Repair of Bone Defect Using Bone Marrow Cells and Demineralized Bone Matrix Supplemented with Polymeric Materials

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Abstract: We present a novel, reverse thermo-responsive (RTR) polymeric osteogenic composite comprising demineralized bone matrix (DBM) and unmanipulated bone marrow cells (BMC) for repair of bone defects. The polymers investigated were low viscosity aqueous solutions at ambient temperature, which gel once they heat up and reach body temperature. Our goal to supplement DBM-BMC composite with RTR polymers displaying superior rheological properties, was to improve graft integrity and stability, during tissue regeneration. The osteogenic composite when implanted under kidney capsule of mice, proved to be biocompatible and biodegradable, with no residual polymer detected in the newly formed osteohematopoietic site. Implantation of the osteogenic composite into a large area of missing area of parietal bone of the skull of rats, resulted in an extensive remodeling of DBM particles, fully reconstituted hematopoietic microenvironment and well integrated normal flat bone within thirty days. The quality and shape of the newly created bone were comparable to the original bone and neither local or systemic inflammatory reactions nor fibrosis at the junction of the new and old calvarium could be documented. Furthermore, combined laser capture microdissection (LCM) technique and PCR analysis of male BMC in female rats confirmed the presence of male derived cells captured from the repaired/ regenerated flat bone defect. The use of active self sufficient osteogenic DBM-BMC composite supported by a viscous polymeric scaffold for purposive local hard tissue formation, may have a significant potential in enhancement of bone regeneration and repair following trauma, degenerative or inflamatory lesion, iatrogenic interventions and cosmetic indications.

Keywords: Bone regeneration; Induced osteogenesis; Bone marrow cells (BMC); Mesenchymal progenitor cells (MPCs); Demineralized bone matrix (DBM); Fibrin glue; Vitrogen collagen gel; Reverse thermo responsive (RTR) polymers.

INTRODUCTION

Cranial and other bone defects can result from trauma, tumor extirpation, bone infections, congenital anomalies or aseptic necrosis of bone. Cranioplastic materials can be artificial, such as metals and acrylic resins, or natural, such as allogeneic or autologous bone grafts. Artificial materials have many favorable properties including availability, strength, and easiness of application. However, being still a foreign body, they may become infected, are brittle, and have stationary size which is especially problematic for application to a growing organism. Theoretically, the best cranioplastic materials should be natural bone grafts presenting biological and mechanical properties of normal bone, participating in normal bone turnover and growth, especially subjected to remodeling capacity. Nevertheless, the use of autografts have definite disadvantages, including donor site morbidity, limited shapes and sizes of the available grafts, as well as tendency to undergo an unpredictable resorption [1-4]. Allogeneic bone grafts are immunogenic, hence requiring life-long maintenance of hazardous immunosuppressive agents, which would be an impractical approach for routine clinical use. Hence, it can be concluded that the existing methods are far from being satisfactory for replacement of cranial defects as well as for replacement of bone defects in other sites.

The most desirable approach is to induce local development of natural bone tissue from autologous mesenchymal progenitor cells (MPCs). It is well established that MPCs, which are capable of extensive proliferation and differentiation into well-defined tissues, including cartilage, bone, tendon, muscle and fat, are present in the bone marrow compartment [5-10]. MPCs need to be adequately induced and conducted to accomplish new tissue formation [11]. Demineralized bone matrix (DBM) combines all the necessary conductive features of a carrier and serves, at the same time, as a natural source of inductive osteogeneic factors [12-14].

Recently, we have demonstrated that the combination of MPCs present in a fresh unmanipulated cell suspension derived from bone marrow and DBM constitute a self-sufficient unit, containing all the essentials for local hard tissue formation in orthotopic or ectopic sites [15]. Being transplanted into the damaged bone site DBM-BMC composite (DBM-BMC) is capable to provide healing through formation of a new bone tissue. We have also shown that induced osteo-hematopoietic sites possess the same main features as normal skeletal bones [16, 17] and that they continue self-maintenance and proper function throughout a period of at least 12 months, corresponding to half the life-span of the experimental mammal.

Taking into consideration that the DBM-BMC composite is designated to provide healing of hard tissues having essential mechanical properties and definite form, the graft should be hard enough to maintain its integrity and stability of shape, while withstanding mechanical influence during the period of tissue regeneration. Therefore, bone formation under the kidney capsule was chosen as the first parameter to examine the efficacy of our new DBM-BMC composite. More over, while being inserted into a bleeding site, the graft has to be protected from partial disintegration leading to deterioration of the shape of regenerating bone. Thus, in order to optimize the properties of a DBM-BMC graft, we propose to support mechanically with a polymeric scaffold (possessing the following features):

1) Compatible with proliferation and differentiation of MPCs, in the course of bone formation; 2) Slowly biodegradable or dissolvable in the body fluids, the degradation time being com-

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patible with the period of new bone formation; 3) Provided in a form allowing the homogeneous mixing with DBM-BMC composite; 4) Renders the DBM-BMC composite sufficiently strong to maintain integrity and shape, as well as to provide biomechanical properties to the transplant during the period of new bone formation; and 5) Non-toxic, non-irritant and non-immunogenic.

Reverse thermo-responsive polymers are required to display low viscosity at insertion time, while a gel or solid consistency develops *in situ*, as they heat up to body temperature. The RTR-displaying polymers used in this study comprise poly (ethylene oxide) and poly (propylene oxide) segments, chain extended using phosgene or adipoyl chloride. Additionally, the performance of an RTR polymer based on a PEO-PPO-PEO triblock (Pluronic F127), modified by binding it to two lactoyl units on each side, and then chain extended using hexamethylene di isocyanate, was also investigated [30]. The purpose of incorporating lactoyl units into the polymeric backbone was to render it biodegradable, due to the well known hydrolytic reactivity of aliphatic polyesters. The various polyether diols were chain extended by reacting their OH terminal groups with the different bifunctional molecules, whereby carbonate, ester or urethane moieties were generated.

Here we report for the first time the development of an advanced composition which comprises a RTR-displaying polymer in addition to DBM-BMC, which seems to be superior to fibrin tissue adhesive glue (Fibrin glue), Vitrogen 100 collagen gel (Vitrogen gel) or pluronic F127 (RTR N7) as a scaffold designated to induce rapid bone formation and provide integrity and shape to osteogenic DBM-BMC graft.

MATERIALS AND METHODS

Animals

C57BL/6 male mice 8-week old and Lewis male rats 10-week old were used as donors of bones for matrix preparation and BMC. Syngeneic mice and rats, respectively, were used as recipients. No less than 5 transplant procedures were performed in each group.The study was conducted in compliance with the international laws on animal experimentation and approved by the Ethical Committee of the Hebrew University Medical School.

Preparation of Demineralized Bone and Tooth Matrix

DBM was prepared as previously described [15]. Rats diaphyseal cortical bone cylinders were cleaned from bone marrow and surrounding soft tissues, crumbled and placed in a jar under magnetic stirring. Bone chips were rinsed in distilled water for 2-3 hrs, in ethanol (70%, 96% and 100% consecutively) for 1 hr, and in diethyl ether for 0.5 hr, then dried under a laminar flow hood, pulverized in a mortar with liquid nitrogen and sieved to select particles between 300 and 450μ. The powder obtained was demineralized in 0.6M HCl overnight, washed to remove the acid, dehydrated in ethanol and diethyl ether and dried. All the procedures were performed at 4°C, to prevent degradation of BMPs by endogenous proteolytic enzymes. Experiments with mice were performed using demineralized tooth matrix (DTM) prepared using the same method from incisors dissected out from adult mice, as previously described [18].

1. Preparation of Donor BMC Suspensions

Femura and tibiae of donor mice or rats were obtained aseptically. The extremities of the bones were removed and bone marrow plugs were mechanically pressed out of the bone cavity with a mandrin. BMC were suspended in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) and prepared for transplantation in a form of a single cell suspension containing $3x10^8$ cells/ml.

Preparation of Vitrogen 100 Collagen gel

Eight volumes of purified collagen solution (3mg/ml Vitrogen 100, Cohesion, Palo Alto, CA, USA), one volume of 10 x phosphate buffer saline (PBS) and one volume of 0.1M NaOH, were added with gentle agitation at 0^oC and pH of the solution was adjusted to 7.0 by the addition of either 0.1 M NaOH or 0.1 M HCI.

Preparation of Human Fibrinogen and Human Thrombin Stock Solutions

Human fibrinogen (Melville Biologics, Inc. NY 11747, 150mg/ vial) was dissolved in 1 ml of sterile double distilled water (DDW) and aliquoted at 3.5mg/ 25ul. Human thrombin (Melville Biologics, Inc. NY 11747, 400 units/ vial) was dissolved in 500ul of sterile DDW and aliquoted at 3.5mg/ 25ml. Both solutions were stored at -20^oC until use.

Preparation of RTR Polymeric Materials

a) Polymer N2. Commercially available Pluronic F127 (Sigma) in 23% (wt/ wt) aqueous solution.

b) Polymer N4. This is a random poly(ether ester) synthesized by coupling poly(ethylene glycol) 6000 (Aldrich) and poly(propylene glycol) 3000 (Aldrich) blocks with adipoyl chloride accordingly to a previous technique [19, 20] in 25% (wt/ wt) water solution.

c) Polymer N7. This is an alternating poly(ether ester) synthesized by coupling poly(ethylene glycol) 6000 (Aldrich) and poly(propylene glycol) 3000 (Aldrich) blocks with phosgene accordingly to a previous technique [21], in 20% (wt/ wt) water solution.

d) Polymer N13. This polymer is based on Pluronic F127 (a PEO-PPO-PEO triblock), bound to two lactoyl units on each side, and then chain extended using hexamethylene diisocyanate, in 15% (wt/vol) water solution.

In order to prepare RTR-exhibiting polymer solutions, the corresponding amount of polymer was dissolved in water at 4^oC. All the systems were low viscosity solutions *in vitro*, at room temperature, with increasing viscous gelation at 37^oC, *in vivo* (Table **1)** [31]

Composition of the Grafts

Grafts were composed of the following ingredients that were mixed *extempore* prior to application *in vivo* in different combinations:

Table 1. Different Types of Reverse Thermal Responsive (RTR) Polymers Used For the Current Study

RTR-Polymer No. #	Contents	Concentrations Used In Vivo
RTG-N2	Commercially available Pluronic F-127	23%
RTG-N4	Block copolymer PEG & PPG with adipoyl unit as the coupling molecule	20%
RTG-N7	Block copolymer PEG & PPG with carbonate unit as the coupling molecule containing 54% PEG	20%
RTG-N13	PluronicF-127 containing 2 Lactic acid unit in each reacting unit of the polymer	15%

(Cohn, D. Sosnik, A. et al. Biomaterials. 2003 Sep; 24(21):3707-14)

Table.1. Reverse thermal responsive (RTR) polymers used for the current study.

Table 1 showing different types of coupling molecules used in the preparation of reverse thermal responsive polymers in DBM-BMC composite for repair of the bone defect for the current study.

1) 20 μl of BMC suspension at a concentration $3x10^8$ cells/ml;
2) 4 mg of DBM, with particles 300-450 micron in size;
3) Supplement solution:
   a) Fibrin tissue adhesive glue (1mg/7! l human fibrinogen and 2units/3! l human thrombin); or
   b) 10 ! l of pH neutralized isotonic Vitrogen gel, or
   c) 10 ! l RTR-displaying polymer solution.

Implantation of DTM-BMC Composite Under the Kidney Capsule in Mice

After general anesthesia of C57BL/6 mice, an incision was performed above the kidney region and the kidney was exposed. A small cut was made in the kidney capsule, and the transplanted material was inserted under the capsule using a concave spatula and the kidney was returned to its normal position. The incision was closed and the skin fixed with stainless steel clips. The transplants consisted of DTM-BMC composite alone or with the supplement of Fibrin glue, Vitrogen gel, or RTR polymer solutions. In the control groups, BMC were transplanted only with the corresponding supplement solutions in order to study their osteoinductive features and ability to substitute DTM. No less than 5 transplant procedures were performed in each group.

Implantation of DBM-BMC Composite into an Experimentally Created Calvarial Defect in Rats

After general anesthesiam of Lewis rats, an incision was performed in the frontal region of the cranium. A full thickness bone defect (6 x 6 mm^2) was created laterally to the sagittal suture using a dental burr. The defect area was either left empty, filled with DBM-BMC composite alone, or with the supplement of Fibrin glue, Vitrogen gel or RTR polymer solutions. In the control groups, BMC were transplanted only with supplement solutions. The defect area was then covered with Fibrin glue, the skin flap was returned into place and fixed with stainless steel clips. No less than 5 transplantations were performed per group.

Analysis of Tissue Samples with Laser Capture Microdissection and Polymerase Chain Reaction

Male BMC were transplanted with DBM into female recipients in order to detect male DNA as a parameter of engraftment, and the newly formed flat bones were checked by polymerase chain reaction (PCR) analysis to confirm the source of the reconstructed tissues at the site of the defect. The new laser capture microdissection (LCM) technology, that allows isolation of individual cells from the tissue sections under precise microscopic control, was used to identify the source of cells (about 100-300 cells per test) [22]. The following extraction procedure was used for direct amplification by PCR. Each Capsure Transfer Film Cap (containing ~ 300-500 laser captured cells) was placed onto a 0.25ml micro centrifuge tube filled with 50ul of Proteinase K digestion buffer [(1mg/ml Proteinase K (Roche Molecular Biochemicals) and 1% Tween20 in TE buffer pH 8.0]. The microcentrifuge tube was then inverted and the digestion buffer was flicked down until all the fluid was in contact with the surface of the Capsure Transfer Film containing the sample. Tubes were then incubated (inverted) at 60 C for 90 minutes and at 45 C for overnight in a dry incubator .Centrifugation was done for 5 minutes at a maximum speed of 6400 rpm (2000 x g) to collect all the fluid and the DNA. The Capsure Transfer Film Cap was removed and visualized under a microscope to verify that all the cellular elements were digested and recovered into the digestion buffer. Inactivation of the proteinase K was done by placing the same microfuge tubes in a dry bath at 95 C for 10 minutes. 35ul from each sample was taken directly for amplification by PCR reaction. The amplification was done in a 50ul reaction buffer containing 1XPCR buffer, 2.25mM $MgCl_2$, 0.1mg/ml BSA, 0.2mM dNTPS, 20 Pmol of rat Y-Sry-specific oligonucleotide primers with the following sequences to regions of Sry gene of the rat "sex determining region" carried on the Y chromosome.*[22]*. Samples were then vortexed, boiled for 5 minutes and chilled on ice before addition of one Unit of Taq Polymerase (MBI, Fermentas).

Table 2. PCR Primer Pairs Used in This Study

Species	Gene	Primer Sets
Rat	Y gene	Forward: 5' primer, 5'-CATCGAAGGGTTAAAGTGCCA-3' Reverse: 5' 3' primer, 5'-TGCAGCTCTACTCCAGTCTTG-3'.

Thirty PCR amplification cycles were carried out in a Thermal Cycler (Company) with each cycle consisting of one denaturation step at 94C for 1 minute, annealing step at 62C for 90seconds, and an extension step at 72C for 2 minutes. Following these 30 cycles of an additional extension was performed at 72C for 5 minutes.The PCR products were analyzed by Electrophoresis on 1.8% agarose gel, followed by ethidium bromide staining to assess the size. The product is a 136 base pair DNA fragment [22].

Histological Evaluation

Tissues obtained at autopsy were fixed in 4% neutral buffered formaldehyde, decalcified, passed through a series of ethanol grades and xylene, and embedded in paraffin. Sections (5-7 microns thick) were stained with picroindigocarmine (PIC). The stain, combining copper carmine, picric acid and indigocarmine gives bright and colourful results. Nuclear structures are stained in red, cytoplasm in varying shades from yellow to green, collagen fibres in blue, the matrix of hyaline cartilage in greyish-blue, muscle tissues from brownish-red to brownish-green, erythrocytes in yellow. Before the decalcification, the harvested calvaria-were scanned and X-rayed.

RESULTS

Examination of the Compatibility of the RTR-Displaying Polymeric Materials with the Process of Induced Osteogenesis in the Sub-Capsular Space of the Kidney

The subcapsular space of the kidney served as a "live test tube" for investigating the influence of the different materials on new bone formation accomplished by the DTM-BMC composite. The reverse thermo-responsive behavior of these polymers is exemplified in Fig. (**1**), for an alternating poly(ether ester) consisting of poly(ethylene oxide) (6,000 dalton) and poly(propylene oxide) (3,000 dalton) segments, chain extended using phosgene. Several RTR polymers, Vitrogen gel and Fibrin glue were therefore, studied in this system for their compliance with desirable criteria. One month after transplantation of DTM-BMC composites supplemented with RTR-displaying polymers (N2, N4, N7 and N13), Vitrogen gel or Fibrin glue, newly formed cortical and trabecular bone, including well-developed marrow cavity and functionally active bone marrow, were observed in all the cases (Fig. **2**). No difference in the developmental level of the ectopic ossicles produced by DTM-BMC composite transplanted alone or with mentioned above supplement materials therefore could be detected. No residual supplement materials were observed in newly developed osteo-hematopoietic sites indicating that Vitrogen gel, Fibrin glue or RTR polymers, were totally resorbed.

BMC transplanted without DTM but supplemented with each of the polymeric materials produced only small ossicles if at all (Fig. **2**). Implantation under the kidney capsule of any of the polymeric materials alone never led to bone formation (data not shown). No inflammatory reaction, fibrosis or any other local or systemic side effects were documented.

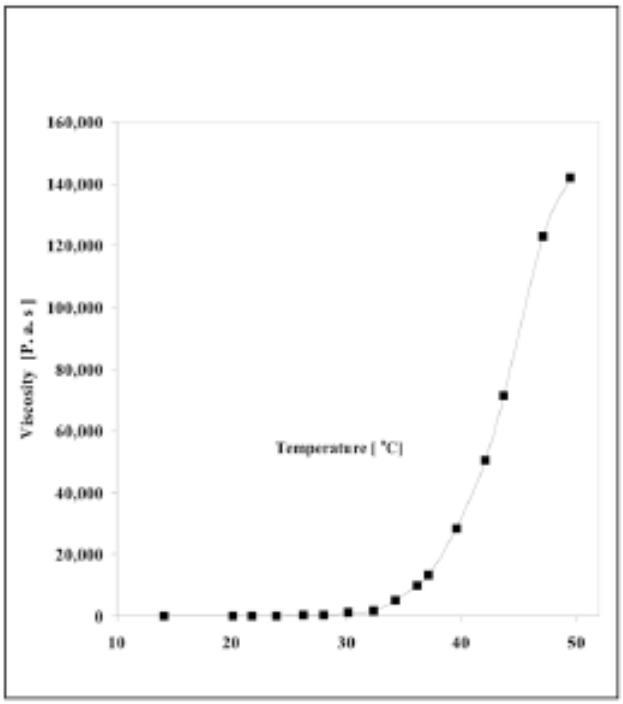

Fig. (1). Evaluation of the viscosity as a function of the temperature of new water-soluble polymers, displaying reverse thermal gelation [(RTR) (data represents for RTR N2)].

The reverse thermo-responsive behavior of the polymers for an alternating poly(ether ester) consisting of poly(ethylene oxide) (6,000 dalton) and poly(propylene oxide) (3,000 dalton) segments, chain extended using phosgene.

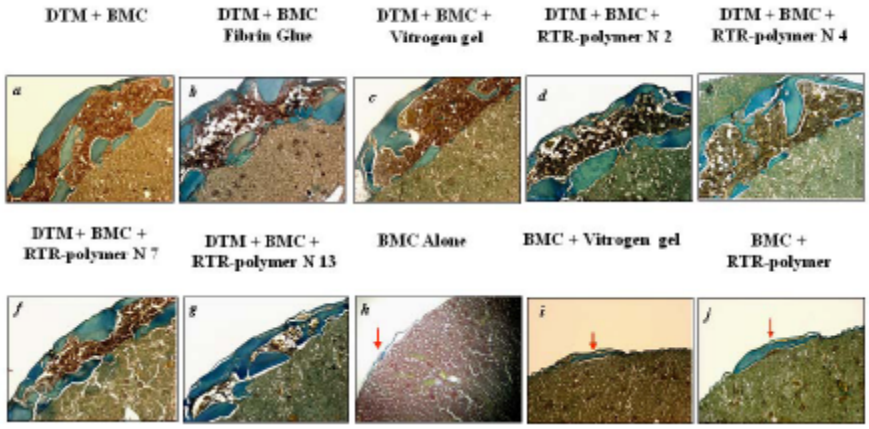

Fig. (2). Study on the influence of Fibrin glue, Vitrogen gel and various polymeric materials on the osteogenic properties of demineralized tooth matrix (DTM) and bone marrow cells (BMC) composition transplanted into the sub-capsular space of the kidney in mice.

Cross sections stained with Picroindigocarmine (-5x).

One month post-transplantation of BMC+DTM alone (Fig. 2*a*), together with Fibrin glue (Fig 2b),Vitrogen gel (Fig. 2*c*), or RTR polymers [(RTR-N 2, 4, 7 & 13) (Fig.2 *d*, *e*, *f*, *g* respectively)], a newly formed cortical and trabecular bone, well developed marrow cavity and functionally active bone marrow are seen in all the cases. No difference in the developmental level of the ectopic ossicles produced by DTM-BMC active complex transplanted with or without aforesaid materials could be observed.

BMC transplanted alone (Fig.2*h*) or mixed with Vitrogen gel (Fig.2*i*) or polymeric material (Fig.2*j*) produced only small ossicles.

Transplantation of DBM-BMC Composite Supplemented with Different Polymeric Materials into the Site of Calvarial Defect

No spontaneous bone regeneration was observed within 30 days after surgery, when the site of parietal bone defect was left unfilled. This strongly suggests that the size of the calvarial defect created was compatible with the definition of non-healing defect. Similarly, when the missing area of parietal bone was filled with BMC together with one of the supplement materials investigated (Fibrin glue, Vitrogen gel or RTR polymers) without DBM no bone formation was observed in most of the cases. Sometimes however a small islands of thin bone developed at the site of transplantation (Fig. **3**). In sharp contrast, when DBM-BMC composite supplemented with Fibrin glue, Vitrogen gel or any of the RTR-displaying polymers (N2, N4, N7 and N13), was transplanted into the missing areas of

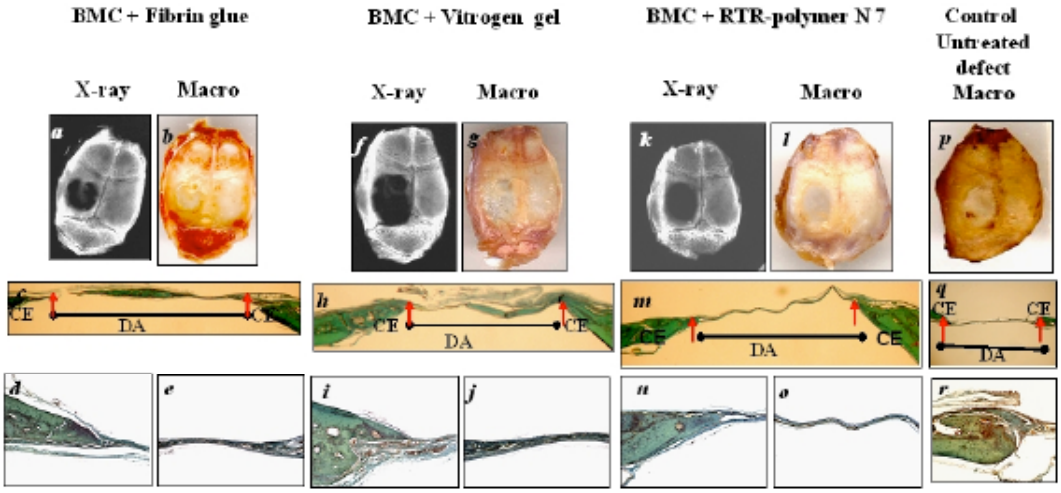

Fig. (3). Correction of experimentally created calvarial defect by transplantation of bone marrow cells (BMC) supplemented with Fibrin glue, Vitrogen gel or RTR polymer. Coronary sections stained with Picroindigocarmine.

X-Ray and Photo- macro and -micrograph (x10) pictures of rats calvaria one month after transplantation of BMC supplemented with Fibrin glue (Fig.3 *a-e*), Vitrogen gel (Fig.3 *f-j*) and RTR –N 7 (Fig. 3 *k-o*) into the area of experimentally created calvarial defect shows absence of bone regeneration, (Fig. 3 *pro*) into the area of untreated experimentally created calvarial defect showing the absence of bone regeneration.

Abbreviations: DA, defect area; CE, cut edge;

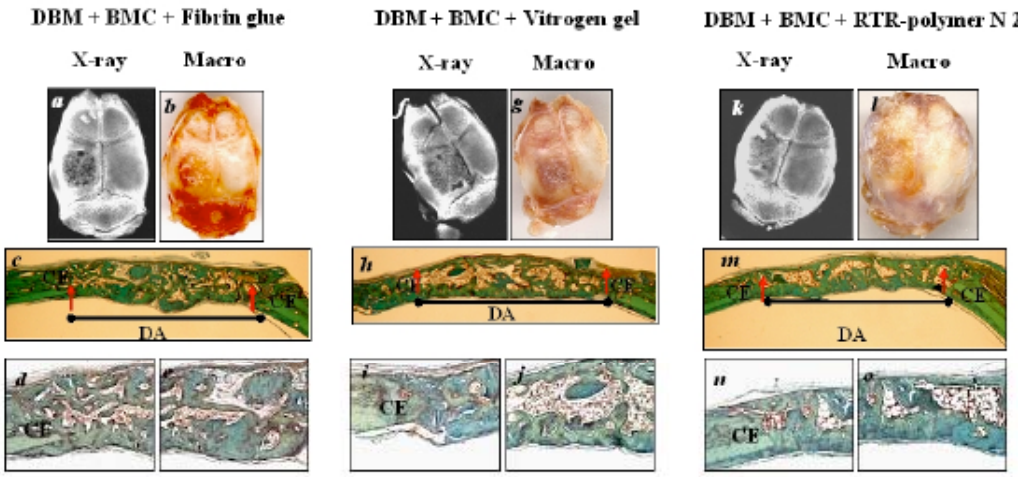

Fig. (4). Correction of experimentally created calvarial defect by transplantation of demineralized bone matrix (DBM) and bone marrow cells (BMC) supplemented with Fibrin glue, Vitrogen gel and RTR-N2. Coronary sections stained with Picroindigocarmine.

X-Ray and Photo- macro and -micrograph (x10) pictures of rats calvaria one month after transplantation of DBM-BMC complex supplemented with Fibrin glue (Fig.4 *a-e*), or Vitrogen gel (Fig.4 *f-j*) or RTR-N2 (Fig. 4 *k-o*) into the area of experimentally created calvarial defect show continuous layer of newly developed bone tissue with hematopoietic areas and active remodeling of the transplanted DBM particles. Cut edge of the native bone can hardly be seen.

Abbreviations: DA, defect area; CE, cut edge;

the parietal bone, extensive remodeling of DBM particles and developing areas of new bone were observed (Figs. **4**, and **5**).

Thirty days post-transplantation, the defect area was reconstituted with a continuous layer of newly developed bone so that the cut edges of the parietal bone could hardly be distinguished from the surrounding new bone tissue. This finding was elaborated by in X-ray and macro pictures and also confirmed by histological studies. It should be pointed out, however that part of transplantations performed with DBM-BMC composites supplemented with Fibrin glue or Vitrogen gel resulted in the creation of a non-uniform bone layer due to the partial loss of grafted material. In contrast, in all cases in which DBM-BMC graft was supplemented with RTR polymers, the missing area in all the animals was fully reconstituted with smooth and uniform layer of newly developing bone, and only small deficiencies were rarely seen.

Furthermore, the shape of the new bone that developed in the damaged area was in exact conformity with the missing bone fragment. No tendency for overgrowing the size of the missing bone or spreading out of the damaged area was observed, thus suggesting that MSC in the DBM-BMC composite retained its remodelling capacity. Neither macroscopically or microscopically visible residues, nor any signs of local reaction or inflammation were observed in any of the experimental groups where polymeric materials were used as a supplement to DBM-BMC composite or BMC, nor in the groups were these materials were implanted alone.

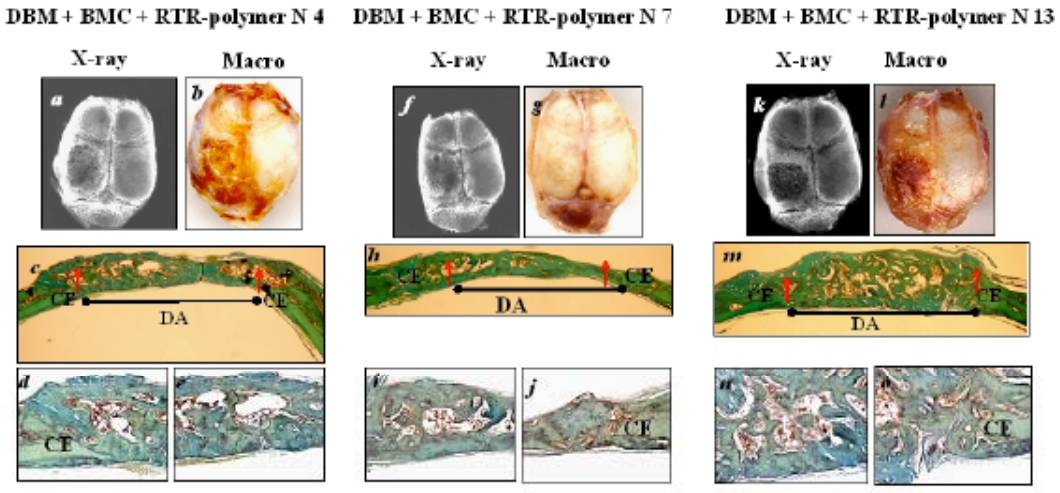

Fig. (5). Correction of experimentally created calvarial defect by transplantation of demineralized bone matrix (DBM) and bone marrow cells (BMC) supplemented with RTR polymers (N4, N7 and N13). Coronary sections stained with Picroindigocarmine.

X-Ray and Photo- macro and -micrograph (x10) pictures of rats calvaria one month after transplantation of DBM-BMC complex supplemented RTR polymers N4 (Fig.6 *a-e*); N7 (Fig.6 *f-j*) and N13 (Fig. 6 *k-o*)into the area of experimentally created calvarial defect show complete regeneration in the area of missing bone. Newly developed bone tissue and residual DBM particles are undergoing the process of remodeling.

Abbreviations: DA, defect area; CE, cut edge;

Determination of the Origin of the Newly Developing Bone After Implantation of DBM-BMC Composite

Male BMC mixed with DBM were implanted in syngenic female rats. The LCM technique was used to procure pure populations of cells from specific microscopic regions of reconstructed tissue sections, in one step, under direct visualization. The cells of interest were punched out by laser beam and transferred to a polymer film. The exact morphology of the procured cells with intact DNA, RNA and proteins was retained and held on the transfer film. PCR analysis of cells captured by LCM from the newly developed flat bone (Fig. **6**) confirmed the presence of donor-derived male cells.

DISCUSION

In the present work we evaluated for the first time the biocompatibility of RTR polymers and their possible use for the mechanical support of a hard tissue implant. In this study we have shown that under the proper conditions, new bone can be induced by bone marrow derived cells added to DBM, when the composite is supported by a proper scaffold. In the first series of experiments, the space under the kidney capsule was selected as the site of transplantation, since this is a place where no bone exists, where new formation of bone can be easily documented. In addition, it has been shown [23] that there are no local mesenchymal progenitor cells that could be induced into osteogenesis. In parallel, the subcapsular

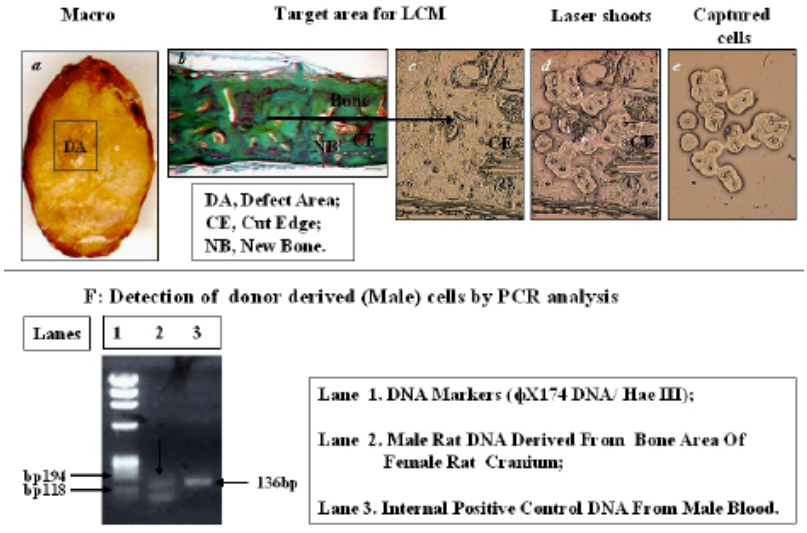

Fig. (6). LCM and PCR analysis of cells obtained from newly formed parietal bone produced by donor male cells in a female recipient.

Photo micrographs of coronary sections through the area of new bone formation. General view with the area of regenerated bone marked (Fig. 7 *a*), target areas for LCM (Fig. 7 *b*) (Picroindigocarmin staining) and (Fig. 7 *c*) (unstained). Laser shots (Fig. 7 *d*). Cells captured for PCR analysis (Fig. 7 *e*). PCR product (a 136-bp fragment) detection by agarose gel electrophoresis. (I) DNA marker (X174 DNA/Hae III); (2) New bone DNA; (3) Control male DNA.

Abbreviations: DA, defect area; CE, cut edge; NB, new bo

space of the kidney supplies all the necessary local conditions supporting the formation of an osteo-hematopoietic complex developing from the implanted cells. Subsequently, we have used a calvarial defect model for testing the capacity of our DBM-BMC complex supported by RTR scaffold to correct an orthotopic bone defect using rat as a pre-clinical model. Our findings suggested that, in addition to the essential DBM and BMC components, stabilization of the composite by the thermo-responsive scaffold generated optimal conditions for the bone regeneration process. It should be especially stressed that the shape of the bone developed in the damaged area was in exact conformity with the missing bone fragment. No tendency for overgrowing the size of the native bone or spreading out of the damaged area was observed. This observation is in agreement with the notion of the remodeling capacity of induced bone. One month post transplantation, the newly formed bone displayed normal structure and histology with well developed marrow cavity and functionally active bone marrow in all recipients. No difference in the developmental level of the ectopic ossicles produced by DTM-BMC active complex transplanted with or without RTR-displaying polymer could be observed, suggesting that the polymer did not interfere with new bone formation.

The general advantage of our approach to purposive bone formation consisting of DBM-BMC graft supported mechanically by a viscous polymeric scaffold containing all the essentials ingredients for local bone development in orthotopic or ectopic sites, the use of unmanipulated, fresh BMCs is a one step procedure. In addition, the advantages of our proposed procedure can be summarized as follows: (1) Creation of a natural bone participating in normal bone turnover and growth and bonding directly to the edges of the damaged native bone without inducing an intermediary layer of connective tissue using a one step procedure; (2) Availability of efficient and technically convenient methodology based on the application of fresh autologous unmanipulated BMC suspension as the source of MPCs, which allows to perform the entire treatment as a single-step procedure, avoiding any preliminary isolation and/or cultivation of MPCs; (3) Feasibility to apply a solution that can be shaped according to the site of a defect, with formation of a biodegradable supportive scaffold gradually replaced by normal bone. Taken together, our methodology provides a possible solution to meet the need for successful repair or replenishment of damaged hard tissues having definite mechanical functions, integrity and stability which using our scaffold could accommodate the shape and structure to be conferred to the shape of the defect, withstanding mechanical forces during the period of tissue regeneration.

To achieve this purpose, the DBM-BMC active complex was modified by adding Fibrin glue, Vitrogen gel or reverse thermo-responsive gelation polymeric materials. Aqueous solutions of reverse thermo-sensitive polymers, display low viscosity at ambient temperature, and a sharp viscosity increase, up to gelation, following temperature increment. Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock (PEO-PPO-PEO), are commercially available as Pluronic^{RTM}, are among the most important families of RTR polymers [24-28]. Among them, Pluronic F127 is the most clinically relevant [28]. Diverse strategies were followed in order to improve the rheological behavior of RTR polymers [21, 30-31]. Polymerizing Pluronic-F127 as well as the biodegradable macromers, had a dramatic effect on the temperature-dependent, rheological behavior of water solutions of these materials. Typically, an eighteen-fold increase in viscosity was achieved when compared to "monomeric" Pluronic F127. Collagen as well as fibrin are known to be highly biocompatible, and collagen in particular is sometimes used as bone graft substitute [32, 33]. Although as shown herewith, several polymers appeared highly compatible with the process of induced bone development by MPCs present in BMC suspension., the new polymers described, N7 in particular and N13 proved most effective as support of the reconstituting bone and bone marrow microenvironment. In general RTR polymer offered more stability to the composite at the implantation site. These polymers were found to be totally biodegradable, showing neither any local or systemic toxicity, nor causing any untoward inflammatory reaction, or secondary fibrosis. Supplementation of DBM-BMC composite with RTR polymeric materials enabling to reach different levels of viscosity seemed preferable for replenishment of damaged hard tissues. Syringeability of RTR polymeric materials at ambient temperature makes their application attractive and especially advantageous, since it allows their introduction into any voluminous or narrow space in the body using minimally invasive techniques. At the transplantation site, as the RTR-displaying polymer acquires the desirable 3-dimensional shape, the viscosity increases due to local heating adjusted to body temperature, thus rendering the final shape and stability of the implant with the sufficient mechanical properties and support.

It can be concluded that transplantation of a composition comprising of BMC, DBM and each of the chosen supplemental polymers can initiate formation of new natural bone with the capacity to grow, bond directly to the edges of the damaged native bone, remodel, seal, and fill holes and inter-osseous spaces, as well as correct defects induced by a surgical procedure or trauma. The new composite can provide the optimal solution for the repair of calvarial defects and most likely defects in other bones as well, with the shape and structure of bone dictated by the local conditions of the transplantation site. Accordingly, DBM-BMC complex, alone or especially in association with RTR polymers, is likely to develop into a useful widely used clinical tool for new bone formation following trauma, degenerative or inflamatory diseases, or iatrogenic interventions.

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