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ORIGINAL PAPER

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Treatment of segmental bone defects in rats by the stimulation of bone marrow osteo-progenitor cells with prostaglandin E_2

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Abstract An alternative to bone grafting is engineered osteo-conductive material that carries osteo-progenitor cells with osteo-stimulant factors impregnated on a malleable osteo-conductive material. We used bone marrow stem cells as the source of osteo-progenitor cells and stimulated them with prostaglandin E_2 using demineralised bone matrix as a carrier. We treated 35 skeletally mature male Wistar albino rats with segmentary radial bone defects using five different treatment groups. Group I received no treatment; the remaining four groups all received a mixture of bone marrow and demineralised bone matrix. In group III, a copolymer was added. In group IV, prostaglandin E_2 and in group V prostaglandin E_2 within a copolymer was added to the mixture. Eight weeks after the surgical procedure, the rats were sacrificed. Radiological and histological evaluation of the radial bone showed that while there was no significant healing in groups I, II and III, there was a significant healing response in groups IV and V.

Résumé Une alternative à la greffe osseuse est un matériel ostéoconducteur portant des cellules précurseurs avec des facteurs ostéostimulant imprégnés dans un matériel malléable ostéoconducteur. Nous avons utilisé des cellules de la moelle osseuse comme source de cellules précurseurs et les avons stimulés avec une prostaglandine E2 qui utilise la matrice osseuse déminéralisée comme porteur. Nous avons traité 35 rats Wistar albinos viril à maturation osseuse, avec un défaut segmentaire du radius, en utilisant cinq groupes de traitements différents. Le groupe I n'a reçu aucun traitement. Les quatre autres groupes ont reçu un mélange de moelle osseuse et une matrice d'os déminéralisé. Dans le groupe III a été ajouté un copolymère. Dans le groupe IV, une prostaglandine E2 et dans le groupe V, une prostaglandine E2 et un copolymère ont été ajouté au mélange. Huit semaines après la procédure chirurgicale, les rats ont été tués. L'évaluation radiologique et histologique de l'os radial a montré qu'il n'y avait aucune guérison notable dans les groupes I, II et III, mais une réponse curative appréciable dans les groupes IV et V.

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Introduction

Autogenous and allogenous bone grafts are widely employed in the treatment of bone defects and non-unions [5]. However, they have certain limitations, and there may be problems associated with their use [1, 3, 10]. An alternative to bone grafting would be to design an osteo-conductive material that carries osteo-progenitor cells. These cells would have to have adequate osteo-stimulant factors and would need to be based on an osteo-conductive material, which itself would be helpful in filling defects.

Bone marrow (BM) cells have the potential to differentiate into bone, cartilage, tendon, muscle and fat tissue both in vivo and in vitro. The osteogenic capacity of BM was first shown in 1869 by Goujon [22]. Since then, it has been used in many studies related to the stimulation of bone healing. The use of BM alone or in combination with a demineralised bone matrix (DBM) can stimulate bone healing [5, 16, 17, 22], but although BM contains progenitor cells, previous observations have indicated that small implants of fresh whole marrow often fail to support bone formation [12].

Bone tissue is rich in prostaglandins, which are usually secreted from the osteoblastic cell line, and prostaglandin E_2 (PGE_2) is the most potent prostaglandin in this respect. The anabolic effects of prostaglandins on bone tissue have been demonstrated in several in vitro and in vivo studies. PGE_2 helps new bone formation and an increase of bone mass by stimulating stem cell differentiation into an osteoblastic cell line, mostly of BM origin [9, 14, 17, 24]. In our study, we used BM stem cells as a source of osteoprogenitor cells in the treatment of segmental bone defects of the rat radius, and we tried to stimulate these cells with PGE_2 using a DBM as the carrier.

Materials and methods

Bone marrow from the assigned group of seven rats was obtained by sacrificing each of these animals with a lethal dose of phenobarbital. The tibias and femurs of both lower limbs were then removed and crushed with sterile pliers [4]. Commercially available human Regenafil (Regeneration Technologies Inc., Alachua, FL, USA) was used as a source of DBM and PGE_2, and this was obtained from Acros Organics (Geel, Belgium, CAS Number 363-24-6).

The copolymer was synthesised from D,L-lactide (Boehringer, Ingelheim, Germany) and ε-caprolactone (Acros) using the method described by the Hacettepe University group [23]. FTIR and ^{1}H-NMR data confirmed the copolymer formation. The actual ratio of D,L-lactide to ε-caprolactone was 88:12 calculated from the ^{1}H-NMR spectrum, which was recorded with an NMR spectrophotometer (Varian, XL-300, USA) at 399.96 MHz and at a constant 18°C temperature (obtained by ^{1}H-NMR). The number and average molecular weights and polydispersity index obtained by a gel permeation chromatography system (Spectra-Physics, P1500, USA) were 112,000, 150,000 and 1.34, respectively. The glass transition and the melting temperatures of the copolymer obtained by a differential scanning calorimeter (Mettler, Model DSC30, Germany) were, respectively, 32.5 and 161.9°C.

Animal studies

With the ethical approval of the Gazi University Research Committee, 42 skeletally mature male Wistar albino rats (aged 6 months and weighing 250–300 g), all obtained from the same source, were used in this study in order to decrease genetic variability. Bilateral radial osteotomies were performed surgically in 35 of these rats. The remaining seven were sacrificed and used as a source of BM cells. The 35 rats were put into five treatment groups with seven in each group. Group I was the control group, and the rats in this group received no treatment. The segmental defects of the rats in group II were treated with the DBM and BM mixture. In group III, the defects were treated with a combination of DBM, BM and the copolymer. Group IV rats received DBM, BM and PGE_2, and group V rats were treated with DBM, BM and PGE_2 in copolymer.

For group II, 0.1 ml BM was applied to 0.1 ml DBM and allowed to 'soak in', the DBM acting like small sponges. The prepared mixture was then used to fill the segmental defect. To keep the BM aspirate fresh, only small amounts of the mixture were prepared at a time. For group III, the BM and DBM mixture was combined in equal parts with copolymer. For group IV, 10^{-7} M PGE_2 was first dissolved in acetone (Sigma, USA), and then this solution was applied to 1 ml DBM. The acetone was allowed to evaporate, and BM was later applied to this mixture as described above. For group V, 2×10^{-5} M PGE_2 was dissolved in 2 ml acetone and then mixed with copolymer. The acetone was allowed to evaporate, and the resulting mixture was then added to the BM and DBM mixture and used as in group IV to fill the segmental defects.

Surgical procedure

The rats were anaesthetised with an intra-muscular (IM) injection of ketamine (100 mg/kg), and an approximate 1-cm bone defect was created in the radius of both 'forearms' [25]. This osteotomy site was then irrigated with 0.9% saline, but no attempt was made to resect the periosteum around the osteotomy site as this had retracted with the overlying muscles. The osteotomy site was then treated following the agreed protocol for each group. As only the radius had been 'osteotomised', no fixation was employed, and the animals used both extremities effectively. Only one rat died during the period of the experiment (within the first 24 h after the surgery), and this was probably due to the anaesthesia. The general health of the rats was monitored daily [8, 11, 19–21]. After the surgery, X-rays of the relevant limbs were taken while the rats were still anaesthetised, and the rats were observed until they recovered.

Eight weeks after the surgical procedure, the rats were sacrificed with an intra-peritoneal injection of pentobarbital (200 mg/kg) [2, 25]. After taking the follow-up A/P X-rays of both forelimbs, they were placed in a container filled with 10% formalin solution and then stored for histological examination. The numerical score grading scale described by Cook et al. was used for the radiological assessment of the forelimbs [6, 15].

To allow histological examination, the harvested tissues were decalcified with 10% formic acid solution with this solution being changed every day for a week. They were next embedded in paraffin, and 5-μm-thick sections were cut through the long axis and stained with hematoxylin and eosin [13]. During the histological examination, the pathologist was unaware to which group each specimen belonged.

Data obtained with the radiological grading scale was analysed with SPSS 10.0 software for Windows with the paired sample test and the Wilcoxon non-parametric two-related sample test.

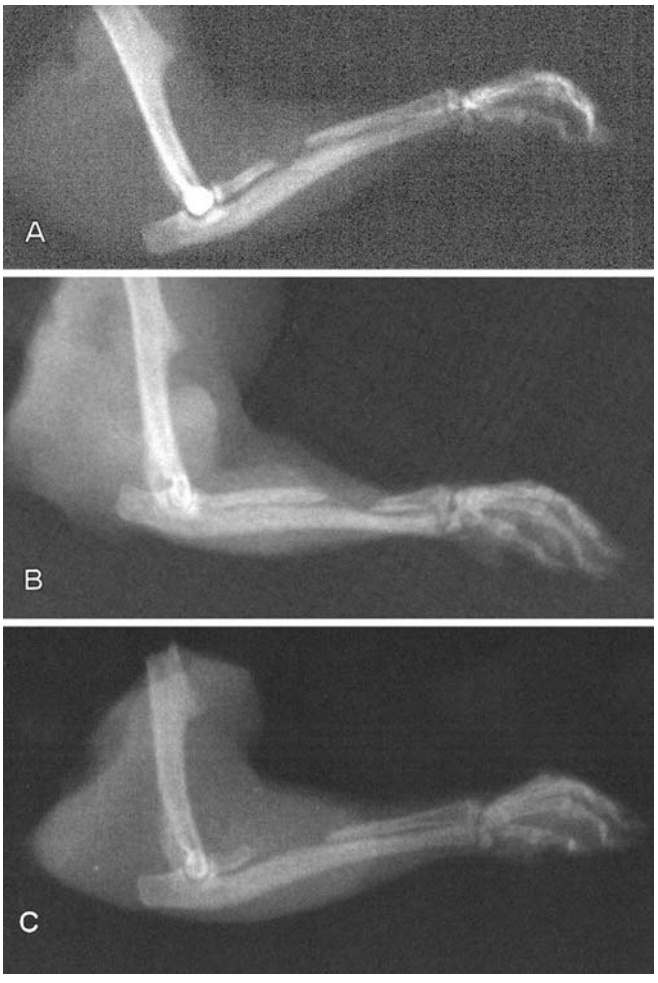

Fig. 1 Radiographs of radii in group I (**A**), group II (**B**) and group III (**C**).

Results

Radiography revealed that there was no significant bone healing in groups I, II and III (Fig. 1). In group IV, the defect in one limb had healed completely while the defects in the six other limbs in this group showed only varied degrees of healing. In group V, there were also varied degrees of radiological healing of the defects (Fig. 2). The mean numerical radiological scores obtained for groups I, II, III, IV and V were: 0.18, 1.4, 0.9, 3.30 and 2.72, respectively, with the values ranging from 1 (0–1), 2 (0–3), 2 (0–3), 4 (6–2) and 4 (5–1), respectively, for all groups. While there was a statistically significant difference between group I ($p<0.05$) and the other groups, there was no difference between group II and group III ($p=0.217$). However, there was a significant difference between group IV and groups II and III ($p<0.05$) but no significant difference between group IV and group V ($p=0.548$).

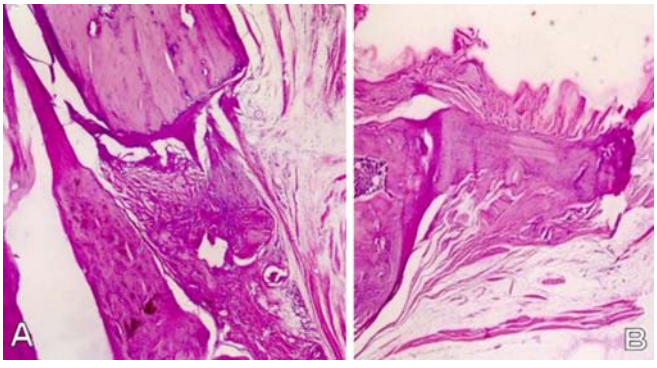

Fig. 3 Osteotomy site in control group I (**A**), and healing response in group II (**B**).

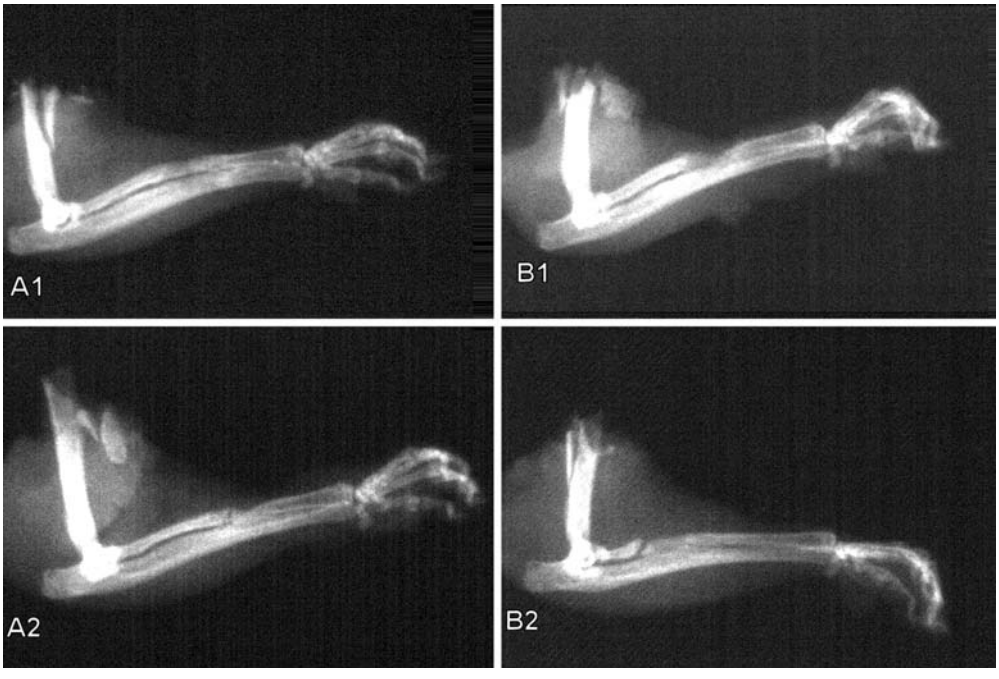

Fig. 2 Radiographs of radius in group IV (**A**) and group V (**B**).

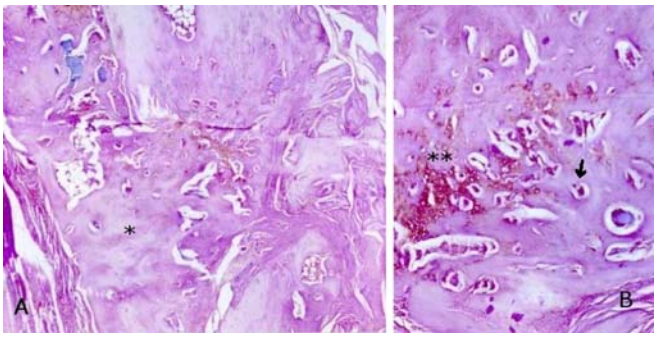

Fig. 4 Prominent increased osteoblastic activity in group IV, osteoblasts (*black arrow*), lacuna formation and enchondral bone formation (**) under high magnification.

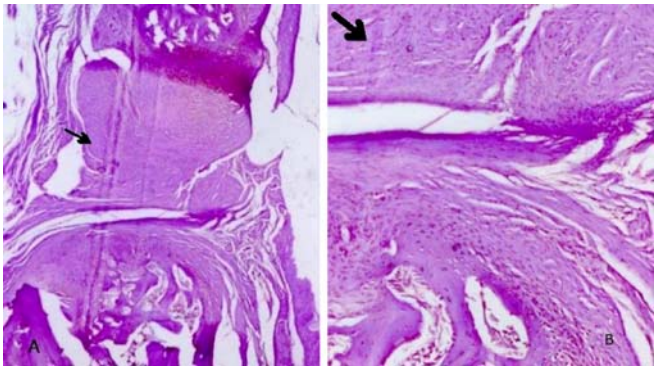

Fig. 5 Pseudo-joint formation with fibrotic bone healing in group V.

Histologically, there were inflammatory changes with increased connective and soft tissue interposition in group I. In group II, soft tissue interposition and an increase in connective tissue elements were the dominant findings (Fig. 3). Fibrous tissue changes were predominant in group III. In group IV, callus formation, pseudo-joint formation, fibro-cartilaginous bone healing and mineralised areas were observed (Fig. 4). The limbs in group V had areas with osteoid and cartilage formation, although the structure of the lamellar bone formation areas was defective, being dominated by fibrous bone healing (Fig. 5).

Discussion

Prostaglandins are biologically active substances, and they are already being used in various medical conditions. Prostaglandins are recognised to be biologically active via four known receptor types: EP1, EP2, EP3 and EP4 [18, 24]. As potent modulators of bone metabolism, prostaglandins have a multi-functional regulatory action. They exert both stimulatory and inhibitory effects. This depends on the receptor type on which they act and on the type of prostaglandin. Prostaglandins are mainly produced by osteoblasts and are found at fracture sites. Application of prostaglandins is known to increase fracture healing.

Stimulating effects of PGE_2 on osteoblastic differentiation is optimal at 100 nM, and this effect is via EP4 receptors [24]. Among all the known PGE_2 analogues, only PGE_2 (dinoprost) is active on EP4 receptors [18]. The presence of PGE_2 on a medium during the first 21 days has been shown to increase the effect of PGE_2 on osteoblastic differentiation [9]. Weinreb et al. [24] reported that PGE_2 exerts its action mainly during the first 24 h, and its application for 21 days does not increase its effects.

According to our in vivo model data, the application of PGE_2 at 10^{-7} M dose to a fracture site significantly stimulates osteoblastic activity either alone or in a sustained-release system. However, the combination of PGE_2 with copolymer induces some fibrotic changes at the site of application. This finding agrees with that of previous studies [7].

The lack of healing in group I allows its use as a control group in studies on segmental bone defects. The absence of any differences in healing response in groups II and III demonstrates that copolymer has no significant stimulating effect. In addition, no copolymer was detected in histological sections at the end of the eighth week. Thus, it seems probable that the degradation products (the oligomers) were thoroughly distributed within the neighbouring tissues [7]. There was no foreign body reaction detected in the histological sections of group III and group V. The callus formation, fibro-cartilaginous bone healing and mineralisation areas observed in group IV agree with the published results of previous tissue culture studies [9, 24].

Our attempts to show whether the application of PGE_2 to osteo-progenitor cells during the first 21 days augments the response to PGE_2 could not be established owing to the observed fibrotic changes in group V. However, it is obvious that the presence of PGE_2 at a fracture site during the first few days of treatment does significantly stimulate osteoblastic activity.

Although there were no problems related to the use of rats as experimental models, the development of pseudarthroses prevented us from performing any biomechanical tests at the end of our study. This suggests that experiments that include biomechanical testing should be made using animals in which more rigid fixation can be achieved. The application of osteo-stimulant materials to fracture sites via carrier molecules raises the question of any inhibitory effects of these materials, and from our experience, we suggest the use of more biologically inert materials, such as starch or natural matrices in future.

The osteogenic capacity of BM cells and the emerging effects of PGE_2 on early osteoblast differentiation suggests their use in engineered bone graft substitutes, possibly combining them with expensive recombinant DNA technology products.

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