



The impact of thickness of resorbable membrane of human origin on the ossification of bone defects: a pathohistologic study

Uticaj debljine resorptivne membrane humanog porekla na osifikaciju koštanih defekata – patohistološka studija

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Abstract

Background/Aim. A wide range of resorbable and non-resorbable membranes have been investigated over the last two decades. The barrier membrane protects the defect from ingrowth of soft tissue cells and allows bone progenitor cells to develop bone within a blood clot that is formed beneath the barrier membrane. The membranes are applied to reconstruct small bony defect prior to implantation, to cover dehiscences and fenestrations around dental implants. The aim of this study was to evaluate the influence of human resorbable demineralized membrane (RHDM) thickness on bone regeneration. **Methods.** The experiment, approved by Ethical Committee, was performed on 6 dogs and conducted into three phases. Bone defects were created in all the 6 dogs on the left side of the mandible, 8 weeks after extraction of second, third and fourth premolars. One defect was covered with RHDM 100 μ thick, one with RHDM 200 μ thick, and the third defect left empty (control defect). The histopathological analysis was done 2, 4 and 6 months after the surgery. In the third phase samples of bone tissue were taken and subjected to histopathological analysis. **Results.** In all the 6 dogs the defects treated with RHDM 200 μ thick showed higher level of bone regeneration in comparison with the defect treated with RHDM 100 μ thick and especially with empty defect. **Conclusion.** Our results demonstrated that the thicker membrane showed the least soft tissue ingrowths and promoted better bone formation at 6 months compared with a thinner one.

Key words:

guided tissue regeneration; mandible; dogs; membranes, artificial.

Apstrakt

Uvod/Cilj. U poslednje dve decenije u brojnim eksperimentalnim i kliničkim studijama opisan je i ispitan veliki broj barijernih membrana, njihovih osobina i uloga u vođenoj koštanoj regeneraciji. Membrane imaju za cilj očuvanje krvnog ugruška formiranog u koštanim defektima alveolarne kosti, sprečavanje urastanja epitelnih ćelija u ugrađeni čvrsti zamenik kosti, kao i bolju fiksaciju postavljenih zamenika kosti. Primenuju se u rekonstrukciji manjih koštanih defekata pre ugradnje implantata, kod dehiscencionih i fenestracionih defekata koji se javljaju kod ugradnje implantata. Cilj rada bio je da se patohistološki ispita uticaj debljine humane resorptivne demineralizovane membrane na osifikaciju koštanih defekata u eksperimentalnoj studiji rađenoj na psima. **Metode.** Eksperimentalna studija, rađena na šest pasa rase nemačkog ovčara po svim etičkim principima, sprovedena je u tri faze. U prvoj fazi izvršena je ekstrakcija drugog, trećeg i četvrtog premolara sa leve strane. Osam nedelja nakon ekstrakcije, formirana su tri defekta na levoj strani mandibule i prekrivena humanom membranom debljine 100 μ , 200 μ , a treći defekt je ostavljen prazan (kontrolni defekt). Patohistološka analiza sprovedena je u periodu nakon dva, četiri, i šest meseci od hirurške intervencije. **Rezultati.** Patohistološkom analizom kod svih šest pasa, ustanovljeno je da su defekti prekriveni humanom membranom debljine 200 μ , imali znatno veći stepen koštane regeneracije u poređenju sa preostala dva defekta. **Zaključak.** Upotrebom humane membrane debljine 200 μ postiže se veći stepen koštane regeneracije.

Ključne reči:

tkivo, vođena regeneracija; mandibula; psi; membrane, veštačke.

Introduction

Guided bone regeneration, a method that has been generated from guided tissue regeneration, is based on the concept of bone separation from the soft tissue, *i.e.* the prevention of apical migration of gingival epithelial and connective tissue into the defect by the application of a barrier membrane which favors the proliferation of regeneration-capable cells and their differentiation into a desired type of tissue¹. Guided tissue regeneration is a procedure relevant mostly to natural teeth, while guided bone regeneration is primarily utilized in implantology, where the core of the problem is insufficient amount of bone for implant placement. Five surgical objectives should be realized for the goal of guided bone regeneration to be attained. This involves the use of an appropriate membrane, primary soft tissue healing, closure and maintenance of the membrane-shielded compartment, adaptation and stabilization of the membrane with adjacent bone, and a sufficiently long period of healing².

The final aim of the membrane as a barrier is the restitution of supporting tissues (bone or cement, or both, and periodontal ligament), where consequences of inflammation or trauma are present.

The properties of membranes for guided bone regeneration have been described by a number of authors³⁻⁵. These involve biocompatibility, appropriate barrier ability (mechanical prevention of soft tissue proliferation), tissue integration, immunologic neutrality, preservation of the space for new alveolar bone, and simplicity of application. Such a membrane must hold out against the masticatory forces and tissue tensions of the flap and prevent the collapse of soft tissues and wound space reduction. The property of integration into the tissue guarantees wound stabilization and inhibits epithelial migration⁶.

Depending on the reaction to their biologic environment, membranes are divided into resorbable and non-resorbable ones.

Non-resorbable membranes maintain their structure and shape in tissues, and their removal therefore requires additional surgical intervention, which is an additional trauma to the patient, with prolonged wound healing, increased costs, and prolonged overall therapeutic management of the patient. Their appliance is bounded because of the need for secondary surgery, high rate of exposure. These factors can bring about high risk of infection⁷.

Resorbable membranes do not require removal after placement, reducing patient discomfort and treatment costs, not to mention the risk of surgical complications. The duration of resorption of these membranes cannot be precisely determined due to their very nature (they can be natural or synthetic), *i.e.* the process of resorption starts as soon as they are placed into the tissue. The literature data about the expected membrane persistence *in vivo* range from 4 weeks to several months⁸.

The ability of collagen to stimulate adhesion, hemostasis, and physiologic degradation of progenitor cells, together with the ability of self-degradation, makes it an ideal material for the construction of membranes. It is also poorly

immunogenic, it induces hemostasis, it is capable of augmenting tissue thickness and interacts with a variety of cells during wound healing^{9,10}. Collagen types I and III of bovine or porcine origin are thus the principal components of most of the commercially available membranes.

Cross-linking increases structural stability and slows down the process of degradation. Collagen cross-linking is performed utilizing physical or chemical agents, such as ultraviolet or gamma radiation, hexamethylene, glutaraldehyde diphenyl phosphorylase, and ribose. By way of cross-linking, *in vivo* rate of resorption of collagen materials is controlled and reduced, and mechanical properties are improved. The essence of the process is the formation of various cross-links between certain amino acids and amino acid and carboxylate groups under the action of chemical or physical agents¹¹.

Membranes with a higher degree of cross-linking stay intact for longer periods of time¹². Studies have shown that premature membrane resorption or its removal can result in incomplete bone healing, and that is why it has been suggested that membranes applied in guided bone regeneration should have the period of degradation of 3 to 9 months, *i.e.* the period required for bone formation².

In the Department of Implantology, a resorbable human demineralized membrane (RHDM) has been developed (patent number 760/02). RHDM is an implantation material of human origin, the structure of which is a barrier to connective tissue ingrowth from the mucoperiosteal flap, and the organic composition of which stimulates osteogenesis of the host bone. RHDM is produced by the combination of physical and chemical methods (demineralization of cortical bone with successive removal of lipoproteins) from the calvarial region of human cadavers. Quantitative analyses (Micro-Qelidel, Hidroxyprolin test) and qualitative analyses (collagenase tests, electrophoresis) have shown that the membrane consists of organic components made of collagen type I. The membrane is sterilized using gamma radiation at the end of the production process, what makes it cross-linked.

The role of different thicknesses of RHDM in the regeneration of bone tissue has been insufficiently studied. Studies, especially histopathologic ones in bone regeneration have not been recorded in the literature, and the need for such studies is obvious.

The aim of this paper was to examine histopathologically the impact of thickness of human resorbable demineralized membrane on the ossification of bone defects in an experimental study on dogs.

Methods

This experimental study was performed in the Department of Implantology, Military Medical Academy (MMA), and the Institute for Medical Research, MMA. The experiment had three phases and involved 6 adult German Shepherd dogs, with the medium body (bw) weight of 24.1 kg and average age 5.1 years, abiding by all the ethical principles, as stipulated in the relevant MMA regulations.

The first phase of the experiment

Combelem (intravenous, 0.3 mL/kg bw) and atropin (subcutaneous, 0.03 mL/kg bw) were used as premedication. Ketamine chloride 5% (intramuscular, 0.3 mL/kg bw) was injected 15 minutes after the premedication.

During this short intravenous anesthesia, the extraction of the second, third, and fourth premolar on the left side of the mandible were performed. Extraction wounds were closed using individual surgical sutures (Dexon 3,0, Davis & Gack).

Postoperative antibiotic therapy consisted of intravenous administration of 1.600.000 IU of crystalline penicillin for two days. The health of experimental animals was controlled daily, while the dogs were kept in separate boxes and fed soft food. The first phase of the experiment ended eight weeks after extraction.

The second phase of the experiment

Eight weeks after the teeth extraction defects were created. Crystalline penicillin (1.600.000 IU) and metronidazole were administered as prophylaxis. After a supracrestal incision and vertical relaxations 2–3 mm away from adjacent teeth, a full thickness flap was elevated. On the left side of the lower alveolar ridge occlusally, bone defects were pre-

pared, using a bone trepan (diametar 4 mm), with copious irrigation with sterile physiologic solution. Three bone defects were prepared. The first defect was covered with a resorbable human demineralized membrane 200 μ thick, the second with a 100 μ thick membrane, and the third was left uncovered (control defect).

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dogs in each instance). Euthanasia was accomplished using the barbiturate thiopental sodium solution (intravenously, 1 g per 30 kg bw). The samples were prepared for histologic analysis. Each sample was cut with a special saw to smaller sections containing the jaw bone defect with appropriate membrane. The cuts were placed in a decalcination solution, and then embedded in paraffin, cut with a microtome in several 5–7 μ thick sections and stained with hematoxyline-eosin (HE), van Gieson, van Kossa, Goldner trichrome, Masson trichrome, PAS, PAS diastase, and toluidine blue.

Results

The preparations were analyzed by light microscopy (Leitz microscope). The following characteristics were analyzed histopathologically: preservation of compact bone tissue, border between soft tissue elements and newly formed bone trabekulas, the presence of osteocytes in the lacunas, osteoblastic reaction, presence of young blood vessels and fibrocytes, maturity of newly formed connective tissue, and degree of defect-filling with new bone tissue.

The following characteristics were estimated by description of histologic samples according to consecutive criterions given in Table 1.

Table 1

The criterions for estimation of histologic characteristics

| Histologic property | Mark | | |
|---|--------------------------|------------------------------|------------------------------|
| | "0" | "1" | "2" |
| Compactness of bone tissue | Not preserved | Partially preserved | Preserved |
| Borderline between soft tissue elements and newly formed bone | Unclear | Partially clear | Clear |
| Presence of osteocytes in lacunas | Empty | Partially complete | Complete |
| Osteoblastic reaction | Absent | Moderate | Marked |
| Presence of young blood vessels, fibroblasts and fibrocytes | Not marked | Partially marked | Marked |
| Maturity of newly formed connective tissue | Young, immature | Partially mature | Mature |
| Defect filling with newly formed bone tissue | Defect filling about 1/3 | Defect filling less than 2/3 | Defect filling more than 2/3 |

pared, using a bone trepan (diametar 4 mm), with copious irrigation with sterile physiologic solution. Three bone defects were prepared. The first defect was covered with a resorbable human demineralized membrane 200 μ thick, the second with a 100 μ thick membrane, and the third was left uncovered (control defect).

Each membrane was shaped to cover the defect completely, extending at least 2–3 mm beyond the defect edges. The wounds were closed using individual surgical sutures.

At the end of each of the surgical procedures, and in two more instances afterwards, an analgesic was administered to reduce postoperative pains. The dogs were fed soft diet to the end of the experiment.

The third phase of the experiment

Sacrificing of experimental animals (euthanasia) was done 2, 4, and 6 months after the surgical intervention (two

The analyzed characteristics are presented in Tables 2–4.

The Kruscal-Wallis and the Wilcoxon rank-sum tests (Wa test) were used for the purpose of statistical analysis of pathohistologic results.

The methods of guided tissue regeneration, depending on the thickness of applied membranes, were classified as follows: I – resorbable human demineralized membrane 200 μ thick (RHDM200); II – resorbable human demineralized membrane 100 μ thick (RHDM100); and III – defects without a membrane (controls).

According to the calculated statistic values of the Wa test, it was obvious that the RHDM200 method achieved better results in comparison with RHDM100 method in the period of 2 months after the surgery in the 4 analyzed characteristics or in 57.14% cases. In the 3 rest characteristics the effects were the same (Table 5).

Table 2

The comparative histopathologic findings 2 months after the surgical intervention

| Property | Method – findings (mark) | | | | | |
|---|--------------------------|--|------------------|---------------------|----------------|------------------------------------|
| | RHDM 100 μ thick | | RHDM 200 μ thick | | Control defect | |
| Compactness of bone tissue | 2 | Preserved | 2 | Preserved | 0 | Not preserved |
| Borderline between soft tissue elements and newly formed bone | 1 | Spots of proliferation of soft tissue elements | 2 | Clear (no ingrowth) | 0 | Unclear, with soft tissue ingrowth |
| Presence of osteocytes in lacunas | 2 | Complete | 2 | Complete | 0 | Empty lacunas |
| Osteoblastic reaction | 1 | Moderate | 2 | Marked | 0 | Absent |
| Presence of young blood vessels, fibroblasts and fibrocytes | 2 | Marked | 2 | Marked | 2 | Marked |
| Maturity of newly formed connective tissue | 0 | Immature | 1 | Partially mature | 0 | Immature |
| Defect filling with newly formed bone tissue | 0 | Less than a third of defect size | 1 | About a third | 0 | Less than a third of defect size |
| Amount: | 8 | | 12 | | 2 | |

RHDM – Resorbable human demineralised membranes

Table 3

The comparative histopathologic findings 4 months after the surgical intervention

| Property | Method – findings (mark) | | | | | |
|---|--------------------------|----------------------------------|------------------|--|----------------|--------------------------------------|
| | RHDM 100 μ thick | | RHDM 200 μ thick | | Control defect | |
| Compactness of bone tissue | 2 | Preserved | 2 | Preserved | 0 | Not preserved |
| Borderline between soft tissue elements and newly formed bone | 2 | Clear (no ingrowth) | 2 | Clear (no ingrowth) | 0 | Unclear, with soft tissue ingrowth |
| Presence of osteocytes in lacunas | 2 | Complete | 2 | Complete | 0 | Empty lacunas |
| Osteoblastic reaction | 2 | Marked | 2 | Marked | 1 | Moderate |
| Presence of young blood vessels, fibroblasts and fibrocytes | 2 | Marked | 2 | Marked | 2 | Marked |
| Maturity of newly formed connective tissue | 0 | Immature | 1 | Immature, spotty transformation to mature tissue | 0 | Immature |
| Defect filling with newly formed bone tissue | 1 | About a two third of defect size | 2 | More than a two third of defect size | 0 | Less than a two third of defect size |
| Amount: | 11 | | 13 | | 3 | |

RHDM – Resorbable human demineralised membranes

Tabela 4

The comparative histopathologic findings 6 months after the surgical intervention

| Property | Method – findings (mark) | | | | | |
|---|--------------------------|--|------------------|--|----------------|--------------------------------------|
| | RHDM 100 μ thick | | RHDM 200 μ thick | | Control defect | |
| Compactness of bone tissue | 2 | Preserved | 2 | Preserved | 0 | Not preserved |
| Borderline between soft tissue elements and newly formed bone | 2 | Clear (no ingrowth) | 2 | Clear (no ingrowth) | 1 | Unclear, with soft tissue ingrowth |
| Presence of osteocytes in lacunas | 2 | Complete | 2 | Complete | 2 | Complete |
| Osteoblastic reaction | 2 | Marked | 2 | Marked | 2 | Marked |
| Presence of young blood vessels, fibroblasts and fibrocytes | 2 | Marked | 2 | Marked | 2 | Marked |
| Maturity of newly formed connective tissue | 1 | Immature, spotty transformation to mature tissue | 2 | Multiplied, mostly mature connective tissue | 0 | Immature |
| Defect filling with newly formed bone tissue | 1 | About a two third of defect size | 2 | Defect filled for the most part filled with newly formed bone tissue | 0 | Less than a two third of defect size |
| Amount: | 12 | | 14 | | 7 | |

RHDM – Resorbable human demineralised membranes

Table 5

The comparative statistics of histological characteristics 2 month after the surgery

| Kruskal-Wallis H0: M1 = M2 = M3 H1: M1#M2#M3 <i>significant level</i> ($\alpha = 0,2$) | Compactness of bone tissue Wilcoxon Wa; <i>significant level</i> ($\alpha = 0,167$); <i>significant interval</i> (7, 3) | | |
|---|--|--------------------------|--------------------------|
| | I – II | I – III | II – III |
| | H0: M1 = M2, H1: M1 > M2 | H0: M1 = M3, H1: M1 > M3 | H0: M2 = M3, H1: M2 > M3 |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Borderline between soft tissue elements and newly formed bone | | |
| H = 4,57/ Hv = 2, $\alpha < 3,71$ + | Wa = 7 + | Wa = 7 + | Wa = 7 + |
| | Presence of osteocytes in lacunas | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Osteblastic reaction | | |
| H = 4,57/ Hv = 2, $\alpha < 3,71$ + | Wa = 7 + | Wa = 7 + | Wa = 7 + |
| | Presence of young blood vessels, fibroblasts and fibrocytes | | |
| H = 0,00/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 5 – | Wa = 5 – |
| | Maturity of newly formed connective tissue | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 7 + | Wa = 7 + | Wa = 5 – |
| | Defect filling with newly formed bone tissue | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 7 + | Wa = 7 + | Wa = 5 – |

(+) presence of a statistically significant difference
 (-) absence of a statistically significant difference

In comparison with the control group, the RHDM200 method was significantly better in 6 analyzed characteristics or in 85.71% cases.

In comparison with control group, RHDM100 method achieved better effects in the 4 analyzed characteristics or in 57.14% cases (Table 6).

According to the calculated statistic values of the Wa test, it was obvious that the RHDM200 method achieved better results in comparison with RHDM100 method in the period of 4 months after the surgery in the 2 analyzed characteristics or in 28.57% cases, which presents a decrease with regard to the period of 2 months after the sur-

Table 6

The comparative statistics of histological characteristics 4 month after the surgery

| Kruskal-Wallis H0: M1 = M2 = M3 H1: M1#M2#M3 <i>significant level</i> ($\alpha=0,2$) | Compactness of bone tissue Wilcoxon Wa; <i>significant level</i> ($\alpha=0,167$); <i>significant interval</i> (7, 3) | | |
|---|--|--------------------------|--------------------------|
| | I–II | I–III | II–III |
| | H0: M1 = M2, H1: M1 > M2 | H0: M1 = M3, H1: M1 > M3 | H0: M2 = M3, H1: M2 > M3 |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Borderline between soft tissue elements and newly formed bone | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Presence of osteocytes in lacunas | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Osteblastic reaction | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ + | Wa = 5 – | Wa = 7 + | Wa = 7 + |
| | Presence of young blood vessels, fibroblasts and fibrocytes | | |
| H = 0,00/ Hv = 2, $\alpha < 3,71$ – | Wa = 5 – | Wa = 5 – | Wa = 5 – |
| | Maturity of newly formed connective tissue | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ – | Wa = 7 + | Wa = 7 + | Wa = 5 – |
| | Defect filling with newly formed bone tissue | | |
| H = 4,57/ Hv = 2, $\alpha < 3,71$ + | Wa = 7 + | Wa = 7 + | Wa = 5 + |

(+) presence of a statistically significant difference
 (-) absence of a statistically significant difference

gery. In the 5 rest characteristics the effects were the same.

In comparison with the control group, the RHDM200 method was significantly better in the 6 analyzed characteristics or in 85.17% cases.

In comparison with the control group, RHDM100 method achieved better effects in the 5 analyzed characteristics or in 71.42% cases, which presents improvement with regard to the period of 2 months after the surgery (Table 7).

The authors also examined and analyzed histopathologically the significance of barrier membranes in bone regeneration in mandibular defects, and found that membrane-covered defects demonstrated significantly better bone healing compared to defects without barrier membranes. The authors stated that barrier membranes increased osteoprogenitor activity of the cells in adjacent bone tissue, enhancing bone regeneration in the mandibular defect. They also found that places regions left uncovered with barrier membrane (control

Table 7

The comparative statistics of histological characteristics 6 month after the surgery

| Kruskal-Wallis H0: M1 = M2 = M3 H1: M1#M2#M3 significant level ($\alpha = 0,2$) | Compactness of bone tissue Wilcoxon Wa; significant level ($\alpha = 0,167$); significant interval (7, 3) | | |
|--|--|-----------------------------|-----------------------------|
| | I-II | I-III | II-III |
| | H0: M1 = M2, H1: M1 > M2 | H0: M1 = M3, H1: M1 > M3 | H0: M2 = M3, H1: M2 > M3 |
| H = 4,57/ Hv = 2, $\alpha < 3,71$ + | Wa = 5 + | Wa = 7 + | Wa = 7 + |
| | Borderline between soft tissue elements and newly formed bone | | |
| H = 3,43/ Hv = 2, $\alpha < 3,71$ - | Wa = 5 - | Wa = 7 + | Wa = 7 + |
| | Presence of osteocytes in lacunas | | |
| H = 0,00/ Hv = 2, $\alpha < 3,71$ - | Wa = 5 - | Wa = 5 - | Wa = 5 - |
| | Osteblastic reaction | | |
| H = 0,00/ Hv = 2, $\alpha < 3,71$ - | Wa = 5 - | Wa = 5 - | Wa = 5 - |
| | Presence of young blood vessels, fibroblasts and fibrocytes | | |
| H = 0,00/ Hv = 2, $\alpha < 3,71$ - | Wa = 5 - | Wa = 5 - | Wa = 5 - |
| | Maturity of newly formed connective tissue | | |
| H = 4,57/ Hv = 2, $\alpha < 3,71$ - | Wa = 7 + | Wa = 7 + | Wa = 7 + |
| | Defect filling with newly formed bone tissue | | |
| H=4,57/ Hv=2, $\alpha < 3,71$ + | Wa = 7 + | Wa = 7 + | Wa = 5 + |

(+) presence of a statistically significant difference
(-) absence of a statistically significant difference

According to the calculated statistic values of the Wa test, it was obvious that the RHDM200 method achieved better results in comparison with RHDM100 method in the period of 6 months after the surgery in the 3 analyzed characteristics or in 42.86% cases, which presents a moderate increase with regard to period the of 4 months after the surgery. In the 4 rest characteristics the effects were the same.

In comparison with the control group, the RHDM200 method was significantly better in the 4 analyzed characteristics or in 57.14% cases.

In comparison with the control group, RHDM100 method achieved better effects in the 4 analyzed characteristics or in 57.14% cases.

Discussion

In the last two decades, numerous experimental and clinical studies have described and examined a large number of barrier membranes, their properties, and roles in guided bone regeneration¹³⁻¹⁸.

In the study of Schenk et al.¹⁹ in 1994, guided bone regeneration was investigated on mandibular defects in dogs.

ones) demonstrated incomplete bone regeneration and the presence of soft tissue elements in the newly formed bone.

The histopathologic findings in our study are almost identical to the findings of Schenek et al.¹⁹, but their study was less informative as to the course and appearance of bone regeneration in mandibular bone defects.

Schenek et al.¹⁹ described microscopically the three categories of bone tissue encountered in the process of regeneration: 1) woven bone – characterized by netlike distribution of bone trabeculas, with plenty of spheric osteocytes and rich blood supply to the connective tissue between bone trabeculas; 2) fibrillar bone – consists of parallel collagen fibers, with less osteocytes. Growing bone forms a primary osteon, positioned in periosteal and endosteal apposition; 3) lamellar bone – a mature bone with lamellar structure.

Based on these findings, the authors concluded that bone regeneration was incomplete after 4 months, and suggested longer periods of healing¹⁹.

In our study, histopathologic analysis of the defects with human resorbable demineralized 100 μ and 200 μ thick membranes two months after surgery, demonstrated a clear-cut borderline between soft tissue elements and compact bone exclu-

sively in the defects covered with 200 μ thick human membrane. Compared to the others, this was also the defect which was best filled with immature connective tissue, rich in fibroblasts, and with numerous newly formed, thin walled, and dilated blood vessels. In the young connective tissue, newly formed shorter and thinner fibrillar bone trabeculas could be observed, in a net-like distribution pattern and with osteoblastic reaction.

Four months after surgery, histopathologic analysis revealed that the borderline between soft tissue and compact bone in both membrane-covered defects was clear, and that there was no proliferation of connective tissue elements. Immature connective tissue was growing mature, and a considerable amount of trabecular bone with pronounced osteoblastic reaction could be seen. After four months, the defect covered with 200 μ thick human membrane was the fullest one (although just slightly more than half of its size).

Six months after the surgery, histopathologic analysis demonstrated that the borderline between bone and soft tissue was clear and without proliferation and ingrowth of soft tissue elements into the bone tissue. In the central area, beneath the compact bone, enlarged, mostly mature tissue could be seen, rich in fibroblasts and fibrocytes, and with numerous young, thin-walled, dilated blood vessels. In the connective tissue, there were thicker and thinner bone trabeculas, with osteocytes in the lacunas, and with marked osteoblastic reaction. Both membrane covered defects were for the most part filled with newly formed bone tissue. The defect covered with 200 μ membrane was almost completely filled.

As for the control defects, a borderline between bone and connective tissue was poorly defined along the whole of its length. Connective tissue elements penetrated deeply into the bone tissue, so that the remnants of bone trabeculas were seen in the connective tissue, having an almost „embedded“ appearance. The region of preserved bone tissue beneath the soft tissue was very narrow as the consequence of proliferation of soft tissue into the bone and its destruction. In animals sacrificed 2 months after surgery, we could see mostly the remnants of bone trabeculas, with empty lacunas and without osteocytes. Between the fragments of bone trabeculas, the growth of immature connective tissue could be observed, with rich blood supply and with numerous fibroblasts and fibrocytes. Four months after the bone defect had been made, clear connective tissue proliferation could be seen, rich in fibroblasts and young blood vessels, as well as a considerable number of new bone trabeculas with the signs of osteoblastic reaction. The defect was for the most part filled with immature, amply vascularized connective tissue. In the sections taken 6 months after sacrificing, in the newly formed, well vascularized connective tissue, a considerable number of thin, shorter or longer fibrillar bone trabeculas were seen, with marked osteoblastic reaction and in a netlike distribution pattern. Soft tissue ingrowth into the bone was obvious. Bone defects were filled to a higher degree compared to the defects seen after 4 months, although not completely as yet; and after 6 months, the defects were filled for more than half of their size, but significantly less compared to the ones covered with membranes.

The histopathologic findings in our experiment demonstrated that the use of membranes as an interface between

soft tissue elements and bone was able to prevent the ingrowth of soft tissue into the new bone, *i.e.* in the space between the bone and membrane, contributing therefore to a better filling of mandibular defects with newly formed bone.

The use of human 200 μ thick membrane, as shown in our study, produced better results than the use of human 100 μ thick membrane, since it showed better results in four properties two months after surgery, in two properties four months after surgery and in three properties six months after surgery, especially contributed to maturation of connective tissue and more rapid defect filling with newly formed bone.

In the literature, the papers dealing with the impact of thickness of resorbable membranes on bone regeneration have been scarce. An attempt at using thicker membranes was published in 2005 by Busenlechner et al.²⁰ The purpose of their study was to examine the potential of a slowly resorbable, prototype trilayer membrane in bone regeneration for alveolar ridge augmentation after the extraction of the first and the second molars in the monkey mandible, and after cavity formation three months after extraction. The animals were sacrificed after 9 months. The study supported the use of slowly resorbable trilayer membrane, since best bone regeneration was achieved with such a membrane and a bone graft. The membrane was created by the addition of a polylactide layer between two layers of collagen in order to prolong the period of membrane degradation and its barrier function. The fragments of polylactide were found on histologic examination even after 9 months. The design of the trilayer membrane can be an important step towards the improvement of stability of a membrane with a certain degree of exposure. In this study the exposure was 8.33%, which was extremely low compared to 43.75% in the study by Sculean et al.²¹ in 1999.

The same prototype of a trilayer membrane was studied by von Arx et al.²² even before the authors mentioned above (in 2002). Their study tried to examine the prototype of a trilayer membrane in combination with different augmentation materials. The study was done on dogs; after the extraction of premolars, defects were made, into which different augmentation materials and membranes were placed. The dogs were sacrificed after four and a half months, and the sections prepared were histopathologically and histomorphometrically analyzed. The best bone regeneration was demonstrated for the prototype trilayer membrane combined with autograft. In spite of these satisfactory results, the authors could not recommend the membrane for clinical use.

In 2009, Kozlovsky et al.²³ histologically compared the biodegradation of Bio-gide membrane placed in one and two layers into the mechanically made defects on the rat calvarias. The results of the study showed that the percentage of membrane degradation was similar in both cases, but markedly more barrier material remained in the tissue even after 9 months, thus suggesting a longer barrier role of the membrane. Single layer membrane could not perform its barrier function for longer period of time. Bilayer membrane therefore performed better in terms of bone regeneration and defect ossification. It should be pointed out that with the second layer micromovements could be reduced, which in turn significantly improve membrane stability.

In a study by Korean authors, the efficacy of bilayer membrane was analyzed with the use of bone grafts in terms of bone resorption. The study was done in rabbits. Blocks of parietal bone were taken from one side, placed on the contralateral side, and covered with membranes. Histologic and histomorphometric analysis was done 2, 4, and 6 months after surgery. The results of the study showed that the use of bilayer membrane reduced graft bone resorption significantly more compared to single layer membrane²⁴.

The results of these few studies examining the membrane thickness, showed that thicker membranes, designed as single- or multilayer structures, demonstrated better barrier performances, maintained longer their presence in the tissue due to their slower biodegradation, and promote better ossification of bone defects, as confirmed by our study as well, since the human resorbable 200 μ thick membrane demonstrated superior regeneration of bone defects.

Conclusion

Based on the results obtained in our study, the resorbable human demineralized 200 μ thick membrane produced a higher degree of bone regeneration compared to resorbable human demineralized 100 μ thick membrane. The use of human demineralized membranes as an interface between bone defects and mucoperiosteal flap soft tissues improved bone regeneration. Bone defects covered with barrier membranes showed better bone healing, although bone regeneration was not complete even after 6 months.

Future studies should investigate the membrane thickness or collagen amounts sufficient to maintain the barrier function beyond 6 months, which is considered the minimal period of time for guided bone regeneration.

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