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Original Article

Histological and histomorphometric analysis of animal experimental dehiscence defect treated with three bio absorbable GTR collagen membrane

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ABSTRACT

Background: Guided tissue regeneration (GTR) allows mesenchymal cells to repopulate the defects. However, there is limited information regarding the efficacy of different membranes. The present study was designed to histologically and histomorphometrically compare three collagen membranes in regenerative treatment of dehiscence defects in dogs.

Materials and Methods: This 8 weeks experimental animal study comprised 4 healthy dogs. 5 x 5 mm periodontal dehiscences were created in each side of the mandible (4 dehiscences in each side of dogs' mandible). In each side, one dehiscence defect was left uncovered as a control site and three other sites were randomly covered with different collagen membranes (Biogide (BG), Biomend (BM), and Cytoplast (CYT)). Histomorphometric and histologic analysis were conducted at 4 and 8 weeks. Data were analyzed using ANOVA, Mann-Withney, Kruskal-Wallis and Fisher 's exact tests ($\alpha = 0.05$).

Results: According to histomorphometric analysis there was a significant difference between treatment and control groups regarding the bone formation and the distance between the reference point and apical end of junctional epithelium (DJE) ($P < 0.05$). At 4 weeks, the maximum amount of bone thickness and height was observed in BG and CYT respectively, and this maximum rate was seen with the use of BG at 8 weeks. It was shown that DJE reached its highest rate in BM and CYT at 4 and 8 weeks, respectively. Organized PDL was formed in treatment groups.

Conclusion: The membrane-treated groups had a statistically significant increase in bone formation and connective tissue attachment compared to control groups. However, there are some differences among experimental groups, which should be considered in GTR treatments.

Key Words: Bone regeneration, collagen membrane, dehicens defect

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INTRODUCTION

According to GTR hypothesis, explained by Melcher, placing a barrier between the overlying gingival tissues and the defect space hinders the faster-moving epithelium and connective tissue from migrating into the wound space, and this provides a great chance for cementum, periodontal ligament, and bone cells to dominate the defect.[1,2] Also, it was declared that membranes can create a space, which stabilize the blood clot and facilitates the progenitor cells' differentiation.[3]

Since Nayman[4] for the first time investigated the capacity of membranes in treatment of human bone defects, numerous studies have evaluated the effectiveness of different membranes in animal[1,2,4-7] and human[8-14] models but unfortunately they were not conclusive enough. Through research of articles

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showed that the use of non-absorbable membranes in treatment of alveolar bone defect is widely accepted among clinicians.[15] Although, polytetrafluoroethylene (PTFE) is the mostly used membranes,[13,16] but difficulty in handling, bacterial contamination and soft tissue irritation have been proposed as its main shortcomings and the use of PTFE has been diminished consequently. Apart from these problems, the need of secondary surgery for removing non-absorbable membranes has restricted their use and absorbable membranes were introduced to the GTR treatments.

Bio absorbable membranes are mainly prepared from dura mater, poly glycolic acid, poly lactic acid and collagen.[17] Promoting progenitor cells' adhesion and chemotaxi and physiologic degradation are indispensable characteristics of an ideal membrane and this properties can be provided in collagen made membranes.[12,13,17,18]

Collagen membranes are mainly produced from type I and III bovine or porcine collagen.[7,12] Collagen fibers provide a structural elasticity during the crystalline phase of bone regeneration[14] and these properties of collagen ensure perfect tissue integration and adequate wound healing.[19] Micro-architecture and cross-links are properties that collagen membranes may differ in and these characteristics define collagen structural durability, stiffness and degradation time.[17] Although, promising results have been shown by the use of collagen barriers, but several complications such as early degradation, epithelial down growth along the material, and premature loss of the material were reported following the use of collagen membranes.[20]

According to all aforementioned statements, there are no appropriate criteria for choosing cell occlusive collagen membranes. Also, there is no conclusive study regarding the efficacy and superiority of different collagen membranes. As a result, this study was designed to histologically and histomorphometrically investigate the efficacy of three types of absorbable collagen membrane of BioMend®, Biogide® and Cytoplast RTM® in treatment of dehiscence defects in canine model.

MATERIALS AND METHODS

This was a 8 weeks experimental animal study which was held with the cooperation of professor Torabinejad research center and this study was approved by the local ethical committee of Isfahan University of medical science.

Four healthy adult native female dogs (12 months old; weighting 20 to 25 kg) were included. Animals were anesthetized using injection of acepromazine 2% (Neurotrano, alfasan, Woerden, Holland; 0.02mL/kg), Ketamine Hcl 10% (Ketamine alfasan, Woerden, Holland; 10 mg/kg). After the injection of atropine 0.1% (Atropine, alfasan, Woerden, Holland; 0.02-0.04 mg/Kg) dogs were intubated and halothane gas (Halothane BP, Nicholas Piramal India Limited, India) was used to maintain the anesthesia. Lidocain (persocaine-E, Lidocaine HCL 2% + Epinephrin1/80000, Daroupakhsh pharmaceutical. Mfg. Co. Tehran, Iran) infiltration was placed in the mucobuccal fold to control the pain and bleeding during the surgical procedure. Oral prophylaxis was performed with chlorhexidine solution 0.2% prior to surgery.

After a sulcular incision from mandibular canine to first molar, a muco-periosteal flap was elevated by an elevator to expose the underlying alveolar bone. Using a carbide bur, bone chisel and curette, four critical size periodontal dehiscences were created in each side of the mandible (8 dehiscences in each dog) by removing 5 × 5 mm of bone and cementum from the roots of the canines and the distal roots of the 2, 3, 4th premolars [Figure 1]. At the apical end of each defect, a notch was made with a half-round carbide bur (No. 2) as a reference point for histomorphometric assessment. Then defects were rinsed by normal saline. In each side, one defect was left uncovered as a control site and three other defects were randomly covered with three commercially available collagen membranes including:

Cytoplast RTM® (CYT): Is a type 1 collagen derived from bovine flexor Achilles tendon (Osteogenics Biomedical, Inc., USA).

BioMend® (BM): Is a type 1 collagen derived from bovine flexor Achilles tendon (BioMend, Zimmer Dental Inc, carlsbad, USA).

Biogide® (BG): Is a type 1 bilayer collagen derived from porcine derma (Geistlich Biomaterials, Inc., Wolhusen, Switzerland).

Each membrane was placed directly on the dehiscence defect according to manufacturer's instruction. Membranes were extended at least 2 mm beyond the defect's borders [Figure 2].

The flap was then repositioned and sutured using 0-3PTFE (Osteogenics Biomedical, Inc., USA).

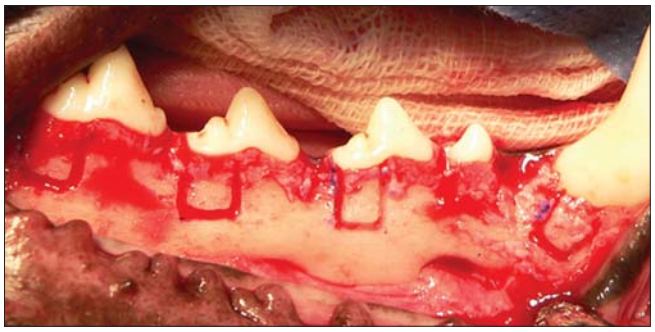

Figure 1: Four dehiscences were created in each side of the mandible

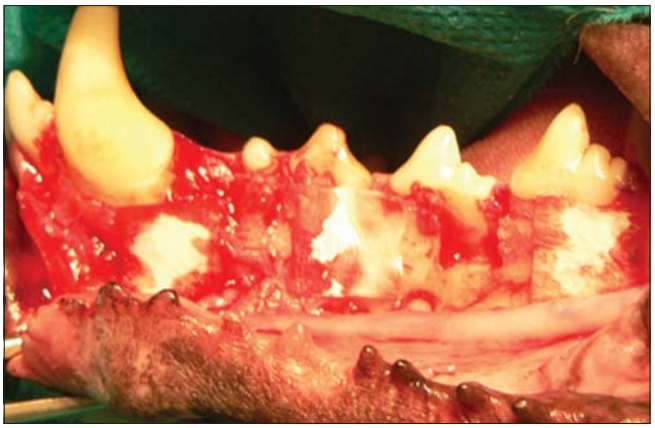

Figure 2: Membranes were placed on the dehiscence defect

Similar procedures were done on another side of each dog's mandible. Totally, 32 periodontal dehiscences were surgically created 8 control and 24 experimental defects. After consciousness, Tramadol 50 mg (Tehran chemie pharmaceutical Co. Tehran-Iran, 5 mg/kg) and ceftriaxone 1gr (Ceftrax, Jaberebne Haian pharmaceutical Mfg.co, Tehran-Iran) were intramuscularly injected to dogs for 7 days, and animals were fed on soft diets for 14 days following the surgery. The operation sites were cleaned with 0.2% chlorhexidine solution twice a day and sutures were removed after 14 days postoperatively. There was no post operative complication, such as sign of infection and abscesses or allergic reactions during the entire period of the experiment.

Histologic and histomorphometric study

Dogs were sacrificed via intravenous injections of ketamine, magnesium sulfate and acepromazineat two time intervals(4 and 8 weeks, two dogs at each time point). The mandibles were then removed and fixed in a 10% buffered formalin solution for 48 h. Each specimen was isolated, rinsed in distilled water, demineralized with nitric acid (solution 5%) for a period of 4 weeks, then dehydrated and embedded in paraffin. Several histologic sections of each defect site were cut in 5 µm thickness buccolingually with a microtome (Accu-Cut SRM. SACURA, Japan). The histological sections were stained with H and E)Hematoxylin and Eosin method) and investigated under optical microscope (Nikon E400, Japan) by a blinded pathologist. Histomorphometric analysis was done to evaluate different parameters with I HMMA (ver. 1, sbmu, Iran) software. Using the apical limit of the notch as a reference point, the following measurements were made: (1) thickness of new cementum (NCt), (2) height of newly regenerated bone (NBh), (3) thickness of newly regenerated bone (NBt) and (4) distance between the reference point and the apical junctional epithelium attachment (DJE).

Structure of the periodontal ligament (PDL) were classified to organized PDL, which characterized by dense connective tissue and regularly oriented fibers from alveolar bone toward cementum surface or disorganized PDL with irregularly oriented fibers according to histological surveys. Then the regenerated PDL was scored based on the following observations:

0: Disorganized PDL 1: Organized PDL 2: More organized PDL.

Also, the inflammatory score was assessed under the optical microscope according to presence of inflammatory cells based on the following observations:

Score 0: < 10% inflammatory cells, Score 1: 10-30% inflammatory cells, Score 2: 30-50% inflammatory cells, Score 3: > 50% inflammatory cells.

Statistical analysis

Data were analyzed using statistical analysis software SPSS 16(SPSS™ SPSS Inc., Chicago, USA). Significant differences among groups were identified by one-way ANOVA with Tukey's post-hoc test, Mann-Withney, Kruskal-Wallis and Fisher 's exact tests ($\alpha = 0.05$)

RESULTS

Histological observations (4 and 8 weeks postoperative)

The histomorphometric observations are presented in Figures 3 and 4.

Chronic inflammatory response was present in treatment and control specimens. All cases showed

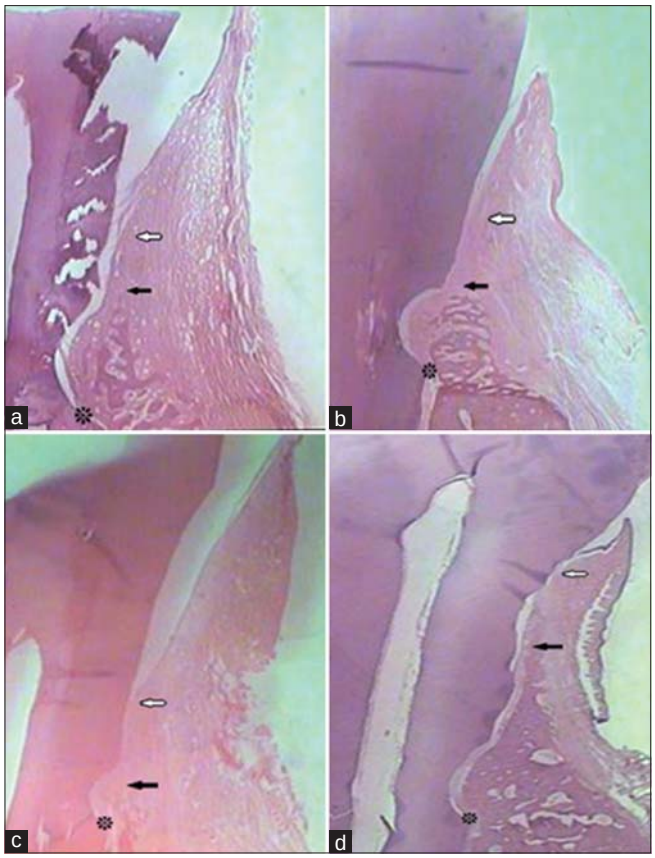

Figure 3: Photomicrographs of periodontal regeneration in different groups at 4 weeks: (a) BG membrane (b) BM membrane (c) Control group d) CYT membrane. H&E; Original magnification ×12 (*)apical notch, (➡) new alveolar crest, (⇨) apical of junctional epithelium

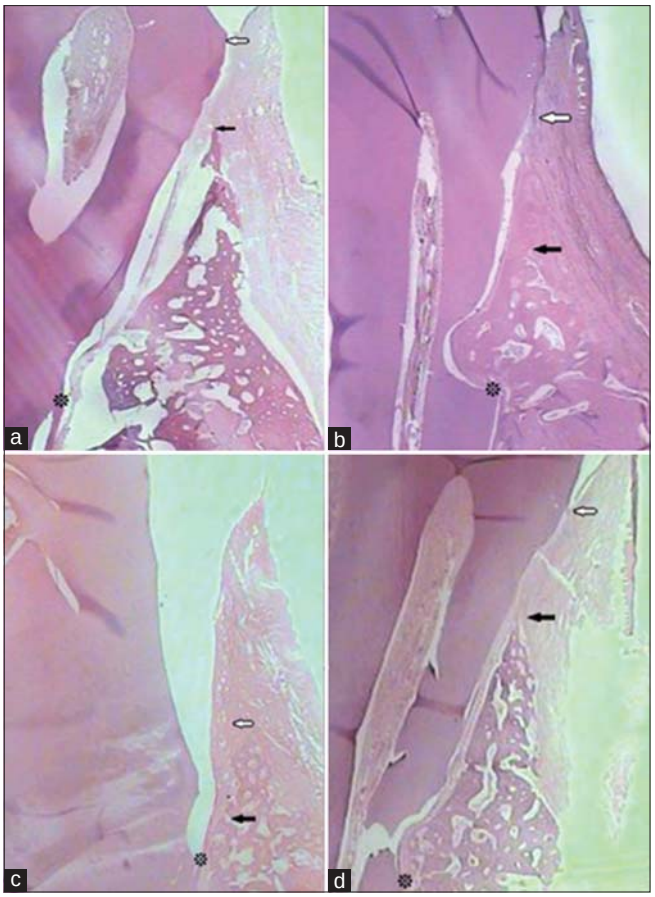

Figure 4: Photomicrographs of periodontal regeneration in different groups at 8 weeks: (a) BG membrane (b) BM membrane (c) Control group d) CYT membrane. H&E; Original magnification ×12 (*)apical notch, (➡) new alveolar crest, (⇨) apical of junctional epithelium

score 0 (less than < 10% inflammatory cells) regarding the inflammatory response except for one control group which showed score 1 (10-30%) over 4 weeks.

Cellular cementum was found in groups which were capable of cementum regeneration.

After 4 and 8 weeks, organized PDL was formed in all groups except for control. The quality of regenerated PDL was more organized after 8 weeks compared to 4 weeks in treatment groups. The quality of regenerated PDL showed significant difference between treatment and control groups at 8 weeks (P = 0/038) [Table 1].

At 4 weeks, the amount of regenerated woven bone was more than lamellar bone (mature bone) in treatment groups. As time elapse, the amount of lamellar bone increased compared to woven bone and it reached to its highest rate in Biomend group. The amount of regenerated lamellar and woven bone showed no significant difference between treatment and control groups ($P_{4\ week\ LB}$ = 0.95 $P_{4\ week\ WB}$= 0.07) [Table 2].

None of the membranes were present after 4 weeks.

Histomorphometric observations (4 and 8 weeks postoperative)

Histomorphometric observations are presented in Table 2.

Although the maximum amount of NCt was regenerated in CYT group, this parameter showed no significant difference between groups in both 4 and 8 weeks ($P_{4\ weeks}$ = 0.06 $P_{8\ weeks}$ = 0.44).

There was a significant difference between all treatment and control groups regarding the mean amount of vertical bone formation (NBh) after 4 and 8 weeks (P < 0.05) except BM at 4 weeks. NBh reached its highest rate in CYT and BG groups after 4 and 8 weeks respectively [Table 2]. Significant differences in treatment groups were observed

between CYT and BM (P = 0.02) at 4 weeks and CYT, BM (P = 0.03) and BG, BM at 8 weeks.

The amount of NBt was statistically different in BG and CYT groups after 4 weeks and BG and BM groups at 8 weeks compared to control. The maximum amount of NBt was obtained in BG group and this amount showed a significant difference compared to CYT and BM groups after 4 weeks (P = 0.000 and P = 0.03). There was no significant difference between all treatment groups regarding the amount of NBt at 8 weeks [Table 2].

It was shown that the amount of DJE was significantly different between all treatment and control groups after 4 and 8 weeks [Table 2]. DJE reached its highest rate in BM and CYT groups after 4 and 8 weeks, respectively. But, there was no significant difference between treatment groups after 4 and 8 weeks ($P > 0.05$).

DISCUSSION

In the present study, three collagen bio absorbable membranes were used. One of the main notable features of membranes is that they preserve the defect space and stabilization of coagulum and hinder the migration of epithelial cells into the defect. To fulfill this aim, membranes structural durability should prevent membranes to collapse into the defect. In the present study, 5 × 5 mm dehiscences were created in the mandible of dogs. In this critical size, membranes are stable enough and do not collapse into the defects.

In the present study, there was significant difference between treatment groups regarding the quality of regenerated PDL at 8 weeks. As time elapse, the more organized PDL increased in treatment groups and this may indicate that PDL maturation requires time and early loss of membranes may jeopardize the maturation process. Also, there was no sign of organized PDL in control group as defects were repopulated by epithelial cells and a true, well-structured PDL was not formed in those defects.

In the present study, the distance between the reference point and apical of junctional epithelial attachment (bone and connective tissue attachment) was assessed histomorphometrically. This distance showed a significant difference between all treatment and control groups but there was no significant difference among treatment groups. The control group showed the least distance and it indicates that in the absence of membrane, the epithelium will down growth the defect. Clinically, this histologic finding can be attributed to an increase in clinical pocket depth. In christgau *et al.*[21] and Stavropoulos *et al.*[22] studies, clinical attachment gain and pocket reduction was observed with the use of bio absorbable membranes but there was no definite histological confirmation for these studies. The present study is in agreement with

Table 1: Histological measurements for newly PDL (*n*=8 specimen measurements per group)

Histological parameter	4 week			8 week		
	Disorganize	Organized	Organized	Disorganize	Organized	Organized^{++}
BG[a]	2	2	0	2	1	1
BM[b]	2	2	0	1	2	1
CYT[c]	3	1	0	1	2	1
CO[d]	4	0	0	4	0	0

P value 4 weeks = 0.181; *P* value 8 weeks= 0.038; BG[a]: Biogide membrane; BM[b]: BioMend membrane; CYT[c]: Cytoplast RTM; CO[d]: Control group; $^{++}$: More organized

Table 2: Histomorphometric measurements for newly formed tissues (*n*=specimen measurements per group)*

Histological parameter	4 week					8 week				
	BG[a]	BM[b]	CYT[c]	CO[d]	*P* value	BG	BM	CYT	CO	*P* value
NCt[1] (mm)	0.27±0.42	0.07±0.09	0.39±0.37	0.007±0.01	0.06	0.25±0.35	0.07±0.08	0.45±0.46	0.017±0.02	0.44
JE[2] (mm)	2.09±0.19	2.69±0.62	2.25±0.29	1.35±0.52	0.006†	2.23±0.6	2.42±0.63	2.85±0.37	1.07±0.32	0.000†
NBh[3] (mm)	1.17±0.45	0.74±0.29	1.59±0.5	0.31±0.41	0.008†	1.78±0.56	1.39±0.09	1.74±0.24	0.32±0.52	0.001†
NBt[4] (mm)	0.56±0.04	0.31±0.18	0.39±0.00	0.1±0.13	0.000†	0.61±0.08	0.5±0.21	0.39±0.16	0.13±0.16	0.04†
LB[5] (%)	14.5±16.25	12.5±15.00	15±10.8	10.00±14.14	0.95	63.75±12.5	63.75±29.81	31.75±40.81	13.00±14.46	0.10
WB[6] (%)	85.5±16.25	87.5±15	85±10.8	40.00±46.90	0.07	36.25±12.5	37.25±29.81	68.25±40.81	62.00±42.92	0.44

*Mean ± standard deviation (confidence interval) BG[a]: Biogide membrane; BM[b]: BioMend membrane; CYT[c]: Cytoplast RTM; CO[d]: Control group; †Statistically significant ($P < 0.05$) compared to control group NCt[1]: Thickness of new cementum, DJE[2]: Distance to the epithelial junction, NBh[3]: Height of newly regenerated bone, NBt[4]: Thickness of newly regenerated bone, LB[5]: Lamellar bone, WB[6]: Woven bone

mentioned studies and can histologically approve the clinical findings.

In the histological surveys, cellular cementum was found in groups, which were capable of cementum regeneration and this is in agreement with Araujo *et al.*,[23] study who also confirmed the formation of cellular cementum in defects with BG resorbable membrane. Although, the cementum thickness was greater in all treatment groups compared to control, there was no significant difference between them and this is in accordance with Gineste L study[11] which showed that there is no significant difference between Biomend treated and control groups regarding the formation of new cementum. According to O'Brien, biodegradable membrane hinders the down growth of epithelium and increase the regeneration rate of cementum and connective tissue attachment.[24] This study also highlighted this statement that in all treatment groups, membranes effectively prevent this movement as it can be seen in new cementum thickness compared to control group.

The mean height of newly regenerated bone (NBh) showed a significant difference between all treatment and control groups ($P < 0.05$) except for BM at 4 weeks. Among the experimental groups, bone height reached its highest rate in CYT and BG groups after 4 and 8 weeks, respectively. This may indicate that CYT membrane can accelerate the bone regeneration process and the regeneration of new bone can be expected in less time with the use of CYT membrane. The minimum amount of NBh was observed in BM group in 4 weeks and this amount increased significantly as time elapse. It may show that bone maturation requires more time in BM group compared to others. This difference between experimental groups may emphasize that the varied properties of these membranes like their pore sizes may affect the pattern of cell immigration and adhesion.

The amount of NBt was statistically different in BG and CYT groups at 4 weeks and BG and BM groups at 8 weeks compared to control.

This finding is in agreement with Stavropoulos *et al.*,[25] and Gineste L.,[11] study that showed collagen membranes regenerated significantly more bone compared to control group. Oh *et al.*,[26] compared the efficacy of Bio-Gide and BioMend Extend membranes for the treatment of implant dehiscence defects and showed that there is no significant difference among groups regarding the amount of new bone fill (BF) at 4 weeks. However, at 16 weeks, it was noted that membrane-treated groups showed significantly higher rate of BF compared to controls.

According to histological examinations, in the membrane-treated sites the bone was regenerated completely at the notches and the new bone was partially regenerated in other sites of dehiscences and this is in accordance with other studies.[11]

Woven bone is a weak poorly organized structure (teimori 21) and it is the first tissue which is formed in bone regeneration process.[15] This is while, for the regeneration of well-structured lamellar bone, hydroxyapatite crystals should be deposited by osteoblast cells. In the second mineralization phase, the mineral contents of lamellar bone and also the size of hydroxyapatite crystals increase and theses phenomenon require time (teimori 21). In the present study, the amount of regenerated lamellar bone increased as time elapse. Also, the control group showed the highest rate of woven bone in 8 weeks.

In the present study, none of the membranes were observed after 4 weeks and this time was less than what was expected by manufacturers. The degradation of membranes maybe explained by the enzymatic activity of macrophages and polymorph nuclear leukocytes of the host.[27] The presence of inflammatory cells can accelerate the degradation process, but in the present study, there was a mild inflammatory reaction at the site and this accelerated degradation time can be attributed to different enzymatic activity of dogs compared to humans. The first 3 to 4 weeks has been regarded as a critical time for appropriate healing. The results of the present study are in agreement with mentioned statements as all membranes regenerated the periodontal structures while there was no sign of any membrane after 4 weeks. So, it can be hypothesized that 4 weeks is the minimum required time for a membrane to be effective.

In all samples after 4 and 8 weeks except for one control specimen, less than 10% inflammatory cells were observed and this indicates that the use of bio absorbable membrane do not induce foreign body reaction and these membranes are biocompatible. This finding is in agreement with Rothamal[28] and Gineste[11] studies which showed that Bio-Gide and Bio-Mend collagen bio absorbable membranes do not initiate any inflammatory or foreign body responses.

Although, all three membranes were successful in regeneration of periodontal attachment apparatus to some extent, but the amount of NCt, DJE, NBt and NBh was different among experimental groups and the reason is yet unknown. It has been mentioned that different structural and physical characteristics in conjunction with variable degradation times may highly affect the regenerative outcome of membranes.[29]

CONCLUSION

The membrane-treated groups had a statistically significant increase in bone formation and connective tissue attachment compared to control groups. The highest rate of vertical and horizontal bone formation was observed in BG group. According to result of the present study, it was concluded that all three collagen membranes were capable of regenerating the lost periodontal apparatus to some extent. It seams that each membrane can be used in specific clinical situation.

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