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Effect of cyclic 3´, 5´ adenosin-monophosphate on osteogenesis embryonic bone cultivated in tissue culture: An autoradiographic study

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SUMMARY

The aim of this study was to evaluate the effect of cyclic 3´,5´adenosin-monophosphate (cAMP) on DNA synthesis of embryonic alveolar bone in tissue culture. Material and methods. Bone fragments were cultured in the medium composed of 80% medium 199, 15% horse serum, 4 mg/ml glucose, 100 μg/ml penicillin using the grid method.The explants were cultured up to 12 days. In the second series, the effect of cAMP in a concentration of 10^{-6} M on bone during 12 days was studied. DNA synthesis was determined by calculating mitotic labelling indices for ^{3}H-thymidine incorporation into cells within cultured explants at 3 to 12 days. The medium was supplemented with 1 microCurie/ml ^{3}H-thymidine for 4 hours and processed for autoradiography. The mitotic labelling index was determined in the histological sections. All values wer presented as mean±standard deviation. Statistical significance was defined by p-values of 0,05 or less. Results. Morphological and statistical analysis showed that there were differences in mitotic incidence (MI) and silver grain densities over osteoblasts in control cultures and with cAMP. The mean value of MI was 4,627±1,001 in control and 7,706±1,188 in the cultures where cAMP was added ($p<0,05$). Conclusion. Thus cAMP inhibited bone resorption and stimulated new bone formation in tissue culture. This study provides a novel concept that may help to identify new therapeutic targets.

Key words: autoradiography, cAMP, osteogenesis, tissue culture.

INTRODUCTION

Recent developments in bone cell biology have greatly changed our conceptions of the regulatory mechanisms of the differentiation of osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation [1]. The studies have shown that lipopolysacharide and inflammatory cytokines such as tumor necrosis factor receptor-alpha and interleukin-1 directly regulate osteoclast differentiation and function [2]. These several findings have opened new areas for exploring the molecular mechanisms of osteoblast and osteoclast differentiation. Transcription factors, Runx2 and Osterix, are found to be essential molecules for inducing osteoblast differentiation [3]. Bone is continuously destroyed and reformed to maintain constant bone volume and homeostasis vertebrates throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively [4].

The differentiation of uncommitted mesenchymal cells into osteoblasts is a fundamental molecular event covering both embryonic development and bone repair. The bone morphogenetic proteins (BMPs) are important regulators of this process [1]. Core binding factor alpha-1 (Cbfa1) is an essential transcriptional regulator of osteoblast differentiation and bone formation, and this process is positively or negatively regulated by a variety of coactivators and corepressors [1]. During the past years, our molecular understanding of osteoblast biology has made rapid progress due to the characterization of the function of one molecule, Cbfa1. Cbfa1 was then shown to regulate the expression of all the major genes expressed by osteoblasts [5]. Consistent with this ability, genetic experiments identified Cbfa1 as a key

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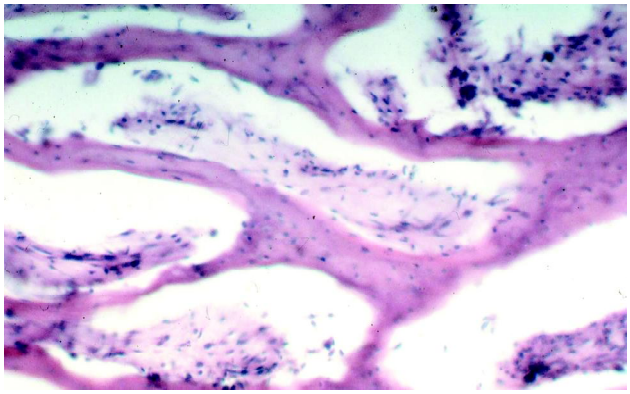

Fig. 1. Control culture of alveolar bone on the 3rd day. Haematoxylin & eosin x 200

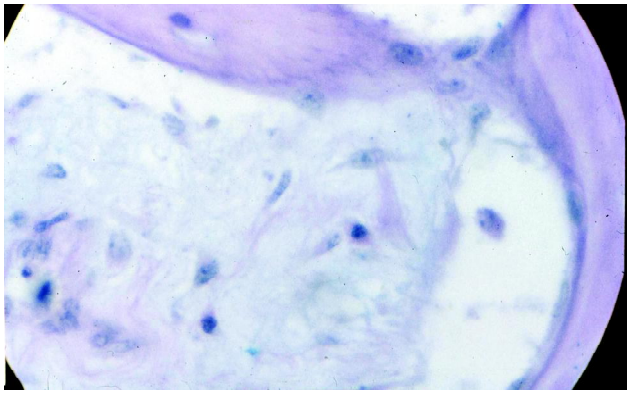

Fig. 2. Control culture of alveolar bone on the 9th day showing continuing resorption of bone and formation of new trabecula of osteoid tissue. Osteoblasts are present in alveolar bone. Haematoxylin & eosin x 200.

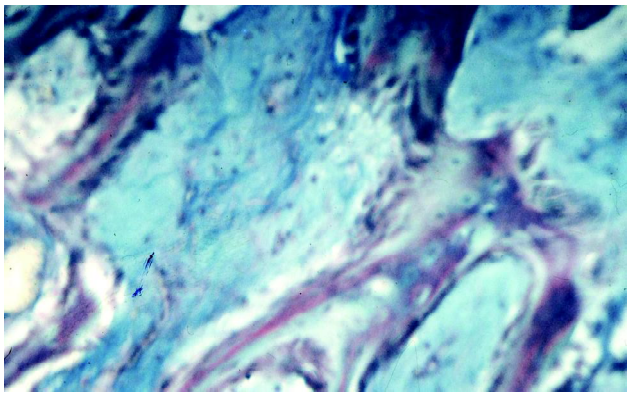

Fig. 3. Control culture of alveolar bone on the 12th day showing newly formed bone surrounded by osteoblasts. Neutral glucosoaminoglucans in mesenchymal cells are in few regions, but quite a lot in preosteoblasts. Stain by Mowry x 200.

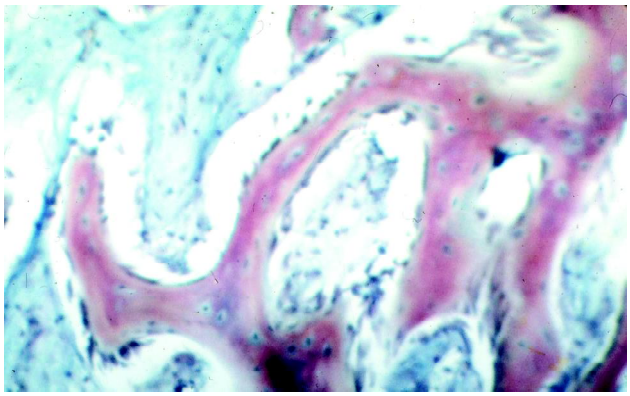

Fig. 4. Alveolar bone culture with cAMP in concentration 10^{-6}M on the 6th day revealed the presence of new osteoid. Osteoblasts are present. The contents of neutral glucosoaminoglucans was higher after cAMP administration. Stain by Mowry x 200.

regulator of osteoblast differentiation *in vivo*. Indeed, analysis of Cbfa1-deficient mice revealed that osteoblast differentiation is arrested in absence of Cbfa1 [6].

Many local and systemic factors have been shown to influence bone resorption and bone formation in tissue culture and could affect bone metabolism *in vivo* [7]. Bone tissue is one of the most active metabolic tissues, capable of exchanging its chemical constituents in a very short time. Our previuos studies have shown that cyclic 3′,5′ adenosin-monophosphate (cAMP) stimulates formation of the alveolar bone in tissue culture [8,9]. It was hypothesized that cAMP could influence proliferation and differentiation of osteoprogenitor cells, and give useful information on the tissue-engineered constructs designed for clinical application. Cyclic AMP stimulations in live cells under both serum-containing and serum-free environments are successfully monitored [10]. Several studies have shown that regulations of tissue blood cAMP levels may prove of therapeutic usefulness in regulating inflammation and healing of tissues after surgery or trauma and the cAMP levels in blood plasma or in tissues may be a markers, estimating the treatment response [11, 12]. Bone tissue engineering, which utilizes osteoprogenitors to form functional bone, offers a promising approach to the repair of bone defects (13)

The aim of this study was to evaluate the effect of cAMP on osteogenesis of embryonic alveolar bone *in vitro*.

MATERIALS AND METHODS

The method consisted of aseptically removing the alveolar bone of 16-17 days mouse embryos in a Hank´s solution. The millipore filter membranes THWP 0,45 μ porosity and 25±5 μ thick were used. The observations on alveolar bone cultures based on a total of 96 culture specimens (48 control and 48 cultures with cAMP). The alveolar bone fragments (1 x 2 mm^3) were placed on the surface of millipore filter resting on a stainless grid so that the medium

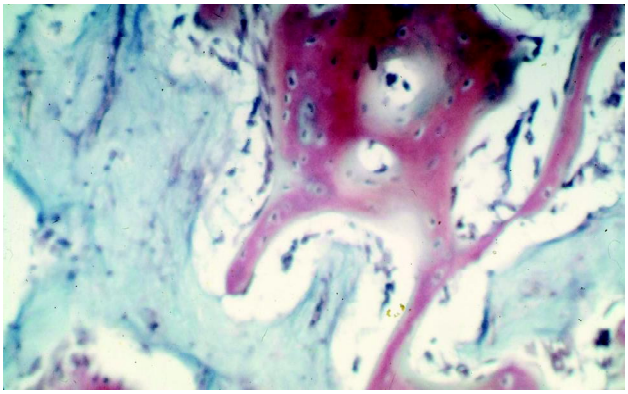

Fig. 5. Alveolar bone culture with cAMP on the 12th day. Note remnants of original bone and new osteoid in variuos stages of formation. Newly formed bone is surrounded by osteoblasts, increase in osteoblasts and mature new bone. Stain by Mowry x 200.

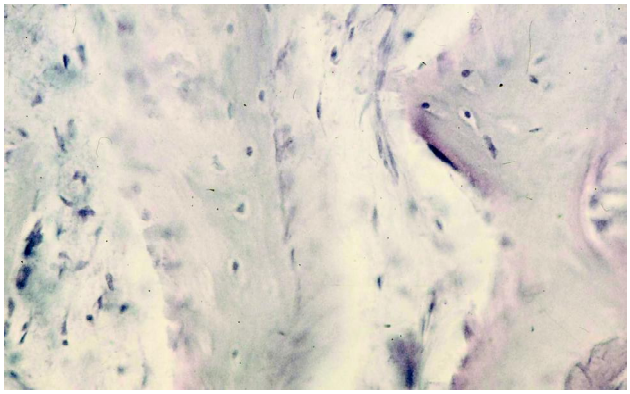

Fig. 6. Alveolar bone culture with cAMP on the 12th day. Osteoblasts with basophilic cytoplasmatic nuclei are situated excentrically, osteocytes are situated in the matrix. Stain by Feulgen x 200.

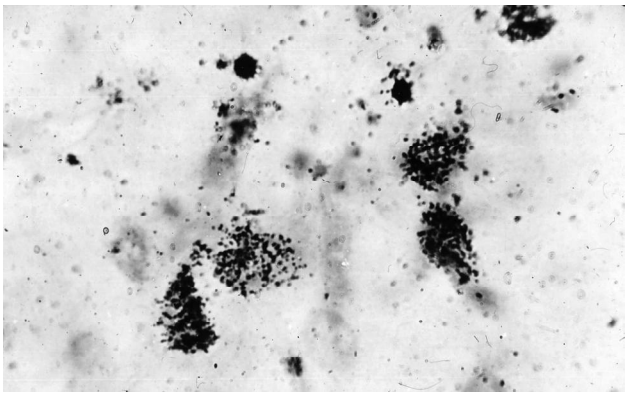

Fig. 7. ^{3}H-thymidine incorporation into osteoblasts nuclei on the 3rd day in control tissue culture. Original magnification: x 1000

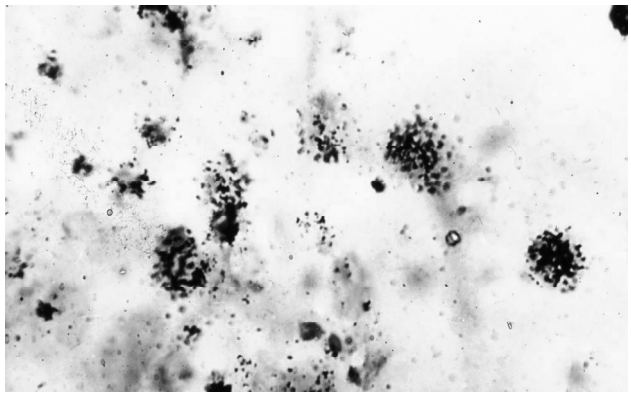

Fig. 8. ^{3}H- thymidine incorporation into osteoblasts nuclei on the 3rd day in tissue culture with cAMP 10^{-6}M. Original magnification: x 1000.

filled the culture vessel (Petri dish) to the surface of the screen. The medium composed of 80 % medium 199 (GIBCO), supplemented with 15 % horse serum, 4 mg/ml glucose, 100 unit/ml penicillin and streptomycin was also added. All the cultures were gassed briefly with a mixture of 50% O_2 and 50% N_2 in a heat-sterilized incubator (Binder GmBH, Germany) at 37º C. The medium was changed after 48-72 hours. The pH was maintained at 7,6. Cultivation was terminated up to 12 days. In the experimental series, the effect of cAMP in a concentration of 10^{-6} M on alveolar bone during 12 days was studied. Every day 4 control bone culture specimens and 4 bone culture specimens with cAMP were removed from the grids and fixed by Bouin. The paraffin-embedded specimens were serially sectioned at a thickness of 5 μ and stained with haematoxylin and eosin and to detect mineralization by Mowry and examined with the light microscope. The cell proliferation rate was determined using a ^{3}H-thymidine assay.

DNA synthesis was determined by calculating the mitotic labeling indices for ^{3}H-thymidine incorporation into cells within cultured explants at 2 to 12 days in control and in cultures with cAMP. Medium of explants was supplemented with 1 μCurie/ml ^{3}H-thymidine for 4 hours and processed for autoradiography. For light microscope radiography 5 μ sections were mounted on glass slides, stained with iron haematoxylin, coated with type M emulsion and exposed in amydol for 3 minutes at 18º C and fixed for 3 min in 24 % sodium thiosulphate.

A cell was to be labeled if ten or more silver grains were localised over the nucleus.Total number of cells were counted, and data were expressed as precentage of cells labeled with tritiated thymidine per time period in tissue culture. The mitoses were counted using a light microscope with a 40x objective and 8x ocular lens, the latter furnished with a grid which at this magnification delineated an area of 312,5 x 312,5 μm in the section. The total number of nucleated cells and the number. of metaphase figures were counted within each area covered by grid. Means and standard deviations of the mitotic indices were calculated for the each group. A metaphase figure was defined as a nucleus with a closely clumped

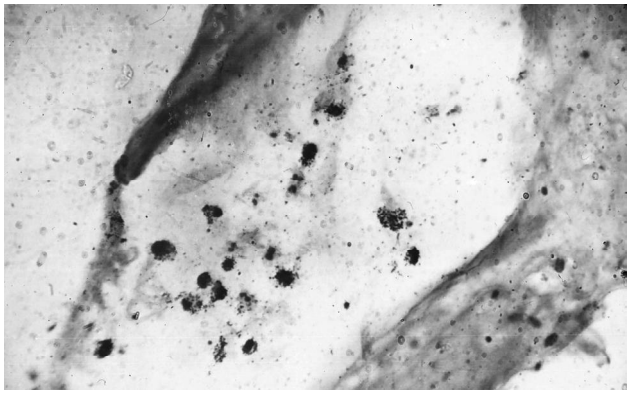

Fig. 9. ^{3}H- thymidine incorporation into osteoblasts nuclei on 12th day in control culture. Original magnification: x 400.

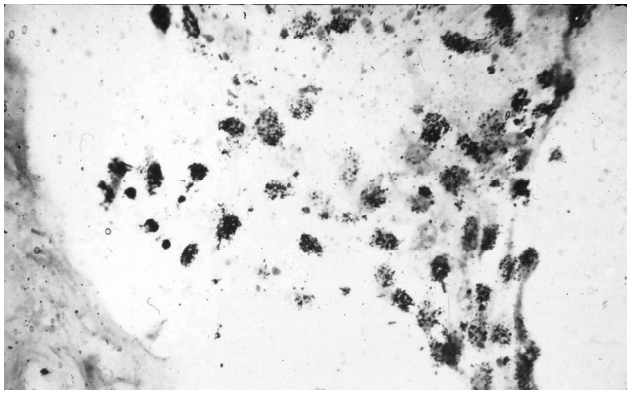

Fig. 10. ^{3}H- thymidine incorporation into osteoblasts nuclei on the 12th day in tissue culture with cAMP 10^{-6}M. Original magnification: x 400.

chromatin mass having an irregular periphery. Mitotic indices expressing the number of metaphases per 1000 cells were calculated separately in control and in cultures with cAMP: The rate of resorption and formation of bone were determined by ocularmicrometer.

The silver grain densities over the osteoblasts and mitotic incidence in control culture and in culture where cAMP was added were calculated and analysed among the groups by Student´s t-test. All values were presented as mean±standard deviation. Statistical significance was defined by p-values of 0,05 or less. Data were the means of four cultures performed under identical conditions.

The study was approved by the Ethical Committee of the Faculty of Medicine, University of Tartu (protocol nr. 73/6 from 21.06.1999).

RESULTS

Alveolar bone resorption was first noted in control culture at about 2 to 3 days (Fig. 1). The resorption continued and proliferation of osteogenous cells started on the 9th day where the new trabeculae have formed in the middle part (Fig. 2). The 12th day revealed the presence of new osteoid, which in some region took on basophilic staining characteristics associated with calcification (Fig. 3).

The addition of cAMP in concentration 10^{-6} M in the medium stimulated bone formation. New osteoid formation was noted earlier on the 6th day and presence of new osteoid partially calcified was observed (Fig. 4). On the 12th day newly formed bone is surrounded by osteoblasts in culture. Remnants of original bone and new osteoid in various stages of mineralization is observed. Newly formed bone is surrounded by osteoblasts. New bone is visible which in some region took on the basophilic staining characteristics associated with calcification (Fig. 5). Alveolar bone culture with cAMP on the 12th day showed more active increase of osteoblasts and new bone formation Osteoblasts with basophilic cytoplasmatic nuclei are situated excentrically, osteocytes are situated in the matrix (Fig. 6).

By determining the ^{3}H-thymidine incorporation we were able to show that the number of DNA synthezising cells and proliferation activity is being increased. Counts showed that the density of silver

Table 1. Silver grain densities over osteoblasts in control cultures and cultures with cAMP in concentration 10^{-6}M

Days after ^{3}H-thymidine administration	Silver grains over osteoblasts in the control culture, Mean±SD	Silver grains over osteoblasts in the culture with cAMP, Mean±SD
2	7,680±3,024*	9,280±2,301*
3	10,080±2,515*	12,400±2,062*
4	24,800±2,677**	43,240±3,218**
5	39,760±5,126*	45,040±5,504*
6	34,400±3.069**	49,600±2,858**
7	38,320±4,250**	51,760±3,467**
8	37,800±2,915**	50,600±2,327**
9	36.080±3,190**	50,360±2,119**
10	38,480±2,190**	48,480±2,044**
11	40,860±3,116**	49,600±2,857**
12	40,218±2,102**	48,587±2,012**

*P>0,05 when compared to the same-day control culture and culture with cAMP.
**P< 0,05 when compared to the same-day control culture and culture with cAMP.

grains over osteoblasts was significantly lower of the 3rd day control (Fig. 7) than that of the 3rd day cultures with cAMP (Fig. 8). There were no significant differences when compared silver grain density over the osteoblasts to the same-day control culture and culture with cAMP on the 2nd, 3rd and 5th days ($p>0,05$), while there were statistically significant differences in silver grain density over odontoblasts from the 6th day. Counts of ^{3}H-thymidine incorporation into osteoblasts on the 12th day showed that the density of silver grains over osteoblasts was significantly higher of the 12th day cultures with cAMP (Fig. 10) than that of the 12th day control (Fig. 9). Newly formed bone surrounded by osteoblasts, increase in osteoblasts and mature new bone was observed. Silver grain densities over osteoblasts in control cultures and in cultures with cAMP in concentration 10^{-6} M are given in the Table 1.

Statistical analysis showed that there were differences in mitotic incidence (MI) of osteogenous cells in control cultures and with cAMP during 12 days in vitro (Table 2). The mean value of MI in osteogenous cells was 4,627±1,001 in control and 7,706±1,188 in cultures where cAMP was added ($P<0,05$).

The results of this study provides a novel concept that may help to identify new therapeutic targets.

DISCUSSION

The advantage of tissue culture is that drug concentration as well as the duration of exposure can be controlled. From the findings of this investigation, it can be concluded that cAMP have stimulated bone formation in tissue culture. Observation of bone cultures revealed that the gap between the remaining portions of the original bone was essentially bridged.

The results confirm a previous study [14] which support mesenchymal cell development into an osteoblastic lineage *in vitro* and when implanted *in vivo*. Bone cell proliferation was examined in embryonic alveolar bone tissue derived osteoblasts by the combined use of autoradiography, histochemistry etc. The developmental sequence has three principle periods – proliferation, extracellular matrix maturation, and mineralisation. Initially, actively proliferating cells, expressing cell cycle- and cell growth-regulated genes, produce a fibronectin/ type I collagen extracellular matrix. A reciprocal and functionally coupled relationship between the decline in proliferative activity and the subsequent induction of genes associated with matrix maturation and mineralization is supported by a temporal sequence of events in which there is an enhanced expression of alkaline phosphatase immediately following the proliferative period, and later, an increased expression of osteocalcin and osteopontin at the onset of mineralisation. Tthe extracellular matrix contributes to both the shutdown of proliferation and the development of the osteoblast phenotype [15, 16].

In our study cAMP showed improved osteogenous cell growth in vitro having an effect on cell differentiation and bone tissue formation. Thus the bone tissue engineering in culture offers a promising approach to the repair of bone defects.

It is stated that calcitonin is a preparation which activates adenylylcyclase and for the result the level of cAMP is increasing in the cell [17]. We hypothesized that decreased intracellular calcium acts by activating calcium-inhibitable isoforms of adenylyl cyclase, increasing cAMP, and stimulating thus osteogenesis [9]. The increased bone formation could be partially explained by the elevated mineralization ability of osteoblasts [13]. Decreased intracellular calcium stimulates adenylylcyclase, resulting in cAMP synthesis. Endothelial cells of blood vessels are one of the sources of osteogenous cells. Cyclic AMP

Table 2. Mitotic activity of osteogenous cells in the control culture and culture with cAMP in concentration 10^{-6} M

Days after 3H-thymidine administration	Mitotic incidence in osteogenous cells control, Mean ±SD	Mitotic incidence in osteogenous cells with cAMP 10^{-6}M, Mean ± SD
1	6,133 ± 1,196	6,345 ± 1,289
2	7,433 ± 1,194	7,552 ± 1,682
3	6,533 ± 1,106	8,200 ± 1,324
4	5,267 ± 1,081*	9,567 ± 1,305*
5	4,500 ± 0,974*	10,100 ± 1,242*
6	3,900 ± 0,923*	10,700 ± 1,264*
7	3,633 ± 0,809*	9,300 ± 1,343*
8	3,200 ± 0,761*	9,933 ± 1,230*
9	2,400 ± 1,133*	8,467 ± 1,137*
10	3,367 ± 0,718*	7,367 ± 1,542*
11	3,633 ± 0,809	5,533 ± 1,137
12	3,833 ± 0,791	4,400 ± 1,070

*P< 0.05 when compared to the same-day control culture and culture with cAMP

reduce oxidative stress and subsequently improve vascular endothelial dysfunction [18, 19].

It was shown that cAMP have a role beyond development and differentiation, to regulate the rate of bone matrix deposition by differentiated osteoblasts. Thus cAMP is an important second „messenger" intracellular mediator not only for osteoblast differentiation but also osteoblast function.

Based on this study, we can conclude that cAMP could influence proliferation and differentiation of osteoprogenitor cells, and give useful cell biology-related information on the bone tissue for clinical application.

CONCLUSION

Nevertheless, a direct stimulating effect of cAMP on the proliferation of osteoblasts is possible. Thus cAMP inhibited alveolar bone resorption and stimulated new bone formation in tissue culture.

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REFERENCES

1. Liu CJ, Chang E, Yu J, Carlson CS, Prazak L, Yu XP, et al. The interferon-inducible p204 protein acts as a transcriptional coactivator of Cbfa1 and enchances osteoblast differentiation. *J Biol Chem* 2005;280:2788-96.
2. Katagiri T, Takahashi N, Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* 2002;8:147-59.
3. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is requiered for osteoblast differentiation and bone formation. *Cell* 2002;108:17-29.
4. Schantz JT, Teoh SH, LimTC, Endres M, Lam CX, Hutmacher DW. Repair of calvarial defects with customized tissue-engineered bone grafts osteogenesis in a three – dimensional culture system. *Tissue Eng* 2003; Suppl.1:113-26.
5. Karsenty G. Role of Cbfa1 in osteoblast differentiation and function. *Semin Cell Dev Biol* 2000;11: 343-6.
6. Ducy P. Cbfa1: a molecular switch in osteoblast biology. *Dev Dyn* 2000;219: 461-71.
7. Ivanov VS, Leibur E. Effect of thyrocalcitonin and sodium fluoride on alveolar bone cultivated in vitro. *Quintessence Int Dent Dig* 1978;9:103-9.
8. Leibur E, Fedorov NA, Ivanov VS. [Effect of cyclic nucleotides on mitotic activity of tooth anlage and alveola.r bone in tissue culture]. *Biull Eksp Biol Med* 1985;99:607-9. Rus
9. Leibur E. [Action of cyclic nucleotides on alveolar bone and tooth germs in tissue culture]. Stomatologiia 1985;64:22-4. Rus
10. Tang Y, Li X, He J, Lu J, Diwu Z. Real-time and high throughput monitoring of cAMP in live cells using a fluorescent membrane potential-sensitive dye. *Assay Drug Dev Technol* 2006;4:461-71.
11. Grower MF, Chandler D, Kramer G, Stow JA. Effects of local anaesthesia on gingival cAMP levels. *J Periodontol* 1979;50:38-42.
12. Leibur E, Tääkre H, Žarkovski A.The role of cAMP in the pathogenesis of periodontal disease. In: Aktualnyje problemy v stomatologii. Moskva, 1989: 40-2. Rus.
13. Yefang Z, Hutmacher DW, Varawan SL, Meng LT. Comparison of human alveolar osteoblasts cultured on polymer-ceramic composite scaffolds and tissue culture plates. *Int J Oral Maxillofac Surg* 2007;36:137-45.
14. Lee YM, Seol YJ, Lim YT, Kim S, Han SB, Rhyu IC, et al. Tissue –engineered growth of bone by marrow cell transplantation using calcium metaphosphate matrices. *J Biomed Mater Res* 2001,54:216-23.
15. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 1990;143: 420-30.
16. Sencimen M, Aydintug YS, Ortakoglu K, Karslioglu Y, Gunhan O, Gunaydin Y. Histomorphometrical analysis of new bone obtained by distraction osteogenesis and osteogenesis by periosteal distraction in rabbits. *Int J Oral Maxillofac Surg* 2007;36:235-42.
17. Glowacki J, Altobelli D, Mulliken JB. Fate of mineralized and demineralized osseous implants in cranial defects. *Calcif Tissue Int* 1981,33:71-6.
18. Ortiz-Capisano MC, Ortiz PA, Harding P, Garvin JL, Beierwaltes WH. Decreased intracellular calcium stimulates renin release via calcium-inhibitable adenylyl cyclase. *Hypertension* 2006;49:45-9.
19. Shah DI, Singh M. Possible role of exogenous cAMP to improve vascular endothelial dysfunction in hypertensive rats. *Fundam Clin Pharmacol* 2006;20: 595-604.

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