Acta Veterinaria (Beograd), Vol. 51. No. 4, 299-310, 2001.

UDK 619:612.751.3

EVALUATION OF COMPOSITE COLLAGEN/HYDROXY-APATITE IMPLANTATION AND NERVE GROWTH FACTOR (NGF) DELIVERY ON NEW BONE INGROWTH

LETIĆ-GAVRILOVIĆ ANKA*, FINI MILENA**,GIAVARESI G **, GIARDINO R **, ABE K ***

*Department of Biochemistry, Faculty of Stomatology, University of Beograd, Beograd, Yugoslavia
**Department of Experimental Surgery, Orthopedic Institute Rizzoli, Bologna, Italy ***Department of Oral Biochemistry, Fukuoka Dental College, Fukuoka, Japan

(Received 5. July 2001)

In this study, a bio-artificial bioresorbable composite consisting of a polymer of collagen/hydroxy-apatite (Col/Hap) enriched with neurotropin - nerve growth factor (NGF), was implanted into the femurs of 73 male Wistar rats, weighing 100-125 g. Implants were left in place for different times. Controls were as follows: a) contralateral femur without any implants; and b) contralateral femur implanted with composite without NGF factor. The rats were euthanized after 1, 10 and 30 days and the implant sites and explants were examined clinically, histologically, by scanning electron microscopy and histomorphometrically.

The results show stimulated periosteal and endocortical woven and lamellar bone formation, which yielded increases in bone mass and decreases in bone marrow. The NGF treatment had greater effects in the femur shafts of older male rats than in younger ones. Additionally, we found that NGF increased remodeling activity in the intracortical region and induced an increase of intracortical cavity number and area by the end of the study. Since no similar studies appear to have been carried out, it is difficult to correlate our findings with other studies where neurogenic factors are delivered by complex polymer composites. We believe that this proposed system has great advantages in tissue engineering and is very suitable as a biomaterial for filling irregular defects in orthopaedic and maxillo-facial surgery, bone replacement and fixation, and as a drug delivery device.

Key words: bone ingrowth, collagen/hydroxy apatite implants, nerve growth factor (NGF)

INTRODUCTION

An essential requirement for the reconstruction of skeletal and connective tissue in tissue engineering is the development of appropriate local and systemic conditions in the tissue to be reconstructed, together with improving the chemical and physical properties of the biomaterial to be implanted.

Development of hybrid composites (copolymers, complexes, hydrogels, blends, etc.) (Bakos *et al.*,1999; Du *et al.*,1998;Kataoka *et al.*, 2001) based on natural and synthetic extracellular bone matrix macromolecules and their open wide spectrum of applications in biomaterials science, has received tremendous attention in bone reconstruction. Furthermorre, various polymer-composites devices have been studied for the sustained release of biologically active molecules (Kataoka *et al.*, 2001;Ripamonti *et al.*,1997;Reddi *et al.*,1998) with a wide range of activities very important for bone development, growth and repair (Lind, 1998;1998a; Kundu *et al.*,1999). Recently, tissue engineering has shown great promise for creating bone alternatives to address and improve the problem of bone deficiency using complex composite implants such as calcium phosphates (CaP) hybridized with natural polymers such as collagen (Horisaka *et al.*,1994), and enriched with osteogenic or other growth factors (Gao *et al.*,1997).

Calcium phosphates (CaP) in different forms, and particularly low-density hydroxyapatite (Hap) together with quickly resorbable -tricalcium phosphate, are generally considered to the materials of choice as bone substitutes (Du *et al.*,1998; Gross *et al.*,1998). While these ceramics have good osteoconductive properties for bone replacement, they are limited by their inherent stiffness, brittleness and low fatigue properties relative to bone mechanics and, because of that, they generally are not sufficiently suitable for bone remodelling (Esposito *et al.*,1998). Hap with its highly interconnected porosity invites in growth of bone into the implant, leading to a more securely fixed and osseointegrated bone implant.

Recently, in order to improve both the mechanical properties of CaP ceramics and also to gain more controllable way of bone growth factor delivery, bioresorbable polymers have been proposed for creating complex nonisotropic composite systems to be used for bone reconstruction (Shikinami and Okuno,1999; Pietzak et a.,1996). Accordingly, bone reconstruction should follow resorption of the polymer and new bone should nucleate all around each grain of the ceramics. Such composites are advocated as carriers for bone growth factors or other biologically active molecules, which could then be released inside the body and have an effect on bone tissue healing (Lind,1998; Kundu *et al.*,1999;Kreuter,1999). Composites, that have been used in the past as carriers for biologically active molecules have met with mixed success (Gross *et al.*,1998; Kundu *et al.*,1999; Yao *et al.*,1999). These carriers, for the most part, release 80-90% of their loaded molecules in the first few hours of immersion into aqueous media, thus making them inadequate for long-term delivery of these biomolecules. This was an additional reason for creating more complex ceramic/ natural polymer composites, with the hope that they will be more useful for growth factor delivery.

Many bone-related biologically active molecules, such as growth factors (Lind,1998;1998; Letic-Gavrilovic *et al.*,2000) are osteoinductive and can induce bone formation starting with early stage mesenchymal cells. Under the influence of diverse biomolecules - nondifferentiated mesenchymal cells may differentiate into early stages of osteoblasts and subsequently into committed and mature osteoblasts. Examples of growth factors having effects on bone and cementum remodelling include: platelet-derived growth factor (PDGF) (Park *et al.*,1998), transforming growth factor (TGF-), acidic and basic fibroblast growth factors (a- and b-FGF), insulin-like growth factors I and II (IGF-I and II), cementum-derived growth factor (CGF) and the bone morphogenetic proteins (BMPs) (Reddi,1998; Ripamonti and Reddi, 1997). The effects of these factors on the process of

osseointegration of the implants are not fully understood, but most researchers agree that the contact between the bioactive surface layer of the implant and the bone is not static but dynamic, and the growth factors play a great role in the process of osseointegration.

The interplay between hard tissue and nerve tissues has been documented at the molecular level by many investigators (Auffray *et al.*,1996; Bjurhalm *et al.*,1988; Sandhu *et al.*,1987; Yada *et al.*,1994; Lerner,1994;1997; Kundu *et al.*,1999), who found neuropeptide containing fibres in bone and functional receptors on bone cells. A neuro-osteogenic "interaction" was accordingly proposed by several authors (Aloe *et al.*,1997; Yada *et al.*,1994; Yanker and Shooter,1982; Lundberg *et al.*,1999; 2001). Nerve growth factor (NGF) is an endogenously-produced neurotrophic factor which plays a crucial role in growth, differentiation, survival and function of neurons in the peripheral and central nervous systems (CNS) (Aloe *et al.*,1999). Recent studies indicate that NGF promotes recovery of several neurological deficits and stimulates wound healing in cutaneous tissues. These NGF effects are mediated by two well-characterized trans-membrane glycoproteins, the high-affinity (trkA) receptor tyrosine kinase and the low-affinity (p75) receptor. There is also consistent emerging evidence indicating that NGF, either alone or synergistically with other biological endogenous mediators, plays a crucial role in cartilagineous and bone tissues (Yada *et al.*,1994; Auffray *et al.*,1996; Bjurhalm *et al.*,1988). Thus, recent studies have shown that exogenous administration of NGF improves fracture healing in laboratory animals, stimulates osteogenesis and increases the rate and quality of fracture repair (Akopian *et al.*,2000). Chondrocytes express NGF and TrkA receptors and NGF is elevated in synovial fluid of patients with chronic arthritis, juvenile chronic arthritis and in cartilage-related diseases. Moreover, this hypothesis is consistent with the observation that the basal NGF levels in osteoarthritis are associated with the distribution of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), which are implicated in this pathology (Bjurhalm *et al.*,1988). These findings indicate that NGF may exert important therapeutic effects on bone regeneration and may have a use in skeletal reconstruction and joint replacement.

The purpose of this paper was to examine a new natural composite, collagen/hydroxyl-apatite (Col/Hap) as a delivery system for neurogenic--osteogenic nerve growth factor (NGF). Bone osseointegration of such a composite implant into natural animal bone, its biocompatibility and neurocompatibility, as well as nerve ingrowth into the implanted composite were analysed. The early responses and dynamics of the cortical bone (femur) response to NGF delivered by the composite of Col/Hap were investigated. The results obtained may help in:

1) The optimisation of new 3D multi-functional natural, bioactive, resorbable composite products as bone substitutes in in-situ shapable forms;

2) The setting up of chemical and physical conditions for a new type of polymeric drug delivery device for sustainable release of growth factors as a key technology for successful tissue engineering.

MATERIALS AND METHODS

Preparation of composite collagen/hydroxyapatite /NGF (Col/Hap/NGF) natural composites

Microporous calcium phosphate hydroxyapatite (Hap) was prepared according to the protocol of Koutsoukos *et al.*,1980. Spherical granules were dried and calcined at 1100^{0}C for 6 h. The porosity of hydroxyapatite particles was very small with the size average of pores around 10 nm. Atelocollagen (Col) was purchased from HYPRO Ltd. (Czech Republic). It is crystalline, native atelocollagen type I, prepared from bovine Achilles tendon in the form of felt, with a noncollagenous peptide content <0.5 wt% and inorganic substances <0.5wt%. The composite material consisted of nine parts of inorganic components by weight and one part of organic component, including a mixture of collagen (92%) and NGF (8%). Before use, the acidic collagen solution was dialyzed against 0.02 M PBS (phosphate bufered saline, pH 7.2) and centrifuged at 48 000 rpm (Ti 70.1, Beckman) for 3 h. The upper two thirds of the collagen solution was collected from the centrifuge tube and used for reconstitution. The solution was mixed with hydroxyapatite (Hap) powders with a weight ratio of 35:65 (collagen to hydroxyapatite) at 43^{0}C. Five ml of the cold mix was added dropwise to 100 ml of olive oil stirring at various speeds at 37^{0}C. Collagen was reconstituted in the droplets and further aged for 1 h in the oil bath. At the end of the incubation, 100 ml of PBS was added to make a suspension of gel beads. The collagen/hydroxyapatite gel beads were collected from the aqueous phase, transferred to a 2.5% glutaraldehyde solution, and incubated at 37^{0}C for three more hours. The cross-linked gel beads were washed repeatedly with 0.02 M PBS. The sizes of the collagen/hydroxyapatite gel beads were controlled by the stirring speed.

Nerve growth factor (NGF) from mouse submaxillary gland was purchased from Sigma, (USA, Product No.72183). The biological activity of recombinant rat NGF-beta was measured in a cell proliferation assay using the factor-dependent human erythroleukemic cell line, TF-1. The declaration was as follows: edotoxin tested; neurobiological tests; neurofilament outgrowth observed at 30 ng/ml; cell culture: free of bacteria, yeasts, moulds and mycoplasma; preparation: by gel filtration and ion-exchange chromatography, sterilized by 0.2 mm-filtration and lyophilized (100 mg) from 1 ml of 5 mM PBS, pH 6.8. Solubility (0.1 mg/ml H2O) clear, colorless.

In vivo biological assessment

Male Wistar rats, weighing 100-125 g, were anaesthetized and the lateral aspect of the thigh shaved and scrubbed with iodine and alcohol. The lateral cortex of the femur was exposed and the periosteum was scraped. A round dental burr, with continuous saline irrigation, was used to create a cylindrical hole perpendicular to the long axis of the femur. The cylindrical implants (1.5 mm O.D. x 2.0 mm length) were tapped into the drilled hole. After implantation, the muscles were allowed to return to their natural arrangement and the skin was sutured closed. A single implant was placed in each femur. Controls were as follow: a) contralateral femur without any implants; and b) contralateral femur implanted with composite without NGF. The rats were euthanized at days 1, 7, 21 and 42 and the implant sites were examined clinically, histologically and histomorphometrically.

Histomorphometric evaluation of Col-Hap-NGF composite bone implant

Implants were left in place for different times, after which termination was performed by cardiac perfusion with Karnovsky's fixative. The bone was dissected to reveal the implant site and subsequently remained in Karnovsky's fixative for another 5 days. One femur from each animal was examined in the scanning electron microscope (SEM). Quantitative evaluations were carried out using a digitizing image analyzing system (DIAS). Total cross-sectional area (T.Ar), marrow area, cortical width, periosteal new bone area, and osteoid surface (O.Pm) were recorded. These parameters were used to calculate the total bone area (TB.Ar), cortical bone area (Ct.Ar), percent cortical area (%Ct.Ar), percent osteoid perimeter (O.Pm/B.Pm), mineral apposition rate (MAR), and bone formation rate per unit of bone surface (BFR/B.Pm). The porosity was defined as intracortical cavities with diameter >30μm. Intracortical cavity area and cortical area were used to calculate the percent porosity area. Forming osteon number (FON) was the sum of single and double labeled surfaces. Forming osteon number, resorption cavity number, and porosity number were used to calculate the forming osteon density (%RCN) and the ratio of the forming osteon number and resorption number (FON/RCN). Results are presented as mean ± SD. The differences within groups were evaluated statistically using one-way analysis of variance (ANOVA). $P>0.05$ was considered significant.

Scanning Electron Microscopy

Representative samples of tissue surrounding composite were processed for Scanning electron microscopy (SEM). Samples were rinsed 3 times with PBS and fixed for 60 minutes with 2.5 % glutaraldehyde and 2% paraformaldehyde in 0.1% cacodylate buffer (pH 7.4). Afterwards, they were postfixed with osmium tetroxide, critical point-dried and sputter-coated with gold-palladium. Morphological analysis and element analysis (KEVEX) were performed by SEM (Etec Autoscan, Etec, Haywood,CA).

RESULTS

Scaning Electron Microscopic Evaluation

Scanning electron microscopy showed a comparable cell morphology on all test surfaces. On all surfaces bone-like tissue formation was observed. SEM observation of the surface of the Col-Hap-NGF composite (Figure 1) showed that the particles of Hap were anchored in the complex of biopolymer matrix and a compact block structure had formed. The particles were completely covered with a film of well developed bone forming cells.

Higher magnification of the osteoblast-like cells revealed the fine lamellar and granular structure on the cell surface. Attached particles of the composite substrate (Fig. 1) could be observed on these cell structures it. The osteoblast-like cells outgrew the Col-Hap-NGF composite substrate and had a close relation with it. In the extra-cellular spaces all around the cells, there were short and thin fibrillar and granular structures, implemented into the extra-cellular matrix and also firmly bound to the nearest osteoblast-like cells. The covered particles were connected by fibrous formations of composite conjugate.

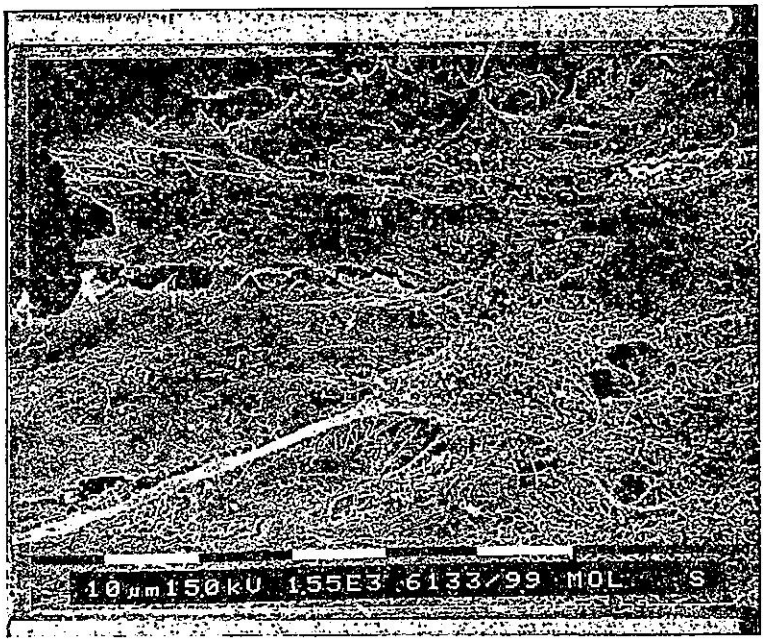

Figure 1. SEM analysis of the surface of the Col-Hap-NGF composite implanted into femur. Compact block structures of biopolymer matrix, covered with a film of well developed bone forming cells could be seen. On these cells, particles attached to the composite substrates can be observed.

Histomorphometric changes at the bone ingrowth surface of Col-Hap-NGF

Femur shafts appeared as follows: (1) 5 of the 11 rats had marrow trabeculae; (2) yellow marrow occupied almost the entire marrow cavity; (3) the periosteal surface contained mainly bone lining cells with few single labels and no double labels; (4) 3 of 4 rats in the pretreatment group had double-labeled endocortical surfaces, whereas aging controls had minimal osteoid and no double label at the endocortical surfaces. No osteoblasts or osteoprogenitor cells were seen at the endocortical surfaces. There were no changes in static histomorphometric parameters among the control groups (Table 1, 2 and 3), with a periosteal labeled perimeter of about 10% and endocortical labeled perimeter of about 23%. Periosteal and an endocortical mineral apposition rate and bone formation rate could not be determined in the 10 day and 30 day controls due to lack of double labels.

Effects of 10 Day composite-NGF implantation

Femur shafts appeared as follows: (1) subperiosteal and endocortical woven bone occurred in three of eight animals; (2) the yellow marrow area was smaller than in controls; (3) two to three layers of osteoprogenitors were seen adjacent to the endocortical surface; (4) a thick layer of osteoid and osteoblasts lined two thirds and one half of the endocortical surfaces, respectively; (5) double-labeled endocortical surfaces occurred in two out of eight rats; (6) double-labeled marrow trabecular surfaces were seen in three of eight rats; and (7) numerous, large intracortical cavities covered with osteoid and labeling were

seen, especially in the zone directly beneath the endocortical surface. Static and dynamic histomorphometric profiles, as compared with controls, were as follows: (1) total tissue area, cortical bone area, and total bone area did not differ; (2) marrow space area did not differ, but the yellow marrow area had decreased to 55%, (3) marrow trabecular area remained unchanged, (4) periosteal and endocortical bone formation surfaces did not differ (Tables 1, 2); (5) several layers of osteoprogenitor cells covered the endocortical surface; (6) osteoid perimeter was 64.5% (30-fold higher) (Table 2), with a thickness of 21.2 mm (20-fold higher); (7) intracortical porosity number increased and percent porosity area was 2.1% (Table 3); (8) the ratio of forming osteon/resorption cavity was 20 (Table 3); and (9) percent remodeling did not differ.

Table 1. Evaluation of the effects of NGF delivered by an implantable composite on bone innervation and osteogenesis. Static histomorphometric changes of the femur shaft

Groups (AN)	T.Ar. (mm^2)	Ct.Ar (%)	T.B.Ar (%)
0 day (6)	6.5 ± 0.3	85.4 ± 0.4	83.7 ± 0.5
10 days cont.(6)	5.9 ± 0.4	82.7 ± 2.1	81.9 ± 2.3
30 days cont,. (6) only composite	6.8 ± 0.5	85.1 ± 1.9	84.0 ± 2.2
10 days NGF (8)	6.7 ± 0.5	85.6 ± 3.9	85.9 ± 4.6
30 days NGF (10)	7.3 ± 0.7	87.7 ± 2.5	92.2 ± 3.7^{abc}

AN, animal number; T.Ar, total tissue area; Ct.Ar, cortical bone area; T.B.Ar, total bone area; ^{a}Vs. 0 day control, $p<0.05$; ^{b}Vs. Control only composite, $p<0.05$; ^{c}Vs. 10 day NGF, $p<0.05$.

Table 2. Evaluation of the effects of NGF delivered by an implantable composite on bone innervation and osteogenesis. Histomorphometric changes at the periosteal and endocortical surface

Groups (AN)	NBF. (mm^2)	MAR (μm/day)	O.Pm (%)
0 day (6)	0/6	0.0 ± 0.0	2.7 ± 5.1
10 days cont.(6)	0/6	0.0 ± 0.0	4.3 ± 3.5
30 days cont,. (6) only composite	0/7	0.0 ± 0.0	2.9 ± 2.3
10 days NGF (8)	4/9	0.9 ± 0.1^{abc}	64.5 ± 15
30 days NGF (10)	10/10	1.6 ± 0.1^{abc}	56.0 ± 11^{abc}

AN, animal number; NBF, new bone (woven+lamellar) frequency; MAR, mineral apposition rate; O.Pm, osteoid surface; ^{a}Vs. 0 day control, $p<0.05$; ^{b}Vs. Control only composite, $p<0.05$; ^{c}Vs. 10 day NGF, $p<0.05$.

Table 3. Evaluation of the effects of NGF delivered by an implantable composite on bone innervation and osteogenesis. Intracortical cavity changes

Groups (AN)	FON (%) (mm^2)	RCN (%) (μm/day)	FON/RCN (%)
0 day (6)	23.2 ± 2.1	12.3 ± 4.7	2.0 ± 0.5
10 days cont.(6)	15.5 ± 4.5	12.9 ± 3.7	1.2 ± 1.1
30 days cont,. (6) only composite	16.0 ± 7.5	14.8 ± 5.3	1.1 ± 0.3
10 days NGF (8)	67.9 ± 8.5 ^{ab}	5.7 ± 2.8 ^{ab}	20.6 ± 4.7 ^{ab}
30 days NGF (10)	71.2 ± 9.8 ^{abc}	2.1 ± 1.0 ^{abc}	33.5 ± 13.4 ^{abc}

AN, animal number; FON, forming osteon number;
FON/RCN, ration of forming osteon number to resorption cavity number;
RCN, resorption cavity number; ^{a}Vs. 0 day control, $p<0.05$; ^{b}Vs.
Control only composite, $p<0.05$; ^{c}Vs. 10 day NGF, $p<0.05$.

Effects of 30 Day composite-NGF implantation

Femur shafts appeared as follows: (1) woven and lamellar bone (Table 1) were apposed to the periosteal surfaces as well as to the endocortical surfaces in ten of ten animals, with a significant increase in osteoid surface (Table 2); (2) newly formed bone on the endocortical surface radiated into the marrow cavity in the form of new lamellar or woven trabecular bone, together with the newly formed bone on the previously existing trabecular surface, filling a large part of the marrow cavity; (3) the yellow marrow area was decreased; (4) a single layer of osteoblasts could be found adjacent to the periosteal and endocortical surfaces; (5) osteoprogenitor cells could be found on the endocortical woven trabecular surface; (6) forming osteon number (Table 3) and osteoid surface and thickness were higher in the experimental group; (7) total bone area, mineral apposition rate and osteoid surfaces were significantly increased in ten out of ten animals.

DISCUSION

Generally, composite systems, recently very popular, comprising inorganic (bioceramic) fibres or particles, and organic polymers, could be divided into different categories depending on their composition, bioresorbability and/or bioreactivity. However, many different types of composites still retain some problems which need to be resolved, such as: 1) rigidity, which is presently less than that of natural cortical bone, 2) degradation rate of high strength polymer, which is presently too low, and 3) bioactivity, which should be increased by adding other osteogenic molecules. While the specific requirements and characteristics for a bioresorbable material will vary with the details of the application, a general set of criteria exist and should be respected for successful implantation. First, the material must be capable of reproducible synthesis to ensure consistent performance in the finished devices. Second, the material should be amenable to a variety of polymer processing techniques, including

extrusion, injection molding, compression molding, and machining. This enhances the versality of the material. Third, the material should retain sufficient strength over time to be effective for clinical therapy. In the case of bioresorbable internal fixation devices, the sum of the time-varying strength of the healing biological union and that afforded by the implant ideally should be equivalent to that of the intact structure. Fourth, during the period the material is in the body, there should be no sustained inflammatory reactions or foreign body responses that necessitate removal. Fifth, the material should completely resorb with no histological evidence of residuals. Sixth, upon complete resorption of the material, there should be little or no physiological histological evidence of the former presence of the implant, that is, the body should "forget" that the implant was ever there. Continuing improvements in the bio-technological performance of currently industrially produced biomaterials, such as composites, by focusing on the fundamental genetic potential of osteogenic cells to respond to these biomaterials, will produce dramatic advances in organ and tissue repair and a better quality of life. Our idea to combine the biologically active composite Col-Hap with a component such as the neuro-molecule NGF, originated from a strong requirement for better cohesion of the implant material to the bone, but also from increasing demands from neurosurgery to improve aesthetic and functional results in patients treated for craniofacial tumours, malformations and traumas. The present study has demonstrated that NGF loaded in composite microsphera and implanted into the femur can increase bone volume, total bone surface and newly formed bone in a gap around the composite-NGF implant. Further studies are needed in more particular clinical situations.

NGF is a protein known to be essential for growth, survival and differentiation of sympathetic and sensory neurons in vertebrates, therefore clinical application of NGF is expected. However, the involvement of NGF in bone metabolism has been only rarely studied (Yada *et al.*,1994; Auffray *et al.*,1996, Kjaer 1998). We found an increase of osteoid surface adjacent to the composite implant as a result of NGF stimulation. Confirming other studies in younger rats, we found stimulated periosteal and endocortical woven and lamellar bone formation, which resulted in increases in bone mass and decreases in bone marrow. These investigations also showed that NGF treatment had greater effects in femur shafts of older male rats than in younger ones. Additionally, we found that NGF increased remodeling activity in the intracortical region and increased the intracortical cavity number and area by the end of the study. Since no similar studies have been carried out, it is difficult to correlate our findings with other studies where neurogenic factors are delivered by complex polymer composites.

The composite consisting of the organic extracellular matrix protein collagen in combination with hydroxyapatite (Col-Hap) could enhance the biological and mechanical (functional) properties of non-metallic implants (Bakos *et al.*,1999). The neurogenic NGF factor was added to this collagen-complex composite in order to generate better bone formation and remodeling which is already, influenced by the nervous system (Bjurhalm *et al.*,1988; Kundu *et al.*,1999). For example, it was reported that kinins and neuropeptides directly or indirectly may modulate the activity of bone cells in physiological and pathological conditions (Lerner, 1994;1998; Lundberg *et al.*, 1999;2001; Bjurhalm *et al.*, 1988). Neuro-osteological evaluation in medicine and dentistry, of pre-natal and post-natal pathological developmental conditions, provides much evidence for neuro-osteological combinations of the central nervous system (CNS) and

osseous development and remodeling (Kjaer,1998). A possible osseous malformation such as a cranio-pharyngeal canal, may thus indicate the site of abnormal brain/cranial base development (Kjaer, 1998). The neurogenic substance NGF is not limited to the peripheral nervous system, but is known to have molecular control over neurogenesis, bone innervation and osteogenesis (Auffray *et al.*,1996; Lundberg *et al.*, 1999;2001). Physiologically relevant quantities of NGF are synthesized and released by various non-neuronal and neuronal cells in mammals. A large amont of this factor is produced in the salivary glands of adult male mice and represents the best available source of NGF (Tanaka *et al.*,1990).

In conclusion our implant system manifested suitable chemical and physical characteristics and favourable tissue tolerance when implanted. No signs of inflammation or cytotoxicity were evident during the period of implantation. It appears that the chemical and physical properties of the composite Col-Hap-NGF affected significantly the process of implant osseointegration. Namely, the suggested composite substratum increased bone ingrowth into the implant. Close contact with bone producing cells, i.e., osteoblasts, was evident. The osteogenic-neurogenic NGF factor significantly affected and helped bone ingrowth into the implanted device. Such an implant system allows various modifications as well as mechanical and chemical adjustments suitable for better bone substitutions in reconstructive surgery. Altogether our system has great advantages over metallic implants, because:

a) it will improve aesthetic and functional results in patients treated for craniofacial tumours, malformations and traumas giving them a better quality of life;

b) it will help the development of new surgical techniques for bone reconstruction of the splanchno- and neuro-cranium;

c)it will permit the achievement of better results in tumour malformative and traumas reconstructions;

d)it will provide better functional, anatomic and morphological restoration of the craniofacial area;

e)it will significantly reduce the bone healing period after implantation;

f)it will offer a new solution to the problem of stress shielding, which often occurs with metallic implants that do not transfer sufficient load to the surrounding natural bone;

g)it will offer a new drug delivery device for sustained release of both neurogenic and osteogenic growth factors, essential in tissue engineering.

Address for correspondence:
Dr Anka Letić-Gavrilović
Fax: 39.06.4440062
e-mail: alg@keika.it

REFERENCES

1. *Akoplan A, Demulder A, Ouriaghli, Corazza F, Fondu P, Bergann P.* 2000.Effects of CGRP on human osteoclast-like cell formation: a possible connection with bone loss in neurological disorders. *Peptides*, 21. 559 - 64.
2. *Aloe L, Bracci-Laudiero L, Bonini S, Manni L.* 1997, The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. *Allerg*, 52:883 - 894.

3. *Auffray I, Chevalier S, Froger J et al.* 1996, Nerve growth factor is involved in the supportive effect by bone marrow derived stromal cells of the factor-dependent human cell line UT-7. *Blood*, 88:1608-18.
4. *Bakos D, Soldan M, Hernandez-Fuentes I.* 1999, Hydroxyapatite-collagen-hyaluronic acid composite. *Biomaterials*, 20(2): 191-5.
5. *Bjurhalm A, Kreicbergs A, Brodin E, Schultzberg M.* 1988, Substance P. and CGRP-immunoreactive nerves in bone. Peptides, 9:165-171.
6. *Du C, Cui FZ,Feng QL, Zhu XD, de Groot K.* 1998, Tissue response to nano-hydroxyapatite/ collagen composite implants in marrow cavity. *J Biomed Mater Res*; 42 (4): 540-8.
7. *Esposito M, Hirsch J-M, Lekholm U, Thomsen P,* 1998, Biological factors contributing to failures of osseointegrated oral implants. *Eur J Oral Sci*; 106(1):527-52.
8. Gao TJ, Lindholm TS, Kommonen B, Ragni P, Paronzini A, Lindholm TC *et al.* 1997, The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep. *Int Orthop*, 21:194-200.
9. *Gross KA, Berndt CC, Iacono VJ,* 1998, Variability of hydroxyapatite-coated dental implants. *Int J Oral Maxillofac Implants*, 13:601-10.
10. *Horisaka Y, Okamoto Y, Matsumoto N, Yoshimura Y, Hirano A, Nishida M et al.* 1994, Histological changes of implanted collagen material during bone induction. *J Biomed Mater Res.* 28(1):97-103.
11. *Kataoka K, Harada A, Nagasaki Y.* 2001, Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv Drug Delivery Rew.* 47:113-31.
12. *Kjaer I,* 1998, Neuro-osteology. *Crit Rev Oral Biol Med* , 9(2):224-44.
13. *Koutsoukos PG, Amjad ZH, Tomson MB, Nancollas GH,* 1980, Crystallization of calcium phosphates. A constant composition study. *J Am Chem Soc*, 102:1553-7.
14. *Kreuter J,* 2001, Nanoparticulate systems for brain delivery of drugs. *Adv Drug Delivery Rew,* 47;65-8.
15. *Kundu B, Khare SK, Singh G,* 1999, Role of polypeptides in the treatment and diagnosis of osteoporosis. *Peptides*, 20:523-37.
16. *Lerner UH, Johansson L, Ranjso M, Rosenquist JB, Reinholt FP, Grubb A,* 1998, Cystatin C, and inhibitor of bone resorption produced by osteoblasts. *Acta Physiol Scand* Sep, 161(1):81-92.
17. *Lerner UH,* 1994, Regulation of bone metabolism by the kallikrein-kinin system, the coagulation cascade, and the acute-phase reactants. *Oral Surg Oral Med Oral Pathol*, Oct;78(4):481-93.
18. *Letic-Gavrilovic A, Scandurra R, Abe K.* 2000, Genetic potential of interfacial guided osteogenesis in implant devices. *Dental Material J*, 19 (2):99-132.
19. *Lind M,* 1998, Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implant fixation. *Acta Orthop Scand*, Suppl, 283:2-37.
20. *Lind M,* 1998, Growth factors: possible new clinical tools. A review. *Acta Orthop Scand*, 67(4):407-17.
21. *Lundberg P, Bostrom I, Mukohyama H, Bjurholm A, Smans K, Lerner UH.* 1999, Neuro-hormonal control of bone metabolism: vasoactive intestinal peptide stimulates alkaline phosphatase activity and mRNA expression in mouse calvarial osteoblasts as well as calcium accumulation in mineralized bone nodules, *Regul Pept*, 85(1):47-58.
22. *Lundberg P, Lundgren I, Mukohyama H, Lehenkari PP, Horton MA, Lerner UH,* 2001, Vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide receptor subtypes in mouse calvarial osteoblasts: presence of VIP-2 receptors and differentiation-induced expression of VIP-1 receptors. *Endocrinol*, 142(1):339-47.
23. *Park YJ, Ku Y, Chung CP, Lee SJ,* 1998, Controlled release of platelet-derived growth factor from porous poly(L-lactide) membranes for guided tissue regeneration. *J Controlled Release*, 51(2-3), 201-11.
24. *Pietzak WS, Sarver D, Verstynen M,* 1996, Bioresorbable implants - practical considerations. *Bone*, 19(1):109S-119S.
25. *Reddi AH,* 1998, Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16:247-52.
26. *Ripamonti U, Reddi AH,* 1997, Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins. *Crit Rev Oral Biol Med*; 8(2):154-63.
27. *Sandhu HS, Herskovits MS, Singh IJ.* 1987, Effect of surgical sympathectomy on bone remodeling at rat incisor and molar root sockets. *Anat Rec*;219:32-38.
28. *Shikinami Y, Okuno M,* 1999, Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part I. Basic characteristics. *Biomaterials;* 20:859-77.
29. *Tanaka E, Habu T, Letic-Gavrilovic A, Abe K,* 1990, Changes in protein secretion by rat submandibular glands in response to Isoproterenol, Alpha-Methylnoradrenaline and Clonidine during postnatal development. *J Dent Res*: 60 (1): 60-66.

30. *Yada M, Yamaguchi K, Tsuji T,* 1994, NGF stimulates differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun*; 205 (2): 1187-93.
31. *Yanker BA, EM Shooter,* 1982, The biology and mechanism of action of nerve growth factor. *Ann Rev Biochem*;51 :845 - 9.
32. *Yao W, Jee WSS, Zhou H, Lu J, Cui L. Setterberg R et al.* 1999, Anabolic effects of Prostaglandin E2 on cortical bone of aged male rats comes mainly from modeling-dependent bone gain. *Bone*:25(6):697-702.

PROCENA UTICAJA KOMPOZITNOG KOLAGEN/HIDROKSI-APATIT IMPLANTATA I FAKTORA RASTA NERAVA (NGF) NA RAST NOVE KOSTI

LETIĆ-GAVRILOVIĆ ANKA, FINI MILENA, GIAVARESI G, GIARDINO R i ABE K

SADRŽAJ

U ovom radu su izneti rezultati primene arteficijelno-prirodnog, bioresorptivnog, kompozitnog inplantata obogaćenog neurofilinom (faktorom rasta nerava NGF). Inplantat je ugrađivan u butnu kost Wistar pacova težine 100 - 125 g a kao kontrole su služile butne kosti istih pacova bez inplantata kao i kosti sa inplantatima ali bez NGF. Mesta ugradnje su ispitivana posle 30 dana i to klinički, histo-morfološki, histološki i skening elektronskom mikroskopijom.

Ovim ogledom je dokazana stimulacija periostalnog i endokortikalnog bujanja uz nastanak koštanih lamela i porast koštane mase praćen smanjenjem zapremine kostne srži. Tretman sa NGF-om je bio efikasniji kod starijih jedinki. Osim toga, NGF je povećavao stepen intrakortikalne remodelacije i broj šupljina u ovom delu kosti. Može se zaključiti da će primena ovakvih inplantata biti od koristi u ortopedskoj i maksilofacijalnoj hirurgiji.