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[54] MIXTURE COMPRISING BONE MARROW CELLS TOGETHER WITH DEMINERALIZED AND/OR MINERALIZED BONE MATRIX AND USES THEREOF IN THE PREPARATION OF COMPOSITIONS FOR THE TREATMENT OF HEMATOPOIETIC DUSIRDERS

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תערובת המכילה תאי מח עצם יחד עם מטריצת עצם נטולת ו/או המכילה מינרלים ושמושיהם בהכנת תכשירים לטיפול במחלות דם

MIXTURE COMPRISING BONE MARROW CELLS TOGETHER WITH DEMINERALIZED AND/OR MINERALIZED BONE MATRIX AND USES THEREOF IN THE PREPARATION OF COMPOSITIONS FOR THE TREATMENT OF HEMATOPOIETIC DISORDERS

Field of the Invention

The present invention relates to compositions comprising bone marrow cells (BMC) and mineralized and/or demineralized, bone matrix (MBM and DBM, respectively) and to their novel uses in bone marrow transplantation in mammals. The compositions of the invention may be particularly effective in restoring the trabecular system and stromal microenvironment, supporting hematopoiesis and developing hematopoietic tissue. When using the compositions and methods of the invention, regenerating hematopoietic tissues originate from the transplanted BMC suspension.

Background of the Invention

A. Transplantation of BMC Into the Bone Marrow Cavity or Extraskeletally

Allogeneic bone marrow transplantation (BMT) involves the transfer of allogeneic marrow stem cells from a healthy donor to a patient in need. Following BMT, patient's bones and hematopoietic niches are reconstituted with donor cells and the entire hematopoietic system, including red blood cells, platelets, nucleated cells, the circulating- and tissue-bound reticuloendothelial system and the entire immune system, are converted to be of the donor origin (1). BMT is widely applicable for treating and correcting a large number of hematopoietic disorders, including a deficiency in any of the bone marrow products, as well as in a need for replacement of abnormal stem cells, in a large number of genetic disorders (2-3). Alternatively, BMT is widely used for cancer therapy and for rescue of patients receiving myeloablative chemoradiotherapy (1). There are many additional potential applications for BMT, such as induction of transplantation tolerance in organ transplantation (4) and as a platform for immunotherapy associated with donor lymphocyte infusions (5-7). Following myeloablative conditioning, BMT can be applied for replacing all host immunohematopoietic system with donor type cells. Alternatively, following non-myeloablative conditioning the role of BMT could be directed to

achieving durable engraftment of donor hematopoiesis side by side with host hematopoiesis for induction of mixed chimerism.

Unfortunately, several limitations restrict the applicability of BMT:

1. Certain hematological diseases for which BMT is indicated may also affect the microenvironment of the marrow and, therefore, stem cell transplantation alone may not be sufficient for complete hematological recovery (8, 9, 10).
2. Efficient consistent engraftment of allogeneic bone marrow cells (BMC), especially purified stem cells or T cell-depleted stem cells, requires transfer of a large number of stem cells which may be difficult to obtain or are unavailable (e.g. cord blood stem cells with limited number of cells; child to adult transplant; etc.) (11,12).
3. Stem cell engraftment in patients with hematopoietic disorders, especially in patients who are treated following mandatory conditioning with irradiation or chemotherapy, is considerably less efficient because of the damaged hematopoietic microenvironment (8,13).
4. Allogeneic BMT as applied to date in patients with hematological disorders is frequently complicated by procedure-related toxicity, poor engraftment and anti-host reaction leading to graft-vs-host disease (GVHD) (14,15).

In view of the above limitations, one of the main objects of the present invention is the improved engraftment of BMC in recipients with damaged hematopoietic microenvironment. As the inventors have recently shown (27) (16), transplantation of BMC within donor hematopoietic microenvironment lowered incidence of GVHD. In addition, when allogeneic BMT is associated with procedure-related toxicity due to use of immunosuppressants for treatment of GVHD, complications could be avoided. Furthermore, improvement in transplantation procedure resulted in reduction of the number of BMC required for transplantation.

In 1990 and 1993 the present inventors have shown that induced bones and hematopoietic microenvironment, developed in mice after ectopic implantation of demineralized bone or tooth matrix, reveal the main properties of skeletal bones (16,17). The following observations were made:

i. The hematopoietic microenvironment in induced bones is capable of supporting fully developed three-lineaged hematopoiesis.

ii. The hematopoietic microenvironment in induced bones is capable of maintaining the proliferative potential of hematopoietic stem cells as could be best documented by colony forming units in the spleen (CFU-S).

iii. *De novo* induced bone and hematopoietic microenvironment are capable of long-term maintenance, remodeling and self-renewal of bone and hematopoiesis without additional application of any exogenous supplement. Once developed, induced bone becomes a permanent structure in ectopic locations with a functionally active hematopoietic niche.

These observations suggest that induction of hematopoietic microenvironment could be a promising approach for correcting stromal and hematopoietic disorders as well as for enhancing hematopoiesis. The BMC transplantation using the composition of the invention, provides appropriate conditions for inducing and maintaining the hematopoietic microenvironment. BMC provide a source of mesenchimal stem cells capable of induced osteogenesis in the presence of DBM. The hematopoietic microenvironment in the induced bones is capable of long-term self-maintenance (17).

It is well known that development and function of the hematopoietic system is supported and regulated by its microenvironment of stromal origin (post-natally, hematopoiesis is totally restricted to skeletal bones) (19, 20, 21).

Engraftment of transplanted BMC is improved when hematopoietic cells are transferred together with the bone transplant (22, 23, 24, 25) or injection of stromal cells of the donor origin (26, 27). In 1999, the present inventors have shown that GVHD is suppressed if hematopoietic BMC are transferred within their own hematopoietic microenvironment (16). Allogeneic bone marrow was transplanted not in the usual form of a single cell suspension, where only hematopoietic stem cells are capable of engraftment, but in the form of an undisturbed bone marrow plug evacuated from the femoral marrow cavity of donor mouse. The donor bone marrow plug was placed under the kidney capsule of the recipient. In this case, which is hardly clinically applicable, mesenchimal cells present in the transplant in the form of undisturbed multi- cellular structures produce ectopic (extraskeletal) microenvironment for hematopoietic cells transplanted within the same bone marrow plug.

However, transplantation of bone osteoblasts or bone marrow plugs has not yet been widely accepted in clinical practice because of many technical limitations.

A major shortcoming for all current BMC transplantation procedures is their inability to simultaneously transfer both hematopoietic cells and stromal microenvironment supporting hematopoiesis. In BMC transplantation procedures currently used in hematological practice, in fact only hematopoietic cells from donor are engrafted in the recipient.

Based on the notion that BMC may provide a source for both hematopoietic cells and mesenchimal stem cells capable of induced osteogenesis, the inventors propose transplantation of a composition comprising BMC and DBM and/or MBM (materials stimulating and supporting osteogenic development of mesenchimal precursor cells), allowing for simultaneous development of hematopoietic and stromal tissues originating from donor BMC transplant.

More specifically, the composition of the present invention (comprising BMC and DBM and/or MBM) is transplanted directly into a bone marrow cavity of the recipient or extraskeletally. This results in *de novo* formation of bone structure, stromal microenvironment, supporting hematopoiesis, and hematopoietic tissue from the same transplanted BMC suspension, all in a one-step procedure.

The one-step BMC transplantation procedure using the composition of the present invention has some major advantages, *inter alia*:

1. Avoiding considerable non-specific loss of hematopoietic stem cells anchored in non-hematopoietic organs when injected intravenously.
2. Improving compatibility and contact between newly formed stromal microenvironment and hematopoietic cells originating from the same donor.
3. Replacing fatty, fibrotic or otherwise abnormal bone medullar spaces with active trabecular system supporting active multilineage hematopoiesis.
4. Feasibility in inducing mixed hematopoiesis with less graft v. host disease (GVHD) and host v. graft disease (HVGR) due to the better compatibility of immunocompetent cells of donor and recipient generated together on the stroma of donor- and recipient-type.

B. Induction of Cartilage Formation

Restoration of a healthy joint surface in a damaged or degenerative arthropathy requires addressing the treatment towards both directions, the cartilage and the subchondral bone.

Various attempts have been made to replace damaged cartilage. These include:

1. Stimulation of bone marrow from subchondral bone to form a fibrotic repair tissue

2. Osteochondral transplantation (allogeneic and autologous).
3. Transplantation of autologous cultured chondrocyte or mesenchimal cells.
4. Combined transplantation of chondrocytes with different kinds of matrices.
5. Artificial implantation of mechanical joints.

Autologous osteochondral graft is restricted to a small area of damaged cartilage, up to 2 cm^2, and could cause morbidity in the donor site. Allogeneic osteochondral graft is immunogenic, hence requires life-long use of undesired, hazardous immunosuppresive agents, which would be an impractical approach for routine orthopedic practice. Transplantation of cultured chondrocytes is cumbersome and most expensive, involving a two-stage procedure. The hyaline-like tissue which is produced after transplantation has suboptimal biomechanical properties (28-31).

Each of these methods has limitations and disadvantages and most of them are expensive and rather impractical. Hence, the goal of adequate restoration of cartilage remains an unsolved problem.

In theory, the most promising approach should involve combined transplantation of cells capable of hyaline cartilage formation and a matrix, providing means for induction/conduction and support of cartilage development and maintenance.

It is widely accepted that the basic requirements for successful application of combined cell-matrix graft are the following:

1. Rich source of progenitor cells capable of differentiation into chondrocytes for continuous repair of "ware and tear" of weight bearing joints.
2. Conductive scaffold for cell attachment should be maintained, leading to development of hyaline cartilage.

3. Conductive scaffold should be non-immunogenic, non-toxic and susceptible to biodegradation simultaneously with the development of new cartilage.
4. Conditions stimulating development of chondrocytes from mesenchymal precursor cells.

Most of the matrices that were tried in combined cell-matrix grafts were either immunogenic or non-biodegradable, the others did not possess conductive or inductive properties needed to support formation of biomechanically strong cartilage. Cells used in combined cell-matrix grafts were in most of the cases chondrocytes, that were already fully differentiated cells, with relatively low metabolic activity and limited self-renewal capacity. Whereas the proliferative capacity of such cells may be sufficient to maintain healthy cartilage, it is certainly insufficient for the development of large areas of hyaline cartilage *de novo*. In addition to being immunogenic, mesenchymal progenitor cell allografts were not combined with optimal supportive matrix. Unfortunately, none of the available modalities fulfill all basic requirements. Hence, in spite of extensive research, the results obtained until now with combined cell-matrix grafts are far from being satisfactory for reliable routine clinical application.

Furthermore, a few publications have related to the combination of BMC and DBM in the osteogenesis process, but not for the re-establishment of hematopoiesis. Thus, for example, Dohi et al. disclose bone and cartilage formation induced by the implantation, in diffusion chamber, of bone marrow cells and DBM in rats [Dohi, Y. et al. (1982) *Journal of Bone & Mineral Research*, **7(10)**: 1173-1180]; Connolly et al. disclose the use of bone marrow transplantation, in combination with DBM, in osteogenic stimulation [Connolly, J. F. (1995) *Clinical Orthopaedics & Related Research*, **313**: 8-18]; and Lindholm et al. refer to the use of BMC in combination with DBM in bone formation in rats [Lindholm et al. (1982) *Clinical Orthopaedics & Related Research*, **171**: 251-255].

The mixture of the invention that comprises BMC and DBM and/or MBM, overcomes the above shortcomings and provides a graft, transplantable into a mammal, which restores and/or enhances hematopoietic microenvironment and hematopoietic tissue originating from the mesenchymal and hematopoietic stem cells present within transplanted BMC.

Summary of the Invention

The present invention relates to a mixture comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM) for use as a graft transplantable into a mammal, for the development of hematopoietic environment originating from the transplantable BMC. Said BMC are allogeneic or said mammal's own.

Generally, said mammal is a human.

The transplantation of said mixture may be into an extraskeletal site, particularly an extraskeletal site is selected from the group consisting of the kidney capsule, muscles, liver and abdominal wall.

In other embodiments, the mixture of the invention is intended for transplantation into a bone marrow cavity.

The mixture of the invention may optionally further comprise bone morphogenic protein (BMP).

The DBM and/or MBM comprised in the mixture of the invention are preferably of vertebrate origin, more preferably mammalian origin, particularly of human origin.

The demineralized or mineralized bone matrix comprised in the mixture of the invention may be in powder form.

Preferably, the particle size of the DBM is about 50 to 2500μ. More preferably, the particle size of the DBM is about 250 to 500μ.

In a further embodiment, the ratio between BMC and DBM in the mixture of the invention is 10 parts of BMC concentrate to 0.5 part of DBM (volume/volume). Preferably, the ratio between BMC and DBM is 4 parts of BMC concentrate to 1 part of DBM (volume/volume).

The mixture of the invention is intended for use in the treatment of a patient suffering from a hematopoietic disorder. In particular, said hematopoietic disorder may be one of a deficiency of stem cells and/or their products, a genetic condition which results in abnormal stem cells and/or products, a hematopoietic disorder of malignant or non-malignant origin, congenital or hereditary bone disorders, bone infections, osteogenesis imperfecta, mucopolysaccharidosis, mucolipidosis and non-neoplastic disorders of the bone.

The invention further relates to the use of a mixture comprising BMC and DBM and/or MBM in the preparation of a composition for the treatment of a hematopoietic disorder, said composition further comprising a pharmaceutically compatible carrier and/or diluent. Optionally, said composition may further comprise BMP.

Said hematopoietic disorder is particularly characterized as being a deficiency of stem cells and/or their products, a genetic condition which results in abnormal stem cells and/or products, or a hematopoietic disorder of malignant origin.

Lastly, the present invention provides a kit for transplantation into a mammal of a mixture as defined in the invention, said kit comprising:

a. a mixture comprising bone marrow cells (BMC) and demineralized, or mineralized, bone matrix (DBM and/or MBM), said BMC being allogeneic or said mammal's own, said mixture further optionally comprising BMP;

b. a BM aspiration needle;

c. an intra-osseous bone drilling burr;

d. a needle with a thick lumen for infusion of viscous mixture;

e. a two-way lumen connector for simultaneous mixing of the mixture with the diluent;

f. a medium for maintaining BMC; and optionally

g. cryogenic means for handling and maintaining BMC or the mixture.

Description of the Figures

Figure 1 Photomicrograph of a kidney section 30 days after transplantation under the kidney capsule.

A: Transplantation of DBM only (Picroindigocarmin Histochemistry - 40X.

B: Transplantation of DBM + BMC (H & E) - 100X.

Notes: Non-degraded DBM particles are present at the site of transplantation (Fig. 1A). Profound degradation of DBM, newly formed bone trabecules and active multi-lineage hematopoiesis may be seen (Fig. 1B).

Figure 2 Photomicrograph of a kidney section 30 days following transplantation under the kidney capsule.

A: Transplantation of BMC only (Picroindigocarmin Histochemistry – 200X).

B: Transplantation of MBM + BMC (BMC (H & E) - 100X).

Notes: No development of bone or hematopoiesis. No transplanted cells at the site of transplantation (Fig. 2 A).

MBM particles at the different stages of degradation and remodeling, new bone trabecules and small areas of active multi-lineage hematopoiesis are seen (Fig. 2 B).

Figure 3 Longitudinal section of an ablated marrow cavity of a locally irradiated rat femur bone – 15 days post implantation (H&E – 100X).

A: Transplantation of DBM only.

B: Transplantation of DBM + BMC.

C: Transplantation of DBM + BMC + BMP.

Notes: Non-differentiated DBM, thin layers of new bone formation and small hematopoietic sites may be observed (Fig. 3A). Partial degradation of transplanted DBM particles, formation of new trabecular bone and several hematopoietic areas are seen (Fig. 3B). Developed bone trabecules and multiple areas of hematopoiesis are observed as well as profound degradation of transplanted DBM particles (Fig. 3C).

Figure 4 Longitudinal section of an ablated marrow cavity of a locally irradiated rat femur bone (H&E – 40X).

A: 15 Days after ablation of femur.

B: 15 Days after ablation of femur and intrafemoral transplantation of BMC.

Notes: No development of trabecular system or structurally normal hematopoietic tissue could be seen in two weeks after ablation of the marrow cavity followed by the local transplantation of BMC (Fig. 4B) or left empty (Fig. 4A).

Figure 5 Longitudinal section of an ablated marrow cavity of a locally irradiated rat femur bone, 25 days after direct transplantation of DBM together with BMC

A: 8X

B: Picroindigocarmin – 100X

C: H&E - 100X

Notes: Extensive development of trabecular system and active hematopoiesis are seen (Fig.5A-C).

Figure 6 Articular cartilage repair following abrasion arthroplasty in rat knee joint (femur).

A: Normal cartilage.

B: Abrasion: day 0

C: Abrasion covered with fibrin glue: day 30

D: Abrasion covered with DBM + BMC + Fibrin glue: day 30

Notes: Extensive development of articular (hyaline) cartilage one month after local transplantation of DBM-BMC mixture into abrased area of interchondylar region (Fig. 6D). In the control (non-transplanted) abrased area only a multilayer connective tissue could be seen (Fig. 6C). Healthy (undamaged) cartilage and damaged area immediately after abrasion are exposed in Figs 6A and 6B, respectively.

Abbreviations:

KC:	Kidney capsule
KP:	Kidney parenchyma
ST:	Site of transplantation
DA:	Area of defect
DBM:	Demineralized bone matrix
MBM:	Mineralized bone matrix
MBM-R:	Mineralized bone matrix remodeling
HT:	Hematopoietic tissue
BV:	Blood vessel
NC:	Normal cartilage
RC:	Regenerated cartilage
NB:	New bone
NBT:	New bone trabecules
CT:	Connective tissue
H&E:	Hematoxillin + Eosin

PIC: Picroindigocaramin

Detailed Description of the Invention

In search for improving BMC and bone transplantation procedures, the inventors have found that using a composition comprising BMC and DBM and/or MBM results in simultaneous development of hematopoietic and stromal tissues (bone, including the hematopoietic microenvironment, and cartilage), originating from the same donor.

Thus, and this is an object of the invention, a BMC suspension, in admixture with DMB and/or MBM, transplanted in a single-step procedure, may be used for treating disorders associated with the malformation, malignancy and/or dysfunction of hematopoiesis and bone marrow stromal microenvironment wherein the transplantation of both stromal and hematopoietic tissues is needed.

It is yet a further object of the present invention to provide the use of a mixture of BMC in admixture with DBM and/or MBM in the preparation of a pharmaceutical composition for transplantation of the BMC containing mesenchymal stem cells supported and stimulated by DBM and/or MBM into a cavity of bone marrow or at extraskeletal sites for formation of new bone with trabecular system and stromal microenvironment supporting hematopoiesis, originating also from the transplanted BMC.

It is yet a further object of the present invention to provide the said composition to improve the engraftment of hematopoietic precursor cells transferred within transplanted BMC for treatment of disorders associated with the malformation, malignancy and/or dysfunction of the bone marrow.

It is an additional object of the present invention to facilitate the BMT also in those cases in which BMT is used for non-hematological purposes, such as induction of the transplantation tolerance for organ transplantation and as a

platform for immunotherapy for donor lymphocyte infusions into the recipient.

The composition of the invention is also aimed at the induction of recipient transplantation tolerance to donor alloantigens, in the setting of allografts or xenografts, when said DBM or MBM is provided into patients bones, orthotopical organelles or in situ in the graft.

It is yet a further object of the present invention is to provide the said composition for transplantation of BMC into damaged joints for the replacement and/or restoration of hyaline cartilage as well as of subchondral bone, originating from the mesenchymal precursor cells existing in the transplanted BMC.

A further object of the present invention is to provide the use of a mixture of BMC in admixture with DBM and/or MBM in the preparation of a pharmaceutical composition for treating damaged or degenerative arthropathy in mammals, associated with malformation and/or dysfunction of the cartilage and/or subchondral bone. The invention may also be used to improve the efficiency of bone regeneration in damaged cranio-maxillo-facial areas, for therapeutic and cosmetic purposes.

The activity of the composition of the invention may be enhanced by the addition of BMP to the mixture comprising BMC and DBM and/or MBM.

DBM is an essential ingredient in the composition of the invention in view of its advantageous ability to combine all the features needed for making it an excellent carrier for mesenchymal progenitor cells.

1. DBM exhibits properties of conductive scaffolding essential for the engraftment of mesenchymal progenitor cells, their proliferation and differentiation in the course of bone and cartilage formation.

2. Further, DBM is the natural source of BMPs (bone morphogenic proteins) active in stimulating osteo- and chondrogenesis, thus fulfilling also the inductive function.
3. DBM is slowly biodegradable, the degradation time being compatible with the period of *de novo* chondro- and osteogenesis.
4. DBM has very low immunogenicity when used as a xenograft, and non-immunogenic at all, when used in allogeneic combinations.
5. DBM is flexible and strong enough to provide biomechanical properties of the joint surface during the period of new cartilage formation.
6. DBM can be provided as an amorphous powder that can be injected locally, without a major surgical intervention, thus avoiding iatrogenic damage to complex joints.

The first four specific features of DBM are of the equal importance for transplantation of the composition of the invention (BMC+DBM) into the bone marrow cavity, extraskeletally and into a damaged joint. The last two features of DBM are especially important for transplantation of the composition into the damaged joint.

(1) Transplantation into the marrow cavity or extraskeletally

The composition of the invention, that comprises BMC and DBM and/or MBM, has the major advantage of transplantation of donor multifunctional population of cells, directly into the marrow cavity of the recipient or extraskeletally, which provides for full development of a self-maintained osteo-hematopoietic complex of the donor origin, within the bone or in an extraskeletal site of the recipient. This new approach of the present invention will further facilitate replacing fatty, fibrotic or otherwise malfunctioning marrow stroma in human long bones (i.e. bones of extremities, with hard cortex and bone marrow cavity, unlike flat bones of skull or ribs. In human adults red (hematopoietic) bone marrow in long bones tends to be replaced for yellow (fatty) bone marrow) with active stromal microenvironment supporting long lasting hematopoiesis. It is

further expected that combined stromal and hematopoietic stem cells transplantation, using a one-step procedure, would expand the applicability of bone marrow transplantation (BMT) in clinical practice, particularly in improving or enhancing of hematopoiesis, inducing transplantation tolerance, etc.

(2) Transplantation into the joint

The idea underlying the procedure of the present invention is that bone marrow cells (BMC) may provide a source for mesenchymal stem cells, that are capable of inducing osteo- and chondrogenesis. Consequently, a new one-step transplantation procedure has been established in which a BMC suspension mixed with demineralized and/or mineralized bone matrix (DBM and/or MBM) powder is administered directly into a damaged joint of the recipient. Such a transplantation procedure, according to the present invention, provides for the formation of new subchondral bone structure and hyaline cartilage originating from mesenchymal cells present in the transplanted BMC.

In designing the experiments, the following approach and basic principles have been applied:

(a) Use of bone marrow cells

Bone marrow (BM) has been used as the source of multipotent mesenchymal stem cells capable of extensive proliferation and differentiation into cartilage, bone, tendon, muscle, fat, etc. (32-35). The inventors suggest that transplantation of multipotent mesenchymal stem cells, and not of differentiated chondrocytes, for remodeling and restoration of a healthy joint structure in arthropathy, is especially important for the following reasons:

(1) Chondrocytes are already fully differentiated cells, with relatively low metabolic activity and limited self-renewal capacity that may be sufficient to maintain healthy cartilage, but is certainly insufficient for the development of large areas of hyaline *cartilage de novo*.

(2) Most frequently, both cartilage and subchondral bone are damaged. Thus, even a successfully developed new hyaline cartilage is unlikely to be maintained for long if the subchondral bone is left damaged. Based on these findings, it was visualized that mesenchymal stem cells of bone marrow, if transplanted under the appropriate conditions, will create a self-supporting osteochondral complex providing healthy joint surface.

(b) Use of demineralized bone matrix (DBM)

It was found that DBM (and/or MBM) provides the optimal carrier in the cases of cartilage and adjacent bone regeneration, since it combines all the essential features for serving as conductive scaffolding, and at the same time provides a natural source for inducer of chondro- and osteogenesis. "Carrier" in this case means supportive material (structure) that is essential for promoting engraftment of mesenchimal progenitor cells and their proliferation and differentiation in the course of bone and cartilage development whenever mesenchimal cells are introduced as cell suspension. Moreover, DBM is mechanically flexible and slowly biodegradable, with the degradation time compatible with the period of *de novo* chondro- and osteogenesis. It is strong enough to provide biomechanical properties of the joint surface during the period of new cartilage formation. In addition, DBM can be provided as an amorphous powder that can be inserted locally, without major surgical intervention, while avoiding iatrogenic damage to complex joints. Furthermore, DBM is a low immunogenic material even when used as a xenograft and if used in allogeneic combination it is non-immunogenic at all (36, 37, 38).

(c) Use of bone morphogenic proteins (BMPs)

BMPs are growth factors that play an important role in the formation of bone and cartilage (39, 40). One of the major advantages of DBM is that it contains natural BMPs that resist the demineralization process. Induction of cartilage and subchondral bone may be enhanced by additional exogenous supply of BMPs that are not species-specific (41, 42), together with DBM

(43). What brings multi- potent mesenchimal stem cells under the influence of DBM to choose between osteogenic and chondrogenic differentiation pathways is not yet clear. It has however been reported that the ratio of cartilage to bone production depends in particular on the site of DBM implantation, which is naturally influenced by the local conditions (44). The most important of these conditions seem to be the local source of mesenchimal cells and the blood supply (45). Low oxygen tension favors chondrogenesis (46), most likely due to the low O_2 tension in poorly vascularized cartilage (47). Taking into consideration that hyaline cartilage is naturally developed and maintained only in the joints, where contact with synovial membranes and lubrication with synovial fluid is available and probably essential, it seems reasonable to assume that the environmental conditions in the joint play a major role in enhancing this type of chondrogenesis.

Lately, a successful substitution of anterior cruciate ligament (ACL) by demineralized cortical bone matrix was reported in a goat model. The remodeling process included new bone formation within the matrix in the osseous tunnels and a ligament-like transition zone developing at the extra-articular tunnel interface (48).

The inventors have shown for the first time that a graft composed of DBM and/or MBM and bone marrow cells transplanted into a joint, led to successful replacement of damaged cartilage and subchondral bone, by providing osteogenesis on the side of contact with bone and chondrogenesis on the free joint surface, according to the physiological environmental conditions favoring osteogenesis or chondrogenesis, respectively.

The procedure of applying the composition of the invention into either an extraskeletal site or into a BM cavity or an articulate joint, comprises the following steps:

1. Selecting the source for BMC. The donor may be of allogeneic type or the BMC may be obtained from the same treated subject (autologous transplantation).
2. Selecting the source of DMB and/or MBM. The DBM is supplied commercially and since it is not immunogenic, there are no limitations for a specific donor. DMB and/or MBM may be in a powder, granules or in a slice form.
3. Preparing a composition comprising a suspension of BMC, DBM and/or MBM, and if so desired BMP, which can be administered either by a surgical procedure, or by non-invasive injection. Alternatively, the composition may be administered so that it is encapsulated within normal tissue membranes. Still alternatively, the composition may be contained within a membranous device, made of a selective biocompatible membrane that allows cells, nutrients, cytokines and the like to penetrate the device, and at the same time retains the DBM and/or MBM particles within the device. Such a membranous device is preferably surgically introduced.
4. Providing a glue, preferably consisting of fibrinogen and thrombin, which is used for fixation of the implant composition at the site of implantation.

Throughout this application various publications are referred to by Arab numerals in parentheses. Full details of these publications are detailed in the List of References preceding the claims. The contents of these references are fully incorporated herein by reference.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention. Those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

Examples

Materials and methods

1. Animals

C57BL/6 male mice 8 weeks old and Lewis male rats with body weight 180-200g were used as the donors of bones (for matrix preparation) and BMC. Mice and rats from the same batches were used as the recipients.

2. Preparation of demineralized bone matrices (DBM)

Demineralized bone matrix (DBM) was prepared as described by Reddi and Huggins (45) with the inventors' modification. Diaphyseal cortical bone cylinders from Lewis rats were cleaned from bone marrow and surrounding soft tissues, crumbled and placed in a jar with magnetic stirring. Bone chips were rinsed in distilled water for 2-3 hrs; placed in absolute ethanol for 1 hr and in diethyl ether for 0.5 hr, then dried in a laminar flow, pulverized in a mortar with liquid nitrogen and sieved to select particles between 400 and 1,000 μ. The obtained powder was demineralized in 0.6M HCl overnight, washed for several times to remove the acid, dehydrated in absolute ethanol and diethyl ether and dried.

Mineralized bone matrix (MBM) was prepared according to the same procedure but the stage of demineralization with HCl was omitted.

Experiments with mice were performed using demineralized tooth matrix prepared from incisors dissected out from adult mice, as previously described (16). In case of rats and bigger animals the cortex of long bones is the appropriate material for preparation of DBM. In mice, however, only DBM prepared from dentine is active enough and produces reproducible results.

All steps, except the drying step, were performed at 4°C to prevent degradation of bone morphogenic proteins (BMP) by endogenous proteolytic enzymes. The matrices were stored at -20°C.

3. Preparation of the implanted material

Preparation of donor BMC suspensions for transplantation:

The femora of donor mice or rats were freed of muscle. Marrow plugs were mechanically pressed out of the femoral canal by a mandrin. Thick single cell suspensions of BMC were prepared after addition to 4-5 femoral plugs of 100 µl RPMI 1640 medium (Biological Industries, Beit Haemek, Israel). The term "thick" relates to a concentrated cell suspension. The number of nucleated cells per femoral bone marrow plug is rather stable (for C57BL/6 male, 8 weeks old, it is about 10^7 cells). Several reproducible verifications have shown that BMC prepared for transplantation in a form of a single cell suspension contains an approximate concentration of $3x10^8$ cells/ml.

Composition of the grafts:

Grafts were composed of the following ingredients in different combinations.

1. BMC suspension 20 µl (concentration $3x10^8$ cells/ml);
2. 4 mg of DBM (or MBM) were used in experiments with rats; 2mg in experiments with mice;
3. 0.5 µg BMP (R & D systems, USA) was added to the mixture in some of the experiments.

The exogenous BMP that is optionally added to the composition of the invention is not a mandatory ingredient. DBM exhibits conductive properties essential for the engraftment of mesenchimal progenitor cells transplanted within BMC suspension, their proliferation and differentiation in the course of bone and cartilage formation. At the same time, DBM is the natural source of BMPs (bone morphogenic proteins) active in stimulating osteo- and chondrogenesis, thus fulfilling also the inductive function. Addition of exogenous BMPs may enhance the efficiency of the induction.

4. Transplantation at a site under the kidney capsule

Anaesthetized rats or mice were used as recipients. A small cut was made in the renal capsule and the transplanted material was inserted using a concave spatula.

5. Local ablation of recipient's bone marrow in the medullar cavity of the bone

Rats were anaesthetized, and right leg was irradiated with 1,000cGy. Radiation was delivered by a Phillips X–ray unit (250 kV, 20 mA) at a rate of 70 cGy/min, using a Cu 0.2-mm filter. The source-to-skin distance was 40 cm.

After local irradiation, a small incision was made over the knee joint of the right leg. The medullar cavity of the femur was entered with a bone marrow aspiration needle and the bone marrow was evacuated. The remainders of the bone marrow were washed out with 3 ml of phosphate buffered saline (PBS) using a syringe with a 19-gauge needle.

6. Implantation of BMC or a mixture of BMC and demineralized (or mineralized) bone matrix into recipient's medullar cavity of the bone

BMC or their mixture with DBM or MBM were implanted into the bone cavity after local ablation of BM. Cells and bone matrix were placed in a

small micropipette tip, that was inserted into the hole in the bone, and transplanted material was pushed inside the bone cavity with a mandrin.

7. Implantation of BMC or a mixture of BMC and demineralized (or mineralized) bone matrix into the area of a local damage in articular cartilage of knee joint

Two kinds of standard artificial damages in articular cartilage and subchondral bone in the rat knee joints were induced as follows:

Following anesthesia, the knee joint was accessed by a medial parapatellar incision, patella temporarily displaced sidewards and:

1. Abrasion (partial thickness defect) was made in the interchondylar region of the femur. The abrased area was about 3x1.5 mm, i.e. approximately one-third of the joint surface.
2. Microfracture drilling (full thickness defect) 1.5 mm diameter x 2.0 mm depth was made in the interchondylar region of the femur.

Both kinds of the defects were filled with DBM (or MBM) in the form of powder (with particle size of 300-450 micron, or in the form of slice) together with BMC suspension, prepared as described in "Preparation of the implanted material" (Material and Methods). In the separate experimental groups only DBM (or MBM) or only BMC were transferred into the damaged area. Transplanted material was fixed in place with fibrinogen-thrombin tissue adhesive glue, patella was returned into its place and the incision was sutured with bioresorbable thread. Skin was closed with stainless clips. In control groups the damaged area was closed only with fibrinogen-thrombin tissue adhesive glue.

8. Histological evaluation

The autopsied material was fixed in 4% neutral buffered formaldehyde, decalcified, and passed through a series of ethanol grades and xylene, then embedded in paraffin. Serial sections (5-7 microns) were performed. One set

of representative serial section was stained with hematoxyllin & eosin, and the other with picroindigocarmin.

Example 1

Transplantation of BMC Together with Demineralized (or Mineralized) Bone Matrix into the Bone Marrow Cavity

A one-step transplantation procedure was established in which a composition of BMC and demineralized (or mineralized) bone matrix powder (DBM or MBM) was inserted into the bone marrow cavity for the formation of a trabecular bone, supportive hematopoietic microenvironment and hematopoiesis originating from mesenchimal and hematopoietic precursor cells present in transplanted bone marrow.

Experimental Data

In research activities that were involved in developing the composition of the invention, it was shown that mesenchimal stem cells persisting in bone marrow (BM) could be induced to form bone and supportive hematopoietic microenvironment when transplanted together with DBM into bone marrow cavity or extraskeletally. In two sets of experiments, one carried out in rats and the other in mice, DBM or MBM were implanted alone or together with BMC supplemented or not supplemented with additional bone morphogenic protein (BMP) (at least 12 transplants per group).

The space under the kidney capsule was selected as the site of transplantation as it has been previously shown that there are no cells which could be induced into osteogenesis and build a bone at least within 2-3 months (49).

In rats, one month after transplantation of DBM (or MBM) without BMC, no signs of new bone formation or hematopoiesis were observed. Implanted matrix particles were still present at the site of transplantation (Fig. 1A). In contrast, in sites where DBM was transplanted together with BMC, newly

formed ossicles with developed trabecular system and active multilineage hematopoiesis were observed (Fig. 1B). Profound degradation of transplanted matrix particles could be seen.

Addition of exogenous BMP (0.5μg/graft) to the graft consisting of DBM and BMC enhanced osteogenesis and development of stromal microenvironment supporting hematopoiesis. Transplantation of BMC alone did not result in development of hematopoietic or bone tissue (Fig. 2A).

Formation of new bone that supports hematopoiesis was also observed when MBM was implanted instead of DBM, together with BMC, under the kidney capsule. However, the bones were considerably less developed, less supported hematopoiesis, and the degradation of transplanted matrix was less prominent (Fig. 2B).

A similar set of experiments carried out in mice gave the same results, i.e. transplantation of DBM or BMC alone did not result in formation of a new bone or hematopoietic tissue under the kidney capsule. However, combined transplantation of DBM and BMC induced development of a new, functionally active osteo-hematopoietic site.

In another set of experiments, carried out in rats, DBM, together with BMC or with BMC and BMP, was implanted directly into the ablated femoral marrow cavity of the locally irradiated leg (at least 6 animals in each experimental group). Two weeks after the intrafemoral implantation of DBM without BMC, new bone development was scarcely observed (Fig. 3A). In contrast, when DBM was implanted together with BMC, newly formed bone trabecules and active hematopoietic tissue were observed (Fig. 3B) Osteo-hematopoietic complex of donor origin was almost fully developed in the recipient's bone marrow, 25 days after intra femoral transplantation of DBM together with BMC (Fig. 5). Addition of BMP (0.5μg/graft) to the graft consisting of DBM and BMC enhanced the development of

osteo-hematopoietic complex (Fig. 3C). When the ablated marrow cavity is left empty, or only BMC are transplanted into it, no development of trabecular system or structurally normal hematopoietic tissue is observed two weeks post- operation (Fig. 4).

Example 2

Transplantation of BMC into the joint together with demineralized (or mineralized) bone matrix

Experimental data

Experiments were carried out to prove that mesenchymal stem cells persisting in BM could be induced to develop hyaline cartilage and subchondral bone when transplanted into the damaged areas of the knee joints, according to the present invention.

In experiments carried out in rats, two kinds of artificial defects abrasion (partial thickness defect) and microfracture drilling (full thickness defect) in articular cartilage and subchondral bone in the interchondylar region of the femur were filled with DBM (or MBM) together with BMC. In the separate group of experimental animals defects in articular cartilage and subchondral bone were filled with DBM (or MBM) alone or only with BMC. The influence of addition of BMPs is also being tested. The implanted material was fixed in place with fibrinogen-thrombin tissue adhesive glue. In the control animals only glue was applied to the defect area.

Fig. 6A presents healthy undamaged articular cartilage in the interchondylar region of the femur in the knee joint of the rat. In Fig. 6B abrasion (partial thickness defect) could be seen immediately after damage.

One month after the operation, when the abrased surface was covered only with fibrinogen-thrombin glue the multilayer of connective tissue cells has been developed (Fig. 6C). In contrast, when DBM powder mixed with BMC

was transplanted into the damaged area, extensive development of hyaline (articular) cartilage is observed (Fig. 6D).

The findings presented herein, indicate that administration of the composition of the present invention into a damaged area of the joint results in generation of new hyaline cartilage and osteochondral complex at the site of transplantation.

Example 3

Transplantation of BMC together with demineralized (or mineralized) bone matrix into the experimentally created calvarial defect.

Experimental data

Experiments were carried out to prove that mesenchymal stem cells persisting in BM could increase the intramembranous development of bone when transplanted together with demineralized (or mineralized) bone matrix into the experimentally created calvarial defect. This can be extended to treat maxillo-facial defects.

Male Lewis rats were anesthetized by intramuscular injection of Ketamine as described previously. An incision was perfromed in the frontal region of the rat cranium. The muscular flap was moved aside and a bony defect (0.5-0.7mm/2) was made in the parietal bone area lateral to the saggital suture of 8-10 weeks old animals using the dental burr. The defect area was either left empty or filled with DBM alone or together with BMC. In the control group the defect was filled only with BMC. In all the groups the defect area was finally covered with fibrin glue. The muscle was returned to place and fixed with one or two biodegradable sutures. The skin was fixed with stainless steel clips and polydene was smeared all around the incised area of the skin. The regeneration process in the cranial defect area will be followed up radiologically and histologically 1,2 and 3 weeks post-transplantation.

Parts of the description that are out of ambit of the appended claims do not constitute part of the claimed invention.

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משרד המשפטים

מסמך זה הינו העתק שנסרק בשלמותו ביום ובשעה המצוינים,
בסריקה ממוחשבת מהימנה מהמסמך המצוי בתיק,
בהתאם לנוהל הבדיקות במשרד המשפטים.

על החתום

משרד המשפטים (חתימה מוסדית).

Claims:

1. A mixture comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM) for use as a graft transplantable into a mammal, for the development of hematopoietic microenvironment originating from the transplanted BMC.

2. The mixture of claim 1, wherein said BMC are allogeneic or said mammal's own.

3. The mixture according to claim 1, wherein the transplantation is into an extra-skeletal site of said mammal.

4. The mixture according to claim 3, wherein the extra-skeletal site is selected from the group consisting of the kidney capsule, muscles, liver, abdominal wall.

5. The mixture according to any one of claims 1 or 2, wherein the transplantation is into a bone marrow cavity.

6. The mixture according to any one of claims 1 to 5, further comprising a bone morphogenetic protein (BMP).

7. The mixture according to any one of claims 1 to 6, wherein the DBM and/or MBM are of vertebrate origin.

8. The mixture according to claim 7, wherein the DBM and/or MBM is of human origin.

9. The mixture according to any one of claims 1 to 8 wherein the DBM and/or MBM are in powder form.

10. The mixture according to any one of claims 1 to 9, wherein the particle size of the DBM is about 50 to 2500μ.

11. The mixture according to claim 10, wherein the particle size of the DBM is about 250 to 500μ.

12. The mixture according to any one of claims 1 to 11, wherein the ratio between BMC and DBM is 10 parts of BMC concentrate to 0.5 part of DBM (volume/volume).

13. The mixture according to any one of claim 12, wherein the ratio between BMC and DBM is 4 parts of BMC concentrate to 1 part of DBM (volume/volume).

14. The mixture according to any one of claims 1 to 13, wherein said mammal is a human.

15. The mixture according to any one of the preceding claims, for use in the treatment of a patient suffering from a hematopoietic disorder.

16. The mixture according to claim 15, wherein said hematopoietic disorder is selected from a deficiency of stem cells and/or their products, a genetic condition which results in abnormal stem cells and/or products, a hematopoietic disorder of malignant or non-malignant origin, congenital or hereditary bone disorders, bone infections, osteogenesis imperfecta, mucopolysacchaidosis, mucolipidosis and non-neoplastic disorders of the bone.

17. Use of the mixture of any one of claims 1-16 in the preparation of a composition for the treatment of a hematopoietic disorder, said composition further comprising a pharmaceutically compatible carrier and/or diluent.

18. The use according to claim 17, wherein said mixture further optionally includes a BMP.

19. The use according to claim 18, wherein said hematopoietic disorder is selected from a deficiency of stem cells and/or their products, a genetic condition which results in abnormal stem cells and/or products, and a hematopoietic disorder of malignant origin.

20. Use of the mixture of any one of claims 1-16 in the preparation of a composition for the induction of recipient transplantation tolerance to donor alloantigens.

21. A kit for transplantation into a mammal of a mixture as defined in any one of claims 1-16, comprising:

a. the mixture as defined in any one of claims 1-16;
b. a BM aspiration needle;
c. an intra-osseous bone drilling burr;
d. a needle with a thick lumen for infusion of viscous mixture;
e. a two-way lumen connector for simultaneous mixing of the mixture with the diluent
f. a medium for maintaining BMC; and optionally
g. cryogenic means for handling and maintaining BMC or the mixture.

משרד המשפטים

מסמך זה הינו העתק שנסרק בשלמותו ביום ובשעה המצוינים,
בסריקה ממוחשבת מהימנה מהמסמך המצוי בתיק,
בהתאם לנוהל הבדיקות במשרד המשפטים.

על החתום

משרד המשפטים (חתימה מוסדית).

A
B
DBM
DBM
NBT
HT
DBM Only
Picroindigocarmin
istochemistry-40X
DBM + BMC
H & E Histochemistry-100X

Fig. 1

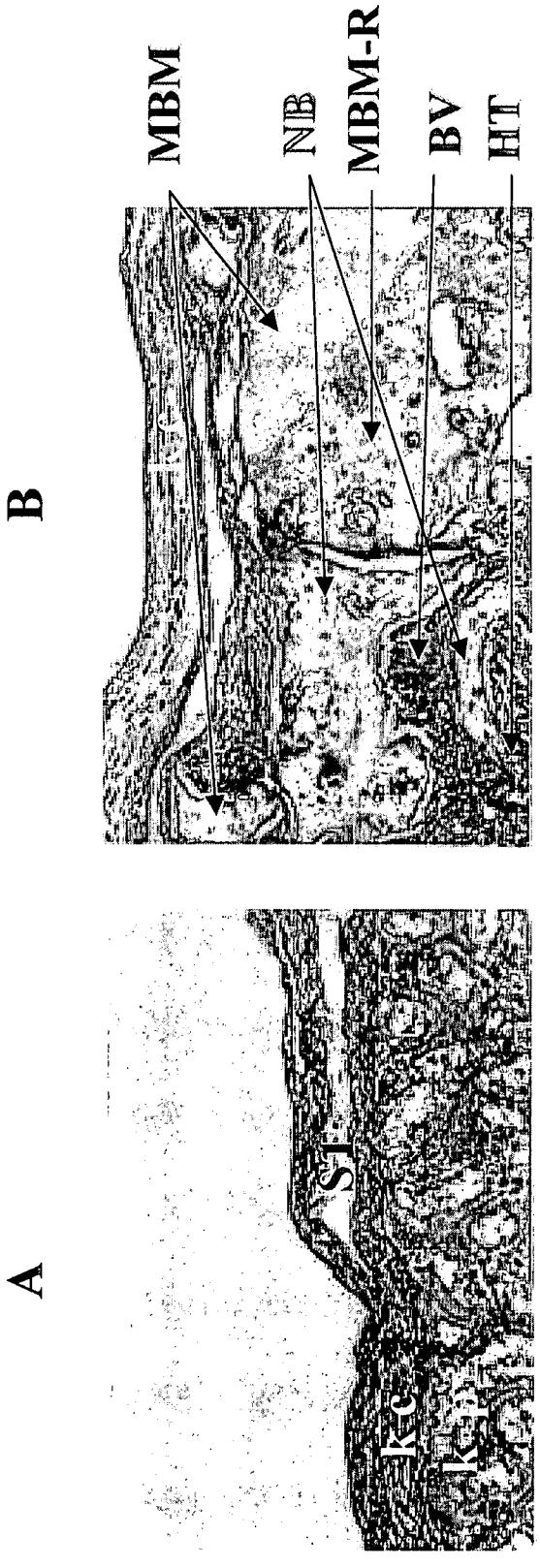

A
B
MBM
NB
MBM-R
BV
HT
SJ
K-c
K
BM C Only
MBM + BMC
Picroindigocarmin
Histochemistry-200X
H & E Histochemistry-100X

Fig. 2

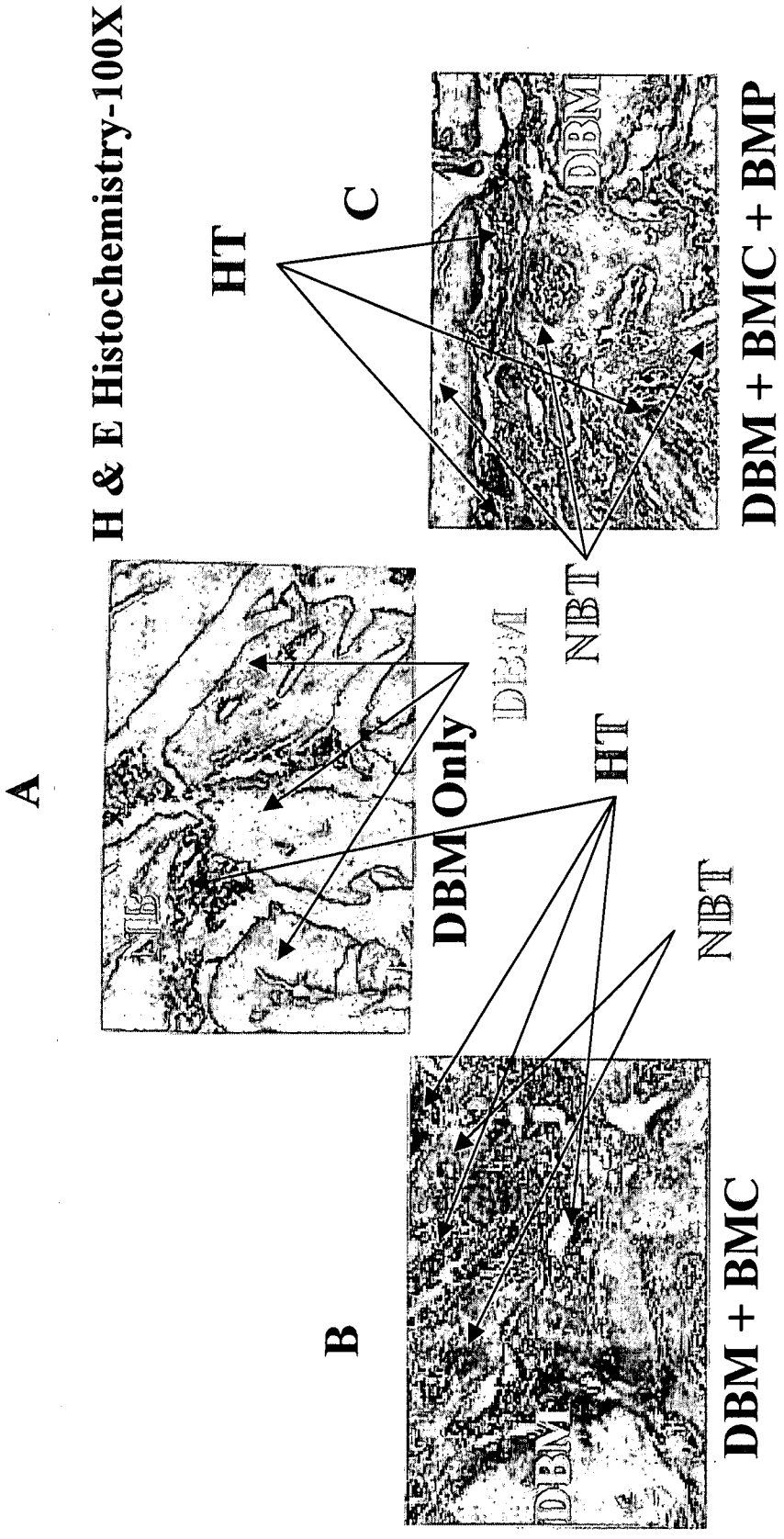

H & E Histochemistry-100X
A
DBM Only
HT
NBT
B
DBM + BMC
C
DBM + BMC + BMP

Fig. 3

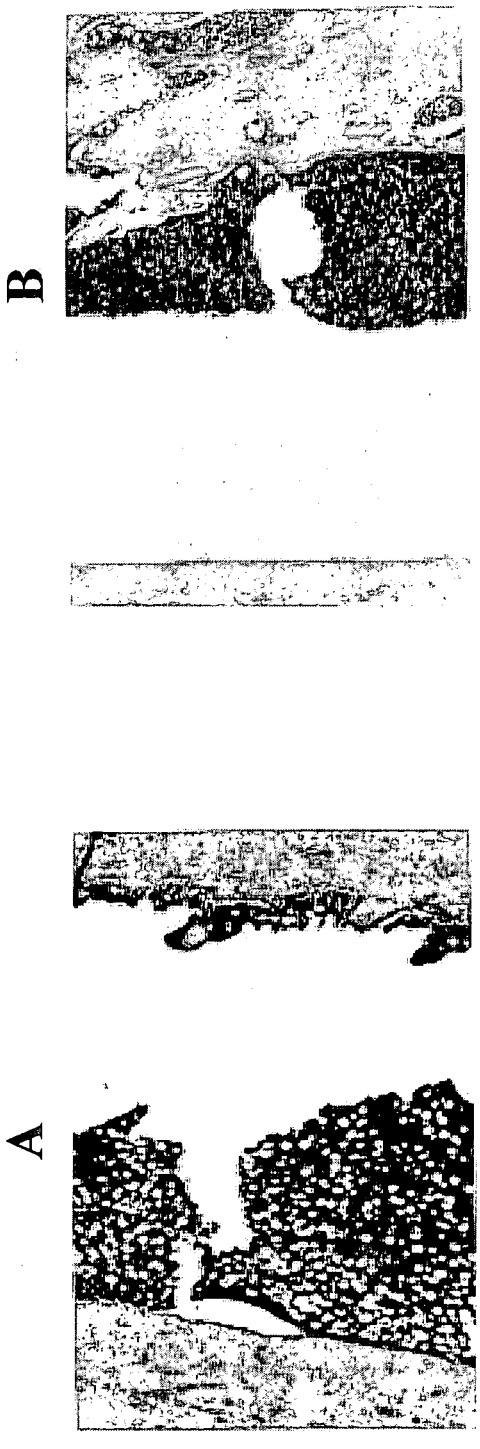

A
B
15 Days after ablation
of femur.
15 Days after ablation
of femur and intrafemural
transplatation of BMC.
H & E Histochemistry-40X

Fig. 4

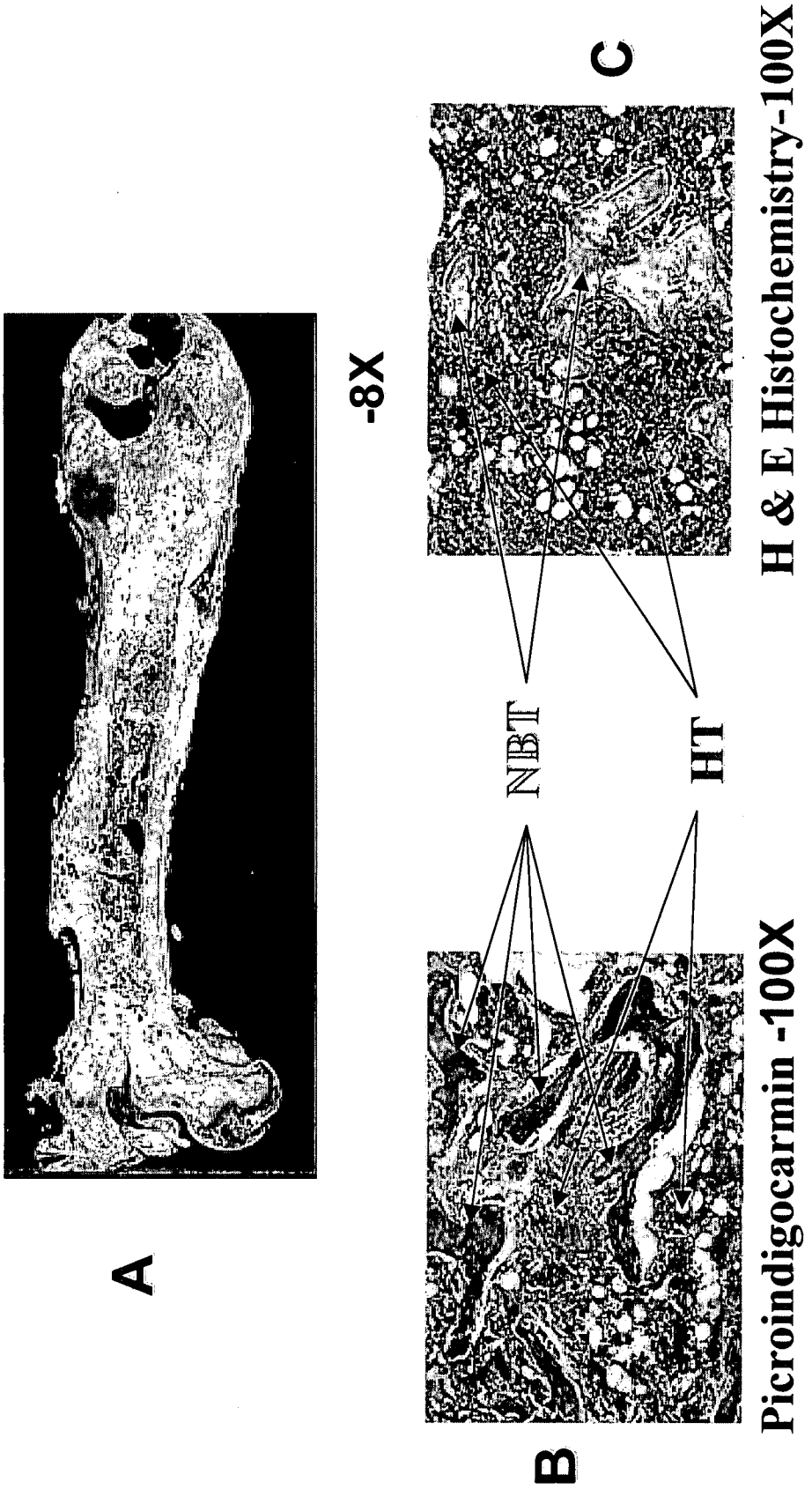
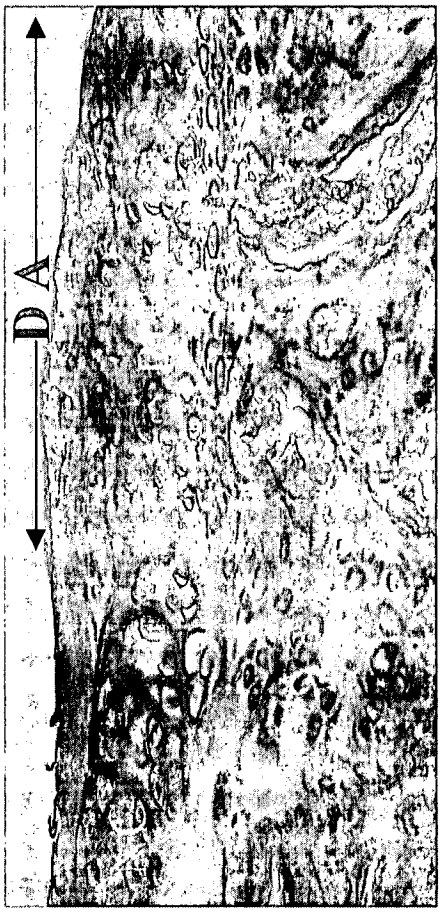
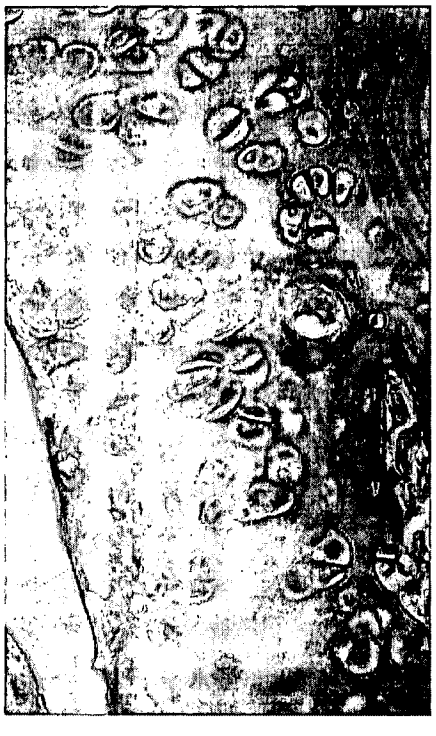

A
-8X
B
NBT
HT
Picroindigocarmin -100X
C
H & E Histochemistry-100X

Fig. 5

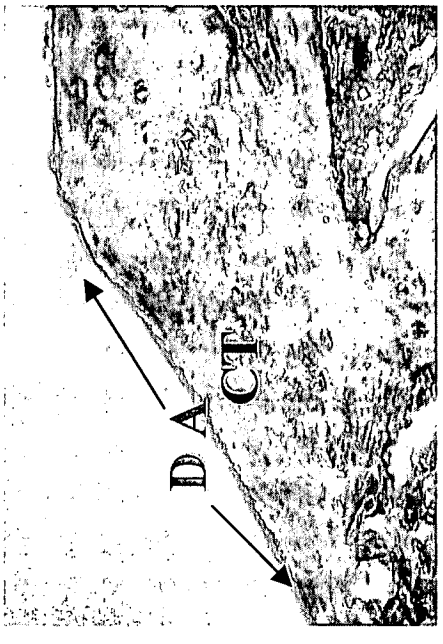
Abrasion Day-0



Abrasion Covered With DBM + BMC + Fibrin Glue Day-30



Normal Cartilage



Abrasion Covered with Fibrin Glue Day-30

Picroindigocarmin -200X

Fig. 6

משרד המשפטים

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בהתאם לנוהל הבדיקות במשרד המשפטים.

על החתום

משרד המשפטים (חתימה מוסדית).