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Clinical Aspects of Gene Expression for Bone Morphogenetic Proteins 2, 4, 6 in Bone Augmentation*

Kliniczne aspekty ekspresji genów dla białek morfogenetycznych kości 2, 4, 6 w augmentacji tkanki kostnej

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A – koncepcja i projekt badania; **B** – gromadzenie i/lub zestawianie danych; **C** – opracowanie statystyczne; **D** – interpretacja danych; **E** – przygotowanie tekstu; **F** – zebranie piśmiennictwa

Abstract

Background. Human jaw bone augmentation is a procedure applied as a part of dental implant therapy. Bone substitutes are very well known and described. The cascade of bone substitute remodeling process leads to bone substitute resorption and new bone formation, called creeping substitution. The most important aspect for this cascade promotion is the activation of stem cells by local or applied growth factors. BMPs are a group of growth factors present in bone allograft (proteins synthesized by the donor) as well as in the newly formed bone (BMPs synthesized by the recipient's cells).
Objectives. The purpose of this study was to evaluate the clinical restoration of jaw bone defects using guided bone regeneration, and analyzed three isoforms, BMP-2, BMP-4 and BMP-6 in newly formed bone tissue, used real time PCR method on the mRNA synthesis stage, i.e. synthesized in response to the graft application.
Material and Methods. 12 patients aged 20–56 years underwent therapy by implanting: 1) allogenic cortico-cancellous bone granules with xenogeneic bovine mineral into bone defects (6 patients); 2) allogenic cortico-cancellous bone granules with synthetic beta-tricalcium phosphate (BTCP) into bone defects (6 patients).
Results. Synthesis of BMP2 and 4 was observed after application of both: natural bovine mineral and synthetic BTCP, but expression of BMP6 was not found. Synthesis of BMP2 was comparable in control tissue and newly formed tissue in the site of bovine mineral application. After BTCP application, the synthesis of BMP2 was significantly lower. Synthesis of BMP-4 was two times higher in the control tissue.
Conclusions. Natural bovine mineral seems to be more effective than synthetic BTCP in bone inducing through the synthesis of BMP2, the main osteogenic growth factor in the human jaw bone augmentation methodology. BMP isoform tests revealed a significant concentration of BMP-2 mRNAs, a lower expression of BMP-4 and trace amounts or no presence of BMP6 mRNA in all newly formed bone samples (**Dent. Med. Probl. 2012, 49, 3, 337–344**).

Key words: bone allograft, human bone augmentation, bovine mineral, BTCP, BMPs.

Streszczenie

Wprowadzenie. Augmentacja kości szczęk jest zabiegiem wspomagającym leczenie implantoprotetyczne. Znanych jest wiele materiałów kościopodobnych i substytutów kości. Kaskada procesu przebudowy, stopniowego zastępowania tych materiałów prowadzi do ich resorpcji i powstania nowej tkanki kostnej. Istotne jest aktywowanie tej kaskady przez pobudzenie miejscowych komórek macierzystych przez miejscowe lub egzogenne czynniki wzro-

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stowe. Białka morfogenetyczne kości są obecne w tkance kostnej przeszczepów allogenicznych (syntetyzowane w organizmie dawcy), a także w nowotworzonej tkance kostnej (syntetyzowane przez komórki biorcy).
Cel pracy. Kliniczna ocena regeneracji uszkodzeń tkanki kostnej szczęk po zastosowaniu metodologii sterowanej regeneracji kości i analiza trzech izoform białek morfogenetycznych kości: BMP-2, BMP-4, BMP-6 w nowo powstającej tkance kostnej, z wykorzystaniem metodologii *real time*-PCR i oceny syntezy mRNA w odpowiedzi na zastosowanie implantu.
Materiał i metody. Badania prowadzono u 12 pacjentów (20–56 lat), którym wszczepiono: 1) kostną macierz allogeniczną wraz z minerałem wołowym (6 pacjentów), 2) kostną macierz allogeniczną wraz z syntetycznym beta-trójfosforanem wapniowym (6 pacjentów). Kość allogeniczna miała postać granulatu (1–2 mm średnicy)
Wyniki. W badaniach 15 eksplantów tkanki kostnej pobranej trepanem o średnicy 3 mm i długości 10 mm z miejsc implantacji poddano analizie rt-PCR. Zaobserwowano syntezę BMP-2 i BMP-4 w przypadku zastosowania obu materiałów: naturalnego minerału wołowego i syntetycznego BTCP. Nie stwierdzono syntezy BMP-6. Instensywność syntezy BMP-2 była porównywalna w kości kontrolnej ektopowej i po zastosowaniu minerału wołowego. Po zastosowaniu BTCP synteza BMP-2 była znacząco słabsza. Synteza BMP-4 była dwa razy większa w kości kontrolnej.
Wnioski. Naturalny minerał ksenogenny jest bardziej skuteczny niż syntetyczny BTCP w procesie kościotworzenia w sterowanej reakcji tkanki kostnej przez ekspresję BMP-2, który okazał się najbardziej skuteczną izoformą BMPs. Mniejszą ekspresję wykazała izoforma BMP-4, nie stwierdzono natomiast obecności BMP-6 lub ekspresja była na poziomie dokładności metody. BMP-6 nie jest czynnikiem niezbędnym w procesie resorpcji, osteogenezy i przebudowy tkanki kostnej, ale być może interferuje z procesem degradacji tkankowej (**Dent. Med. Probl. 2012, 49, 3, 337–344**).

Słowa kluczowe: przeszczep kostny, augmentacja kości u ludzi, minerał wołowy, BTCP, BMPs.

Advanced methods of bone augmentation using growth factors and/or stem cells constitute one of the most rapidly developing areas of oral implantology, especially when bone quantity is not sufficient.

The crucial role of bone morphogenetic proteins (BMPs) in craniomaxillofacial and neurological surgery were discussed [1, 2], but the efficacy of a different methodology is still under investigation.

A recent significant breakthrough in tissue engineering therapy was the application of isolated CD34+ bone marrow stem cells [3]. Their total amount of approximately 1,000,000 per 1 ccm of transplant appeared to be too small to achieve detectable bone tissue regeneration, the more so that the proposed recombinant human growth factors BMP-2, BMP-4 and lately BMP-7 and EGF-2 require also, as mitogens, a significant local stem cell pool [4–6]. BMP-2 has been acknowledged as one of the most effective growth factors in dental therapy and has a synergistic effect with BMP-4 [7]. Both of these factors in a recombinant form of rhBMP2 are already used in clinical conditions for bone tissue augmentation, e.g. in USA, and so is rhPDGF BB in periodontal regeneration. However, the action of the above factors is known to be of a cascade nature, and there is feedback because PDGF is a mitogen, while BMP-2 and BMP-4 plays the role of a morphogen [8–11]. Bone morphogenetic proteins stimulate human stable cell lines, human mesenchymal stem cells *in vitro* [12–13] as well as in animal studies [14]. The other known BMP-6 influenced on macrophages cell lines [15], which suggest other, maybe opposite action in bone to BMP-2, or BMP-4. From a clinical point of view, it was confirmed that synthetic human recombinant BMP-2 can enhance bone formation and osteointegration in implant therapy in sinus lift procedure [16] as well as in orthopaedic surgery [17].

The aims of the study included: assessment of a clinical restoration of jaw bone defects using guided bone regeneration, and expression analysis of three bone morphogenetic protein (BMP) isoforms, BMP-2, BMP-4 and BMP-6 in bone tissue induced by augmentation in dental implant treatment.

Material and Methods

For this purpose, guided bone regeneration (GBR) methods were used. This technique was used in 12 patients (men) aged 20–56 years by implanting: 1) allogenic cortico-cancellous bone granules (50% of volume) with rapidly resorbable synthetic beta-tricalcium phosphate (BTCP) (50% of transplant volume) into bone defects in 6 patients (Fig. 1 top); 2) allogenic cortico-cancellous bone granules (50% of volume) with slowly resorbable xenogenic bovine mineral (50% of transplant volume) into bone defects in 6 patients (Fig. 1 bottom).

The main volume of transplants consisted of allogenic bone in the form of granules taken from one lot from the Central Tissue Bank. Each implantable material that was used made up 50% of the 3 cm^3 of transplant volume. Patients were given pharmacotherapy: Sumamed 1 × 0.5 mg daily for 6 days, metronidazole 3 × 0.25 g daily, Aescin 3 × 1 daily (antioedema action), analgesics (Ketonal) and a probiotic (Trilac) for 6 days.

Sutures were removed 10 days after surgery in all patients. 6 months following implantation of

guided bone regeneration material, cylinder bone samples were collected using a trephine bur during implant bed preparation after muco-periosteal flaps were elevated (Fig. 2). 18 samples in total (10 samples collected after implantation of allogenic bone + bovine mineral, 8 samples collected after implantation of allogenic bone with BTCP) were placed in extraction reagents for BMP-2, –4 and –6 RNA isoforms.

Real Time-PCR Test

Tissue Sampling

Samples (5 in each group) collected after the explantation was immediately placed in tubes and immersed in 1 ml of TRI-reagent (Sigma-Aldrich), a liquid preventing the RNA from being destroyed by RNA-ses and enabling isolation of RNA. Tubes were transferred to –22°C and after initial freezing moved to –70°C until further processing. Applied methodology was described by Chomczynski et al. recently [18, 19].

Total RNA Extraction

Tissues with TRI-Reagent in which they were stored were individually homogenized in cryomill in tubes washed with DEPC-treated water to inactivate RNA-ses. For total RNA extraction a method was applied with the use of TriReagent (Sigma-Aldrich), TRI Reagent is an improved version of the single-step total RNA isolation reagent developed by Chomczynski [18, 19]. Homogenized tissue was placed in 1.5 ml centrifuge tube (Eppendorf). TRI--Reagent was added up to 1 ml. Tubes were shaken in an ambient temperature for 5 minutes. Then, the samples were allowed to stand for 5 minutes. 0.2 ml of chloroform was added (Sigma-Aldrich). Tubes were shaken vigorously and allowed to stand at room temperature for 10 minutes. Then, tubes were centrifuged at 12 000 g at 4°C for 15 minutes. Aqueous phase was transferred to a fresh tube (Eppendorf) and 0.5 ml of isopropanol (Sigma-Aldrich) was added. Samples were mixed and allowed to stand at room temperature for 10 minutes, then centrifuged at 12 000 g at 4°C for 15 minutes. Supernatant was removed and the pellet was washed by adding 1 ml of 75% Ethanol (POCH). Samples

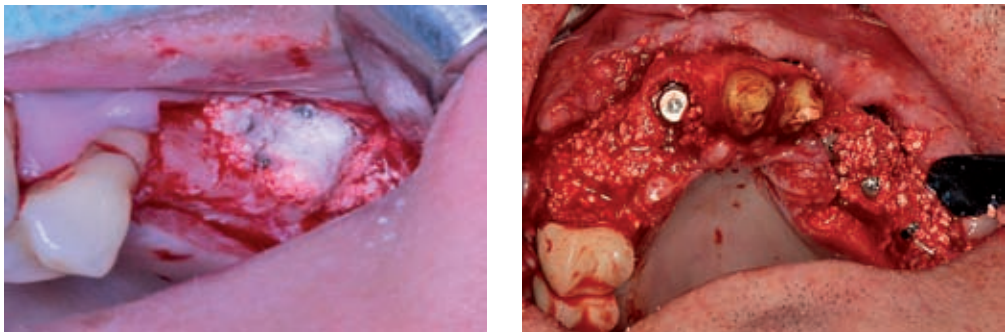

Fig. 1. Jaw bone augmentation – surgical procedure graft consist of: BTCP + allogenic bone granules (up), xenogenic mineral + allogenic bone granules

Ryc. 1. Augmentacja kości szczęk – zabieg chirurgiczny: przeszczep zawiera: BTCP + granulat kości alogenicznej (góra), minerał ksenogenny + granulat kości alogenicznej

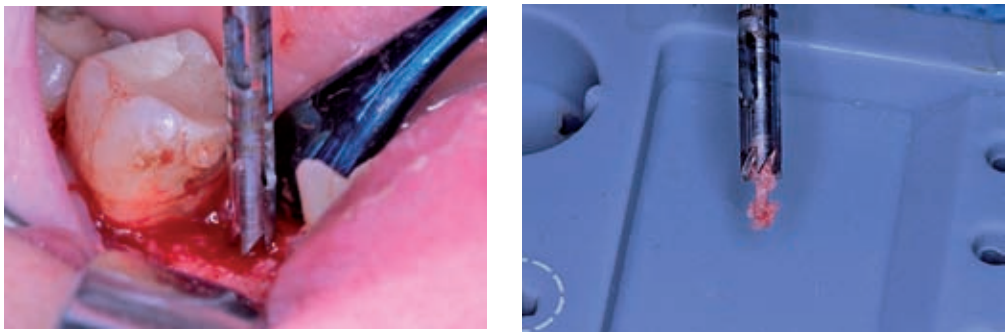

Fig. 2. Augmented jaw bone site: (core) bone microbiopsy taken by the trephine before implant installation/sample of augmented bone tissue inside the trephine

Ryc. 2. Augmentowana tkanka kostna szczęk: biopsja tkanki kostnej pobrana trepanem przed instalacją implantu próbka–rdzeń tkanki kostnej wewnątrz trepanu

were vortexed and centrifuged at 7500 g at 4°C for 5 minutes. Supernatant was removed and the samples dried in ambient temperature for 20 minutes. The pellets were resuspende in 0.5 ml water for molecular biology (Sigma-Aldrich) and the amount of RNA was measured with the use of Biophotometer (Eppendorf) in order to optimize the volume of sample used for reverse transcription.

Reverse Transcription

For obtaining cDNA further used for real time PCR procedures, High Capacity cDNA transcription Kit (Applied Biosystems/Life Technologies) was applied according to the manufacturer's protocol. Kit components included: buffer, dNTP mix, random primers, reverse transcriptase, RNAse inhibitor, nuclease-free water. Solution of RNA isolated in the first step was added to the reverse transcription mix in concentrations allowing us to obtain up to 2 ug of RNA per reaction tube which was controlled with the use of a spectrophotometer (Biophotometer, Eppendorf). Reverse transcription reaction was performed in thermal cycler (Realplex^2, Eppendorf) according to the program recommended by the producer of the reverse transcription kit. Tubes with the products of reverse transcription were stored overnight in –22°C for further processing.

Real Time PCR

Real time PCR was carried out with the use of TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems/Life Technologies) according to the manufacturer's protocols, on Realplex 2 cycler (Eppendorf). A single reaction tube contained 1 ul of TaqMan Gene Expression Assays, 9ul of cDNA template, 10 µl of TaqMan Gene Expression Master Mix. Target genes were *BMP-2, BMP-4, BMP-6*. Reference gene was HPRT (hypoxanthine-guanine phosophoribosyltransferase). 40 cycles were performed, each including a 15 s phase at 95°C and 1 min phase at 60°C.

Results and Discussion

Clinical examination showed total wound healing in all patients 6 months after surgery. There are no differences in the gingival profile after the application of different grafting materials. A radiological and histological study will be publishing soon. During the next stage of implant therapy, all bone preparation was similar, the differences in hardest of bone core during bone microbiopsy by the trephine were not noticed. Implants were installed in augmented sites with good primary stabilization in all patients.

PCR Results

According to the manufacturer's instructions, CT values above 40 were considered as negative, i.e. no amplification of cDNA could be confirmed.

Analysis of detection pattern of BMPs RNA after implantation of guided bone regeneration materials revealed (Table 1, Figs. 3–5):

The expression of BMP-2 and BMP-4 genes was higher than the expression of the house-keeping gene HPRT in case of both applied materials: bovine mineral, BTCP as well as the control, i.e. orthotopic autogenous bone.

The expression of BMP-6 gene was in all cases lower than the expression of HPRT and target genes for BMP-2,4, reaching values on the edge of detectability.

BMP-2 RNA amount was comparable in case of orthotopic autogenous bone and bone formed after bovine mineral application; its expression was significantly lower in case of the use of BCTP as an osteoconductive scaffold.

The expression of BMP-4 RNA was two times higher in autogenous bone in comparison to bone formed after application of BTCP and bovine mineral.

Allogenic bone granules, characterised by minimal osteogenic properties, were used with synthetic (BTCP) or natural (bovine mineral) implanted materials for promotion of bone formation.

The results achieved indicate that in humans:

Bovine mineral seems to be more effective than BTCP in bone inducing properties through synthesis of BMP-2, the main osteogenic growth factor in human jaw bone augmentation methodology

BMP isoform tests revealed significant concentration of BMP-2, lower expression of BMP-4 and trace amounts or no presence of BMP-6 mRNA in all induced bone samples

BTCP has shown low biological efficacy in the bone augmentation process, which occur through the inducing of expression of BMPs isoforms in the environment of orthotopic autogenic jaw bone as well allogenic bone granules. This observation could be explained by BTCP properties i.e. fast resorbability.

The role of BMP-2 and BMP-4 in osteogenesis was confirmed, while BMP-6 isoform seems not to be involved in bone regeneration

Both implanted scaffolds: synthetic BTCP and natural bovine mineral are well described as osteoconductive materials with no osteoinductive properties. However, when implanted with allogenic bone granules into the autogenic jaw bone

Table. 1. Ct values observed in tissues obtained after application of bovine mineral and BTCP and control material – autogenous bone (minus values of the ΔCt indicate an expression lower than the expression of the reference gene)

Tabela 1. Wartości Ct zaobserwowane w tkankach pozyskanych po zastosowaniu minerału bydlęcego, BTCP (beta trójfosforanu wapnia) i materiału kontrolnego – kości autologicznej (wartości ujemne Ct świadczą o ekspresji większej od ekspresji genu referencyjnego)

	Average Ct				ΔCt		
Type of material (Rodzaj materiału)	BMP2	BMP4	BMP6	HPRT	BMP2	BMP4	BMP6
Autogenous bone (Kość autologiczna)	34,0	33,3	36,5	36,3	–2,3	–3,0	0,2
Bovine/xenogenous mineral (Minerał bydlęcy)	36,0	37,0	41,0	38,5	–2,5	–1,5	2,5
BTCP (Beta trójfosforan wapnia)	33,5	33,0	40,0	34,5	–1,0	–1,5	5,5

site – can improve not only bone shape/profile, but also cascade of new bone formation through osteogenic growth factors.

The importance of BMPs for the process of osteogenesis was proved in several animal studies. Heterotopic bone formation in an animal model can be induced by the expression of BMP-2, 4 and acts on local stem cells present in the muscle tissue, out of periosteum [20].

BMPs are a morphogen in the following processes: embryonic bone development, skeletal growth, bone remodeling, bone fracture healing as well as bone graft remodeling. The number of local stem cells for osteogenesis that respond to BMPs is not known, the application alone of 1 million human stem cells per graft for the treatment of bone defect – is not effective [3]. It seems that also local BMPs concentration plays an important role. The golden standard, i.e. autologous bone implantation is the best type of bone graft but in that case an additional donor site is needed. Allogenic bone, from a tissue bank, has very low osteoinductive properties, and the concentration of BMPs is estimated in picograms. The application of a combine graft: allogenic bone with bovine mineral or with BTCP still is advantageous as it filled the de-

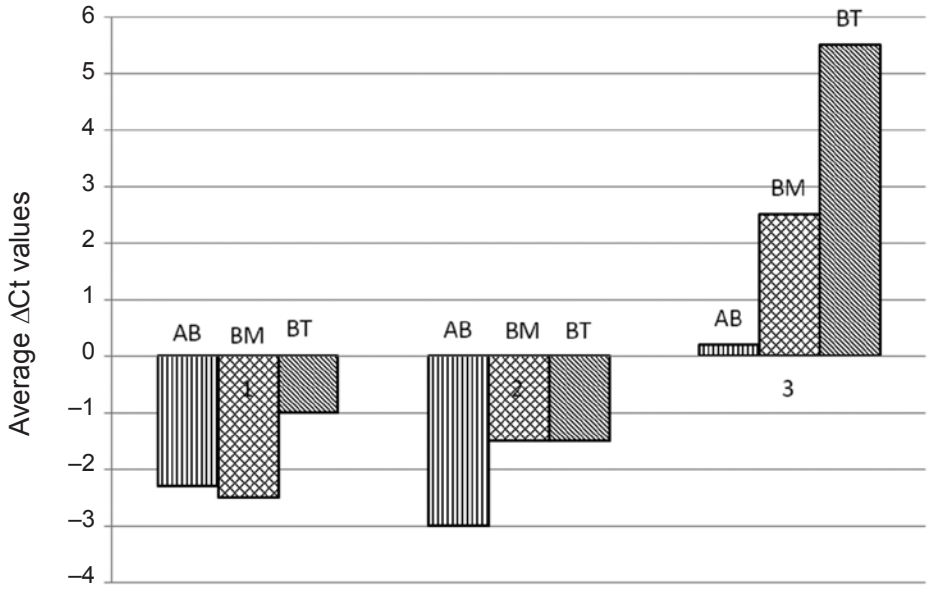

Fig. 3. Average ΔCt values as determined by real-time PCR by subtracting the average HPRT Ct value from the average Ct for each target gene. Series: 1 – BMP2, 2 – BMP4, 3 – BMP6; AB – autogenous bone, BM – bovine mineral, BT – BTCP (minus values of the ΔCt indicate an expression higher than the expression of the reference gene)

Ryc. 3. Średnie wartości ΔCt wyliczone z wartości obserwowanych w *real-time* PCR, uzyskane przez odjęcie średniego Ct dla genu HPRT od średniej wartości Ct dla każdego genu badanego. Serie: 1 – BMP2, 2 – BMP4, 3 – BMP6; AB – kość autologiczna, BM – minerał bydlęcy, BT – BTCP, (beta-trójfosforan wapnia, wartości ujemne Ct świadczą o ekspresji wyższej od ekspresji genu referencyjnego)

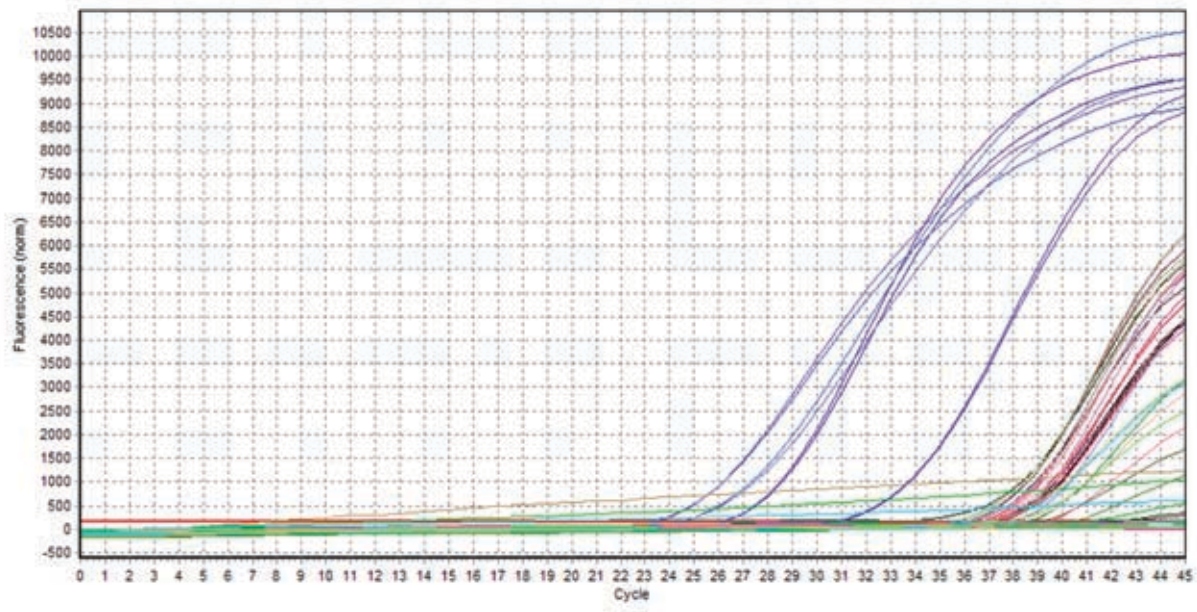

Fig. 4. Collective diagram. cDNA amplification for BMP-2, BMP-4, BMP-6 (target genes) and HPRT (reference gene) sequence. The study samples underwent 45 PCR cycles. Detection (Ct) above 40 indicates a number of present transcripts on the verge of detectability

Ryc. 4. Diagram zbiorczy. Amplifikacja cDNA dla genów badanych BMP-2, BMP-4, BMP-6 oraz genu referencyjnego HPRT. Badane próbki podlegały 45 cyklom PCR. Detekcja (Ct) powyżej 40. cyklu jest traktowana jako graniczna, nie potwierdza obecności badanej sekwencji

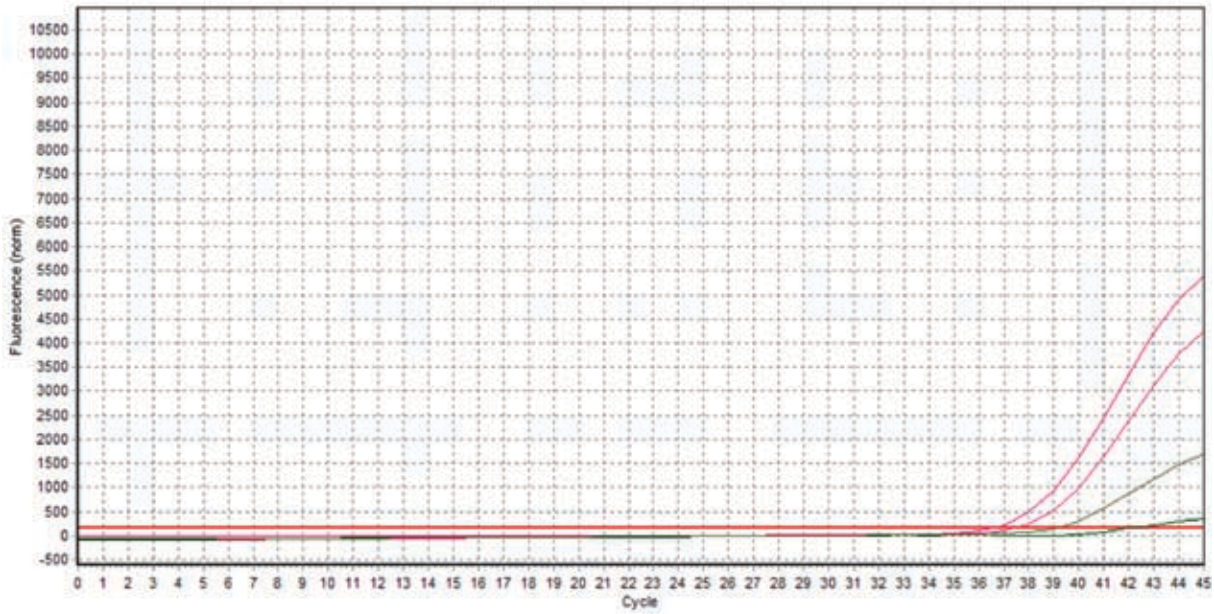

Fig. 5. A typical result from amplification observed in the study BMP-2 and BMP-4 transcripts detectable, BMP-6 transcripts undetectable

Ryc. 5. Typowy wynik amplifikacji obserwowany w badaniu. Transkrypty BMP-2, BMP-4 oznaczalne, BMP-6 nieoznaczalne

fect better within bone spaces than soft, gingival tissue. That type of a graft can induce wound healing in a faster and biologically more effective manner. It seems that BTCP undergoes a fast degradation, a process that does not take place normally in a newly formed bone. It is possible that the surface of BTCP granules does not attract cells/stem cells as opposed to natural bovine bone mineral which shows better cell adhesion and spreading properties. The degradation of BTCP may not in-

volve the activity macrophages/monocytes osteoclastic cell line and hence BMP-6 pathways that are hypothetically co-dependent on the presence of macrophages/monocytes osteoclastic cell line in the site of tissue remodeling. It seems that the degradation of BTCP can proceed via simple dissolution and without or with a poor activation of macrophages. Author hypothesis is that BMP-6 can interfere in bone resorption. The clinical and radiological (not publish yet) results of authors study confirm the observation that the bovine mineral is refractory to resorption. The role of BMP-6 in bone metabolism is still not clear; BMP-6 was not expressed significantly in the human skeleton [21]. The biological and genomic bases for creeping substitution of the allogenic, xenogenic and alloplastic grafts are different. It seems that their combination allows for better perspectives for oral, regenerative medicine [22–23].

The authors concluded that though the application of BMPs is successful in orthopaedic and maxillofacial surgery, human recombinant BMPs are still not available for the dentists. An alternative source of BMPs is autologous bone, considered as a golden standard – this, however, applied seldom, rather in maxillofacial cases. The combination of allogenic bone from a tissue bank with alloplastic or xenogenic materials, especially long-resorbed materials is an effective method for bone augmentation in small/dental bone defects.

The project was approved by Bioethical Committee WUM.

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