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Healing of Bone Defects by Guided Bone Regeneration (GBR): An Experimental Study

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ABSTRACT

Bone defects may be regained by osteogenesis, osteoinduction, osteoconduction or guided bone regeneration. Each one of these four biologic processes may work independently but theoretically combination of any of these processes will give better results. 72 experimental rats were classified into four groups where creation of two parietal critical size defects (5 mm) were done. One defect served as a control and the other as a test. The test sides were managed either by Polytetrafluoroethylene (PTFE) membranes (guided bone regeneration) (n = 18), PTFE membranes and Medpor (n = 18), PTFE membranes and demineralized bone matrix (DBM) (n = 18) and lastly by autogenous bone grafts (n = 18). Rats in each group have been euthenized at three-time period 3, 6 and 9 weeks. Specimens were collected, fixed, decalcified and prepared for histological examination. Control defects showed no healing. The defects managed by PTFE (GBR) membranes alone or with DBM (Osteoinduction) showed complete bridging by bone at six weeks. The defects managed by PTFE membranes and Medpor (Osteoconduction) showed complete Osseo-integration of the Medpor at 9 weeks. Lastly the defects managed with autogenous bone grafts showed incorporation at three weeks. Guided bone regeneration (PTFE) membranes work very well provided that the space was maintained. Medpor (osteoconductive substance) maintained the space while the PTFE prevented fibrous integration. Demineralized bone matrix (osteoinductive) failed to induce early bone formation under the PTFE membranes. Autogenous bone graft is still the proper solution apart from its well known complications.

INTRODUCTION

Reconstruction of a bony defect is one of the major problems that continued to perplex reconstructive surgeons for centuries. Many methods were tried to solve the problem but the pitfalls of each method led to the trial of the other. The earliest attempt to repair bony defects was reported in the Edger Smith papyrus to be around 2000 BC **[1,2]**. Metals were used to manage bony defects at this time. In 1682 Van Meekren **[3]** tried xenografts with a reported success **[4-7]**. This was followed by the use of allografts. But after knowing about the immune response to the allografts, surgeons shifted to the use of autografts. But this of course was faced with the limited availability of donor sites-especially in children -and the risk of morbidity. By introduction of the mechanism of creeping substitution by Barth **[8]**, the structure of the substance to close a bony defect was suggested to be osteoconductive. Osteoconduction alone may require a long time to heal a defect and the addition of osteoinductive materials are expensive and their use is still under investigation. Osteogenesis is the formation of new bone from osteocompetent cells. Osteoconduction is the formation of new bone along the scaffold of a biologic or alloplastic substance where the bone forming cells originate from pre-existing host osteocompetent cells. Osteoinduction is the formation of new bone by differentiation and stimulation of mesenchymal cells by bone inductive proteins **[9]**.

A common obstacle for successful bone healing using any of the previously mentioned techniques is that in contrast to osteogenesis, connective tissue formation occurs rapidly. Therefore, based on the hypothesis put by Dahlin et al. **[10]** and Nyman et al. **[11]**, that different cellular components in the tissue have varying rates of migration into a wound area during healing, the idea of guided tissue regeneration using a membrane technique, emerged. Thus, by placing an inert membrane barrier over the defect in close contact with the bone tissue, a closed space is created between the bone and the membrane. Since the membrane acts as a

mechanical barrier preventing the in-growth of connective tissue into the defect, the only cells that are allowed to repopulate the wound area are cells originating from the surrounding bone. Thus, osteogenesis is able to occur without interference from other tissue types [12].

One important thing which allowed good comparison between the results of different studies was the standardization of what is called the critical size defect (CSD). Schmitz and Hollinger, [13] defined it as the defect that is large enough to preclude spontaneous healing in a certain area during the life time of an animal. In animal research a defect that will not heal over the duration of the study is a CSD [14]. The CSD of rats of this study was found to be 5 mm.

Many researches [15,16] were performed to investigate the principle of guided bone regeneration. These researches succeeded in answering a lot of obscure questions in this field, but still other questions need to be answered as what is the effect of adding osteoconductive and osteoinductive materials in the closed space between the bone and the membrane? Is it better to put osteoinductive substances as Bone Morphogenic Protein in between non-resorable membranes or to add them to the bio-resorable membranes used for guided bone regeneration? Can custom made membranes be used to achieve a desired shape and volume? This is the aim of this experimental piece of work.

MATERIAL AND METHODS

Seventy-two skeletally mature Sprague-Dawley rats were the material of this study. Their body weight ranged from 300-500 gm. They were housed at the animal cages of the Plastic Surgery Micro-vascular Laboratory at Ain Shams University (Cairo, Egypt) with 12 hours light/dark schedule.

All surgical procedures were performed with aseptic techniques. All rats were anesthetized by inhaling ether for short time just till hypnosis and then they were weighed. Freshly prepared intraval (Sodium thiopental) was injected intraperitoneally in a dose of 5 mg/kg of body weight and Ketamine Hydrochloride (Ketalar) was injected intramuscularly in a dose of 10 mg/kg of body weight. A single dose of penicillin was given intramuscularly just before the onset of the operation.

Each rat was placed in the prone position and the scalp was shaved, prepped with Betadine (10% povidone iodine) and draped with sterile towels. Skin incision was done longitudinally and the periosteum was elevated over the parietal bones. Critical-size bone defects were created using biopsy punches 5 mm in diameter on each side of sagittal suture. Care was taken to avoid injury of the underlying dura. One defect served as an experimental side and the other one as a control.

The seventy-two rats were divided into four groups (18 rats/group). The rats in each group were further divided into three groups (6 rats/group) depending on the duration of the experiment (3, 6 and 9 weeks) (Tables 1 & 2).

Group 1: Guided Bone Regeneration (PTFE) membrane group:

Two polytetrafluoroethylene membranes covered the defect on the test side. One membrane was applied over the dura (Fig. 1) and the other under the periosteum. The non-resorbable Polytetrafluoroethylene membranes (IMTEC biobarrier membranes) were porous (pore size 5.0 μm) and flexible. The control side was left uncovered by membranes.

Group 2: Osteoconductive (PTFE + Medpor) group:

Two polytetrafluoroethylene membranes covered the defect on the test side with Medpor (high density porous polyethylene) implant filling the defect in-between. Medpor implant, which is osteoconductive, was cut to the size of the defect (5 mm) using the same sized biopsy punch and was pressed fit into the defect. The control side was left neither filled nor covered.

Group 3: Osteoinductive (PTFE + DBM) group:

Two polytetrafluoroethylene membranes covered the defect on the test side with demineralized bone matrix (DBM) filling the defect in-between. The autogenous demineralized bone matrix was prepared in laboratory by treatment of the undemineralized bone with 0.5M HCl (25 mEq/gm) for three hours at room temperature followed by sequential washings in sterilized distilled water, absolute ethanol and anhydrous ether [17]. The control side was left neither filled nor covered.

Group 4: Autogenous bone graft group:

The calverial bone taken during creation of

the defect on the control side was pressed fit into the test side as an autogenous bone graft. The defect on the control side was left empty.

For euthanasia (killing the animals) after three, six and nine weeks, each animal received an overdose of ether inhalation till death. We collected the specimens by excising the whole skull vaults cautiously using a bone cutter. The specimens included the sites of the two defects. The specimens were fixed in 10% formaline, decalcified in 5% trichloroacetic acid, dehydrated in an ascending grade of ethyl alcohol, cleared in zylol and embedded in parablast. The sections were cut serially at 6 μm thickness. The sections were stained with haematoxylin and eosin and Masson's trichrome stains [**15**]. This stain is mainly used to stain the collagen fibers type I present in bone collagen and fibrous tissue. Qualitative analysis of the tissue filling the defects histopathologically was done in all groups.

RESULTS

All rats included in the study were recovered uneventfully. All rats gained weight. Clinically, there were no signs of any inflammatory reactions at the operation site that may suggest synthetic membrane rejection. No neurological insults such as paralysis, convulsion, respiratory distress or signs of pain were observed. Microscopically, no round cells, multinucleated giant cells or macrophages were found to infiltrate the defects (Tables 3 & 4).

A- *By naked eye:*

At time of harvesting the specimens, all control groups showed covering of the defects by thin, soft, flexible and translucent membranes. Polytetrafluoroethylene covered defects were thick but soft at three weeks, firm at six weeks and hard at nine weeks. When Medpor (high density porous polyethylene) was added, the defects were hard from the start. When demineralized bone was added, the defects were thick but soft at three weeks, firm at six weeks and hard at nine weeks. As regards auto grafts, they were hard but mobile at three weeks and fixed at six and nine weeks.

B- *Histologically:*

1- *Results of the control sides:*

The control sites are bridged with collagenous fibers that stain pink (acidophilic) with haematoxylin and eosin (H&E) and light green with Masson's trichrom stain (specific stain for collagen fibers type I) (Fig. 2). Fibers run in wavy bundles, which branch. Associated with fibroblasts and fibrocytes, which are branching cells with abundant basophilic cytoplasm. Very minimal new bone formation is hardly seen at the edges of bone defects. This picture is the same however the specimens harvested at three, six or nine weeks.

2- *Results of double PTFE group membrane (GBR):*

At three weeks, the defects were bridged with a thin layer of newly formed cancellous bone associated with mesenchymal membrane formed of widely separated, pale staining stellate cells. The cytoplasmic processes were interconnected with very few collagen fibers (Fig. 3). The cells have oval relatively large nucleus. Endothelial cells forming blood vessels are also seen. The new bone is immature showing uneven acidophilic staining of matrix. Lamellae are irregularly arranged and contain abundant osteocytes. The (PTFE) membranes surround the newly formed bone on both sides and are covered from outside by fibrous connective tissue layers.

At six weeks, defects are completely bridged by new cancellous bone, which shows a combination of mature and immature characters. At nine weeks, the defects are completely bridged by new bone, which is hardly differentiated from the original bone. It has a mature character (even acidophilic staining of its matrix, regularly arranged lamellae and fewer osteocytes) and surrounded by membranes that are covered by fibrous tissues. Sections stained with Masson's trichrom stain show green lamellae of new bone (filled with lacunae), which branch and anastomose. Membranes are covered from outside with faint green connective tissue fibers.

These findings prove that guided bone regeneration occurred in the gaps protected by double PTFE membranes as early as three weeks in rats.

3- *Results of use of porous high-density polyethylene (Medpor) covered by the double PTFE membranes:*

At three weeks, the gap between the two PTFE membranes shows lucent areas of unstained polyethylene and islands of newly formed

cancellous bone lamellae, which are still immature. These bone lamellae extend from the edge of the original bone through the lucent areas (i.e. inside the Medpor) and between the lucent areas and the membranes on both sides. No obvious fibrous connective tissues are found in the gap. On the other hand collagenous fibers and fibroblasts are found outside the PTFE membranes.

At six weeks, the gap, between the double polytetrafluoroethylene (PTFE) membranes, shows greater formation of cancellous bone lamellae (Fig. 4). Parts of the bone lamellae are mature while the others are immature. Bone lamellae extend both inside Medpor and between Medpor and the membranes. No fibrous connective tissues are seen inside the membranes but covering them from outside. At nine weeks, the gap shows the greatest bone formation. Bone lamella are mature extending inside Medpors and between them and the membranes.

Instead of having the whole defect filled with bone the results show that the osteoconductive material remained in-between the newly formed bone. This will affect the quality of the newly formed bone.

4- *Results of use of demineralized bone matrix covered by the double PTFE membranes:*

At three weeks, the gaps between the two PTFE membranes show mesenchymal tissues formed of widely separated pale staining stellate cells with interconnecting cytoplasmic processes (Fig. 5). Stellate cells have oval relatively large nucleus. There are endothelial cells forming blood vessels within this membrane. Very minimal amount of newly formed bone is noticed at the edge of the original bone. This newly formed bone is immature. Fibrous connective tissues cover the PTFE membranes from outside.

At six weeks, the gaps are bridged by thin trabeculae of immature bone parallel to each other with some mesenchymal-like cells in-between. At nine weeks the bone trabeculae become mature. Fibrous connective tissues cover the membranes from outside.

5- *Results of use of autogenous bone grafts:*

At three weeks, the gaps between the original bone and the autogenous bone grafts show newly formed immature cancellous bone lamellae. The junction between the newly formed bone and the transplanted bone shows abundance of giant multinucleated cells (osteoclasts) on high magnification. No fibrous connective tissues are found at the junctions.

At six weeks, the gap between the original bone and the autogenous bone grafts are filled with newly formed cancellous bone lamellae partially mature and partially immature. No fibrous connective tissues are found in the gaps. At nine weeks, the sites of the junctions between the original and transplanted bones are hardly detected because they are formed of mature bone, very similar to the original one. No fibrous connective tissues are seen in the gap.

Table (1): Total number of defects in each group.

			Test (left) side	Control (right) side
Group 1	PTFE group	GBR	18 defects	18 defects
Group 2	PTFE + Medpor	Osteoconduction	18 defects	18 defects
Group 3	PTFE + DBM	Osteoinduction	18 defects	18 defects
Group 4	Autogenous bone graft	Osteoconduction + Osteoinduction + Osteogenesis	18 defects	18 defects

PTFE = Polytetrafluoroethylene.
DBM = Demineralized bone matrix.
GBR = Guided bone regeneration.
Test side covered with PTFE membranes with nothing in-between in group 1.
With Medpor in-between in group 2.
With DBM in-between in group 3.
Test side covered with autogenous bone grafts in group 4.
Control sides left uncovered and unfilled in all groups.

Table (2): Number of test defects in each group based on duration of the experiment.

		3 weeks	6 weeks	9 weeks
Group 1	PTFE group	6 defects	6 defects	6 defects
Group 2	PTFE + Medpor	6 defects	6 defects	6 defects
Group 3	PTFE + DBM	6 defects	6 defects	6 defects
Group 4	Autogenous bone graft	6 defects	6 defects	6 defects

Table (3): Results by naked eye (inspection and palpation).

		3 weeks	6 weeks	9 weeks
Group 1	PTFE group	Thick but soft	Thick and firm	Thick and hard
Group 2	PTFE + Medpor	Thick and hard	Thick and hard	Thick and hard
Group 3	PTFE + DBM	Thick but soft	Thick and firm	Thick and hard
Group 4	Autogenous bone graft	Hard and mobile	Hard and fixed	Hard and fixed
Control	Uncovered & unfilled	Thin, soft and translucent	Thin, soft and translucent	Thin, soft and translucent

Table (4): Results by histological examination of the defects.

		3 weeks	6 weeks	9 weeks
Group 1	PTFE group	Mesenchyme + immature bone	Immature + mature bone	Mature bone
Group 2	PTFE + Medpor	Lucent areas + immature bone	Lucent areas + immature + mature bone	Lucent areas + mature bone
Group 3	PTFE + DBM	Mesenchyme + immature bone	Immature + mature bone	Mature bone
Group 4	Autogenous bone graft	Immature + mature bone	Immature + mature bone	Mature bone
Control	Uncovered & unfilled	Mesenchyme	Fibrosis	Fibrosis

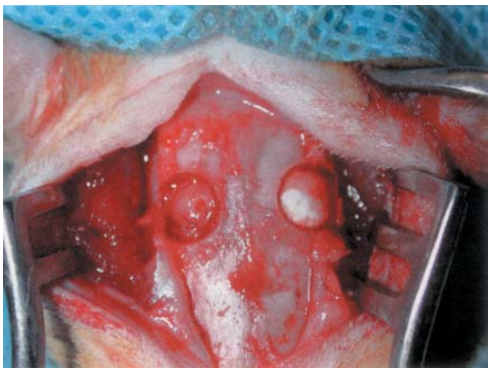

Fig. (1): An intra-operative photo shows a circular non-resorable polytetrafluorethylene membrane (6 mm in diameter) over the dura on the left side. One millimeter of the membrane is under the inner bony edge of the defect.

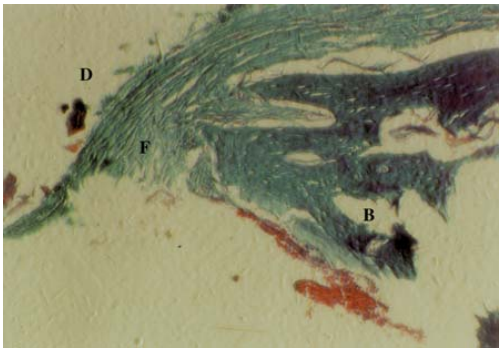

Fig. (2): A photomicrograph represents healing of the defect in the control group at nine weeks. It shows old bone (OB), New Bone (NB) and fibrous tissue (F) (Massion's trichome X 100).

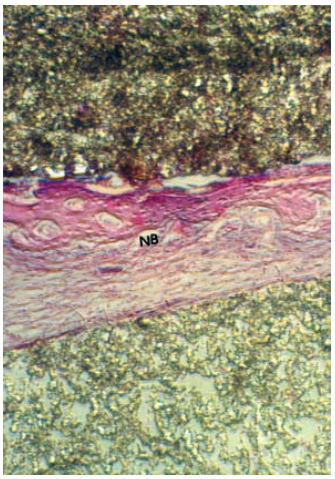

Fig. (3): A photomicrograph represents healing of the defect in the double PTFE membrane group at three weeks. It shows old bone, new bone and the mesenchyme (Hx & E X 100).

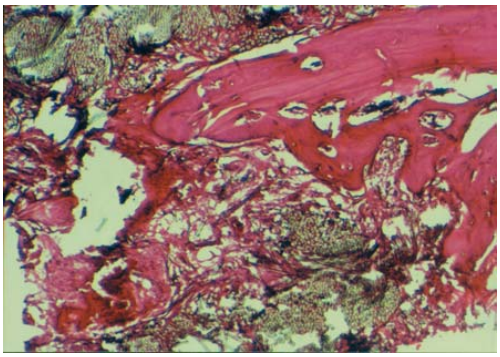

Fig. (4): A photomicrograph represents healing of the defect filled with high density porous polyethylene (Medpor) and covered with double PTFE membrane group at 6 weeks (Hx & E X 100).

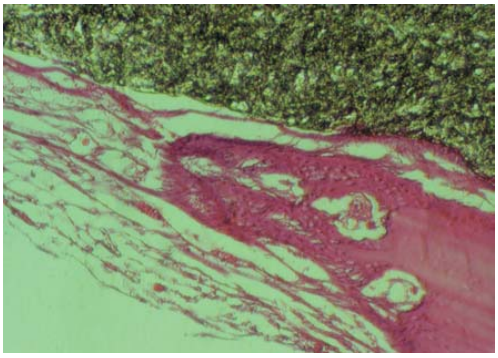

Fig. (5): A photomicrograph represents healing of the defect filled with demineralized bone matrix (DBM) and covered with double PTFE membrane. It shows old bone, new bone and mesenchyme (Hx & E X 100).

DISCUSSION

Bone defects are significant clinical problem within plastic surgery, orthopedics, oral surgery as well as periodontology. Examples include bone defects in the facial skeleton, resulting from trauma, cysts or periapical bone resorption. In reconstructive surgery, there is often a need to create new bone. A main obstacle for successful bone healing and the formation of new bone is that, in contrast to osteogenesis, connective tissue formation occurs rapidly. Thus, ingrowth of soft tissue may disturb or totally prevent osteogenesis from taking place in a defect or wound area. This in turn will lead to anatomical aberrations and functional disturbances, often requiring re-entery operations **[16]**.

The animal model in this study was the critical size cranial defect in rats. Takagi and Urist, **[18]** and Hollinger et al. **[19]**, mentioned that the critical size cranial defect in skeletally mature rats is 8 mm. On the other hand, Bosch et al., **[20]** found that the critical size defect in this model is only 5 mm. In our study we used 5 mm defects one on each side of the sagittal suture and the results proved that 5 mm is a critical size defect because it healed with fibrous tissue when left empty and healed with bone when the guided bone formation membranes were added (Group I).

The mechanical hindrance to connective tissue proliferation into a bony defect can be of profound importance for unimpeded bone healing. In this work, the defects managed with guided bone regeneration alone, showed complete bony healing (without the addition of any osteoinductive or osteoconductive materials) at three weeks. This clarified the significance of hindering the penetration of the surrounding connective tissue proliferation into the defect thus paving the way for bone regeneration to close the defect from the surrounding bone edges.

To test the effect of combing osteoconductive materials to guided bone regenerating membranes, we chose Medpor (high density porous polyethylene) as an osteoconductive material (Group II).

In our study when we covered Medpor implants with Polytetrafluoroethylene membranes, complete bridging with bone occurred at 9 weeks. These results are similar to those of Schliephake et al., **[21]**. In their study, the results showed that in the groups without membranes, bone formation inside the hydroxyapatite blocks was confined mainly to the layers in contact with bone, while bone formation was almost absent in the layers beneath the soft tissue covering. Actually, in this study, Medpor served as a space maintainer in-between the membranes in addition to its osteoconductive property. The same results were achieved by Hopper et al. **[22]**, who used fibrillar lactic acid as an osteoconductive material.

It was thought that by addition of demineralized bone matrix (DBM) (Group III) under the two layers of the polytetrafluoroethylene membranes will have a promotive effect, but this was not the case at 3 weeks (the time by which the bone induction should have been completed). It was found that mesenchymal cells occupying the defect with few bone at the periphery less than that achieved by the membranes alone. These results are contradictory to the synergistic effect of adding DBM (demineralized bone matrix) in-between biodegradable polymer membrane, mentioned by Kleinschmidt et al., **[23]**.

Absence of any cartilaginous tissues and presence of mesenchyme adjacent to the new bone indicated that the demineralized bone matrix did not work as an osteoinductive material and that healing occurred by intramembranous ossification. Reddi and Cunningham **[24]**, mentioned that osteoinduction proceeds through a cascade of interrelated events similar to those occurring during endochondral ossification.

Our failure to achieve this synergistic effectmentioned by Kleinschmidt et al., **[23]** - might be explained by either one or combination of two possibilities. Either the concentration of the BMP (bone morphogenetic protein) in the demineralized bone was not enough to induce bone formation or the presence of membranes prevented access of inducible mesenchymal cells to the vicinity of the defect where DBM is located. The presence of the membrane confined the defect space to be accessible only to mesenchymal cells from the adjacent bone edges and marrow cavities and therefore limited activator mediated cellular interaction. Reddi and Cunningham **[24]**, mentioned that each step of osteoinduction (chemotaxis, mitosis and differentiation) needs certain concentration of BMP. Therefore in our study the concentration might have not been enough to cause differentiation.

The results of this work confirmed that the autogenous bone grafts still the main source to bridge a bone defect. They provided the scaffold for osteoconduction, bone morphogenic protein for osteoinduction and surviving cells for osteogenesis. For from the disadvantages of their use as regards the limitation and donor site morbidity, the results of this study showed that they are the optimum solution for closure of bone defects **[25,26]**.

This study showed that osteopromotive membranes alone can allow bone regeneration, if they did not collapse although regeneration occurs in a delayed but predictable pattern. Osteoconductive materials help in maintaining

the space under the osteopromotive membranes, while the membranes inhibit fibrous integration. Osteoinductive materials failed to show any additive effect to the membrane either due to the low concentration of bone morphogenetic protein (BMP) in the demineralized bone or failure to contact enough inducible mesenchymal cells. It appears, at least from this study that guided bone regeneration per se is sufficient to bridge a defect provided that the defect was maintained. On the other hand osteoconduction and osteoinduction did not add too much to the guided bone regeneration. Lastly autogenous bone graft stood the test of time as regard managing a bone defect.

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