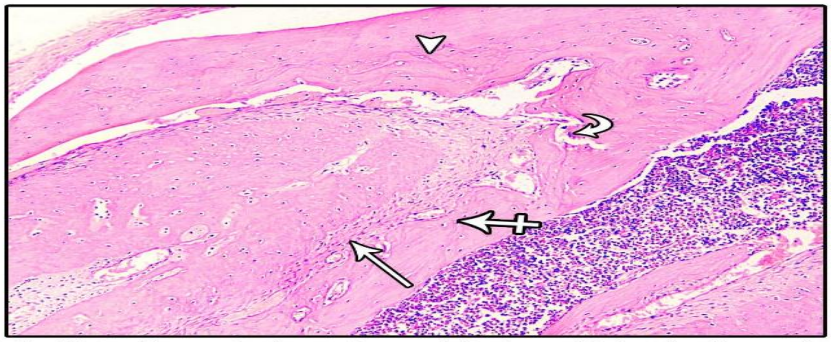
Results:-

Clinical finding:
Animals continued to feed normally and the wounds of all animals had primary wound closure, and no ingrowth of oral or skin epithelium into the defect or surrounding tissue occurred. Also, defects healed with no signs of infection.

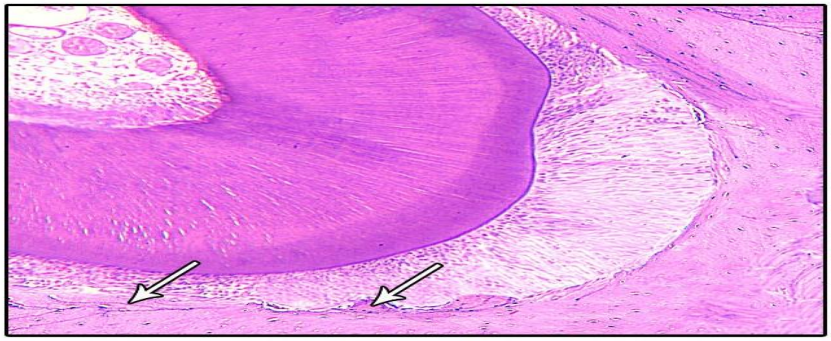
Light microscopic finding:
Haematoxyline and Eosin stain (H&E) results:
Control group (G1):
The 1st month after defect induction, the microscopic examination revealed that there was disorganization of periodontal ligament fibers and minimal regeneration of newly cellular bone formed with howships lacuna marked by reversal lines and resorptive activity (Fig.1). While, two months after defect induction, the microscopic examination revealed that there was mild spaced more organized periodontal ligament fibers, with increase maturity of bone and no signs of previous resorption (Fig.2).

EMD group (G2):
One month after treatment of the induced defect with EMD, the microscopic examination revealed that there was disorganization with wide dimension of periodontal ligament fibers, with minimal formation of new bone within the defect (Fig.3). While, two months after treatment of the induced defect with EMD, the microscopic examination revealed that there was a well-organized periodontal ligament fiber regained their normal dimension, with newly mature and organized bone filling the defect (Fig.4).

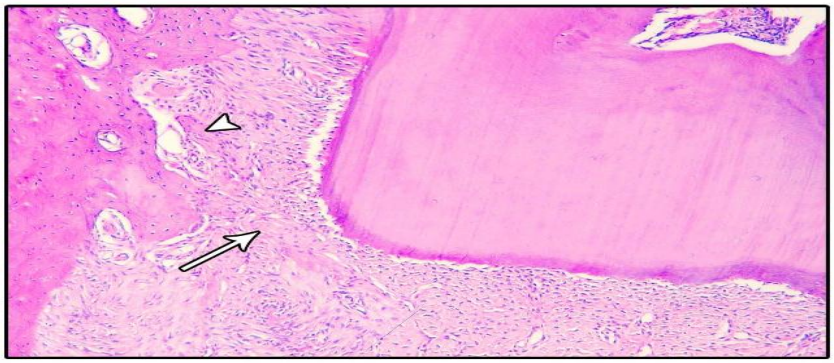
EMD+PRF group (G3):
One month after treatment of the induced defect with combination of EMD and PRF, the microscopic examination revealed that there was minimal disorganization of periodontal ligament fibers, and the newly immature formed bone has wide spaced osteocyte lacunae (Fig.5). While, two months after treatment of the induced defect with combination of EMD and PRF, the microscopic examination revealed that there was more organized periodontal ligament fibers, and more mature bone with multiple reversal lines and increase bone density which indicated by decrease bone marrow cavities (Fig.6).



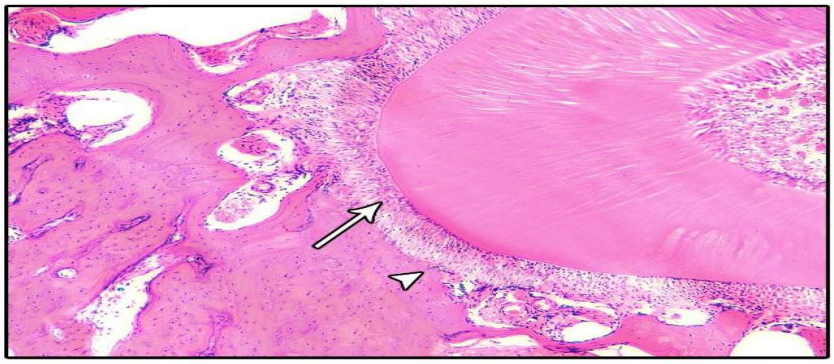
(Fig.1) : Photomicrograph of control group (after 1 month) showing disorganization of periodontal ligament fibers (arrow) and minimal regeneration of newly cellular bone formed (crossed arrow) with howships lacuna (curved arrow) marked by reversal lines (arrow head) and resorptive activity. **(H&E x100)**



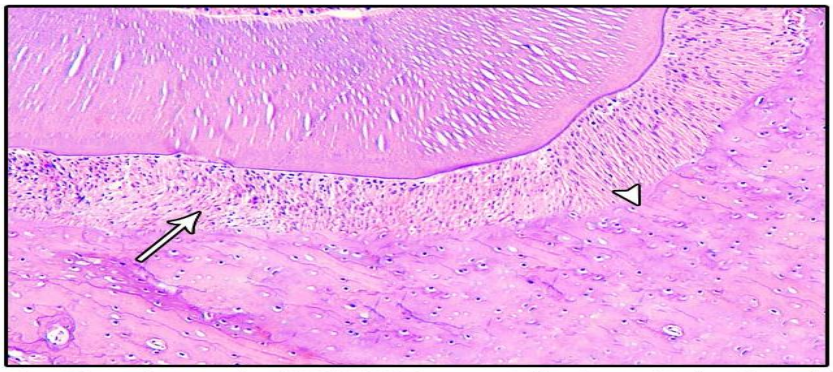
(Fig.2) : Photomicrograph of control group (after 2 months) showing mild spaced more organized periodontal ligament fibers, with increase maturity of bone and no signs of previous resorption (arrows). **(H&E x100)**



(Fig.3) : Photomicrograph of EMD group (after 1 month) showing disorganization with wide dimension of periodontal ligament fibers (arrow), with minimal formation of new bone (arrow head). **(H&E x100)**

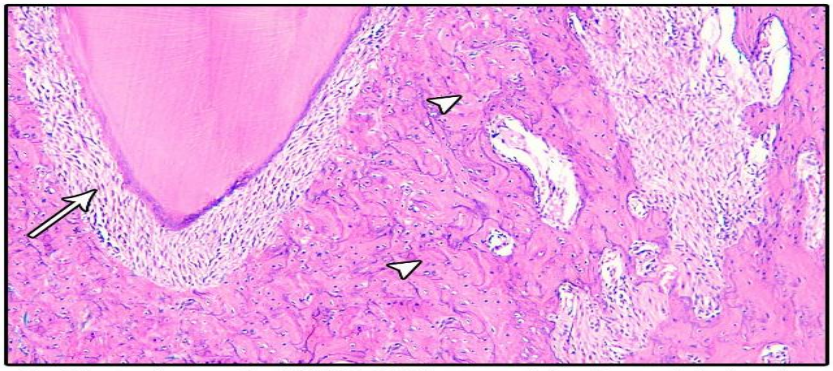


(Fig.4) : Photomicrograph of EMD group (after 2 months) showing well-organized periodontal ligament fiber regained their normal dimension (arrow), with newly mature and organized bone formation (arrow head). **(H&E x100)**



(Fig.5) : Photomicrograph of EMD+PRF group (after 1 month) showing minimal disorganization of periodontal ligament fibers (arrow), with newly immature formed bone has wide spaced osteocyte lacunae (arrow head)

(H&E x100)



(Fig.6) : Photomicrograph of EMD+PRF group (after 2 months) showing more organized periodontal ligament fibers (arrow), with more mature bone with multiple reversal lines and increase bone density which indicated by decrease bone marrow cavities (arrow heads).

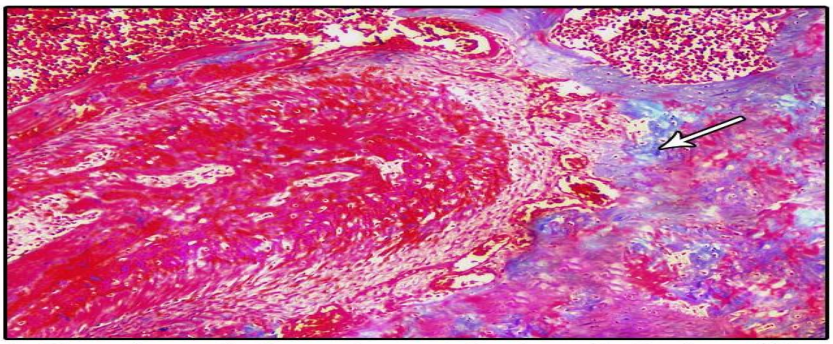
(H&E x100)

Masson's trichrome stain:
Bluish color of bone indicated immature bone with more collagen fibers, while red color of bone means it is more mature bone and little collagen fibers.

Control group (G1):
The 1st month after defect induction, the specimens revealed that there was cellular woven bone formation and increased collagen fibers content percent area of 3.039 ± 0.7720 (Fig. 7).While, two months after the defect induction, the specimens revealed that there were more mature bone and decrease collagen fibers content percent area of 2.969 ± 0.6740 (Fig.8).

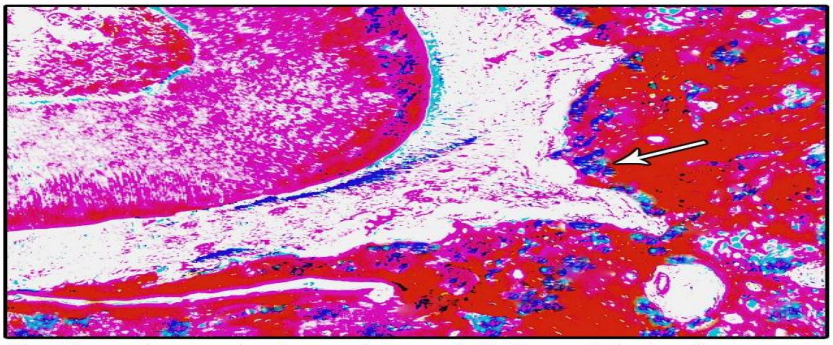
EMD group (G2):
One month after treatment of the induced defect with EMD, the specimens revealed that there was minimal new bone trabecular, the collagen content percent area of 1.016 ±0.3916 (Fig.9). While, two months after treatment of the induced defect with EMD, the specimens revealed that there was increase bone maturity when compared to 1st month, with decreased collagen fibers percent area of 0.9031 ±0.2204 (Fig.10).

EMD+PRF group (G3):
One month after treatment of the induced defect with combination of EMD and PRF, the specimens revealed that there were newly formed mature bone, and decreased the collagen fibers percent area of 0.4084± 0.1103 (Fig.11). While, two months after treatment of the induced defect with combination of EMD and PRF , the specimens revealed that there was increased bone maturity with traces of the collagen fibers, collagen amount in the bone is less when compared to G2 and G3, the collagen content percent area of 0.2217 ±0.06403 (Fig.12).



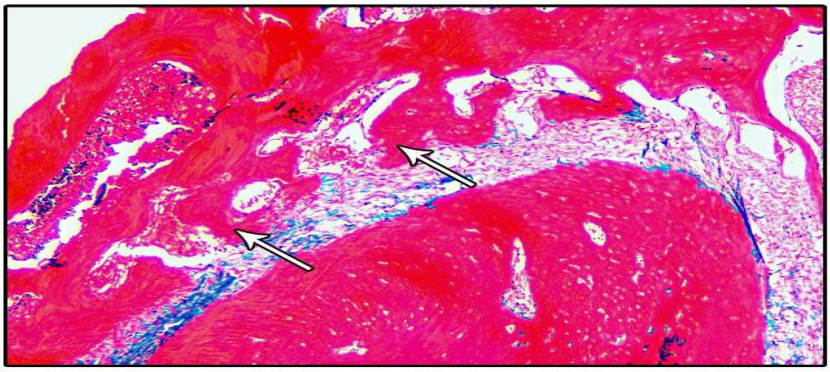
(Fig.7) : Photomicrograph of control group (after 1 month) showing cellular woven bone formation and increased collagen fibers percent area of 3.039 ± 0.7720 (arrow).

(Masson's trichrome x100)



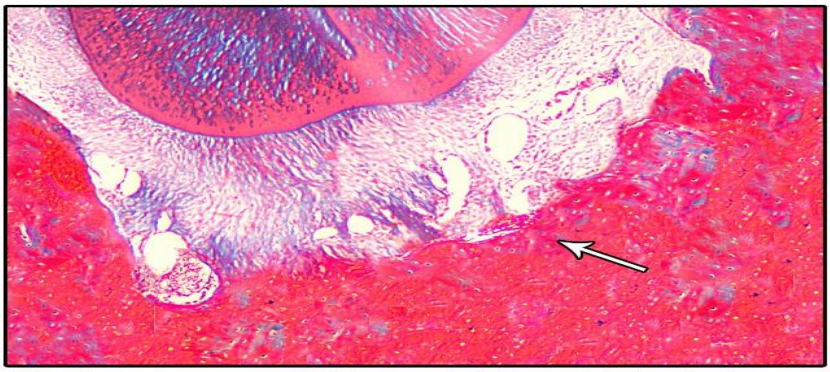
(Fig.8) : Photomicrograph of control group (after2 months) showing more mature bone and decrease collagen fibers percent area of 2.969 ± 0.6740 (arrow).

(Masson's trichrome x100)



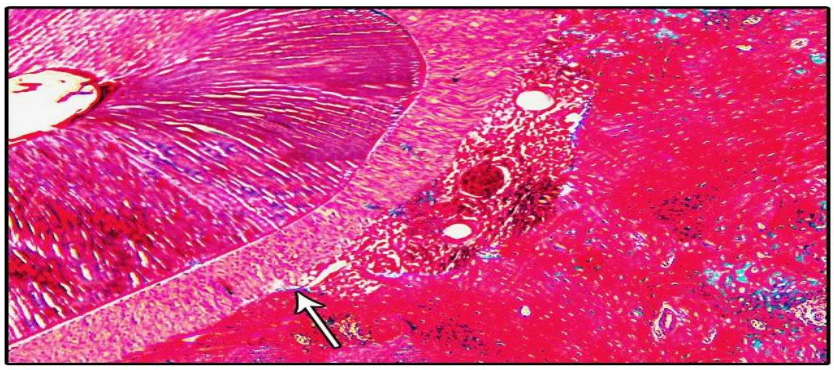
(Fig.9) : Photomicrograph of EMD group (after1 month) showing minimal new bone trabecular (arrows), the collagen fibers percent area of 1.016 ±0.3916.

(Masson's trichrome x100)



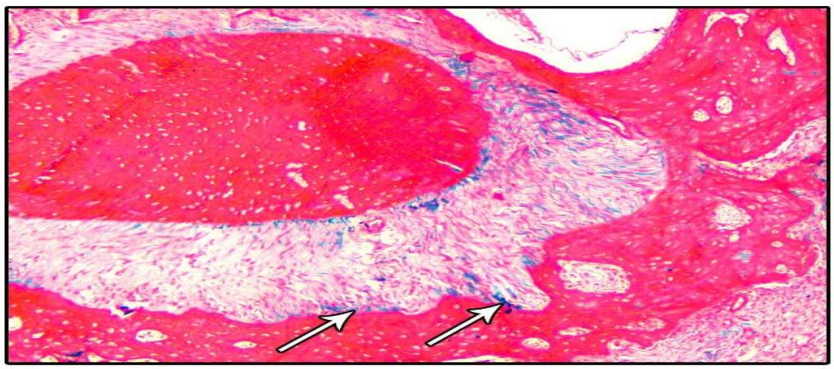
(Fig.10) : Photomicrograph of EMD group (after2 months) showing increase bone maturity (arrow) with decreased collagen fibers percent area of 0.9031 ±0.2204.

(Masson's trichrome x100)



(Fig.11) : Photomicrograph of EMD+PRF group (after1 month) showing newly formed mature bone (arrow) , and decreased the collagen fibers percent area of 0.4084± 0.1103.

(Masson's trichrome x100)



(Fig.12) : Photomicrograph of EMD+PRF group (after2 months) showing increased bone maturity with traces of the collagen fibers percent area of 0.2217 ±0.06403 (arrows).

(Masson's trichrome x100)

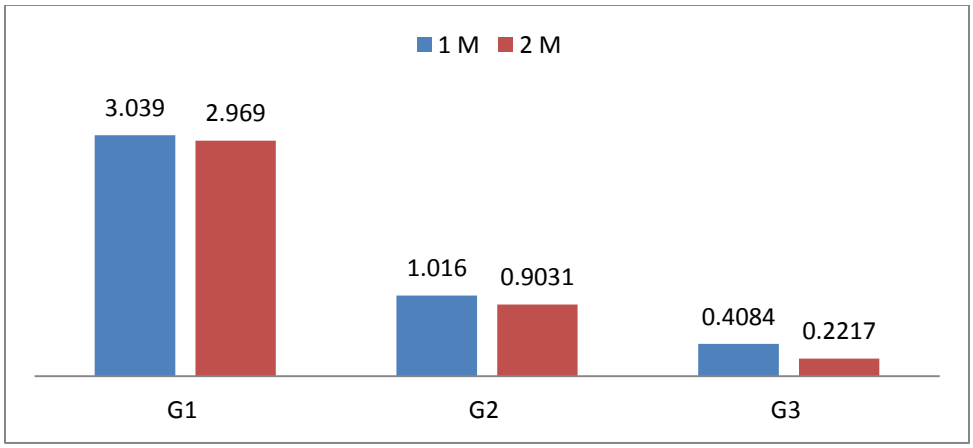
Statistical results:

1. Table 1 One way ANOVA showed significant difference in mean value of collagen in all study groups after one and two months treatment (P=<0.001*). For the control group (G1), the mean value and standard deviation of collagen was 3.039 ± 0.7720 after one month treatment and 2.969 ± 0.6740 after two months treatment.
2. It was also shown the mean value and standard deviation of collagen in EMD group (G2) was 1.016 ± 0.3916 after one month treatment and 0.9031± 0.2204 after two months treatment. In addition EMD group (G2) showed significant difference with control group (G1) after one and two months treatment (P=<0.001*).
3. The mean value and standard deviation of collagen in EMD+PRF group (G3) was 0.4084 ± 0.1103 after one month treatment and 0.2217 ± 0.06403 after two months treatment. As well EMD+PRF group (G3) showed significant difference with control group (G1) after one and two months treatment (P=<0.001*). Also EMD+PRF group (G3) showed significant difference with EMD group (G2) after one month treatment (P=0.02*) and after two months treatment (P=<0.005*).

Table 1:-Comparison of percent area of collagen among different studied groups after one and two months treatment:

		Control (G1)	EMD (G2)	EMD+PRF (G3)	P
1 Month	**Mean**	**3.039**	**1.016**	**0.4084**	**<0.001***
	±SD	**0.7720**	**0.3916**	**0.1103**	
	Posthoc		**P1=<0.001***	**P1=<0.001* P2=0.02***	
2 Months	**Mean**	**2.969**	**0.9031**	**0.2217**	**<0.001***
	±SD	**0.6740**	**0.2204**	**0.06403**	
	Posthoc		**P1=<0.001***	**P1=<0.001* P2=0.005***	

SD: standard deviation **P**: Probability ***: significance <0.05.
Test used: One way ANOVA followed by post hoc tukey test.
P1: significance relative to control Group (G1).
P2: significance relative to EMD Group (G2).



Histogram showing means value percent area of collagen in all groups after one and two months treatment.

Discussion:-

Periodontitis is a chronic condition where bacterial biofilms leads to host responses within periodontal tissues, and induces inflammatory response resulting in breakdown of the connective tissue and loss of supporting structures of the teeth.[24] The most important goal of periodontal treatment is to regenerate the tissues damaged due to inflammation and disease. To achieve success in periodontal regeneration therapy, the sealing of the junctional epithelium, the insertion of new connective tissue fibers, the formation of new cementum, and the alveolar bone restoration are needed.[25] Techniques applied in periodontal regeneration therapy include the use of tissue barriers (membranes) and bone and/or bone substitute graft materials such as autografts, allografts, xenografts and alloplasts.[26]

Periodontal regeneration still in challenges due to the unique and complex tissue structures. A new era in periodontal management using combined biological mediators to stimulate the processes required for the regeneration of periodontal tissues.[27] Therefore, the aim of the present study was to investigate the efficacy of combination of enamel matrix derivative with platelet rich fibrin on the regeneration of periodontal tissues.

In this study, rat model was used due to the similarities of periodontal molar anatomy of rats to human peridontium.[28] Since histological studies are not possible in human because of the need to save the teeth and the surrounding periodontium.[29] So, using animal model in periodontology for testing periodontal regenerative procedures are necessary because of the controlled quantitative histological analysis is required to evaluate the quantity, quality, and extent of the newly formed supporting tissues which require large blocks to be available.[30] Furthermore, periodontal induction defect was created according to **King et al 1997** [22] whom reported that this type of induced defect is useful to study periodontal regeneration and wound healing.

However, to the best of our knowledge, there is no enough information found in the literature from in vivo studies about the use of PRF in combination with EMD and the demonstration of their histological effects on the regeneration of lost periodontal tissues. Enamel matrix dervites (EMD), and platlete rich fibrin (PRF) were used as regeneration promoting materials in this study design. Study groups used in order to clarify the effect of each tested material when used alone (as in G2: EMD) and when used in combination with PRF (as in G3: EMD+PRF) and compare their results together and to the control group (G1). Therefore, in this study, PRF was used in combination with EMD to test whether this combination could/couldn't achieve better regeneration outcomes of lost periodontal tissue. Additionally, to test whether this combination could improve and/or accelerate the periodontal regeneration in surgically created defects in rats.

Histological observation in EMD treated group showed that was disorganization with wide dimension of periodontal (PDL) fibers, with minimal formation of new bone within the defect after one month post treatment, and was a well-organized PDL fiber regained their normal dimension, with newly mature and organized bone filling the defect after two months post treatment. These results could be explained as EMD plays a significant role in cell production and in growth factor creation. Furthermore, used EMD alone (G2) showed more regeneration in periodontal tissue with statistically significant difference when compared to control group (G1). This could be explained by the role of EMD in growth factor production and proliferation of osteoblast and periodontal ligaments cells.[4, 32, 33] In addition, EMD group result showed statistically significance difference with EMD+PRF group. This result was in agreement with that of **Correa et al 2016.**[31] They reported that less defect fill in non-treated control group compared with EMD treated group.

The results of our study showed that periodontal tissues showed the best regeneration of PDL fibers with more mature bone formation in the EMD+PRF group (G3) when comparing to other groups. The histological finding showed that there was minimal disorganization of PDL fibers, and the newly immature formed bone has wide spaced osteocyte lacunae after one month post treatment, and more organized PDL fibers, and more mature bone with multiple reversal lines and increase bone density with decreased bone marrow cavities after two months post treatment. This could be explained by the role of amelogenin protein in promoted both PDL and bone cell attachment, and ability of PRF to proliferation of a variety of cells including endothelial cells, gingival fibroblast, chondrocytes, and osteoblasts, there by heavily promoting tissue repair and angiogenesis.

This result was in agreement with **Turkal et al 2016.**[34] This clinical trial used PRF and EMD in the treatment of intrabony defect. According to these results, PRF+EMD combination (G3) has been found to be a promising regenerative material in treatment of periodontal defects. It has showed statistically significant difference when comparing with control group (G1) and EMD group (G2).

Within the limitations of this study was small number of animals used to participate in this experimental study. Thus, large animal numbers and histological blocks are recommended to confirm our results. Also, early two weeks and long term follow up is recommended to show early as well as late changes of tissue regeneration in response to these different treatment modalities.

Conclusions:-

Application platelet rich fibrin in combination with biological mediators such as enamel matrix protein in periodontal treatment improve regeneration of periodontal tissues.

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