The Effects of Bio-resorbable Barrier Membrane containing Safflower Seed Extracts on the Periodontal Healing of One-Wall Intrabony Defects in Beagle Dogs

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This certifies that the dissertation thesis of Won-Seok Song is approved

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2003년 7월

저자 씀

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ABSTRACT

Background: Recently many efforts are concentrated on the regeneration potential of materials used in oriental medicine. In some experiments, there have been many evidences that these materials effect on bone regeneration. Among these, Safflower seeds have been used for the treatment of blood stasis, bone fracture and osteoporosis in traditional Korean medicine. They are known to have anti-inflammatory effects by improving blood circulation. The objective of this study is to evaluate the periodontal tissue regenerative effects of the bio-resorbable barrier membrane (poly lactide glycolic acid electro-spun nonwoven membrane) containing Safflower seed extracts applied to surgically created one-wall intrabony defects in beagle dogs.

Materials & Methods: One-wall intrabony defects were surgically created at the bilateral mandibular second (mesial & distal sides) and fourth (mesial side) premolars, randomly assigned, the surgical control group received a flap operation only, while the experimental groups received guided tissue regenerative procedure with bio-resorbable membrane (PLGA) and received guided tissue regenerative procedure with bio-resorbable membrane containing Safflower seed extracts (SSE/PLGA). The subjects were sacrificed 8 weeks after the operation, and comparative histological examination was done.

Results: New cementum formation was 2.49±0.41 mm in the surgical control group, 3.22±0.35 mm in the PLGA group, and 3.67±0.82 mm in

the SSE/PLGA group. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The amount of infrabony cementum was 1.75±0.06 mm, 2.40±0.33 mm and 2.70±0.81 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively; the PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The value of the suprabony cementum was 0.73±0.48 mm, 0.82±0.21 mm and 0.97±0.09 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively; significant differences were not observed among the different treatments. The amount of new alveolar bone formation was 1.74±0.25 mm, 2.36±0.30 mm and 2.64±0.74 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. A significant difference was exhibited between the surgical control group and other groups ($p<0.05$). Root resorption was often observed, but ankylosis was not present.

Conclusion: Surgical application of poly lactide glycolic acid nonwoven membrane with or without Safflower seed extract promoted the regeneration of alveolar bone and cementum in intrabony periodontal defects.

Key words: Safflower seeds extracts; Bio-resorbable membrane; Regeneration of periodontal tissue; one-wall defect; new cementum; infrabony cementum; suprabony cementum; new alveolar bone.

The Effect of Bio-resorbable Barrier Membrane Containing Safflower Seed extracts on the Periodontal Healing of One-Wall Intrabony Defects in Beagle Dogs

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Ⅰ. Introduction

The ultimate purpose of periodontal therapy is the elimination of the cause of the disease and the structural and functional regeneration of lost periodontal tissue. Although it is very difficult to attain this goal, recent advances in periodontal wound healing concepts encourage hope reaching it.

In the performance of conventional periodontal therapy, it has been reported that the rapid apical migration of the gingival epithelium inhibits new attachments. (Caton et al., 1980; Caton et al., 1980)

Therefore, the surgical methods that can avoid repopulation of the cells interfering with a new attachment except periodontal ligament cells have been designed. Surgery with various kinds of bone grafts (Kim et al., 1998; Kim et al., 1998; Kim et al., 1998; Kim et al., 1996) and root

conditioning (Lowenguth et al., 1993) have produced some clinical success. However total regeneration of the complete periodontal tissue is limited. Melcher has suggested that periodontal tissues may regenerate depending on the cell types that migrate onto root surfaces, and that periodontal ligament cells are capable of regenerating periodontal tissues (Melcher, 1976), Nyman et al. have shown cementum regeneration with principal fiber insertion using a cellulose acetate filter in human case (Nyman et al., 1982). Gottlow et al. proposing the term "guided tissue regeneration" (GTR) procedure, have shown regeneration in several intrabony defect cases using expanded polytetrafluoroethylene (ePTFE) membranes (Gottlow et al., 1986). Although guided tissue regenerative procedures have improved the amount of new attachment formation, the results remain unpredictable and show limited new cementum and bone formation.

Therefore, investigators have used membranes in conjunction with osteoinductive materials such as autogenous cancellous bone or decalcified freeze-dried bone allograft (DFDBA) (Blumenthal et al., 1990). But fresh autogenous cancellous bone has problems associated with harvesting, while DFDBA has problems associated with possible contamination of disease (Buck et al., 1989), displacement from the graft site due to pressure from the overlying flaps and limited capacity to promote horizontal bone formation (Bower et al., 1989). Recent studies on the regeneration of periodontal tissue have focused not only GTR, but also various growth factors (Caffesse et al., 1993; Giannobile et al.,

1998; Terranova et al., 1987) and enamel matrix derivatives (Hammarstrom, 1997; Heiji et al., 1997) to induce the proliferation and differentiation of the cells related to periodontal regeneration. The use of growth factors with stimulatory effects on cell migration and proliferation, and synthesis of matrix components by the cells has been proposed to promote periodontal regeneration. However, the specific effects of growth factors on periodontal ligament fibroblast activities are not fully understood and the best mode of their application to promote periodontal regeneration remains to be identified. (Cho et al., 1995)

Recently efforts are concentrated on the regeneration potential of materials used in oriental medicine. In some in vitro and in vivo experiments, there have been many evidences that these materials have an effect on bone regeneration. (Kim et al., 2000)

Among these natural materials, Safflower (*Carthamus tincotorius* LINNE) seeds have been used for the treatment of blood stasis, bone fracture and osteoporosis in traditional Korean medicine. Safflower is an annual herbaceous plant belonging to a composite of chrysanthemum and has been used to promote the blood circulation, to resolve blood stasis and to control pain in oriental medicine (Kutsuna et al., 1988). Huh et al. reported that a Safflower seed fraction extract stimulate the formation of calcification nodules, and also has an increasing effect on mRNA expression of alkaline phosphatase and bone sialoprotein in periodontal ligament cells and MC3T3-E1 cells (Huh et al., 2001). Kim et al. suggested that among the segments extracted sequentially from

powdered Safflower seed with hexane, chloroform, MeOH, the water-soluble segment shows the best activity for inducing the formation of calcification nodules in osteoblast, and had the best bone formation capacities. Also they showed that Safflower seed extracts have an effect initially on the length and radiopacity at the newly formed bone area when applied to calvarial defects in Sprague-Dawley rats (Kim et al., 1996). Despite of the encouraging results, a controlled investigation into the effects of the Safflower seed extracts on the regeneration of periodontal tissue has yet to be conducted.

Periodontal regeneration of lost supporting tissues ideally requires a barrier membrane which is not only osteoconductive, acting as a scaffold on which new bone is formed, but also one which contains an osteoinductive agent to promote both new alveolar bone and cementum formation (King et al., 1998). So, at the present study, poly lactide glycolic acid (PLGA, copolymer of poly glycolic acid and poly lactic acid) nonwoven membrane was used as a barrier membrane and Safflower seed extracts (SSE) were used as an osteoinductive agent. The objective of this study is to evaluate the periodontal tissue regenerative effects of the bio-resorbable membrane containing extracts from Safflower seeds applied to surgically created one-wall intrabony defects in beagle dogs.

Ⅱ. Materials & Methods

A. Animals

Six 2-year-old Beagle dogs, approximate weight 15 kg, were used. The dogs exhibited intact dentition with a healthy periodontium. The selection of animals, management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea. The animals were fed a soft diet throughout the study to reduce chance of mechanical interference with healing during food intake.

B. Bio-resorbable membrane and SSE constructs

PLGA is a copolymer of poly glycolic acid (PGA) and poly lactic acid (PLA). PLGA is a biodegradable high-molecular material that widely used and has hydrophilic property. It is possible to control the time to resolve. For making the PLGA electro-spun nonwoven membrane, PLGA chip was melted at 240℃ and high voltage was applied between pressured shooting nozzle and revolving drum. After the PLGA was accumulated on the surface of rotating drum, took up the PLGA. In the pilot study, heat treated (annealing) at 130℃ nonwoven membrane had slow disintegration velocity and more stable property than that were treated at 110℃ or 120℃. These heat treated membranes were used for

barrier membrane.

Powdered Safflower Seeds were extracted with n-hexane, the remnant was extracted with chloroform, methanol and 70% ethanol. The methanol extract(saf-M) was divided into the chloroform layer, water layer and the insoluble layer. Among the segments, the saf-M-W segment that showed the best activity in the formation of calcification nodules in osteoblasts was selected. In the pilot study, the efficacy of fractions with various concentrations was evaluated in terms of cytotoxicity and the effect on osteoblast activity (Huh et al., 2001). On the basis of the results, appropriate concentration(10㎍/㎖) was chosen. PLGA nonwoven membrane was soaked, dried at room temperature and used for guided tissue regenerative procedures.

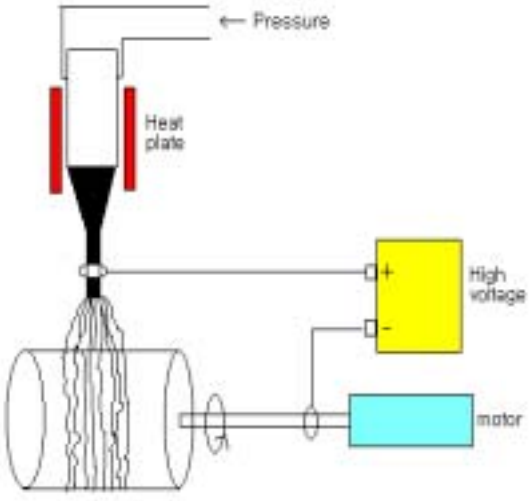

Figure 1. A schematic diagram of electro-spun nonwoven process.

C. Experimental design

Surgically created defects in the surgical control group were given the flap operation only. Defects in the PLGA group were received guided tissue regeneration (GTR) procedure with PLGA nonwoven membrane. And defects in the SSE/PLGA group were received GTR procedure with PLGA nonwoven membrane containing SSE.

D. Surgical protocol

The surgical procedure was performed under general anesthesia induced by an intravenous injection of atrophine (0.04 mg/kg; Kwangmyung Pharmaceutical Ind. Co. LTD. Seoul, Korea) and intramuscular induction with a compound of xylazin (Rompun, Bayer Korea Co., Seoul, Korea) and ketamine (Ketara, Yuhan Co., Seoul, Korea), followed inhalation (Gerolan, Choongwae Pharmaceutical Co., Seoul, Korea). Routine dental infiltration anesthesia (2% lidocaine hydrochloride with 1/80,000 epinephrine) was used at the surgical sites. The mandibular first and third premolars were extracted in advance of the experimental surgery and the extraction sites were allowed to heal for 8 weeks.

At reconstructive surgery, the buccal and lingual mucoperiosteal flaps were elevated and 4×4 mm one-wall intrabony defects were surgically created with burs at the mesial and distal aspects of the mandibular second premolars and mesial aspects of the fourth premolars. Following

throughout root planing, a reference notch was made with a round bur on the root surface at the base of defect. Each bilateral intrabony defects received one of the three experimental conditions: GTR with SSE/PLGA membrane, GTR with PLGA membrane only or root planing only. Experimental conditions were rotated among the defects sites in subsequent animals. A nonwoven membrane was adapted above the alveolar crest with approximately 3 mm extension over the defect bone margins. Gingival flaps were adapted to cover the membrane and suturing was accomplished without tension. The sutures were removed 7 days after surgery.

E. Post-surgical management

Post-surgery management included the administration of antibiotics intramuscularly (Tetracycline HCl, Chongkundang Pharmaceutical Co., Seoul, Korea), a soft diet and a daily topical application of a 0.12% chlorhexidine solution (hexamedine, Bukwang Pharmaceutical Co., Seoul, Korea).

F. Clinical and Histologic procedures

The animals sacrificed 8 weeks after the first surgical procedure by an intravenous injection of concentrated sodium pentobarbital. Block sections including the surgical sites were removed, rinsed in saline, and

fixed in 10% buffered formalin for 10 days. Subsequently, the block sections were decalcified in 5% nitric acid for 7~8 days and embedded in paraffin. 5 ㎛ thick serial sections were made in the mesiodistal direction. The four most central sections from each block were stained with hematoxylin/eosin and examined using light microscopy.

G. Analysis

1. Histologic Analysis

A PC-based image analysis system (Image-Pro Plus, Media cybernetics, Silver Spring, MD, USA) was used to observe the experimental sites with regards to junctional epithelium migration, the regeneration of connective tissue, the formation of new bone and new cementum, root resorption, ankylosis, and the states of implanted materials, etc.

2. Histometric Analysis (Figure 2)

For the histometric analysis, the CEJ and the notch were used as reference points. The histometric parameters were:

▪ Defect height : the distance from CEJ to the base of the reference notch.

▪ Junctional epithelium migration : the distance from CEJ to the apical

extension of the junctional epithelium.

▪ Connective tissue adhesion : the distance from the apical extension of the junctional epithelium to the coronal extension of cementum regeneration.

▪ Cementum regeneration : the distance from the base of the reference notch to the coronal extension of the newly formed cementum on the root surface.

- Infrabony cementum regeneration : the regenerated cementum where there are perpendicularly inserted fibers or where it is lined with cementoblast-like cells.

- Suprabony cementum regeneration : the regenerated cementum above the intrabony cementum regeneration, where there are horizontally oriented fibers or it is barely lined with cementoblast-like cells. It is not easy to distinguish the cementoid tissue and the true cementum using light microscope.

▪ Alveolar bone regeneration : distance from the base of the reference notch to the coronal extension of the newly formed alveolar bone along the root surface.

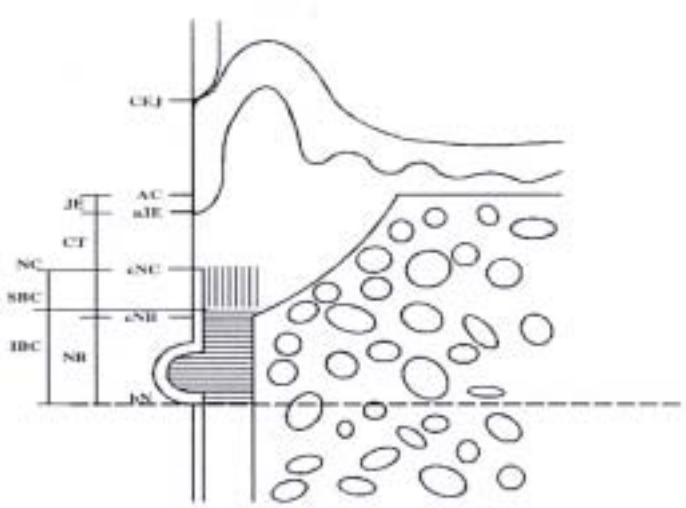

Figure 2. A schematic diagram depicting the experimental design, the landmarks and the parameters used in histometric analysis.

CEJ: cementoenamel junction
aJE: apical extent of junctional epithelium
cNB: coronal extent of new bone
JE: junctional epithelium migration
NC: new cementum regeneration
IBC: infrabony cementum regeneration
AC: alveolar crest
cNC: coronal extent of cementum
bN: base of the reference notch
CT: connective tissue adhesion
NB: new bone regeneration
SBC: suprabony cementum regeneration

H. Statistical Analysis

Histometric recordings from the 4 sections in each block were used to calculate the mean scores for each defect (mean±SD). The data were used to test for differences among the experimental groups using Kruskal-Wallis test. The Mann-Whitney U test was used for a comparison between groups.

Ⅲ. Results

A. Histologic observations

Surgical control group: The junctional epithelium migrated apically and the collagen fibers were observed in a irregular or loose pattern beneath the junctional epithelium. Inflammatory cell infiltration was minimal in the most of defects. But some inflammatory cells were observed at connective tissue area and near the junctional epithelium. Above the reference notch, new bone and new cementum had formed along the root surface. But the amount of new bone was smaller than that of other groups and the length of new cementum was shorter than that of other groups. The thickness of cementum was thickest at the notch area and became thinner coronally. The periodontal ligament fibers were usually oriented in a parallel direction but in some areas they were perpendicular to the root surface.

PLGA group: The amount of junctional epithelium migration was similar to that of surgical control group. Inflammatory cell infiltration was minimal. Residual PLGA nonwoven membrane remnants were observed at the connective tissue area in some specimens. The arrangement of the periodontal ligament fibers were shown more regular and dense pattern than that of the surgical control group. The amount of newly formed bone and cementum was increased compared to surgical control group. The thickness of cementum was thickest at

the notch area and became thinner coronally, too.

SSE/PLGA group: The amount of junctional epithelium migration was shorter than other groups. Inflammatory cell infiltration was minimal in the most of defects. Residual PLGA nonwoven membranes were observed at the connective tissue area in some specimens. The amount of new alveolar bone regeneration was greater than that of the surgical control group. The border line between the new bone and the old bone was indistinguishable. Osteoblast-like cells were arranged along the new bone surface. The amount of new cementum increased significantly along the root surface compared to surgical control group. The thickness of cementum was thickest at the notch area and became thinner coronally. In the infrabony cementum area, cementoblast-like cells were closely arranged and fibers embedded perpendicularly. On the other hand, above this cementum area, the suprabony cementum, a thin cementum layer was observed and fibers showed parallel orientation. Cementoblast-like cells were rare. Therefore, it is believed that the cementum layer with perpendicularly embedded fibers is the true cementum in the newly formed cementum. The periodontal ligament was shown more regular pattern than that of surgical control and denser fiber arrangement.

All groups maintained a good periodontal ligament space. Limited root resorption was observed in the majority of defects and appeared greater in the connective tissue immediately interfaced with root dentin than in the tissue covered by new cementum. None of the specimens showed the sign of ankylosis.

B. Histometric observations (Table 1, Figure 3)

The defect height averaged 4.34±0.41 mm, 4.66±0.27 mm and 4.81±0.18 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. There was no significant differences among groups.

The junctional epithelium migration was 0.62±0.80 mm, 0.51±0.50 mm and 0.21±0.74 mm, respectively, for the surgical control group, the PLGA group, and the SSE/PLGA group, without statistical significant differences among groups.

The amount of connective tissue adhesion was 1.24±0.24 mm, 0.94±0.13 mm and 0.97±0.15 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. Significant difference was observed between PLGA group and surgical control group ($p<0.05$).

The amount of cementum regeneration was 2.49±0.41 mm, 3.22±0.35 mm and 3.67±0.82 mm for the surgical control group, the PGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The amount of infrabony cementum averaged 1.75±0.06 mm, 2.40±0.33 mm and 2.70±0.81 mm for the surgical control group, the PGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The suprabony cementum averaged 0.73±0.48

mm, 0.82±0.21 mm and 0.97±0.09 mm, respectively, for the surgical control group, the PLGA group, and the SSE/PLGA group, without significant differences among groups.

The amount of alveolar bone regeneration was 1.74±0.25 mm, 2.36±0.30 mm and 2.64±0.74 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). There was no significant differences between the PLGA group and SSE/PLGA group at any items.

Table 1. Histometric analysis (mm)

	Surgical Control group	PLGA group	SSE/PLGA group
	Mean ± SD	Mean ± SD	Mean ± SD
DH	4.34 ± 0.41	4.66 ± 0.27	4.81 ± 0.18
JE	0.62 ± 0.80	0.51 ± 0.50	0.21 ± 0.74
CT	1.24 ± 0.24	0.94 ± 0.13 *	0.97 ± 0.15
NC	2.49 ± 0.41	3.22 ± 0.35 *	3.67 ± 0.82 *
IBC	1.75 ± 0.06	2.40 ± 0.33 *	2.70 ± 0.81 *
SBC	0.73 ± 0.48	0.82 ± 0.21	0.97 ± 0.09
NB	1.74 ± 0.25	2.36 ± 0.30 *	2.64 ± 0.74 *

DH : Defect height
JE : Junctional epithelium migration
CT : Connective tissue adhesion
NC : New cementum
IBC : Infrabony cementum
SBC : Suprabony cementum
NB : New bone

* : Statistically significant difference compared to surgical control group. $p<0.05$

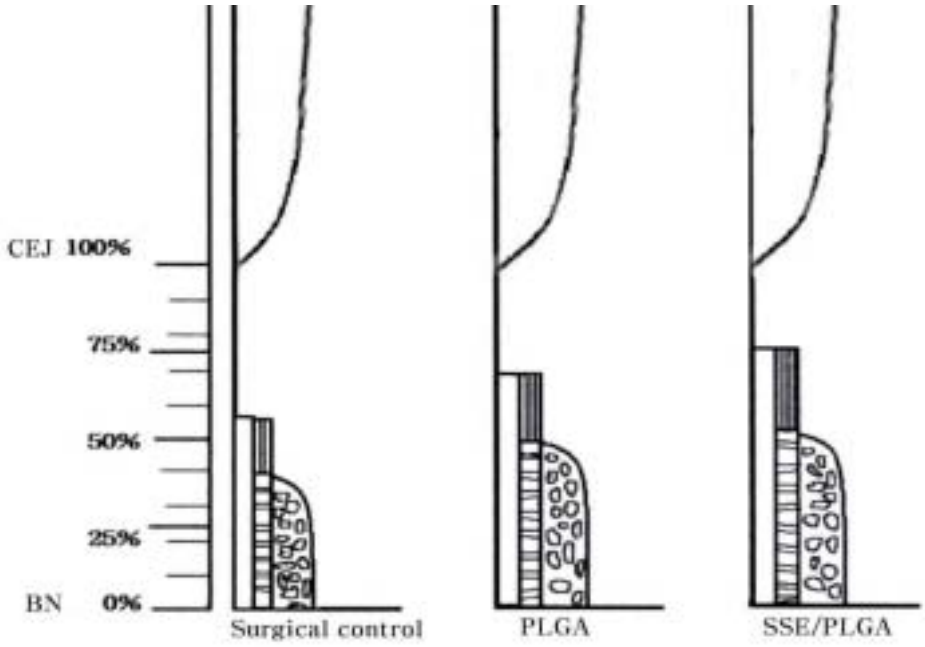

Figure 3. Periodontal healing illustrated as percentage of the defect height.
CEJ: cementoenamel junction, BN: base of notch

□ : new cementum
▥ : suprabony cementum
▤ : infrabony cementum
⁘ : new bone

Ⅳ. Discussion

True periodontal regeneration means healing after periodontal treatment that results in the regain of lost supporting tissues including new acellular cementum attached to the underlying dentin surface, a new periodontal ligament with functionally oriented collagen fibers inserting into the new cementum and new alveolar bone attached to the periodontal ligament. But complete and predictable true regeneration is still a goal that is difficult to attain (Heiji et al., 1997). A variety of therapeutic techniques, such as root planing and curettage, gingivectomy and flap procedures including transplantation of various materials into defects, have been used to obtain regeneration of the periodontium. However total regeneration of complete periodontal tissue is limited. This may be due to primary population of the wound by tissues with poor regenerative capacities (Melcher, 1976). Various bone grafts have been utilized to solve intrabony defects. But, fresh autogenous cancellous bone has problems associated with harvesting limitation in the amount of grafts that can be obtained. DFDBA has problems associated with possible contamination and transmission of disease (Buck et al., 1989), displacement from the graft site due to pressure from the overlying flaps and limited capacity to promote horizontal bone formation (Bower et al., 1989). And DFDBA contains little bone morphogenetic proteins and has osteoconductive properties. Previous studies have shown epithelium to migrate apically between the root and

adjacent tissues, preventing regeneration. The periodontal ligament contains undifferentiated mesenchymal cells with the potential to differentiate into cementoblasts and osteoblasts (Boyko et al., 1981). Gottlow et al. have shown that cells from the periodontal ligament have the potential to regenerate new attachment if they are given preference in populating the wound (Gottlow et al., 1984; Gottlow et al., 1986). Various filters and membrane materials have been used to delay the gingival epithelium and connective tissue from entering the wound. These treatments have resulted in the formation of substantial amounts of new attachment, but they do nothing to improve the migration of periodontal ligament cells within the defect, nor do they encourage regeneration of lost alveolar bone (Blumental et al., 1990). And these therapies have limited efficacy on periodontal regeneration of especially large-sized, multiple bony defects. In the early 1980s, Urist et al. isolated the proteins responsible for osteoinductive activity in cancellous bone and decalcified freeze-dried bone into a partially purified form (Urist et al., 1984; Urist et al., 1982). These bone morphogenetic proteins(BMPs) are a set of growth and differentiation factors acting on early osteoprogenitor cells to differentiate into mature osteoblasts, resulting in the formation of new bone and new cartilage when implanted in animals. Recently both rhBMP-2 and rhBMP-7 have been shown to increase the regeneration of alveolar bone, cementum and connective tissue attachment lost due to periodontal disease (Sigurdsson et al., 1995). rhBMP-2 when incorporated in a collagen gel promoted a

significant increase in early new bone formation and a trend towards increased cementum formation. However, additional unwanted bone formation occurred beyond the defect on the adjacent cortex at some distance from the site of application (King et al., 1998). In addition to BMPs, the use of growth factors with stimulatory effects on cell migration and proliferation, and synthesis of matrix components by the cells has been proposed to promote periodontal regeneration in animal studies (Caffesse et al., 1993). However, the specific effects of growth factors on periodontal ligament fibroblast activities are not fully understood and the best mode of their application to promote periodontal regeneration remains to be identified.

Recently interests in the search for a new non-toxic, biodegradable material that would be free from any side effect has been growing. Therefore many efforts are concentrated on the regeneration potential of materials used in oriental medicine. In some in vitro and in vivo experiments, there have been many evidences that these materials have an effect on bone regeneration (Kim et al., 2000). Among those natural materials, Safflower (*Carthamus tincotorius* LINNE) seeds have been used for the treatment of blood stasis, bone fracture and osteoporosis in traditional Korean medicine. Safflower is an annual herbaceous plant belonging to a composite of chrysanthemum and has been used to promote the blood circulation, to resolve blood stasis and to control pain in oriental medicine (Kutsuna et al., 1988; Shen et al., 1994).

Safflower seed is composed of various components, and these

components may have a synergic effect on the required efficacy, but because of its possibility as antagonist, the individual components need to be separated and purified. In this experiment, it was used the segment saf-M-W, which stimulated the activity on calcification nodule formation in osteoblast among segments extracted with various organic solvents. Powdered Safflower Seeds were extracted with n-hexane, the remnant was extracted with chloroform, methanol and 70% ethanol. The methanol extract(saf-M) was divided into the chloroform layer, water layer and the insoluble layer. Among the segments, the saf-M-W segment that showed the best activity in the formation of calcification nodules in osteoblasts was selected. In the pilot study, the efficacy of fractions with various concentrations was evaluated in terms of cytotoxicity and the effect on osteoblast activity (Huh et al., 2001). On the basis of the results, appropriate concentration for periodontal ligament cells was 10 ㎍/㎖. A previous study using rat, suggested that a Safflower seed extract at this concentration had a positive effect on bone density, size and length compared to the control when it was applied to calvarial defects that were surgically created (Kim et al., 2000)

To apply liquid phase Safflower seed extract into the 1-wall intrabony defect, a carrier required. PLGA nonwoven membrane was used as a carrier. PLGA is a copolymer of PGA and PLA. PLGA is a biodegradable high-molecular material that widely used and has hydrophilic property. It is possible to control the time to resolve. PLGA

nonwoven membrane has several advantages compared to other barrier membranes. First of all, there are many porosities in PLGA membrane, so profitable for blood circulation and tissue integration. And the increased specific surface area because of many porosities makes the membrane contact the tissue more widely. Also, the membrane has flexibility, so easy to manipulate. In addition, there is an economic benefit than previously used membranes. PLGA nonwoven membrane absorbing osteoinductive materials maintained the osteoinductive effect for a long time by preventing material washout, releasing them into the defects. The surface of PLGA membrane soaked the SSE was somewhat sticky. The thickness of membrane was irregular from 50 to 100 ㎛, because it was made by hands. An ideal carrier would be easy to soak and manipulate, does not provoke an immune reaction, maintains the space by supporting the flaps and does not hinder cell differentiation and vascular infiltration (Sigurdson et al., 1996). The PLGA membranes acted as a suitable carrier system for the Safflower seeds extracts as well as barrier membrane.

Dogs are commonly used in studies of new attachment. However it has been found that when surgically created defects are used to test implants in dogs, such lesions have a tendency for spontaneous regeneration (Bluementhal et al., 1986) Therefore, in this experiment, one-wall defects with only interproximal bony wall which had minimal self-healing capacities were used to examine the efficacy of SSE as an implantation material in periodontal regeneration. The defect

configuration is a primary variable in periodontal regeneration. More walls mean better osteogenesis, since an increase in number of walls bring about an ample supply of osteogenic cells (Mellonig, 1984). Furthermore, the more walls of a defect facilitate firmer fixation of the implanted materials and easier suturing, which in turn helps suppress epithelial migration. The use of surgical defects was decided based on the previous report showing that the healing process shows no difference between the surgical defects and the attachment loss caused by natural disease or ligation (Wikesjo et al., 1991), despite of the previous report that unoperated controls are necessary to assure that healing seen in grafted defects was the result of the grafts and not the capacity of the lesions to regenerate spontaneously (Bluementhal et al., 1986).

The observation period in the histological studies in animals has varied from 2 weeks to 3 or 6 months. In many studies (Kim et al., 1998; Polson et al., 1982; Sigurdsson et al., 1995), 8 week healing interval has been required to evaluate bone regeneration of space maintaining biomaterials, and Choi et al. reported that there was no significant differences in bone regeneration by BMP between 8 and 24 week interval (Choi et al., 2002). Therefore, 8 week healing period is sufficient to observe the initial healing process.

In the present study, there is no significant difference in junctional epithelium migration among groups. The value in the SSE/PLGA group was smaller than other two groups, it was not significant and showed

rather irregular pattern. In the previous reports (Kim et al., 1998; Wikesjo et al., 1991), high rate of migration was seen in the surgical control group, however the different outcomes were observed at this experiment.

In connective tissue adhesion, statistically significant difference was seen between PLGA group and surgical control group ($p<0.05$). But the gap of two value was not so big and the amount of connective tissue adhesion of SSE/PLGA group was rather big than that of PLGA group. Though the three groups showed a little difference in connective tissue adhesion, PLGA and SSE/PLGA groups had a tighter distribution of collagen fibers and parts of them showed functional arrangement, while the surgical control group had relatively loosely distributed collagen fibers with their orientation varying from random to parallel adjacent to the roots.

New bone regeneration was measured 54.9%, 50.6% and 40.1% of the defect height for the SSE/PLGA group, PLGA group and the surgical control group respectively, with statistically significant difference between the surgical control group and the other groups ($p<0.05$). No significant difference was shown between PLGA and SSE/PLGA groups. In previous studies with BMP in dogs, Choi et al. reported 65-68% of bone regeneration in three-wall defects (Choi et al., 2002), Wikesjo et al. (Wikesjo et al., 1991) and Sigurdsson et al. (Sigurdsson et al., 1995) reported 86-96%, 94% of regeneration respectively, in supraalveolar defects. In a PDGF study, Cho et al. reported 80% of

bone fill in the Class III furcation defects (Cho et al., 1995). Although, no standardization of the periodontal defects configuration among studies was done, the value that obtained at this study was too small. On the contrary, the surgical control group yielded a higher rate of regeneration than the 0% by Wikesjo et al. and 14% by Sigurdsson et al., both of whom used horizontal defects. Results were not to be enough, but Safflower seed extract and PLGA nonwoven membrane showed statistically significant positive effects distinctly on the regeneration of bone. The border line between the new bone and the old bone was indistinguishable. The newly formed bone was frequently lined with osteoblast-like cells and osteoids, so continued bone formation after observation period was expected. Studies have shown that healing of large ungrafted defects and defects filled with osteoconductive material is usually preceded by bone ingrowth from edge of the defect (Lindholm et al., 1994). However, when an osteoinductive agent is used in conjunction with an osteoconductive material the new bone also develops in the central parts of the defect (Ripamonti et al., 1993; Ripamonti et al., 1992). But in this study, the coronal thinning of new bone was observed in all groups, it was suggested that SSE and PLGA nonwoven membrane might not act as a good osteoinductive material and osteoconductive barrier.

The amount of new cementum was measured about 76.3%, 69.1%, 57.4% of the defect height for the SSE/PLGA group, PLGA group and the surgical control group respectively, with significant difference

between the surgical control group and the other groups ($p<0.05$). No significant difference was shown between PLGA and SSE/PLGA groups. In previous BMP studies in dogs, Choi et al. reported 54% in three-wall defects, Sigurdsson et al. reported 40% regeneration in supraalveolar defects. In comparison, this study showed more regenerated cementum. Defects treated with GTR using PLGA nonwoven membrane and/or SSE showed high value than surgical control sites. It is thought that PLGA nonwoven membrane effects the regeneration of cementum by acting as a barrier inhibiting epithelial migration by stabilizing wounds, inducing cells from periodontal ligament, promoting regeneration of new cementum (Bluementhal et al., 1986; Yaffe et al., 1984). The infrabony cementum regeneration was measured 56.1%, 51.5% and 40.3% of the defect height for the SSE/PLGA group, PLGA group and the surgical control group respectively, with statistically significant difference between the surgical control group and the other groups ($p<0.05$) The percentages of suprabony cementum were 20.2%, 17.6% and 16.8% of the defect height for the SSE/PLGA group, PLGA group and the surgical control group respectively. The value of each group was somewhat similar and no significant difference was shown. Usually, the cementum did not include obvious embedded cell structure and a cementoid layer or cementoblast-like cells were difficult to demonstrate (Sigurdsson et al., 1995). In this study, from the notch to the crest, regenerated cementum appeared cellular, usually covered by cementoblast-like cells and

cementoid materials. But above the crest, cementum with few cellular elements formed a thin strip along the root surface.

In all three groups, the perpendicular periodontal fiber orientation was found mostly where the bone was adjacent to the new cementum. This result suggests that the suprabony site in a periodontal defect is healed by the fibers of a gingival origin, while the infrabony site is healed by the fibers of periodontal ligament origin (Barney et al., 1986). This difference in wound healing accounts for why a suprabony site is more likely to lose structural, functional continuity in the case of a relapse.

Root resorption appeared to be a common sequela of repair in experimental periodontal defects (Wikesjo et al., 1991). Most teeth in the present study exhibited superficial resorption, appearing more pronounced when the connective tissue was directly opposed to dentin rather than when the root surface was covered by cementum. This is in agreement with the suggestion that cementum matrix formation may prevent resorption (Schroeder, 1992). But no relevant correlation among groups was detected.

No root ankylosis was seen any of specimens. It was regarded as a consequence of extensive bone regeneration and often occurs in sites of fast osteogenetic development without the regeneration of the periodontal ligaments (Card et al., 1987). Usually, periodontal ligament cells are far faster in repopulation at the root surface than osteogenic cells.

Overall, it was thought that SSE and PLGA nonwoven membrane to

a certain extent promote the formation of new bone and new cementum. However, further analysis of how it promote periodontal regeneration and components of Safflower seed extract is required. Also, more effective delivery system and the specific application protocol for clinical use should be established.

Ⅴ. Conclusion

Recently interests in the search for a new non-toxic, biodegradable material that would be free from any side effect has been growing and many efforts are concentrated on the regeneration potential of materials used in oriental medicine. Safflower seeds have been used for the treatment of blood stasis, bone fracture and osteoporosis in traditional Korean medicine.

The objective of this study is to evaluate the periodontal tissue regenerative effects of the bio-resorbable barrier membrane (PLGA electro-spun nonwoven membrane) containing Safflower seed extracts applied to surgically created one-wall intrabony defects in beagle dogs. One-wall intrabony defects were surgically created at the bilateral mandibular second and fourth premolars. Surgically created defects in the surgical control group were given the flap operation only. Defects in the PLGA group were received GTR procedure with PLGA nonwoven membrane. And defects in the SSE/PLGA group were received GTR procedure with PLGA nonwoven membrane containing SSE. The subjects were sacrificed 8 weeks after the operation, and comparative histological examination was done. The results are as follows:

1. The defect height averaged 4.34±0.41 mm, 4.66±0.27 mm and 4.81±0.18 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. There was no significant differences

among groups.

2. The junctional epithelium migration was 0.62±0.80 mm, 0.51±0.50 mm and 0.21±0.74 mm, respectively, for the surgical control group, the PLGA group, and the SSE/PLGA group, without statistical significant differences among groups.

3. The amount of connective tissue adhesion was 1.24±0.24 mm, 0.94±0.13 mm and 0.97±0.15 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. Significant difference was observed between PLGA group and surgical control group ($p<0.05$).

4. The amount of cementum regeneration was 2.49±0.41 mm, 3.22±0.35 mm and 3.67±0.82 mm for the surgical control group, the PGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The amount of infrabony cementum averaged 1.75±0.06 mm, 2.40±0.33 mm and 2.70±0.81 mm for the surgical control group, the PGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$). The suprabony cementum averaged 0.73±0.48 mm, 0.82±0.21 mm and 0.97±0.09 mm, respectively, for the surgical control group, the PLGA group, and the SSE/PLGA group, without

significant differences among groups.

5. The amount of alveolar bone regeneration was 1.74±0.25 mm, 2.36±0.30 mm and 2.64±0.74 mm for the surgical control group, the PLGA group, and the SSE/PLGA group, respectively. The PLGA group and the SSE/PLGA group were significantly different from the surgical control group ($p<0.05$).

Surgical application of PLGA nonwoven membrane with or without Safflower seed extract promoted the regeneration of alveolar bone and cementum in intrabony periodontal defects.

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Ⅶ. 국문요약

성견의 1면 골내낭에서 홍화 추출물 함유 생체분해성 차단막의
치주조직 재생효과

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송 원 석

연구배경: 최근에는 동양의 의학에서 사용되어 온 재료들의 재생 능력에 대해 많은 연구가 이루어지고 있다. 몇몇 실험에서는 이런 재료들이 골 재생에 영향을 미친다는 많은 증거들을 보였다. 이런 재료들 중 홍화씨는 전통 한의학에서 울혈이나 골절, 골다공증 등의 치료에 사용되어져왔으며 혈액 순환을 촉진시켜서 항염작용을 나타내는 것으로 알려져 있다.

이 연구의 목적은 홍화씨 추출물을 함유한 생체 분해성 차단막(PLGA 부직포)을 beagle dog에서 외과적으로 형성한 1면 골내낭에 적용시켜서 치주조직의 재생에 미치는 영향을 평가하는 것이다.

재료 및 방법: Beagle dog에서 하악 양측의 제2 소구치의 근원심면과 제4 소구치의 원심면에 4×4mm 크기의 1면 골내낭을 외과적으로 형성하고, 임의로 위치를 선택하여 대조군에는 치은 박리 소파술만 시행하고, 실험1군에는 PLGA 부직포를 이용하여 조직 유도 재생술을 시행하고 (PLGA군), 실험2군은 홍화 추출물을 함유한 부직포를 이용하여 조직 유도 재생술을 시행한 후 (SSE/PLGA군) 봉합하였다.

실험동물은 술 후 8주에 희생시키고, 조직학적 검사를 시행하였다.

결과: 새로운 백악질의 형성은 대조군에서는 2.49±0.41 mm, PLGA군에서는 3.22±0.35 mm 그리고 SSE/PLGA군에서는 3.67±0.82 mm로 각각 측정되어, PLGA군과 SSE/PLGA군은 대조군과 통계적 유의차를 보였다 ($p<0.05$). 이 백악질 중 신생골 하방의 백악질 형성의 양은 대조군, PLGA군 그리고 SSE/PLGA군에서 각각 1.75±0.06 mm, 2.40±0.33 mm, 2.70±0.81 mm로 측정되었고, PLGA군과 SSE/PLGA군은 대조군과 통계적 유의차를 보였다 ($p<0.05$). 골 상방의 백악질 형성은 대조군, PLGA군 그리고 SSE/PLGA군에서 각각 0.73±0.48 mm, 0.82±0.21 mm, 0.97±0.09 mm로 측정되었고, 세 군간에 통계적 유의차는 보이지 않았다. 치조골 형성의 양은 대조군에서는 1.74±0.25 mm, PLGA군에서는 2.36±0.30 mm 그리고 SSE/PLGA 군에서는 2.64±0.74 mm로 각각 측정되어, PLGA군과 SSE/PLGA군은 대조군과 통계적 유의차를 보였다 ($p<0.05$).

결론: 홍화 추출물을 함유한 PLGA 부직포를 외과적으로 형성한 치조골 결손부에 적용시킨 경우 새로운 백악질 형성 및 치조골 재생이 촉진되었고, 또한 홍화 추출물을 함유하지 않은 PLGA 부직포를 이용한 경우에도 치주 조직 재생 유도 능력을 보여, PLGA 부직포를 이용한 치료가 소실된 치주 조직의 재생에 유용함을 보였다.

Ⅷ. Figure Legends

Figure 1. A schematic diagram of electro-spun nonwoven process.

Figure 2. A schematic diagram depicting the landmarks and parameters used in histomorphometric analysis.

Figure 3. Periodontal healing illustrated as percentage of the defect height.

Figure 4. Photomicrograph of the surgical control section. Epithelial downgrowth, connective tissue adhesion, new bone and cementum formation above the notch is observed. (Hematoxylin & eosin; original magnification×20)

Figure 5. Photomicrograph of the surgical control section in figure 3. New bone and new cementum formation is shown. Periodontal ligament is mainly organized with irregular and loose collagen fibers. Fiber adhesion shows parallel orientation along the root surface in the supracrestal region(arrow heads). (H&E×100)

Figure 6. Photomicrograph of the surgical control section in figure 3. Junctional epithelium is migrated apically and suprabony cementum formed a thin strip along the root surface. Irregular and loose fiber pattern in connective tissue area is observed. (H&E×100)

Figure 7. Photomicrograph of the PLGA section showing epithelial downgrowth, connective tissue adhesion and new bone formation above the notch. (H&E×20)

Figure 8. Photomicrograph of the PLGA section in figure 6. A slight amount of new bone and new cementum above the notch

is shown. On the cementum layer, there are closely arranged cementoblast-like cells (arrow heads) and fibers embedded perpendicularly. (H&E×100)

Figure 9. Photomicrograph of the PLGA section in figure 7.
Periodontal ligament fibers are embedded perpendicularly to new bone and new cementum. (H&E ×400)

Figure 10. Photomicrograph of the PLGA section in figure 6.
Suprabony cementum formed a thin strip along the root surfaceand fiber adhesion showed parallel orientation along the newly formed cementum in the supracrestal region(arrow heads). Root resorption is observed. (H&E×100)

Figure 11. Photomicrograph of the PLGA section in figure 6.
Junctional epithelium is migrated apically. Few inflammatory cells are shown. (H&E×100)

Figure 12. Photomicrograph of the SSE/PLGA section showing new bone formation above the notch. (H&E×20)

Figure 13. Photomicrograph of the SSE/PLGA section in figure 11.
Obliquely or perpendicularly oriented new periodontal ligament is observed between new bone and new cementum. (H&E×100)

Figure 14. Photomicrograph of the SSE/PLGA section in figure 12.
Periodontal ligament fibers embedded perpendicularly. (H&E×200)

Figure 15. Photomicrograph of the SSE/PLGA section in figure 13.
Periodontal ligament fibers are embedded perpendicularly to new bone and new cementum. (H&E×400)

Figure 16. Photomicrograph of the SSE/PLGA section in figure 11. Suprabony cementum formed a thin strip along the root surfce and fiber adhesion showed parallel orientation along the newly formed cementum in the supracrestal region(arrow heads). (H&E×100)

Figure 17. Photomicrograph of the SSE/PLGA section in figure 11. Junctional epithelium is migrated apically. Superficial root resorption is served at the connective tissue is directly opposed to dentin. (H&E×100)

Ⅷ. Figures

Figure 4 Control (H&E×20)

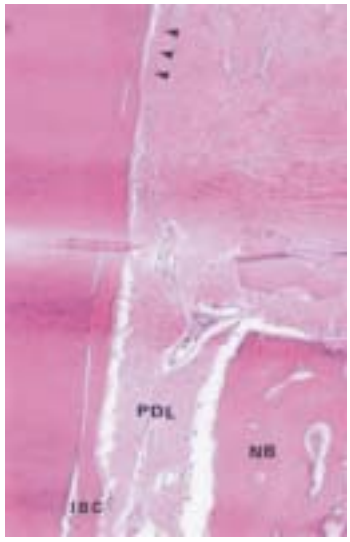

Figure 5 Control (H&E×100)

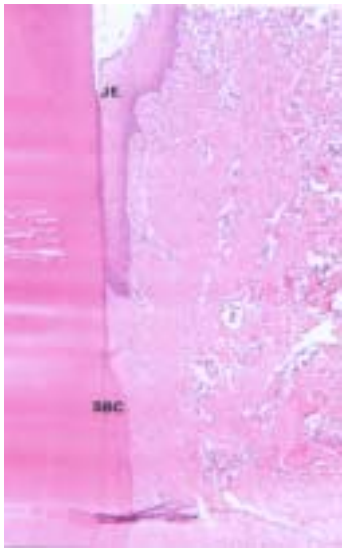

Figure 6 Control (H&E×100)

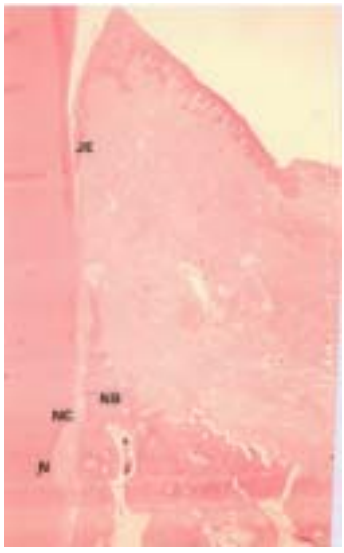

Figure 7 PLGA (H&E×20)

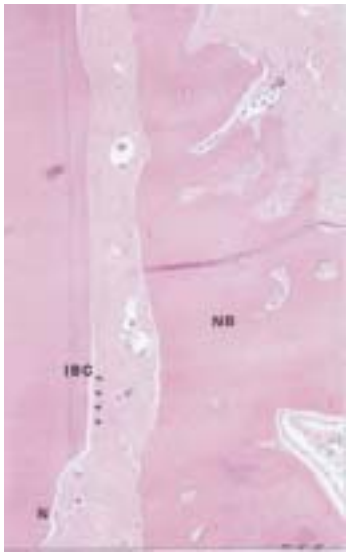

Figure 8 PLGA (H&E×100)

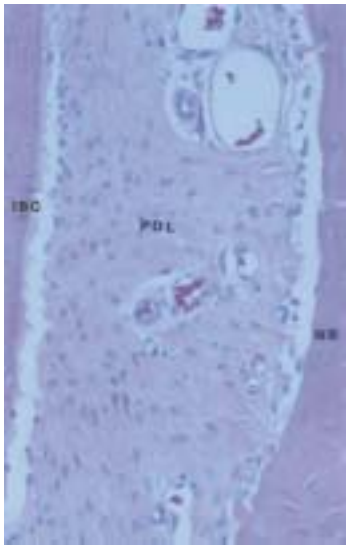

Figure 9 PLGA (H&E×400)

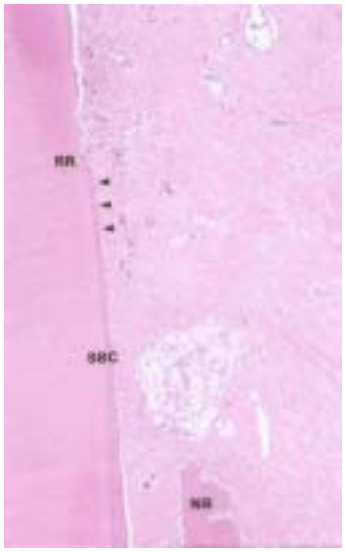

Figure 10 PLGA (H&E×100)

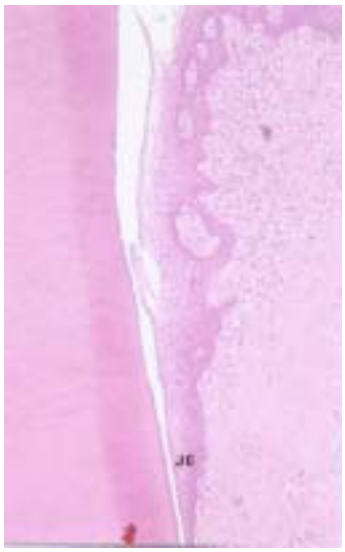

Figure 11 PLGA (H&E×100)

Figure 12 SSE/PLGA (H&E×20)

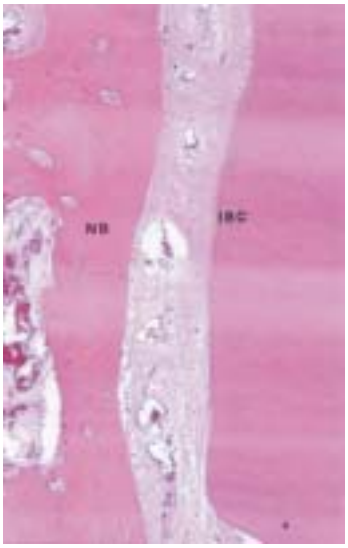

Figure 13 SSE/PLGA (H&E×100)

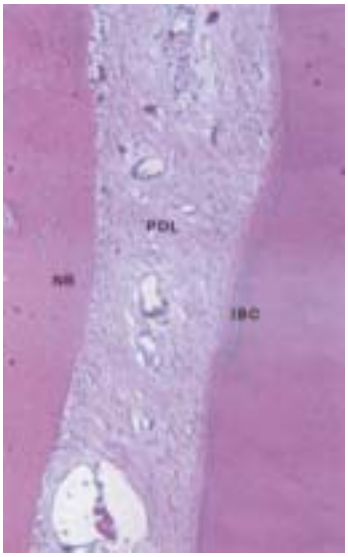

Figure 14 SSE/PLGA (H&E×200)

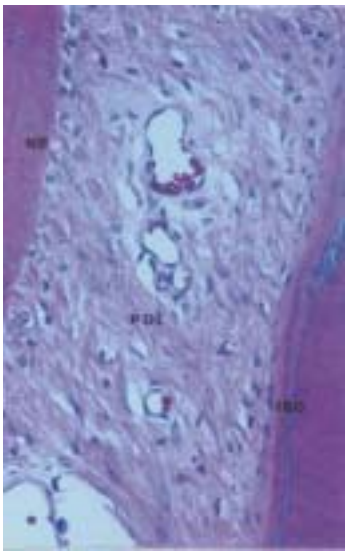

Figure 15 SSE/PLGA (H&E×400)

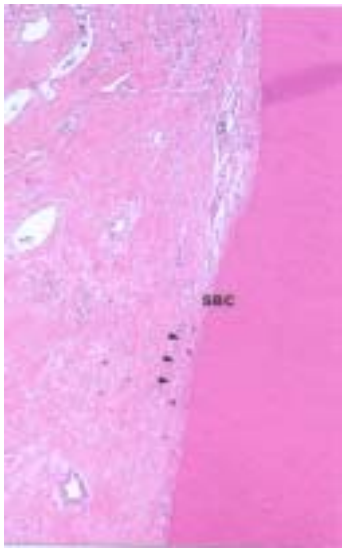

Figure 16 SSE/PLGA (H&E×100)

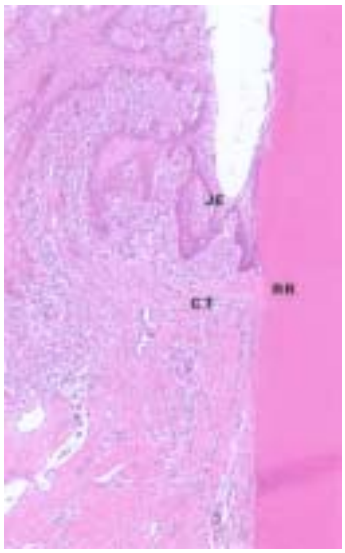

Figure 17 SSE/PLGA (H&E×100)