Histological Evaluation of the Effect of Melatonin Gel in the Treatment of acute one-wall intrabony defect in Dogs

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Abstract: Recently, importance has been given to the use of melatonin for predictably obtaining periodontal regeneration. This experimental study was conducted to investigate the resulting histological regeneration after the use of melatonin gel in the treatment of induced periodontal one-wall intrabony defects in Dogs. ***Methods:*** One-wall infrabony defects (4x4mm) were surgically created in the mesial aspect of second premolars bilaterally (split mouth study) in 8 dogs. Each intrabony defect underwent one of 2 treatment modalities: melatonin gel/ collagen sponge (experimental site group I) or placebo gel (methyl cellulose)/ collagen sponge (control site group II). Four animals (8 defects 4 defects from tested side and 4 from control side) were sacrificed with an overdose of anesthesia at one month post-surgically and block sections (8 specimens) of the defects were collected for histological and histometric examinations. At 3 months, the other four animals were sacrificed to obtain another 8 block sections for the same purpose. **Results:** ***At one month,*** melatonin treated specimens showed moderate amount of newly formed bone, newly formed cementum, poorly organized PL fibers, with no epithelial down growth was observed. On the other hand, the surgical control specimens showed epithelial down growth along the root surface, and minimal amount of bone formation at the apical part of the defect. ***At three months***, histological results of group I (melatonin treated group) revealed true periodontal regeneration that demonstrated similar features to the native periodontal structures found apical to the notches; no epithelial down growth was observed. Well organized, functionally oriented periodontal ligament fibers were observed with plumps of fibroblast cells after melatonin treatment. Surgical control group showed similar histologic features that recorded at one month with limited amount of bone and osteoid tissue confined to the apical portion of the defect. **Conclusion**: Results of the present study indicated that, the use melatonin is advantageous in stimulating periodontal regeneration.

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Keywords: Melatonin; One-wall infrabony defects, Periodontal regeneration.

Introduction

Periodontal disease (PD) is an inflammatory process affecting the alveolar bone, gums, and periodontal ligament. **(**1**).** The damage of periodontal tissues results from a direct effect of the toxic products released by the bacteria, and from the action of the immune system stimulated by the bacterial infection (2). An important feature in PD is the generation of free radicals, some of which derive from the bacteria themselves, and others originate from the immune response **(**3). Free radicals generated by the phagocytic cells, e.g., neutrophils and macrophages, migrate to the inflammation site, and significantly damage the gingival tissue (4,5). The imbalance between the pro-oxidant and antioxidant systems may lead to a further oxidative attack and substantial deterioration of the periodontal tissues (6,7).

Melatonin (N-acetyl-5-methoxytryptamine) is one such powerful hormone derived from an essential amino acid tryptophan (8). It has various local and systemic functions playing a critical role in controlling inflammatory reactions. It is predominantly synthesized and secreted from pineal gland and other extrapineal sources like retina, gastrointestinal tract, lens, and immune system cells (9).

Melatonin is a noteworthy free radical scavenger and a broad-spectrum antioxidant (10,11,12). Several studies proved that melatonin could directly neutralize a variety of reactive oxygen species (ROS) including superoxide anion radical O-2, Hydrogen peroxide H2O2 and the hydroxyl radical –OH (13,14). Increased ROS scavenging by melatonin and its metabolites in the inflamed area would be beneficial in reducing the degree of tissue damage. Additionally, melatonin influences fibroblast activity and bone regeneration by promoting osteoblast differentiation and bone formation and suppression of bone resorption (15, 16). Moreover, it stimulates the synthesis of type I collagen fibers (17).

Furthermore, melatonin mediated down regulation of RANKL-mediated osteoclast formation

and activation. Furthermore, melatonin was shown to increase the expression of bone sialoprotein as well as other essential bone marker proteins including alkaline phosphatase ALP and osteocalcin, in addition, stimulate both osteoblast differentiation and mineralization (18). Those positive effects of melatonin and its derivatives on inflammatory mediators and bone cells may be beneficial in improving periodontal health (1,19).

Yamazaki *et al.*,(20) reported that, daily Intraperitoneal injection of melatonin increases peri-implant osteogenesis in the rabbit femurs, suggesting that melatonin is a useful agent to stimulate bone wound healing. Melatonin also has been shown to inhibit the inflammatory enzyme cyclooxygenase-2 (COX-2). Melatonin reportedly binds to the active sites of COX-1 and COX-2 indicating that it may act as a natural inhibitor of the function of these enzymes and thereby be an endogenous inhibitor of inflammation (21, 22) .

Cutando *et al.,* (16) proved that salivary melatonin levels may vary according to the degree of periodontal disease. A negative association was found between salivary melatonin levels and periodontal disease severity. Consequently, decreased saliva and melatonin production with age predisposes older individuals to increased risk of oral and periodontal diseases (19).

A recent study (23) showed that melatonin strongly suppresses nitric oxide (NO) and interleukin-6 (IL-6) production induced by lipopolysaccharide (LPS) from *P. intermedia*, a major cause of inflammatory periodontal disease

Despite the multiple properties of melatonin, its effect in periodontal health warrants further investigation. So the present study was conducted to evaluate the effect of melatonin on the healing of surgically induced one-wall infrabony defect in dogs.

Animal Model selection*:*

A total of 8 adult male mongrel dogs weighing from 20-25 kg were selected from Physiology Department, Faculty of Medicine, Tanta University to be used in this study.

Gel preparation

Melatonin gel was prepared from melatonin powdered at a concentration of 1% (Amon Pharmaceutical, Ltd),in the faculty of Pharmacy Phytochemistry department.

Surgical protocol

- The animals were anaesthetized using sodium thiopental IV (12 mg/kg b.wt.). Bilateral mandibular first premolar P1 were extracted prior to experimental surgery and the extraction sites were allowed to heal for 2 months.
- After healing buccal mucoperiosteal flaps were elevated, and 1- wall intrabony defects (4x4mm) were surgically created with burs in the mesial aspect of second premolars bilaterally(split mouth study) fig (1). -A reference notches were created with a round bur on the root surfaces one at crest of the alveolar bone and another one at the base of the defect to act as a guide for histologic evaluation. The roots surfaces were carefully scaled, planned, flushed with sterile saline, and then dried with sterile gauze.

Animals grouping and treatment:-

Each intrabony defect underwent one of two treatment modalities: melatonin gel/ collagen sponge (experimental site group I) or placebo gel (methyl cellulose)/ collagen sponge (control site group II). A sterile collagen sponge was cut into 3 x 3 mm, and soaked in 1ml of melatonin gel, or placebo gel according to the treatment modality. The soaked sponges were fitted into the created defects, and closure of the wound areas were performed by interrupted interdental suture fig (1).

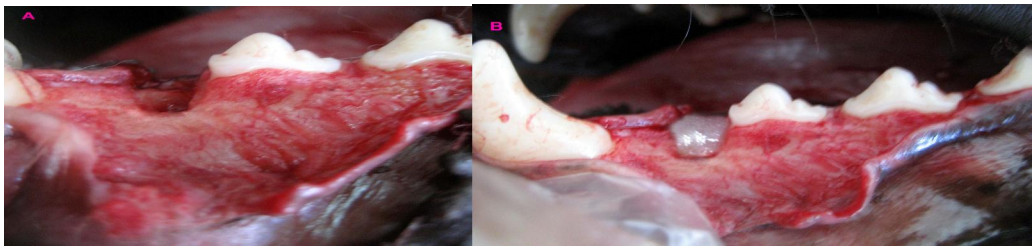

Fig 1:a) Shows 1-wall intrabony defect surgically created in the mesial aspect of the lower second premolar, b) melatonin gel in a collagen sponge implanted in the defect area.

Postsurgical care:-

After the operative procedures the dogs received Voltaren‡ 25 mg IM every 12 hours for pain control for 2 days. Intra-muscular administration of antibiotics, an intramuscular injection of penicillin (1.5 mL – 150,000 IU) in the first two days postoperatively after that mixed with dog's food for 7 days. The dogs were kept in separate cages, and a daily topical application of 0.12 % CHX solution during the healing period was performed. The animals were observed daily until suture removal and at least twice weekly thereafter and they were fed a

soft diet throughout the study evaluation period to reduce the chance of mechanical interference with healing during food intake

Histopathologic Examination:-

The animals were sacrificed for histological examinations according to the following order:

- Four animals were sacrificed at one month post-surgically (4 dogs/ 8 defects).
- Four animals were sacrificed at 3 months post-operatively (4 dogs/ 8 defects). Scarification was done with an overdose of anesthesia.

The treated experimental mandibular P2 teeth (16 defects) with the surrounding bone were dissected free mesially and distally, fixed in 10 % formalin for 10 days, decalcified in EDTA, dehydrated in ascending grades of ethanol (70%, 80%, 90%, 100%) and cleared with xylene. Specimens were then kept in paraffin wax (the paraffin wax melting point should not exceed more than 56 ºc to avoid the burning of the tissues) for 24 hours and then embedded in paraffin blocks. All formalin-fixed-paraffin-embedded tissues were serially cut mesio-distaly with rotary microtome at 5 µm thickness. The mesio-distaly sections should represent the central part of the furcation. The sections were then placed on glass slides and incubated overnight at 60ºc, after which they were rehydrated in xylene, rinsed in ethanol, and then water. The slides were stained with basic hematoxylin stain and counterstained with eosin for contrast and evaluation.

Histomorphometric analysis:- Fig (2).

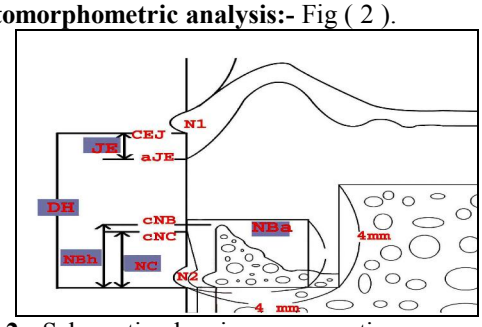

Fig 2: Schematic drawing representing parameters for histometric analysis. CEJ: cemento-enamel junction, aJE: apical end of junctional epithelium, cNC: coronal extension of new cementum, cNB: coronal extension of new bone, DH: defect height, JE: junctional epithelium migration, NC: new cementum, NBh: new bone height , NBa: new bone area in percentage.

For histometric analysis of the tissues formed during the healing of the infrabony defects, the histologic sections were photographed and digitized with a light microscope ♪ coupled to a digital camera♪♪, at a lenses magnification of ×25 that connected to a monitor and a personal computer, using the fornix of the furcation and the root notches performed on the mesial and distal root surfaces as reference points (24).

Linear and area measurements were performed after verification of anatomical landmarks at high magnification, and by outlining the borders of the various structures with a mouse cursor.

This analysis was performed under blinded conditions by one of the coworkers not involved in the study.

-The following measurements were performed to describe the defect and the tissues in the defect:-

1. The total area of the furcation defect (TDA) were measured in millimeters squared.
2. The defect height (DH): the distance between the two reference notches in millimeters .
3. Percentage value of newly formed bone height (NFBH) (new bone height / defect height × 100). (% NFBH= NFBH/ DH× 100).

♪ . Olympus BX50, Optical Co. LTD , Japan. ♪♪: C5060WZ, Sony Electronic Inc., Tokyo, Japan.

4. The percentage value of area of newly formed bone (NFBA) (newly formed bone area/ total defect area ×100). (% NFBA= NFBA/TDA× 100).
5. The percentage value of the newly formed cementum NFC :The distance from the coronal extent of the new cementum to the apical extent of the second notch NFC /defect height x 100).
6. The relative proportion (the percentage value) of the newly formed periodontal tissues NFPL, representing an area coronal to the notch by measuring the length of the newly formed periodontal ligament LNFPL / length of the of the root from the fornix of the furcation to the bottom of the notches(furcation circumference FC) ×100. (% NFPL= LNFPL/FC× 100).
7. Junctional epithelium (JE): The distance from the coronal extent of the junctional epithelium to the apical extent of junctional epithelium/ DH X 100. (% EDG = LEDG/ DH ×100).

The statistical tests used for data analysis:

The collected data were organized, tabulated and statistically analyzed using SPSS soft ware statistical computer package version 16. For quantitative data, the mean and standard deviation were calculated. Chi-square test used for comparing the significance of the effect of the treatment modalities (surgery+ melatonin gel, surgery only (control) on histometric measurements of regenerated tissues after treatment.

Results

Histological observations

At one month post-operatively, the newly formed woven bone trabeculae were thin, exhibited

numerous osteoblasts and osteocytes and narrow marrow spaces. Area of osteoid tissue was also observed with no evidence of epithelial down growth in melatonin treated specimens Fig (3). The histometric results at one month are shown in table (1) fig 5. The defects treated with melatonin were not completely filled by newly formed bone with mean NFBH 49.75 ±4.11 of the defect height. The mean NFBA occupied about 41 ± 4.54 of the defect area. (Fig 3).

On the other hand, the control treated defects were filled by granulation tissues with epithelial downgrowth (healing by long junctional epithelial) along the side of the root extend to about 10.93 ±1.65 of the defect height, with minimal amount of newly formed bone reaching the notch area with mean NFBH (25.75 ±4.34) and (NFBA 23.75 ±4.78) and inflammatory cells infilteration was observed Fig (4).

Healing of melatonin group revealed a thin layer of acellular cementum with length of (39 ± 4.54) as compared to the control treated group (26.25 ± 7.50) but there is no significant differences was observed at one month (P>0.05). Compared to the control treated group melatonin treated group showed well organized periodontal ligament fibers with no inflammatory cells. Taken together, the melatonin treated group showed superior significant healing features as regard NFBA, NFBH, and NFPL with no epithelial down growth than control group (P<0.05) Table (1).

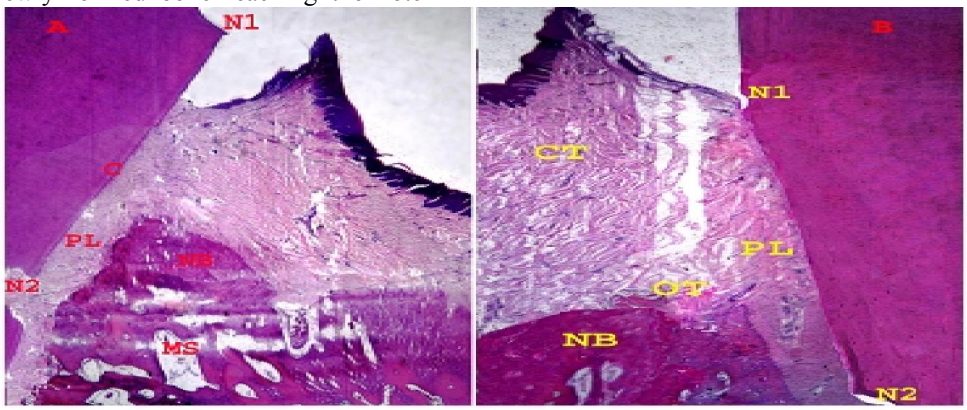

Fig 3:A) photomicrograph of melatonin treated defect after 1 month post-operatively, showing not well organized periodontal ligament fiber PL, limited amount of new bone NB (H&E X40) . B) Another photomicrograph, showing a thin layer of acellular cementum AC, and new bone above the notch NB lined by area of osteoid tissue OT , moderately organized highly cellular periodontal ligament fiber PL. (H&E X40)

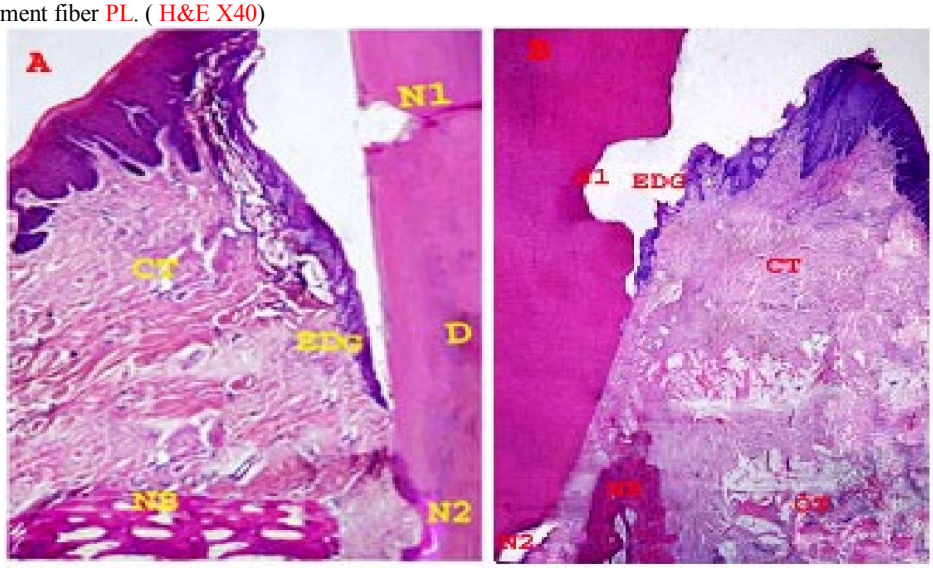

Fig 4 :A) Photomicrograph of control treated defect after 1 month postoperatively, showing epithelial down growth EDG, connective tissue adhesion CT, limited amount newly formed bone reaching the notch area NB, slight amount of new cementum (H&E X40).B) Another photomicrograph showing epithelial down growth EDG, connective tissue adhesion CT, limited amount newly formed bone reaching the notch area NB, osteoid tissue OT, chronic inflammatory cells infiltration (H&E X40).

Table (1): effect of different treatment modalities on (%) histometric measurements of tested parameters at one month post-operatively.

Parameters tested	Melatonin treated group I N=4	Control treated group II N= 4	X^2	*P*. value
	Mean ±SD	Mean ±SD		
% NFBA	41 ± 4.54	23.75 ± 4.78	**5.647**	**0.011***
% NFBH	49.75 ± 4.11	25.75 ± 4.34	**9.537**	**0.002***
% NFC	39 ± 4.54	26.25 ± 7.50	**2.735**	**0.072**
% NFPL	29.37 ± 4.26	14.75 ± 2.06	**11.720**	**0.001***
% EDG	0 ± 0	10.93 ± 1.65	**15.963**	**0.001***

NFBA = newly formed bone area NFBH = newly formed bone height NFC = length newly formed cementum NFPL = length of newly formed periodontal ligament EDG = length Epithelial down growth

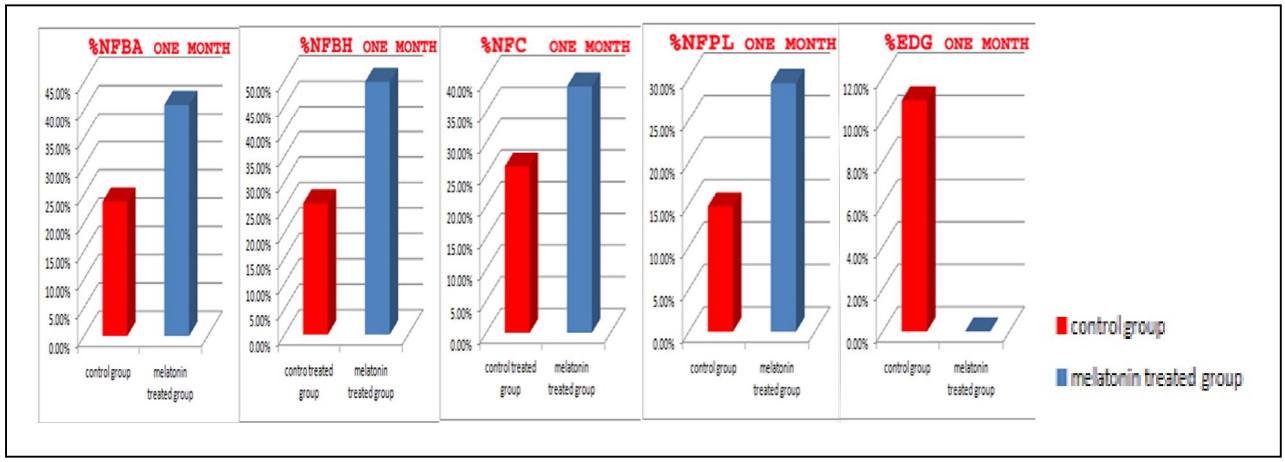

Fig 5 : shows the effect of treatment modalities on % histometric measurements at one month post-operatively.

Three months postoperatively:

Healing was clearly observed in this period. Melatonin treated group showed new attachment with no evidence of epithelial downgrowth. Thick new lamellar bone filled almost the defect completely with narrow marrow spaces (Fig 6, 7). Resting line of bone deposition was also noted (Fig 7). Thin layer of acellular cementum with cementoblasts aligned along the cementum was observed in certain area. Highly vascular, cellular (plumps of fibroblast cells) and well organized periodontal ligament fibers were also observed (Fig7). On the other hand, three months post operatively, control treated group showed epithelial growth. The newly formed cementum disturbed, deficient and thin. In the middle portion of the lesion there was little amount of bone regeneration, this bone tissue seemed to be growing due to the great number of cells (osteoblast, osteoid tissue) and blood vessels Fig (8).

Data analysis showed significant differences between the treated group regarding the newly formed bone height (82.55 ± 2.06 and 33.75 ± 8.53mm for melatonin and control treated groups respectively) *P*<0.01. In addition, inter-group analysis demonstrated that melatonin treated group presented superior length of newly formed cementum (78.90 ±3.72 and 33.25 ± 2.98 for melatonin and control treated groups respectively) *P*<0.01.

Concerning the newly formed bone area melatonin treated group showed significant amount of newly formed bone area than the control treated group (83.72 ± 3.71 and 34.75± 4.64 respectively) *P*<0.01. Data analysis additionally demonstrated that the extension of periodontal ligament was greater for melatonin treated group (table 2, Fig 9). By comparing the two experimental groups with respect to epithelial down growth, data analysis showed no epithelial down growth for melatonin while (6.75 ± 0.64 mm) for control treated group (*P*<0.01).

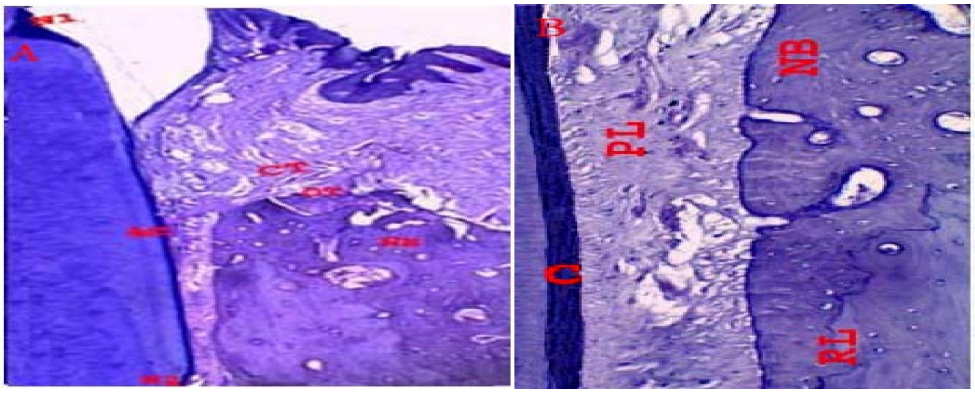

Fig 6: Photomicrograph of melatonin treated defect after 3 month postoperatively, showing a thin layer of acellular cementum C, and new bone above the notch NB, functionally organized highly vascular periodontal ligament fiber PL. (H&E X40)B) Higher magnification shows highly vascular and cellular functionally oriented periodontal ligament fiber PL, Reversal line also observed RL, and newly formed lamellar bone NB (H&E X200).

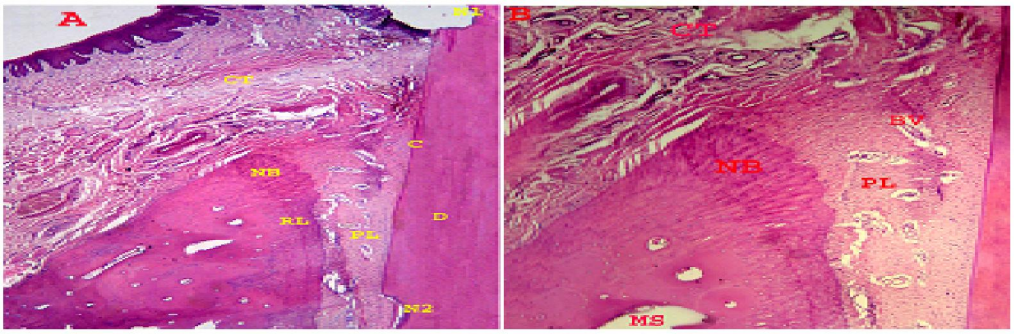

Fig 7:A) Photomicrograph of melatonin treated defect after 1 month postoperatively, showing a thin layer of acellular cementum AC, and new bone above the notch NB lined by area of osteoid tissue OT , moderately organized highly cellular and vascular periodontal ligament fiber PL. (H&E X100). B) Higher magnification of the previous section A (H&E X200).

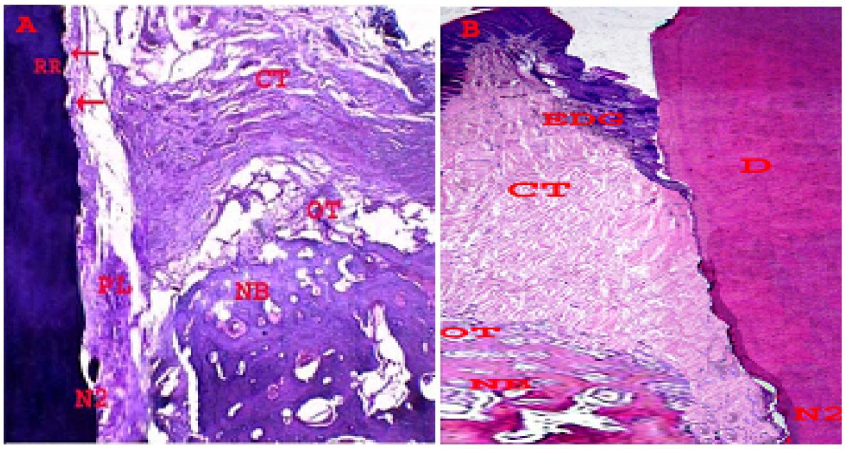

Fig 8: Photomicrograph of control treated defect after 3 month postoperatively, A) showing not well organized periodontal ligament fiber PL, limited amount of new bone NB covered by layer of osteoid tissue OT, area root resorption RR (H&E X100). B) Another section show epithelial down growth EDG, limited amount newly formed bone reaching the notch area NB, and area of osteoid tissue was observed, slight amount of new cementum (H&E X40).

Table (2): Effect of different treatment modalities on (%) histometric measurements of tested parameters at three month post-operatively.

Parameters tested	Melatonin treated group I N=4	Control treated group II N= 4	X^2	*P*. value
	Mean +SD	Mean +SD		
% NFBA	83.72 + 3.71	34.75+ 4.64	12.446	0.001*
% NFBH	82.55+ 2.06	33.75+ 8.53	10.325	0.002*
% NFC	78.90 + 3.72	33.25+ 2.98	17.466	0.001*
% NFPL	80.75+ 3.50	30.50+ 3.41	17.883	0.001*
% EDG	0 + 0	6.75+ 0.64	14.325	0.001*

NFBA = newly formed bone area NFBH = newly formed bone height NFC = length newly formed cementum NFPL = length of newly formed periodontal ligament EDG = length Epithelial down growth

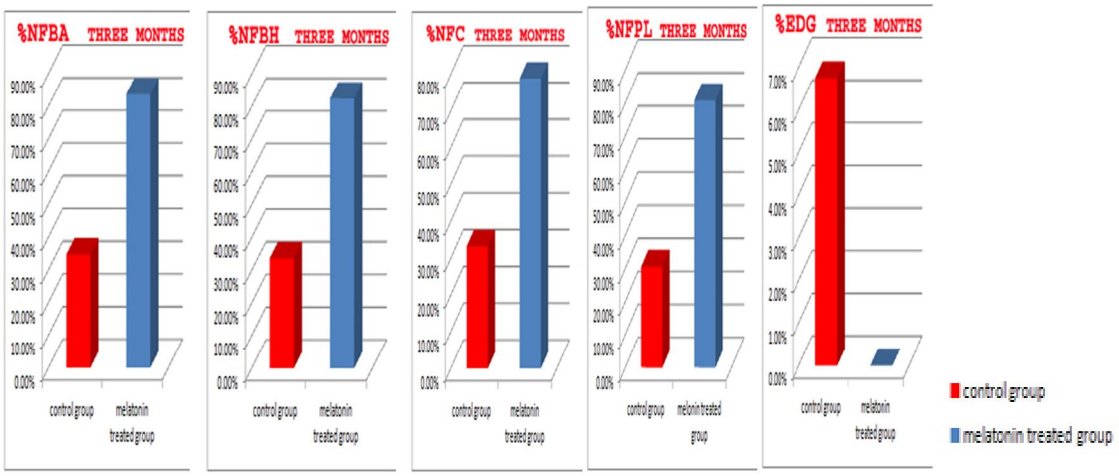

Fig 9 : shows the effect of treatment modalities on % histometric measurements at three months post-operatively

4. Discussion

Free radicals have been implicated in many pathophysiologic processes of the oral cavity (25, 26). It was demonstrated that salivary melatonin excretion has a protective role (1). Although melatonin known as a biomimic agent which act as antioxidant, immune enhancing, promoter of fibroblasts proliferation and bone remodeling , it is still not widely used and applied in treatment of periodontal diseases. The present study was carried out to evaluate the histological and histometrical observation of the effect of melatonin 1% gel on the treatment of one wall infrabony defect as compared to placebogel.

Melatonin a noval indolamin has been found to be a significant modulator of the metabolism of calcium, and prevents osteoporosis and hypocalcemia in certain cases, probably due to its interaction with other bone regulatory factors, such as parathormone, calcitonin or prostaglandins (27,28)

Melatonin is non toxic when administered in local or systemic forms (29). Additionally melatonin reduces the progression of alveolar bone destruction as demonstrated in many studies that there is a link between melatonin and bone metabolism, where melatonin act as a local growth factor (18, 15, 30).

Since histological evaluation remains the only reliable method to determine the efficacy of periodontal therapies (31), therefore, the present study was employed in an attempt to evaluate the healing of surgically created infrabony periodontal defects in dogs after treatment with melatonin gel in a collagen sponge histologically and histomor-phometrically. It is well accepted that healing of intrabony defects is positively correlated to the number of bone walls limiting the defect. One and 3 wall intrabony defects appear to be reproducible models to evaluate candidate technologies for periodontal regeneration (32). The box-type one-wall intrabony defect model in dogs is a well-established model and has been used to evaluate the effect of particular biomaterials on periodontal regeneration (32, 33).Therefore, in this study,1-wall defects with

only an interproximal bony wall which had minimal self-healing capacities were used.

The standardized surgical defect size in acute model allows equal conditions for healing. In addition, this defect is less time and money consuming (34). Furthermore, this model is suitable for investigation of new material, drugs or substances to determine their safety and establish the risk of adverse reactions during periodontal repair process (35). Isidor *et al.,* (36) demonstrated that, there is no difference regarding the reformation of connective tissue attachment whether this has been lost because of periodontal disease or mechanically removed (acute defect).

To apply gel phase strontium into1-wall infrabony defect, a carrier is required; therefore, collagen sponge was used as a carrier. Its fibrillar meshwork structure makes it a conductive scaffold for colonization by host cells from periodontal ligament, and it also act as a chemotactic to periodontal ligament cells (37,38).

In the current study, there was no evidence of unusual reactions such as aberrant healing, ankylosis or root resorption. This finding confirmed the clinical safety of melatonin gel as regenerative material. It is also explained the potential effect of melatonin gel on stimulating the coronal migration of periodontal ligament cells, which repopulated on the root surface, and prevented ankylosis by a relatively higher rate of new cementum formation and PDL space maintenance (39).

At one month of wound healing, it is worthy noted that, melatonin treated defects showed aspects that could indicate that; the regenerated tissues were in a process of formation and/or remodeling. These included a line of non-mineralized matrix around the bone tissue, presenting an osteoid tissue characterizing a process of bone apposition was also seen.

On the other hand, in the control defects, the amount of new attachment and bone formation was very limited and unpredictable. Additionally, migration of the junctional epithelium along the root was also observed. Similar findings are obtained from extensive histologic animal studies demonstrating that, following flap surgery alone, the healing is mainly of a reparative type (long junctional epithelium) (40,-41**).**

Shedding the light on the histologic findings of 1-wall infrabony defects receiving melatonin at three months, revealed an impressive coronal bone formation, almostly filling the defects were observed. These observations were confirmed by the results of the histomorphometric analysis as it is found that the sites treated with melatonin showed amount of new bone of (3.89±0.09,) when compared with the control treated sites (1.17±0.63).

These results supported the positive augmentation regenerative effect of melatonin gels obtained through their unique mode of action as a matrix enhancement factors by creating a positive environment for fibroblast proliferation, osteoblasts differentiation matrix synthesis, increased alkaline phosphatase and osteocalcine production (**18, 42**). Additionally, it was demonstrated that, in vitro application of melatonin up regulated the expression of OPG and down-regulated the expression of RANKL in mouth osteoblast line cells MC3T3-E1 consequently, inhibition of osteoclasts maturation, differentiation, and activation (15). Another important contributing factor for the marked amount of newly formed bone may be related to the collagen sponge which acts as a scaffold for colonization by PL cells and space maintaining material preventing flap collapse (**39**). It may also prolong the osteoinductive effect of melatonin gel by preventing the washout and provide the slow release into the defect (**43**).

In this regard, several studies have shown that melatonin stimulates the proliferation and differentiation of human osteoblasts *in vitro*,, as well as the synthesis of type I collagen and other proteins of the bone matrix (28,42). Melatonin, at micromolar concentrations, promotes the proliferation of human mandibular (HOB-M) cells, and of a human osteoblastic cellular line (SV-HFO); this effect is dose dependent, with the maximal effect occurring at a concentration of 50 µm (17).

Results of this study confirmed by **Tresguerres *et al.,* (44)** who showed that direct local application of melatonin 3 mg around dental implant in the tibia of the rabbit significantly increased bone density.

Regarding cementum formation, at one month, although results of the melatonin treatment showed that there was no significant differences as compared to control treated group but its predictive potential for cementum regeneration can't be neglected at this period. At 3 months a regular layer of cementum was observed on the root surface in the test defects, this means that melatonin stimulate the formation of cementum late at one month and reached its peak at three months.

Insertion of functionally oriented connective tissue fibers with no epithelial downgrowth indicating new attachment, this newly formed insertion was greater in the defects treated with melatonin gel than in the control treated sites, which showed repair by long junctional epithelium and more connective tissue adhesion as indicated by histomorphometric measurements (6.75±0.64).

This can be explained by that, melatonin may stimulate a rapid formation of a connective tissue seal that is supposed to have the ability to block the epithelium migration and allow for repopulation of the previously contaminated area by periodontal ligament cells and by the creation of space by collagen sponge allowing migration of periodontal ligament cells and bone cells on the denuded root surface which is necessary for periodontal regeneration (**45**). This finding also could be explained by the ability of melatonin gel to act as cytostatic agents on epithelial cells which suppresses the downgrowth of junctional epithelium onto the dental root surfaces.

Melatonin, a remarkably new drug showed a predictive efficacy in accelerating periodontal regeneration pathway. With the main limitation of this study (the number of animals and studied defects), it is strongly hoped that, further investigations with higher statistical power will be needed to provide crucial information for a better understanding and clarifying of the efficacy of melatonin therapy as a regenerative treatment modality for periodontal defects. Also a more effective delivery system and a specific application protocol for its clinical use should be developed (e.g membranes, chips, ..etc).

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