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Bone Regeneration of Mandibular Defects with Osteopromotive Membranes and Recombinant Human Bone Morphogenetic Protein-7 on Rabbits

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RhBMP7 AND e-PTFE MEMBRANES IN MANDIBLE RECONSTRUCTION

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BONE REGENERATION OF MANDIBULAR DEFECTS WITH OSTEOPROMOTIVE MEMBRANES AND RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-7 ON RABBITS,

Objectives: This study evaluates whether recombinant human Bone Morphogenetic Protein-7 in collagen as a carrier (rhBMP-7/ACS) might be an alternative for conventional bone grafting reconstructive surgery, to promote bone regeneration in conjunction with expanded polytetrafluoroethylene (e-PTFE) "osteopromotive" membranes.
Material and Methods: This study was carried out in the University Clinic Center Sarajevo, Bosnia and Herzegovina, from 2003 to 2005. A standardized critical-size mandibular defect was created surgically on 14 New Zealand Rabbits. 7 animals (control) were treated with a bicortical iliac crest autologue grafts. In the other 7 (test), the defect was covered with membrane and filled with rhBMP-7/ACS alone. A local flap of masseter muscle was previously placed in the all test defects. The healing period was 2 months. Alkaline phosphate activity was evaluated at day 0,5,14,21,and 30.3-D. CT scans were obtained at day 30 and analyzed for bone mineral density (BMD). At day 60 the animals were sacrificed for clinical evaluation of bone reconstruction and histological evaluation of representative bone biopsies.
Results: There was a remarkable difference in the ALP activity between the Test and Control group. Significantly higher values of ALP activity was detected at day 21 on the test group compared at day 30 on the Control group. There was no remarkable difference in BMD between groups, with values over 213 mg/cm3 defined as calcified bone. The clinical evaluation of the gross specimens at sacrifice showed complete bone reconstruction in 5 of 7 test animals. Only 2 of 7 control animals reestablished bone continuity. The histological analysis supported these findings.
Conclusions: It was concluded that the rhBMP-7/ACS and membrane was strongly osteoinductive. Although e-PTFE membrane prevented the degradation of the collagen carrier, thus strongly reducing the availability of rhBMP, the local muscle flap placed in the defect supplemented the environment with membranous non-occlusive macrophages and plasma cells necessary for the collagen degradation.

Background

Numerous diseases, damaging traumatic, developmental and postresection etiology result in exposed craniofacial skeleton. Over time, requests for the treatment of such diseases resulted in the development of numerous operative procedures for achieving better functional and esthetic results. The most successful method for bone reconstruction currently used in clinical practice is autologous bone graft or vascularized bone.[1] However, the limited availability of donor tissue as well as morbidity at the donor sites are the constraining factors associated with this type of graft. Consequently, different material and methods are highly desirable. Recently, two new techniques to promote bone healing were introduced; the osteopromotive membrane technique, and local delivery of growth-stimulatory factors, both with a high rate of success in preclinical experiments.

In 1965 Urist described ectopic bone formation after the implantation of demineralizied bone matrix in intramuscular sites in rats. The factor responsible for this was later named bone morphogenetic protein

(BMP) [2]. Wozney et al 1988 subsequently succeeded in cloning a cDNA of human BMP that enabled mass production of recombinant human BMP (rhBMP). BMP belongs to the larger TGF-beta Cytokines family and some BMPs, (rhBMP-2, rhBMP-4, and rhBMP-7) each as single substances, have been shown to induce bone formation in several different species when using the segmental defect model.[3, 4,5,6,7,8,9,10] In previous research Smajilagic A. et al 2005 also report that rhBMP-7 promotes bone formation in vivo, displaying induction of the presented stem cells. Conversely bone marrow contains osteoprogenitor stem cells, therefore we considered and demonstrated, in an previously experiment, that the implantation of cells that respond to growth factor as rhBMP-7 and bone marrow construct, promoted bone healing and regenerated full thickness of the segmented mandible defect on rabbits.[11] Placement of the osteopromotive membrane improves bone healing in craniofacial bone defect[12,13]which is not a new concept. Ollier et all 1897 was the first to demonstrate the role of periostal membranes in bone regeneration. This technique, and Guided Bone Regeneration (GBR) principles determined from this concept[14], implies that a skeletal defect is physically isolated from the surrounding soft tissues by means of a barrier membrane that prevents soft-tissue interference with osteogenesis, providing a so-called bone-forming chamber in which osteogenic components are released from the bone ends.[15, 16] Combining rhBMP as growth-stimulating agents has been shown to result in significant enhancement of the bone-healing rate beneath the membrane.[17,18] However, few reports have explored the relationship between these two concepts. The purpose of this study was to explore whether rhBMP-7 alone, in collagen as a carrier (ACS), could be a potential osteoinductive substance as an alternative for autologue transplantation and if the concept of combination with GBR would be advantageous in this respect.

Material and Method

This study was performed under the Clinic for Maxillofacial Surgery, University Clinic Center, Sarajevo, from 2003 to 2005. Prof.dr.S.Vukicevic of the Medical Faculty University in Zagreb, Croatia donated rhBMP-7/ACS construct.

A total of 14 White New Zealand Rabbits, age 9 months with mean body weight of 3,5 kg, were kept under standard laboratory conditions with free access to tap water and standard pellets, General anesthesia was induced by administering 1mg Ketamine Hydrochloride i.m. (Veterinary Ketalar 50), and maintained by isoflyrane during surgery. A submandibular placed incision was made on shaved and disinfected skin to expose the mandible. Using a low speed trephine, mounted in a standard dental drill, a 20mm wide full thickness portion of the mandible was resected. The resection was positioned proximally from incisors and distally to the angle of the mandible **(Fig. 1A.)**. The surgical field was irrigated with sterile saline continuously during drilling to reduce thermal damage. Defect were stabilized with a titanium mini plate and screws (Aescolap, Germany). On 7 rabbits (test) the defects were treated with a concentration of 100-micrograma rhBMP-7 soaked in absorbable collagen sponge (ACS), commercially available as "Helistat". 1x2 cm size ACS particles were prepared. The local flap of masseter muscle was repositioned in the defect **(Fig. 1B.)** and then buccally and lingually covered with the expanded polytetrafluoroethylene (e-PTFE) osteopromotive membranes (Gore-Tex), fixed with interrupted absorbable sutures. The surgical site was closed in layers. On the second group of 7 rabbits (Control) the defects were treated with bicortical autologue block bone graft harvested from an iliac crest. Fixation for remaining bone fragments was achieved with titanium mini plate and screws (Aescolap, Germany), and closed in layers. Clinically autologues graft was used as a control, taking in consideration fact that this transplantation method established as a standard method for any bone reconstructive procedure currently practiced.

Postoperative Enteroflyxatine 1g daily was administered for three days.

Evaluation of Alkaline Phosphate (ALP) enzyme activity from periphery blood circulation at day 0,5,14,21 and 30 was analyzed as a significant marker of osteoblast function.

Blood samples were donated from the ear artery and ALP values were expressed in the mmol/l. Bone mineral density (BMD) was analyzed from Siemens CT scans, obtained at day 30. During CT scanning the animals were sedated with the 0,5 mg. Ketamine Hydrochloride i.m. CT BMD analysis on newly formed tissue inside the defect was done using GENAINE 2,0 software (Bon. Alyse Ltd) (**Fig.2 A, B**). Expression of BMD values is in mg/cm3. The software specified densities over 213 mg/cm3 as calcified bone.

After two months the animals were sacrificed for a clinical evaluation of the defect. Specimens of the newly formed tissue from the defects were harvested for histological analysis. The specimens were placed in

10% buffered formalin for 2 weeks, dehydrated with a graded ethanol series and embedded in methylmethacrylate. The specimen block was sectioned in 50-60 micro.m. slices and each section was stained with toluidine blue for visualization under a light microscope.

Results

All animals survived the experimental period. Immediately after surgery the animals were fed a normal diet and masticatory force was loaded on the test sites and bone graft sites. Macroscopic local infection was detected on all animals that received rhBMP-7/ACS and e-PTFE to the defects during the examination period. On the second group treated with the bone graft, infection was detected on 5 animals. The average value of the ALP enzyme activity was significantly higher 21st day postoperatively (39,42 mmol/l) compared with 14th day postoperatively (21,48 mmol/l; t-test=2,381,p<0,06) on rhBMP-7/ACS treated animals. The autologue bone graft group showed significantly higher average values 30th postoperatively day (51,22 mmol/l) compared with 21st postoperatively day (31,29 mmol/l; t-test=3,230,p<0,01) (**Tab.1, 2.**)

Average BMD values, 30th postoperatively day was 392 mg/cm3 from the saggital and 452,2 mg/cm3 from the caudal projection for rhBMP-7/ACS e-PTFE treated sites.

Bone graft treated sites showed average values of the BMD 585,86 mg/cm3 from the saggital and 578,83 mg/cm3 from the caudal projections. There were no statistical differences compared with rhBMP-7 treated group (t=1,0839 and t=1,717), and all values were over 213 mg/cm3.

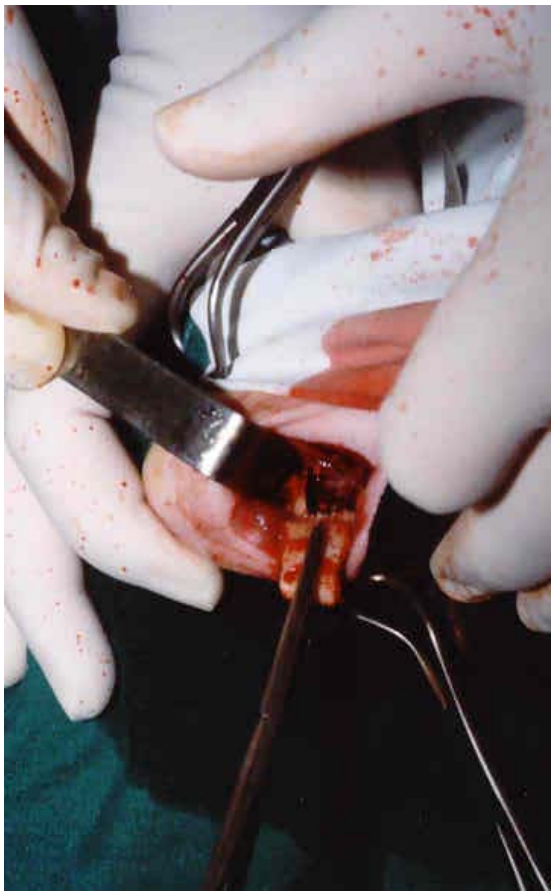
Table 1: Average Values ALP Enzyme Measurement in Determinates Time Intervals

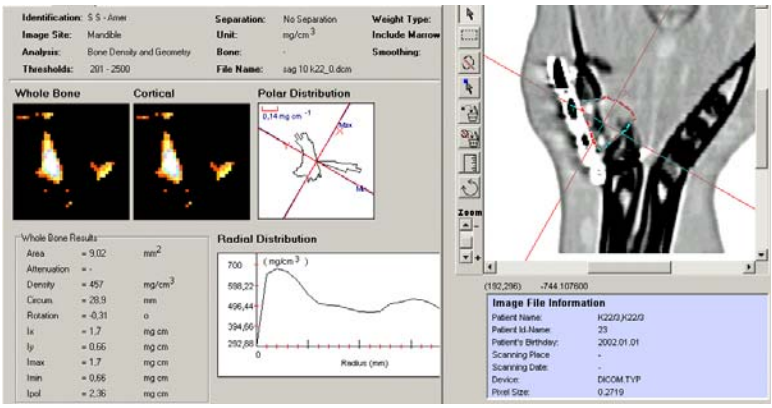
	x S.D.	rhBMP-7/ACS & e-PTFE	Crest Iliac Bone Graft	t-test Significances Between Sites
Preop.	x S.D.	36,08 19,03	26,00 11,81	t= 0,63
5th day	x S.D	17,92 4,66	18,93 8,95	t = 0,143
14th day	x S.D	21,48 9,81	22,67 10,81	t = 0,107
21st day	x S.D	39,42 15,09	31,29 12,31	t = 0,547
30th day	x S.D	44,99 30,92	51,22 11,40	t = 0,253

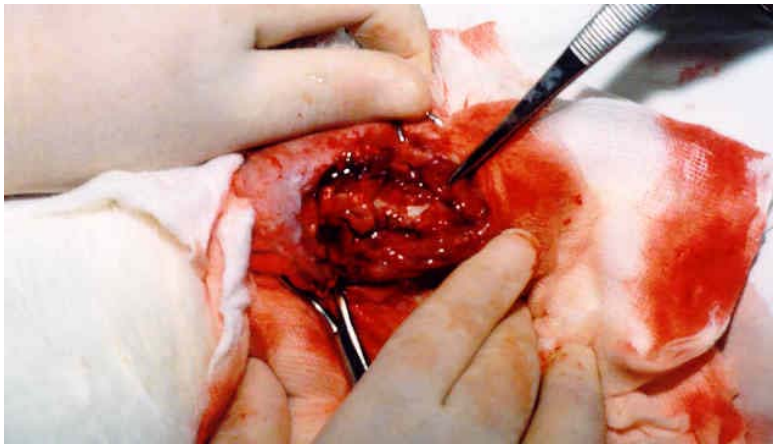
Table 2: Determination Significance Differentiation in Average Values ALP Enzyme Between Time Intervals. (d- Average Differentiation)

	rhBMP-7/ACS & e-PTFE		iliac crest bone graft	
Intervals	**d**	**t-test**	**d**	**t-test**
5th day : preop.	-22,51	t=3,244 **p<0,05**	-7,07	t=1,603.
14th day : 5thday	3,95	t=1,225	3,75	t=0,668
21stday : 14thday	17,945	t= 2,381 **p<0,06**	8,62	t=1,810

30thday : 21stday	5,57	t=0,728	19,93	t=3,230 **p<0,01**







The clinical evaluation of the gross specimens at sacrifice day 60 showed complete bone reconstruction of defects in 5 of 7 animals treated with rhBMP-7/ACS. Histology analysis showed newly formed bone marrow and plentiful osteoblastic and fibroblastic cells. **(Fig. 3)** No sign of necrosis was detected.

The bone graft treated animals showed reestablished bone continuity in 2 of 7 sites. Histological analysis of tissue in this group was represented with lymphocytic infiltration and signs of bacterial infection with necrosis, in most cases.

Discussion

New knowledge and intercorrelations about the relationship between growth factors, cell type and intercellular signal mechanisms offer redefinition of the latest strategies involved in biologically engineered bone procedures. Recently several strategies have been developed for achieving therapeutic effects in tissue, with an engineering approach. The present investigation was designed to gain information on a new approach by delivering growth factor as an alternative to the conventional bone grafting method. This investigation assessed whether a combination of tissue engineering concept and a GBR concept would result in an improved effect. A significant increase in ALP activity at day 21 postoperative on the rhBMP-7 treated sites compared with day 30 obtained in the bone graft treated sites showed stronger osteoinductive effect with the alternative therapeutic approach. It was demonstrated that at day 30 rhBMP-7 alone, covered with the e-PTFE, produced bone tissue inside the defect detectable by BMD analysis. There were no statistically different average values of BMD compared with bone graft values at day 30. These early results demonstrated that rhBMP-7/ACS alone has the capacity to be an adequate alternative to autologous bone graft in clinical trials. Most of the previous research involved with osteoinductivity problems used bone graft or cancelous bone enrichment with growth factors (rhBMP) to improve

their osteoinductivity in the treatment of non-critical size defects. In this research we avoided any type of bone transplant and the advantages of this new approach could be zero donor morbidity, avoidance of unnecessary operative procedures and short operative times. The success of our approach was confirmed after two months. Construct rhBMP-7 showed superior therapeutic affect by completely bridging the defect with newly formed bone in 5 cases compared to bone graft treated sites demonstrating this in only 2 cases **(Fig. 4A, B.)**. Histological section of the rhBMP-7 induced tissue at postoperative day 60 showed newly formed bone tissue with macrophages and plasma cells. It has been suggested that the presence of macrophages and other inflammatory cells is essential to supply the collagenases necessary to release rhBMP from a collagenous carrier. Our results negated previous research that showed non-synergistic or delayed effect collagen as a carrier and osteopromotive membrane. For example: Zellin and Linde 1997 founded that rhBMP-2 was highly osteoinductive in rat mandible defects when delivered in a poly lactide-coglycolide acid (PLGA) carrier under the membrane than in collagen as a carrier. An explanation may be that the presence of an e-PTFE membrane prevents the degradation of the collagen with their non-occlusive effect for macrophages and plasma cells, thus strongly reducing the availability of rhBMP.[19] Taking into consideration these notions we first successfully repositioned local muscle flaps into the defect under the membrane to enrich the local environment with the macrophages, plasma cells and stem cells. Our results also confirmed previous findings that non-occlusion of the membrane for these cells could be the main reason for lack of success. We argued in favor of collagen and believed the degradation of PLGA impeded osteogenesis.[20]

Previous studies reported a dose dependent increase in bone induction by rhBMP.[21, 22,23] However, recent results of the long-term studies showed that using a higher dose of the morphogenes can produce extensive osteoclastic activity and reabsorbtion of produced bone.[13,24]To minimize the effective dose of rhBMP, a combination with osteopromotive membrane was applied in our study. According to the results by Gordh et all.,1999, which showed that high doses rhBMP-2/ACS mixed with cancelous bone graft without membrane covering on calvarian defects in Lewis rats resulted in loss of graft size, but membrane covered grafts demonstrated complete integration after 20 weeks, we deduced that a combination with membrane could be useful. The advantage of using membrane in our study was to keep osteoinductive substance in place, and also the excellent results obtained with moderate dose of rhBMP-7 could be because of added action of growth stimulatory proteins. Immediately after surgery animals in the study were fed a normal diet and this overload of chewing could jeopardize the outcome of the result. Up to now the crucial statements for successful reintegration of the bone graft were stability and fixation without any moving. This may be the reason for the poor result demonstrated in the bone graft treated animals in our study. These rules could, however, be overweighed by new ideas. RhBMP-7/ACS construct showed stronger osteoinductive capacity and membrane facilitation in their action outside these unfavorable conditions offering a more successful result. This theory could be of great importance for future clinical trials. Human Beings demonstrate slower rates of metabolism than rodent species, particularly in older people with lower numbers of available stem cells. This could produce a non-response or failed action of the growth stimulatory proteins. Using stem cells directly from all available resources, such as circulation and bone marrow from the bone fragments, and repositioning vascularized local muscle flap exposed to the growth factors covered in membrane, preventing them dissolving, improves the results of tissue engineering concepts. The same concept could be applied for any damaged tissue regeneration such as cardiac muscle, but only adequate growth factors and their combinations and concentration have to be considered. This concept is more advantageous that current concept with combination in vivo and in vitro procedures using isolation and cultivation of autologues stem cells and their retransplantation. This new concept avoiding and ethnic dilemmas faced when using embriogenetic stem cells manipulation.

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