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HISTOMORPHEMITC EVALUATION OF THE EFFICACY OF AMNIOTIC CHORION MEMBRANE IN MANAGEMENT OF GRADE II FURCATION DEFECTS (IN VITRO STUDY)

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ABSTRACT

INTRODUCTION: Treatment of furcation defects especially critical size defects remains a considerable challenge in clinical practice. Treatment modalities vary according to the type and location of the defects. Regenerative procedures are one of the treatment modalities in furcation involvement.

OBJECTIVES: The aim of this study was to evaluate histomorphometrically the regenerative potential of amnion chorion membrane (ACM) on the management of induced grade II furcation defects and compare it to collagen membrane.

MATERIAL AND METHODS: This study was conducted on eight adult male Mongrel dogs, about 18-24 months old, weighing approximately 18-24 Kgs. On the left sides; the created defects were filled with β-tricalcium phosphate + hydroxy apatite and covered by amnion chorion membrane (study group). While on the right sides; the created defects were filled with the β-tricalcium phosphate + hydroxyapatite then covered by collagen membranes (control group).

RESULTS: ACM has shown superior results in bone formation when compared to control group treated with resorbable collagen membrane.

CONCLUSION: ACM is an effective, easy to handle, safe, and time saving membrane that can be used in periodontal regeneration and management of grade II furcation defects.

KEY WORDS: Amniotic chorion membrane – Critical sized defects - Grade II furcation defects - Regenration.

RUNNING TITLE: Efficacy of amniotic chorion membrane in management of furcation defects.

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INTRODUCTION

Periodontitis is one of the most common diseases characterized by the destruction of connective tissue and alveolar bone support following an inflammatory host response secondary to infection by dental plaque bacteria. It is now generally agreed that almost all forms of periodontal disease occur as a result of mixed microbial infections within which specific groups of pathogenic bacteria coexist (1). Uncontrolled progression of inflammatory periodontal disease ultimately results in attachment loss sufficient enough to affect bifurcation or trifurcation of multi rooted teeth (2). Bacterial plaque and the inflammatory consequences resulting from its long term presence represent the primary etiologic factor in the development of furcation defects. Dental caries and pulpal involvement (2), chronic trauma from occlusion (3), and iatrogenic factors such as; over hanging restorations can also lead to furcation involvement (4).

Debridement of furcation area by routine periodontal instrumentation is very difficult due to its complex anatomic morphology. Therefore, furcation involvement in molars represents one of the major challenges to manage in clinical periodontics (5).

Attempts to treat furcation lesions have led to therapies ranging from non-surgical periodontal therapy, such as scaling and root planning (SRP), to surgical root debridement, root resection, hemisectioning, regenerative therapy, and attempts in tissue engineering (6). Generally, advanced grade II furcation involvement in mandibular molars and buccal furcations in maxillary molars can be successfully treated by regenerative procedures (7).

The ultimate goal of the regenerative procedures is to exclude the epithelium and the gingival corium from the root to delay epithelial down growth during healing and to provide an opportunity for progenitor cells of the periodontal ligament and bone to repopulate previously diseased root surfaces using guided tissue regeneration materials (GTR) (8).

Clinical studies had shown that guided tissue regeneration (GTR) can improve the response of advanced grade II furcation defects to therapy by means of pocket reduction, gain in clinical attachment levels and bone defect fill. The improvement in these clinical parameters plus the potential of creating new attachment leaded to the consideration of GTR approach as the treatment of choice in furcation defects (9).

Among the regenerative procedures which had been used for treating grade II furcation involvement was the use of a composite bone substitute graft combined with a non-absorbable or bio absorbable barrier membranes and a coronally positioned flap (10). Other modalities as osteogenic protein -1 (OP-1) which is a member of the transforming growth factor beta super family and enamel matrix derivative had also been used (11). Amniotic chorion membrane (ACM) which contains a multitude of growth factors has also been used (12).

Amniotic chorion membrane (ACM) which is a placental based membrane, is composed of amniotic membrane (AM), and chorion membrane. Unlike other barrier membranes, ACM is biologically active due to the presence of bioactive proteins and growth factors (GF) that hasten granulation tissue formation and act as a bioactive matrix that facilitates cell migration. Wound healing property is further enhanced by the physiological seal obtained with the gingiva (12).

Immunohistochemical (IHC) staining of amniotic chorion membrane showed intense concentrations of laminin and laminin-5 in the barrier. Laminin-5 has an affinity for binding gingival epithelial cells (13,14). ACM provided a protein enriched matrix which naturally hastens cellular migration across the exposed portion of the barrier. These unique biological properties allowed ACM to be left exposed to the oral environment (12).

ACM was used for tissue engineering in the reconstruction of occular surface, management of Steven Johnsons syndrome, chemical burns, nerve regeneration, skin reconstruction, endothelial cell cultivation, local drug delivery, and as a GTR membrane in treatment of periodontal osseous defects, gingival recessions, and furcation defects (12).

In spite of this membrane's safety, few studies had been conducted to evaluate GTR procedures using ACM augmented with bone graft in the management of furcation defects.

MATERIALS AND METHODS

Materials

- BioXclude, Amnion chorion Membrane (ACM) (20x30mm) allograft. (SNOASIS Medical, Llc, 1905 Sherman St #245, Denver, Co 80203, USA.)
- Hypro-Sorb F, Atelo-Collagen Type I membrane (15x20x0.2 mm). (Bioimplon GmbH, Friedrich-List-Str. 27, 35398 Giessen, Germany.)
- BoneMedik - DM Bone, β-tricalcium phosphate with hydroxyapatite in the ratio of (40:60), alloplast. (META BIOMED Co,270 Osongsaengmyeong1-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungbuk, Korea.)

Animals Selection

- Eight adult male Mongrel dogs (*Canis familiaris*), about 18-24 months old, weighing approximately 18-24 Kgs were selected for this study.
- The animals were adapted to the housing conditions during the study period at animal house in the Medical Research Institute, Alexandria University.
- The number of animal had been estimated according to sample size calculation made in Biomedical Information and Medical Statics department, Medical Research Institute, Alexandria University using NCSS & PASS program. (NCSS/PASS software - 329 North 1000 EastKaysville, Utah 84037 USA).
- A split mouth design was applied.

Methods

Surgical creation of the defects

- All procedures were performed under general anesthesia using intramuscular injection of a combination of 0.1ml ketamine hydrochloride (KEPRO, 17-7421 Maagdenburgstraat, Deventer, Netherlands.) and 0.05 ml xylazine hydrochloride (EIPICO, 10th Of Ramadan City, Egypt.) for each 100 gm body weight (15, 16).
- Sulcular incisions were performed, followed by the reflection of mucoperiosteal full thickness flaps buccally on the mandibular third and fourth premolars (P3 & P4) in the right and left jaw quadrants (17).
- Grade II critical sized furcation defects were created in the mandibular premolars of each dog (17,18).
- Defects were about 4mm horizontally and 5 mm vertically in both sides.
- Root conditioning was done.
- A split mouth design study was used in all dogs.
- On the left side; the created defects were filled with β-tricalcium phosphate + hydroxy apatite and covered by amnion chorion membrane (study group).
- While on the right side; the created defects were filled with the β-tricalcium phosphate + hydroxyapatite and then covered by collagen membranes (control group).
- Releasing incisions were made to obtain a tension free closure; flaps were then repositioned and closed with silk sutures.

Postoperative Care

The animals received:

- Antibiotic (Amoxicillin 500mg (EIPICO, 10th Of Ramadan City.)) that was given intramuscularly in the first day, and then mixed with dog's food for seven days.
- Non-steroidal anti-inflammatory drugs (Ibuflex 400mg; (SEDICO,1st. industrial zone,6th of October City P.O. Box 43, Egypt.)) that was given intravenously in the first day.
- Dogs were fed on soft diet in the postoperative period to reduce the possibility of local trauma to the operated sites (19).
- Dogs were euthanized by overdose intravenous injection of thiopental sodium (Anapental (SIGMA, District 6, 6 October City, Egypt.)) given intravenously.

Animal sacrifice

The dogs were sacrificed at two different time intervals

- At one month post-operatively.
- At two months post-operatively.
- The mandible was dissected out divided into two segments then preserved in 10% buffered neutral formalin for histological processing.

Histological procedure

- Segmenting: The jaws was washed under running water and segmented, each segment contained an experimental tooth, investing bone and the surrounding soft tissues.
- Fixation: Segments of the specimens were fixed in 10% neutral buffered formalin for five days.
- Washing: After fixation, the specimens were washed in running water to remove formalin.
- Decalcification: 8% trichloro-acetic acid was used to decalcify the specimens, the acid was changed every two

days for three weeks and the specimens were tested for completion of decalcification by passing a fine needle into the jaw bone, if it passed without any resistance it meant that the decalcification process was complete.

- Washing: when decalcification was complete the specimens were washed by running water for at least 12 hours to remove all traces of acid.
- Dehydration: Specimens were gradually dehydrated in ascending concentrations of ethyl alcohol (50%, 70%, 90%, and 95%) and two or three changes of absolute alcohol to ensure that the water was completely replaced by alcohol.
- Clearance: The specimens were passed from alcohol through two changes of xylene, which is miscible with both alcohol and paraffin because paraffin and alcohol are not miscible.
- Infiltration: After xylene had completely replaced the alcohol in the tissue; the specimens were infiltrated with paraffin wax by placing them in a dish with melted embedding paraffin in a constant-temperature oven regulated to about 60° C until the xylene in the tissue was replaced by paraffin. This took about 12-24 hours to ensure complete paraffin infiltration.
- Embedding and cutting: The specimen was removed from the dish after complete infiltration by paraffin and embedded in the center of a block of paraffin, the hardened paraffin block was mounted on a paraffin coated wooden cube then the specimen was clamped on a precision rotary microtome adjusted to cut through the entire mesio-distal plane of the teeth in a serial of 5µm section.
- Mounting: The ribbons of wax sections were mounted on the coating of clean glass slides with a thin film of albumin adhesive. The slides were placed into 60 0C constant temperature furnace to ensure that the sections adhered to the slides.
- Staining :Sections were stained with:
  - Harris Hematoxylin and Eosin Stain (H&E) for general examination and evaluation of healing.
  - Gomori's trichrome stain to examine the new bone formation and collagen organization. (20)

Steps of measuring the height of newly formed inter-radicular bone

1. A straight line was drawn on each photograph from the beginning of the furcation area till a line at the most apical end of the created defects.
2. This line was drawn to set a scale of 5 mm on the photograph converting image pixels into millimeters.
3. Another straight line was drawn from the most coronal end of the newly formed inter-radicular bone till the apical line at the notches.
4. The length of this straight line represented the height of the formed inter-radicular bone.

Steps of measuring the percentage of the formed bone surface area

1. A rectangle with standardized dimensions (2 x 1.5 cm) was drawn on each photograph containing the inter-radicular regenerated bone, parts of the two adjacent roots and PDL tissues to be measured using the using image J 1.46r software (LOCI, University of Wisconsin).
2. The total surface area of this selected region was measured by choosing region of interest (ROI) manager, from tools from analyze and the measurement was recorded.
3. The surface area occupied by the bone marrow, parts of the PDL and adjacent two roots of the tooth were selected using the wand tracing tool and the measurement was recorded.
4. The latter measurement was subtracted from the measurement of the whole rectangle (total surface area). Then its percentage to the total surface area was calculated.

Evaluation of the regenerative outcome

Histomorphometric analyses

Histomorphometric analysis of the percentage of the total surface area of the newly formed interradicular bone and its height was carried out using Image J 1.46 software (21) on photos taken from sections prepared at specific depth of the blocks.

Statistical analysis

The data were analyzed by using descriptive statistical analysis. Measurements from the two variables (inter-radicular bone height and percentage of bone surface area) were collected from the images. Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.

RESULTS

Histomorphometric results

Data obtained from the histomorphometric analysis regarding all parameters showed comparable results in both groups at the two observational periods with overall higher values for the study groups.

1. The height of newly formed inter-radicular bone in pixels

a. At 4 weeks: (Table 1, Figure 1)

In the study group the mean value of the height of the newly formed inter-radicular bone was (**56.46 ± 7.09 pix**), while the control group revealed a mean of (**34.23 ± 6.33 pix**) with a subsequent highly significant difference between two values (P <0.001).

b. At 8 weeks: (Table 1, Figure 1)

The study group showed a mean of (**29.60 ± 4.77 pix**), while the control group showed a mean of (**31.85 ± 5.13 pix**) which created a significant difference **p =0.18** in favor of study group.

Table (1): Comparison between the two studied groups according to the height of newly formed inter-radicular bone after 4 and 8 weeks in pixels.

	Study group	Control group	P Value
4 weeks (mean-SD)	56.46±7.09	34.23±6.33	0.0001*
8 weeks (mean-SD)	31.85±5.13	29.60±4.77	0.18*

p: p value for comparing between the studied groups
*: Statistically significant at $p \leq 0.05$

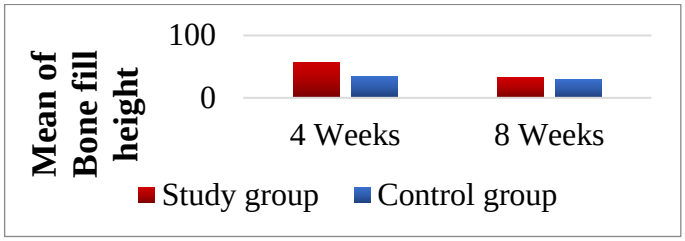

Figure (1): Comparison between the studied groups according to the mean value of the height of newly formed inter-radicular bone at the two observation periods (Pix).

2. The percentage of the formed bone surface area

a. At 4 weeks: (Table 2, Figure 2)

In the study group the mean of the percentage of the formed bone surface area was (**42.69± 5.34 %**), while for the control group it was (**38.77± 2.22 %**) with a subsequent highly significant difference (**P =0.038121**).

b. At 8 weeks: (Table 2, Figure 2)

The study group showed a mean value of (**60.98± 6.72 %**), while the control group showed a mean of (**52.20± 5.52 %**) with subsequent highly significant difference (**P = 0.006369**).

Table (2): Comparison between the two studied groups according to the mean value of percentage of the formed bone surface area at 4 and 8 weeks.

	Study group	Control group	P Value
4 weeks (mean-SD)	42.69±5.34%	38.77±2.22 %	0.038121*
8 weeks (mean-SD)	60.98±6.72 %	52.20±5.52 %	0.006369*

p: p value for comparing between the studied groups
*: Statistically significant at $p \leq 0.05$.

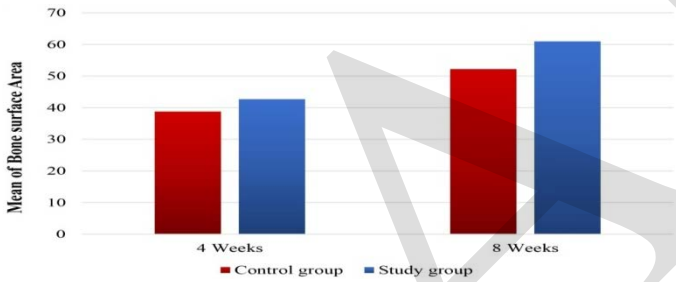

Figure (2): Comparison between the two studied groups according to the mean value of the percentage of the formed bone surface area at the two observation periods.

DISCUSSION

The present study was carried out on eight adult male Mongrel dogs to evaluate histomorphometrically the regenerative potential of amnion chorion membrane in the management of induced grade II furcation defects and compare it to resorbable collagen membranes. A split mouth design was conducted in all dogs. On the left sides; the created defects were filled with β-tricalcium phosphate + hydroxy apatite and covered by amnion chorion membrane (study group). While on the right sides; the created defects were filled with the β-tricalcium phosphate + hydroxyapatite and then covered by collagen membranes (control group). After one and two months, tissue blocks containing the studied teeth with the regenerated periodontium were obtained and examined histologically and histomorphometrically (22,23).

In the current study, an animal model was selected in order to evaluate periodontal regeneration histomorphemetriclly. This is in accordance with the World Workshop of Periodontics' statements in (1996) (24) which stated that the use of animal histology is crucial for proof of periodontal regeneration. Therefore, histological examination is considered the most accurate method to determine the true extent of periodontal regeneration (23,25). Moreover, dogs were chosen for this study due to the resemblance of the dento-alveolar architecture of dogs to that of humans (26).

In the current study, critical sized defects were created (5mm. in the vertical component and 4mm. in the horizontal component) (27). These defects can be defined as the smallest defects in a particular bone of certain animal species that will not heal spontaneously during the life time of the animal (28).

The defects were surgically created (acute-type defects) to ensure that they were all of a standardized critical size to permit standardized healing conditions (29).

The selection of biological mediator as ACM in management of critical size grade II furcation involvement was due to presence of biologic growth factors, which may enhance periodontal tissue regeneration (14), in this study ACM was used with Biphasic Calcium Phosphate (BCP), as the addition of the biological factors present in ACM to the properties of BCP may enhance bone formation, stimulate angiogenesis, and osteogenesis (30). The need for filler under the soft membranes will prevent their collapse in the defects.

To assure that healing occurred under similar conditions and to standardize all the factors between the study and the control groups a split mouth design was carried out in this study to exclude any genetic and tissue factor variations between different dogs (25).

Histomorphermtric evaluation was performed after one and two months. These specific time intervals as stated by Reddy et al; (21) had been reported to allow comprehensive examination of the different phases during defect healing.

In the present study, the post-surgical clinical response showed absence of any symptoms as pain, infection, discomfort and no evidence of allergic reaction throughout the follow up period providing the evidence of biocompatibility of the materials employed in the current study (31).

Also the anti-inflammatory properties of ACM may decrease the influx of inflammatory cells and inflammatory mediators to the wound area. (23).

In addition to that, ACM released inhibitors of matrix metalloproteinases (MMPs), e.g: 1, 2, 3, and 4, which were released by infiltrating neutrophils and macrophages (12). Furthermore, the presence of interleukin-10, and interleukin-1 receptor antagonists and endostatin inhibit endothelial cell proliferation, angiogenesis, and tumor growth, leading to inhibition of inflammation (24). Moreover, the matrix of amniotic membrane stroma had the ability to suppress pro-inflammatory mediators e.g: interleukin-1α and interleukin-1β (25, 32).

The results of the current study revealed that ACM membrane exhibits easy handling during manipulation compared to collagen membrane, which may be due to its standardized shape, size, and thickness of about (300 micrometers), compared to traditional collagen membranes' thickness of (700-800 micrometers) (33). Therefore, it does not require chair side fabrication (14). As well as, ACM membranes were easier to apply, due to the self -adhering properties, so they do not require sutures for fixation. This property may be due to the

presence of adherent molecules as laminin and fibronectin (19,24), in addition to the presence of elastin which made it elastic and self-adherent. All these properties allow ACM to ultimately mold to the defect anatomy and adapt to the contours around the roots' surfaces (14,34).

Among the other biomechanical properties of ACM was the presence of interstitial collagens, which helped the membrane to be resistant to various proteolytic factors (19). This was one of the causes which allows ACM to be left exposed to the oral cavity. These properties of ACM facilitated its use, and save time (22).

In the current study, examination of histological sections obtained from tissue blocks of the experimental and control groups revealed a great difference between the latter and the former groups (35). Where the healing and regeneration were occurring in the experimental group was better than that of the control group at both of the two observation periods regarding vascularization and bone formation (36). This result may be due to the presence of significant amounts of laminin and laminin-5 (19, 25). Additionally, chorion matrix contained abundant growth factors, such as keratinocyte growth factor (KGF), basic fibroblast growth factor (b-FGF), and transforming growth factor beta (TGF- β), that promoted periodontal regeneration (20), and provided a natural environment for accelerated healing (14).

Various defects in all groups presented bone surrounding the graft particles. However, in the study group the defects were filled by a higher density bone with a more evident mineralized bone area with islands of highly cellular bone marrow.

This histological results was confirmed by the histomorphemtric result in the current study where the height of newly formed inter radicular bone showed that ACM accelerated bone regeneration significantly nearly twice than the control group at the week 4 while nearly the same at 8 week. The percentage of the formed bone surface area in both study and control groups showed a slight increase in 8 week than 4 week at both study and control group.

The possible explanation of the results in test group may be due to the presence of various growth factors in ACM, which can stimulate bone formation (22), and also because the basement membrane of AM served as a safe and suitable bed for the growth of cells. (23,25). Therefore allowing for more rapid bone formation in test group at the start of the study.

The improvement of horizontal and vertical furcation components may be attributed to the bone inductive potential of ACM, due to its ability to upregulate the recruitment of mesenchymal progenitor cells which demonstrated osteogenic and adipogenic differentiation (37). At the same time, ACM showed excellent acceptability with bone grafts by demonstrating excellent containment of the material and its resorption without the formation of voids (26).

Amniotic membranes (AM) had the ability to produce β-defensins, which are a major group of antimicrobial peptides and an integral part of the innate immune system (28,29). Anti-viral properties are exhibited by the presence of cystatin E, the analogue of cysteine proteinase inhibitor (25,38).

The current results were also in agreement with Kumar et al., (26) who used AM with hydroxyapatite (HA) bone graft to manage contained interdental defects, and found a significant gain in bone fill at test group from baseline to 24 weeks. They concluded that AM has the potential to function as a barrier for GTR and can act as a matrix for periodontal regeneration.

Similarly Kothiwale et al., evaluated the efficacy of DFDBA and bovine derived xenogeneic bone graft with AM in treatment of Grade II furcation defects, and showed significant improvement in bone fill. (39,14)

When compared to collagen membrane, ACM also demonstrated many unique properties such as that it possessed a variety of proteins that provided a bioactive matrix to facilitate wound healing (22). Also, the presence of collagen in ACM which was well tolerated and biabsorbable, has hemostatic properties and encourages migration of adjacent autogenous connective tissues which represent a successful regenerative outcome of the membrane (26).

Therefore, ACM was believed to be beneficial in periodontal regeneration as it contained abundant growth factors, and it up regulated stem cell recruitment allowing these cells along with the soluble bioactive proteins in the membrane to enter into the wound space, thereby, accelerating healing, decreasing inflammation and then allowing for periosteum to participate in bone healing (12,40).

Regarding the improvement achieved by ACM in management of critical size grade II furcation defects, ACM membrane may hold promise for regeneration and treatment of one of the most challenging clinical conditions.

CONCLUSION

Amnion chorion membrane was an effective, easy to handle, safe, and time saving membrane that can be used for periodontal regeneration.

Conflict of interest

The authors report no conflict of interest relevant to this article.

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