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# The influence of transforming growth factor beta-1 proven in autologous omental graft on the healing of critical size defects in rabbit radius

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**ABSTRACT**

Fracture healing is a complex physiological process. Multiple factors regulate this cascade of molecular reactions, affecting different sites in the osteoblast lineage, through various processes such as: migration, proliferation, chemotaxis, differentiation, inhibition, and extracellular protein synthesis. The omentum is a serous membrane made up of a lattice of blood vessels and fat. Basically, it is a highly vasculated organ with a rich source of angiogenic factors that promote the growth of blood vessels into whatever tissue it is placed close to. Recent studies have revealed that the omentum, apart from being a great source of various growth factors, also contains omnipotent stem cells that can differentiate into a variety of cell types. The study was carried out on 16 adult male New Zealand rabbits in the same condition. A large segmental defect was created in the radius of each animal in groups A and B. In group B the defect was filled with a piece omental fat tissue. The animals were euthanized 56 days after the operation and the bones removed for histomorphometric analysis. Histomorphometric analysis was performed. The osteoblast interface (Ob.S/BS) proved to be the statistically significant parameter (P = 0.005). An osteoblast interface was found in the treated group in contrast to the control. The surface of trabecula covered with the osteiod and osteoblast interface showed a high degree of positive linear correlation, in both the control and the treated group. Our study shows that the statistically significant osteoblast interface leads to the conclusion that omental fat tissue has a certain influence on bone turnover, especially on the formation of the newly-created bone.

**Key words**: rabbit, omentum, fat tissue, bone, healing

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**Introduction**

Adipose tissue is considered to be the largest endocrine gland, because it produces free fatty acids, hormones, growth factors, and cytokines, such as leptin, adiponectin, resistin, VEGF, HGF, IGF-1, angiogenin, IL-6, TNF-α, and angiopoietins. (NEELS et al., 2004; RUPNICK, 2002). Growing adipose tissue contains a diversity of cell types including adipocytes, adipose stromal cells (ASCs), endothelial cells, and inflammatory cells (REHMAN et al., 2004). The diversity of heterogeneous cell populations determines the expression of multiple growth factors and cytokines, that either individually or jointly regulate vessel growth, although little is known about the functional interplay between these factors (CAO, 2005).

Adipocytes develop from mesenchymal cells via a complex cascade of transcriptional and non-transcriptional events. Adipocyte differentiation is a complex process accompanied by coordinated changes in cell morphology, hormone sensitivity and gene expression, that have been studied primarily in murine preadipocyte cell lines. In the literature, adipose-derived stromal cells have been termed ‘‘pre-adipocytes” and there is a growing appreciation that they are multipotent, with chondrogenic, neurogenic and osteogenic capabilities.(ZUK et al., 2002). ASCs secrete high levels of a number of angiogenic factors (BOULOUMIE et al., 2001). ASCs (adipose stromal cells) represent a major area of interest for research and have a potential role as building blocks for clinical cell therapy. Growth factors are also a key interest in regenerative medicine. The BMPs are part of the transforming growth factor beta (TGF- β) superfamily, and have been shown to take part in bone and cartilage formation, fracture healing and repair of other musculoskeletal tissues. (MATTHEWS, 2005). The osteoinductive growth factors rhBMP-2 and -7 are in clinical use. They induce ossification by recruiting mesenchymal cells from surrounding tissues and differentiating these cells towards osteoblasts. (SAMARTZIS et al., 2005).

The TGF- β family contains three closely related mammalian isoforms:TGF β 1, β 2, and β 3, that arose by duplication of a common ancestor. TGF- β 1 regulates a broad range of biological processes, including cell proliferation, cell survival, cell differentiation, cell migration, and production of extracellular matrix (ECM). The combined actions of these cellular responses mediate the global effects of TGF-β1 on immune responses, angiogenesis, wound healing, development, and bone formation. Bone formation by TGF-β1 is promoted through chemotactic attraction of osteoblasts, enhancement of osteoblast proliferation and the early stages of differentiation, with production of ECM proteins, stimulation of type II collagen expression and proteoglycan synthesis by chondrocyte precursor cells (PEPPER, 1997; LETTERIO and ROBERTS, 1998).

This study aims to investigate the use of omental fat tissue in order to improve new bone formation in nonunion defect of rabbit radius filled with autologous omental graft.

**Materials and methods**

The research was conducted on samples of the omentum of five rabbits, which were previously euthanized with introcardiac injections 0.3 ml/kg per weight of the preparation T61 (Hoechst, Munich, Germany). The euthanasia was conducted according to the Law and with observance of regulations on the ethics of animal welfare standards of the Republic of Croatia and the European document on keeping and handling laboratory animals: "Directive for the Protection of Vertebrate animals used for Experimental and other Purposes" (86/609/EEC). For immunofluorescence determination of TGF-β1 molecules in the omentum, we used omentum samples of 1 cm3 size and fixed them in 10% formaldehyde, buffered in phosphate puff (pH 7.2) The rabbit omentum samples, fixed in 10% buffered formaldehyde, were dehydrated by immersion in 70 % ethanol (for 2 days), 96% ethanol (for one hour) and 100% ethanol (twice per hour). The processed omentum tissue was immersed for one hour in the mixture of chloroform and pharaplast (1:1) at a temperature of 56 degrees, and then fitted in pharaplast. After cooling, the blocks were cut with microtom (Reichert-Jung, Germany) into parts 6-7μm thick.. The deparafying of cuts was carried out by dipping in two changes of xylol (for ten minutes), in declining concentrations of ethanol (100%, 96%, 80%, 70%; all for five minutes) and two changes of distilled water (for five minutes).

*The sample processing.* The omentum samples of rabbits fixed in 10% buffered formaldehyde were dehydrated by immersion in increasing concentrations of alcohol and were fitted in pharaplast according to the procedure previously described. The blocks were cut with microtom.. The treated cuts were subjected to the indirect immunofluorescence method. The cuts were incubated with primary antibody for two hours at room temperature. We used the mouse monoclonal antibody TGF-β1 (Mouse monoclonal anti-TGFβ-1 antibody, R&D Systems) diluted in 1:5 PBS. After flushing in PBS puff (three times for five minutes), the cuts were incubated with a secondary antibody. We used goat against mouse antibody, conjugated with fluorescein (Goat F(ab')2 Anti-Mouse Ig's, Fluorescein Conjugate) diluted in 1:10 PBS. The cuts were flushed in PBS (three times for five minutes) and in distilled water (for five minutes). After immunofluorescence, the tissue cuts were dehydrated in a series of increasing concentrations of ethanol, cleared with immersion in xylol and kept in a freezer for two days. The samples obtained were processed with the help of fluorescentic microscope Olympus BX 51 under magnification of 400. By observing the samples with the assistance of the fluorescentric microscope, the fluorescence was determined which was clearly confirmed by the test results of TGF-β1 of the rabbit omentum (Fig. 1). The sample analysis was conducted at the "Ruđer Bošković" Institute.

*Animal procedure.* Sixteen male New Zealand rabbits, 2.50 ± 0.30 kg, were kept in the same living conditions (room temperature 20 ± 1 °C, relative humidity 55 ± 5%). They

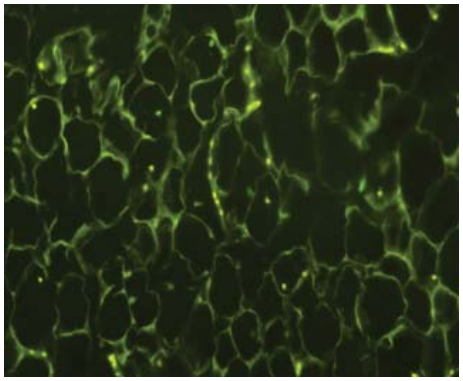

Fig. 1. Immunofluorescence confirms the presence of TGF-β1 in the omentum

were fed the standard diet and administrated water ad libitum. The animals were denied food for 12 h preoperatively, but had access to water. The animals were anaesthetized by injecting 5 mg/kg of xylazine and 40 mg/kg of ketamine intramuscularly.

The euthanasia was conducted according to the Law and the ethics and standards of the animal welfare of the Republic of Croatia and the European document regulating the keeping and handling of the laboratory animals: "Directive for the Protection of Vertebrate Animals Used for Experimental and other Purposes" (86/609/EEC).

Sixteen rabbits were divided into two groups. A large segmental defect was created in the radius of each animal in both A and B groups. In group B the defect was filled with a piece of omental tissue fat; a small piece of omental fat was obtained from a 3 cm mid-abdomen incision.

Ketofen® was administered intramuscularly for 3 days to control pain. On days 2, 9 and 16 before sacrifice, each rabbit was injected intramuscularly with fluorochromes demeclocycline (20 mg/kg), alizarin complexone (30 mg/kg), and calcein (20 mg/kg), respectively. All fluorochromes were purchased from Sigma (Deisenhofen, Germany).

The animals were euthanized 56 days after operation and the bones removed for histomorphometric studies. The bone samples assigned for histomorphometry were embedded undecalcified in methylmetacrylate. Sections 5 μm thick were cut with a 2040 outcut microtome (Reichert-Jung, Heildelberg; Germany) and stained with Toluidine blue and Goldner-Mason. The sections were analyzed using an Opton microscope. The nomenclature follows the recommendations of the American Society for Bone and Mineral Research.

**Results**

Eight weeks after the operation, the histological as well as radiological findings showed that the greater omentum graft provoked significant bone activity with

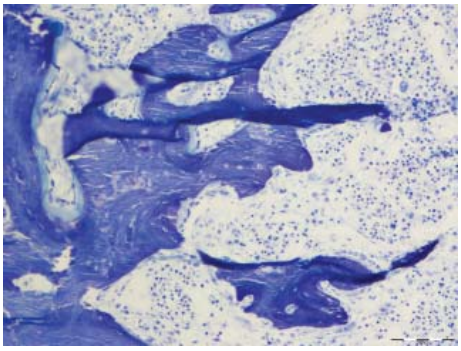

Fig. 2. The specimen without free omental graf, no bone turnover rate is observed Toluidin blue staining, ×10

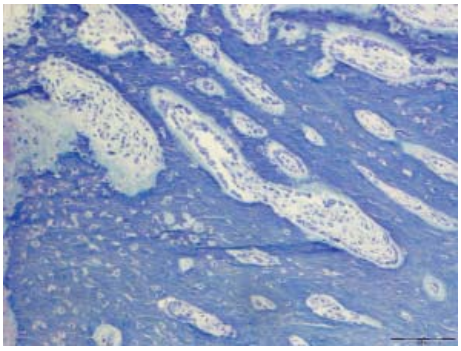

Fig. 3. Specimen with free omental graft, significant bone activity with predominational bone forming present, Toluidin blue, ×10

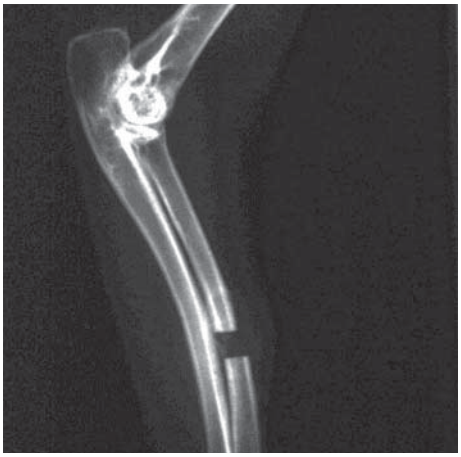

Fig. 4. By observation of the radiographs of the control group, no signs of new callus formation could be noticed.

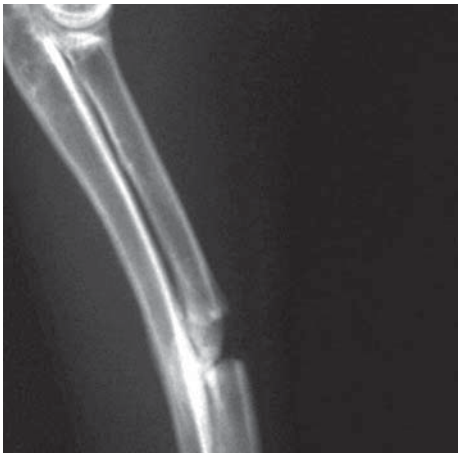

Fig. 5. By visual observation of radographs it is noticable that the bone healing process on the large segmental defect was more intensive in the treated group after 8 weeks. The greater bone callus volumen was observed in the treated group

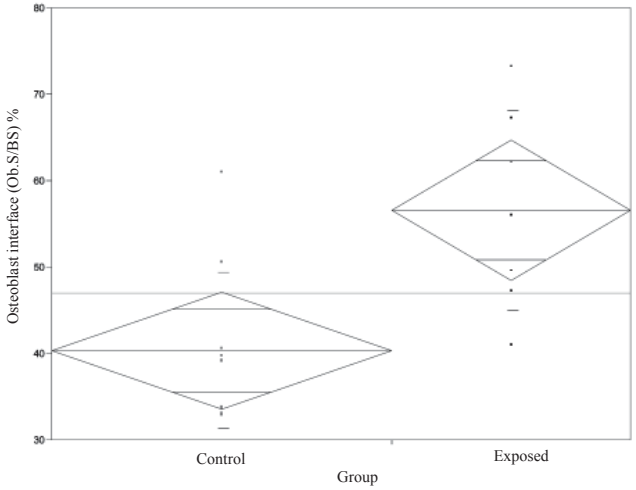


Fig. 6. Levence test has proved that osteoblast interface (Ob.S/BS) is statistically significant parameter (P = 0.005)

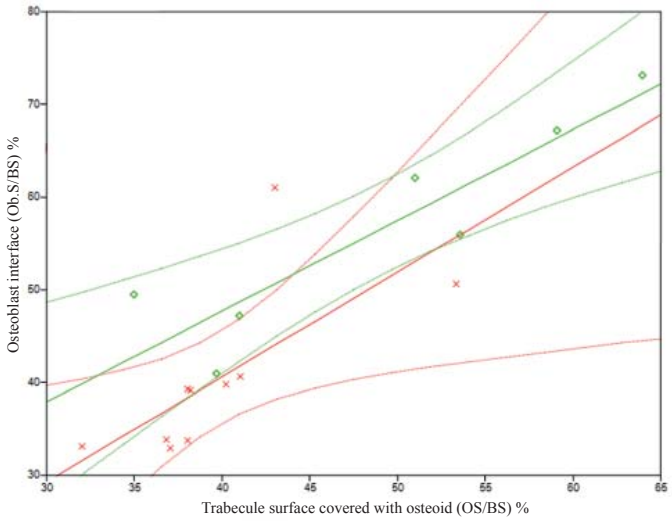


Fig. 7. The trabecular surface covered by osteoid and osteoblast interface showed a high degree of positive correlation, both in the control r(10)00.701, P = 0,024, marked red on the figure and the treated group r(7) 0,915, P = 0,004, marked green on the figure

predominatial bone forming, and bone turnover rate with osteoblast domination (Fig. 3, 5), while in the control group the bone activity was low and no bone turnover rate was observed. (Fig. 2, 4).

The Levence test for equal variance showed that in the case of osteoblast interface (Ob.S/BS) the samples had equal variance. Therefore, analysis was performed on these samples (Analysis of Variance, ANOVA).The osteoblast interface proved to be a statistically significant parameter ($P = 0.005$). The osteoblast interface was found in the treated group in contrast to the control one. The surface of the trabecula covered with osteiod and osteoblast interface showed a high degree of positive linear correlation in both the control and the treated group.

**Discussion**

Repair of bone defects caused by severe trauma, resection of tumors, and congenital deformities remains a major challenge to surgeons. As a gold standard for the treatment of bone defects in clinically treated patients, autologous bone grafts are usually limited by considerable donor site mobility and the available supply of tissue that can be harvested (BURCHARDT, 1983; YOUNGER and CHAPMAN, 1989). Bone marrow stromal cells (BMSCs) are the commonly used seed cells for bone engineering (BRUDER et al., 1998). Recent reports indicate adipose tissue as a novel source of multipotent stem cells, which can be used in pharmacological studies and clinical praxis. Growing adipocytes produce a dozen angiogenic factors, including leptin, VEGF, FGF-2, HGF, IGF, TNF-α, TGF-β, placental growth factor (PlGF), VEGF-C, resistin, tissue factor (TF), neuropeptide Y (NPY), heparin-binding epidermal growth factor, and angiopoietins (VOROS et al., 2005).

Osteoblasts originate from common progenitors, which are capable of differentiating into other mesenchymal cell lineages, such as chondrocytes, myoblasts, and bone marrow stromal cells including adipocytes. During the differentiation process from mesenchymal progenitors, various hormones and cytokines regulate osteoblast differentiation. Cbfa1 plays important roles in skeletal development at two stages, for commitment to skeletal lineage cells and for maturation of osteoblasts in postnatal development (YAMAGUCHI et al., 2000).

The formation, deposition, and mineralization of bone tissue are executed by the osteoblasts that differentiate from mesenchymal precursor cells. The key transcription factor that drives the mesenchymal precursor cell toward the osteoblast lineage and controls bone formation is Runx2 (Cbfa1), and it regulates the expression of all known marker genes expressed by the osteoblast (DUCY et al., 1997). Runx2 binding sites are found in the promoters of several bone formation markers, including collagen 1, ALP, osteopontin, RANKL, and osteocalcin (STEIN et al., 2004).

TGF- β1 is covered up in a latent form by bone cells and is stored in the ECM. Active, resorbing osteoclasts are capable of activating TGF-β1, which in turn attenuates further bone resorption by impairing osteoclastogenesis and promotes bone formation through chemotactic attraction and stimulation of proliferation and differentiation of osteoblast precursors. The *in vitro* effects of TGF-β1 on cells of the osteoblast and osteoclast lineage depend greatly on factors such as cell differentiation stage, cell density, TGF-β1 concentration, the presence of serum, and other culture conditions. TGF-β1 increases bone formation *in vitro* mainly by recruiting osteoblast progenitors and stimulating

their proliferation, thus expanding the pool of committed osteoblasts, as well as by promoting the early stages of differentiation (ALLISTON et al., 2001; MAEDA et al., 2004).

Induction of osteoblast-specific gene expression in these cells requires coordinated action between Runx2 and BMP2-induced Smad5 (LEE et al., 2000). In the early differentiation stage, TGF- β1 induces the expression of Runx2 in combination with BMPs. Despite these positive reports, the osteoinductive capacity of TGF- β1 is rather weak when compared with that of the BMPs. As yet, no clinical application has been developed for TGF- β1, whereas BMP-2 and BMP-7 have already been used in clinical trials and have proven their efficacy in the healing of critical-sized fibular defects and tibial nonunions in humans (GEESINK et al., 1999; FRIEDLAENDER et al., 2001). In an *in vivo* experimental model of fracture healing, BMP-2 induced differentiation of mesenchymal cells into osteoblasts and chondrocytes during intramembranous bone formation and early chondrogenesis, whereas TGF-β1 expression correlated with active differentiated osteoblasts and chondrocytes during chondrogenesis and endochondral ossification (SI et al., 1997).

In bone, TGF- β1 plays an important role in keeping the balance between the two tightly regulated processes of bone resorption and subsequent bone formation (MUNDY, 1991).

TGF-β also has many other roles, including promoting angiogenesis (BOLANDER, 1992), differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts (JOYCE et al., 1990; SANDBERG et al., 1993) regulating cartilage matrix calcification; and stimulating osteoblast activity and intraosseus wound regeneration (MUNDY, 1993; TALLEY et al.,1995). According to our investigation the results showed a significant difference between the treated group and the control group in relation to the parameters of bone turnover and mineralization, as manifest in the presence of the statistically significant activity of osteoblasts on the bone defect. Moreover, the results showed the absence of dissociation between osteoblast and osteoclast activity, which means that normal balance between deconstruction and new bone formation was sustained, whereas the increased osteoblast activity was noticeable only on the bone defect (SMOLEC et al., 2010).In conclusion these results indicate that omental fat tissue transplant application

stimulates bone healing, which opens the possibility of wide clinical application of the omental fat tissue in reconstructive surgery.

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**SAŽETAK**

Cijeljenje loma kosti složeni je fiziološki proces. Više faktora regulira ovu kaskadu molekularnih reakcija utječući na osteoblaste kroz razne procese kao što su migracija, proliferacija, kemotaksija, diferencijacija, inhibicija i ekstracelularna sinteza proteina. Omentum je serozna membrana koju čini mreža krvnih žila i masnog tkiva. To je izrazito vaskulariziran organ koji je ujedno bogat izvor angiogenih faktora koji potiču rast krvnih žila u bilo kojem tkivu na koje ga se položi. Nedavna istraživanja su otkrila, da omentum osim što je obilan izvor različitih faktora rasta, također sadrži omnipotentne matične stanice koje se mogu diferencirati u razne tipove stanica. Istraživanje je obavljeno na 16 odraslih mužjaka novozelandskog kunića istog statusa. Na radijusu svake životinje, podijeljene u skupine A i B, učinjen je veliki segmentalni defekt. U skupini B defekt je bio ispunjen komadićem (isječkom) masnog tkiva omentuma. Životinje su bile eutanazirane 56. dana nakon operativnog zahvata, a kosti su zatim izuzete za potrebe histomorfometrijske analize. Učinjena je histomorfometrijska analiza. Sloj osteoblasta (Ob.S/BS) pokazao se kao statistički značajan pokazatelj (P = 0,005). Sloj osteoblasta ustanovljen je u pokusnoj skupini u usporedbi s kontrolnom skupinom. Površina trabekula pokrivena osteoidima i slojem osteoblasta pokazala je visok stupanj pozitivne linearne korelacije i u kontrolnoj i pokusnoj skupini. Naše istraživanje pokazuje da sloj osteoblasta statistički značajno povećan, te se može zaključiti da masno tkivo omentuma ima stanoviti utjecaj na obnavljanje kostiju, naročito na formiranje novostvorene kosti.

**Ključne riječi:** kunić, omentum, masno tkivo, kost, cijeljenje