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Original Article

The effect of AMP kinase activation on differentiation and maturation of osteoblast cultured on titanium plate

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KEYWORDS
5′ adenosine monophosphate-activated protein kinase;
Acadesine/AICA riboside;
Dental implant;
Osteoblast

Abstract *Background/purpose:* 5′ Adenosine monophosphate-activated protein kinase (AMPK) is known as an enzyme that maintains intracellular homeostasis and has various biological activity. The purpose of this study is evaluation effect of AMPK activation on implant prognosis.
Materials & methods: MC3T3-E1 osteoblast-like cells were cultured on titanium using a 24-well plate. The experimental group was divided into the following 3 groups: (1) the normal culture group (control group), (2) the osteogenic induction group, and (3) the osteogenic induction + AMPK activation group. The cell counts were measured; real-time PCR was used to assess the expression of ALP and Osterix as osteogenic related genes at Day 0,7,14 and 21 after experiments. Additionally, ALP activity and calcification were assessed.
Results: The results of the real-time PCR assessments revealed that the expression of ALP, which is a marker for the initial stages of calcification, was significantly increased by AMPK activation compared to the normal culture or osteogenic induction. A significant increase was also observed in the expression of Osterix, which is a marker for the later stages of calcification. Because significant increases were observed in ALP activity and calcification potential, this suggested that AMPK activation could elicit an increase in osteoblast calcification potential.

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Conclusion: AMPK activation promotes implant peripheral osteoblast differentiation and maturation and enhances calcification. Our results suggest that AMPK activation may help to maintain implant stability.

Introduction

Implants are already extensively used in the clinical setting in dentistry, and have become established as a prosthetic treatment option for tooth loss. Titanium implants achieve osseointegration, and therefore have a dramatically higher long-term success rate than previous implants.[1] Much research was conducted on modifying the surface properties of implants and those studies resulted in the high success rate for implants that we have today.[2–5] In addition, in order to tackle the problem of inadequate bone mass, various bone creation methods, such as bone guided regeneration and maxillary sinus floor elevation, have been proposed, and the indications for implant therapy have expanded.[6–8] Various other methods of improving the results achieved with implant therapy have also been attempted, including photofunctionalization to modify the implant surface and the use of inducing factors for bone regeneration such as BMP2, and the use of osteoblasts differentiated from mesenchymal cells derived from bone marrow and/or lipids.[9]

AMPK is known as an enzyme that maintains intracellular energy homeostasis: among other things, it promotes mitochondrial synthesis and is involved in energy production, and plays a central role in the intracellular uptake of glucose and lipids. It is known to be activated by diabetes medications, as well as under conditions in which the body is under strain, such as hypoxic stress or hypoglycemia.[10,11] Burkholder et al. reported that AMPK is activated in response to stress in skeletal muscle, and induces the regeneration and/or growth of injured muscle.[12] As far as the relationship between AMPK and osteoblasts is concerned, it has been reported in the results of multiple studies that osteoblast differentiation and maturation is promoted by the activation of AMPK.[13–17] Similar effects can be expected in the osteoblasts around the periphery of the implant, as well. In addition, the AMPK activator Acadesine/AICA riboside (AICAR) has been reported to regulate muscle mass by, for example, strengthening and/or preventing the atrophy of skeletal muscle mass.[18–20] Lijuan et al. reported that AICAR exhibits therapeutic efficacy in acute lymphoblastic leukemia.[21] Eric et al. reported that AICAR exhibits therapeutic efficacy in acute T-cell lymphoblastic leukemia.[22] AICAR is therefore expected to have various pharmacological effects, including the treatment of leukemia and increasing skeletal muscle mass. Fernanda et al. reported that research of drug treatments for osteoblasts in the periphery of implants revealed that the addition of melatonin to human osteoblasts (MG63) cultured on titanium disks promoted osteoblast growth and differentiation.[23] Kanazawa et al. reported that metformin activated the AMPK in osteoblasts (MC3T3-E1), resulting in ALP activation and an increase in calcification potential.[14] Xiao et al. reported that the AMPK in rat osteoblasts on titanium implants is activated by adiponectin in a diabetic environment, which results in the osteoblasts being activated.[24] However, extremely little research has been done to assess the direct effect of AICAR on the osteoblasts in the implant periphery. Therefore, in order to assess the potential for AMPK activity to strengthen implant bone binding, we investigated the effects of AMPK activation on osteoblasts on the implant surface. The aim of our research was to confirm whether or not AICAR has any effectiveness as a supplemental therapy for maintaining implant stability.

Materials and methods

Cell culturing

MC3T3-E1 mouse osteoblast-like cells were cultured on titanium plates in αMEM containing 1% penicillin-streptomycin and 10% bovine fetal serum (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) in 5% CO_2 at 37 °C. Titanium plates that were 10 mm by 10 mm and 1 mm thick (Nantoh Co., Ltd., Shizuoka, Japan) were used in this experiment. After it had been confirmed that the cells were growing confluently, the titanium plates were placed in the wells of 24-well plates. Each well was inoculated with cells at a density of 1.0×10^5 cells/l. After culturing for 48 h, the medium was replaced with a serum-free medium, and the cells were placed in a starved state for 24 h. The control group was cultured in a normal medium, and the osteogenic induction medium group (the OG group) was cultured in α-MEM containing 10% FBS, 1% penicillin-streptomycin, and 10 mMβ-glycerophosphate. The osteogenic induction medium + AMPK activation group (the OG + AICAR group) was cultured in the same medium as the osteogenic induction group, with AICAR (1 μM) added. During the experiment, the media were replaced twice a week.

Cell growth test

Cell growth was measured using cell counting kit-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan). In each group, cell counting kit-8 solution was added in an amount of 50 μL to each of the wells of the 24-well plates on Days 0, 7, 14, and 21, and the reactions were allowed to occur for 4 h at 37 °C in 5% CO_2. We transferred 100 μL of the eluate to a 96-well plate, and measured the absorbance at 405 nm using a multiplate reader (the 1420 Multilabel Counter ARVO MX; PerkinElmer, Waltham, MA, USA).

RNA isolation and measurement of gene expression levels by quantitative RT-PCR

RNA was totally extracted from the cells using Isogen (Nippon Gene, Toyama, Japan) for reverse transcription on Days 0, 7, 14, and 21 in each group. SuperScript II reverse transcriptase (Invitrogen, Waltham, MA, USA) was used to synthesize the cDNA. For the detection of mRNA expression, gene-specific primers were used based on the cDNA nucleotide sequence. QRT-PCR analysis of the target mRNA was performed using the SYBR Prime Script RT-PCR kit made by (Toyobo Co. Ltd., Osaka, Japan) and the ABI7500 RT-PCR system (Applied Biosystems, Waltham, MA, USA). The test was run for 40 cycles under the following reaction conditions: degeneration for 30 s at 95 °C and then 5 s at 95 °C, 10 s of annealing at 60 °C, and 35 s of elongation. Beta-actin was used as the internal standard for controlling variation in the amplitude associated with differences in the starting mRNA concentration. The cycle threshold (Ct) was defined as the fractional cycle count. The gene expression level was expressed relative to the beta-actin expression level, and the delta (delta Ct) method was used to calculate the fold change for each sample.

Assessment of alkali phosphatase activity

The TRACP & ALP Assay Kit (Takara Bio Inc., Shiga, Japan) was used to measure alkali phosphatase activity. The cells on the titanium plates in the 24-well plates were washed twice with a normal saline solution (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan), and 500 μL of a cell extraction solution (a normal saline solution containing 1% NP-40) was then added to each well. An alkaline phosphatase buffer solution was then added to each well in an amount of 50 μL, and allowed to react for 15—60 min at 37 °C. NaOH (0.5 M) was then added to each well in an amount of 50 μL, an amount equivalent to the substrate, and 100 μL of the eluate was transferred to a 96-well plate and the absorbance at 405 nm was measured using the 1420 Multilabel Counter ARVO MX (PerkinElmer). Samples were recovered from each of the control, OG, and OG + AICAR groups on Days 0, 7, 14, and 21.

Assessment of calcification potential

Alizarin red stain (Iwai Chemicals Company Ltd., Tokyo, Japan) was used for the calcification of the MC3T3-E1 cells. For each group, the media in the 24-well plates after culturing were removed on Days 0, 7, 14, and 21 and the cells were washed with PBS. The cells were fixed for 10 min in a neutral buffered formalin solution and then removed, after which an alizarin red solution was added in an amount of 0.5 mL/well and staining performed for 30 min at room temperature. The alizarin red solution was then removed, and the plates washed using purified water and observed under an optical microscope. After the photo image was obtained water was removed from the wells, a calcified nodule extraction solution was added in an amount of 0.5 mL/well, and the plate was agitated for 10 min to elute the stain. The eluate was transferred in an amount of 100 μL to a 96-well plate, and the absorbance was measured at 405 nm using 1420 Multilabel Counter ARVO MX (PerkinElmer).

Statistics

Values of real-time PCR, ALP activity and calcification potential was standardized number of cells at each time course of experiments. Data are expressed as the mean ± the standard error of the mean (SEM). Differences were analyzed using one-way analysis of variance (ANOVA). P values < 0.05 were considered significant.

Results

Assessment of the growth of MC3T3 cells on titanium plates

The cell counting kit-8 was used to measure the number of cells. No significant differences were found among the groups on Days 7 or 14. On Day 21, the numbers of cells were slightly decreased, but no significant differences among the groups were found (Fig. 1).

Analysis of expression levels of genes associated with osteoblast differentiation by real-time PCR

The expression levels of the genes of the MC3T3-E1 cells on the titanium plates were analyzed using real-time PCR. The ALP expression levels were significantly higher in the OG + AICAR group than in the other 2 groups on Day 7. No significant differences among the 3 groups were found subsequently, on Days 14 or 21, although the expression levels tended to be lower (Fig. 2, A). No significant differences among the 3 groups were found in the expression levels of Osterix on Days 7 or 14. On Day 21, the expression level in the OG + AICAR group was significantly higher than that in the other groups (Fig. 2, B).

Assessment of alkali phosphatase (ALP) activation

ALP activation in the OG + AICAR group was significantly higher than that in the other 2 groups on Day 7. On Day 14, ALP activity was significantly higher in the OG group than in the control group, and ALP activity was significantly higher in the OG + AICAR group than in either the control group or the OG group. On Day 21, ALP activity tended to have decreased in all of the groups, and no significant differences among the groups were found (Fig. 3).

Assessment of calcification potential

The cells on the titanium plates were stained using alizarin stain. The stain concentration increased with the passage of time for the cells in the OG group and the cells in the OG + AICAR group. The increase in the OG + AICAR group was particularly notable (Fig. 4, A). Alizarin dye quantification resulted in no significant differences being found among the groups on Day 7. On Days 14 and 21, a significant increase in the degree of calcification was observed in the OG group compared to the control group. On Days 14 and

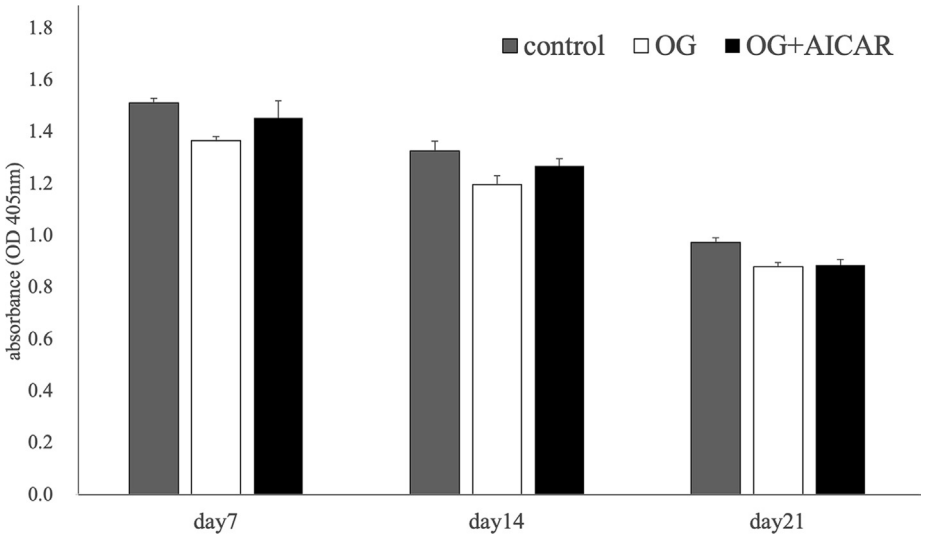

Figure 1 Cell proliferation assay of MC3T3-E1 cells cultured on titanium plate. Counting cells was performed with Cell Counting Kit-8 measuring 405 um absorbance at days 7, 14, and 21 after experiments in control group, OG group and OG + AICAR group. (*p < 0.05, compared to the control group).

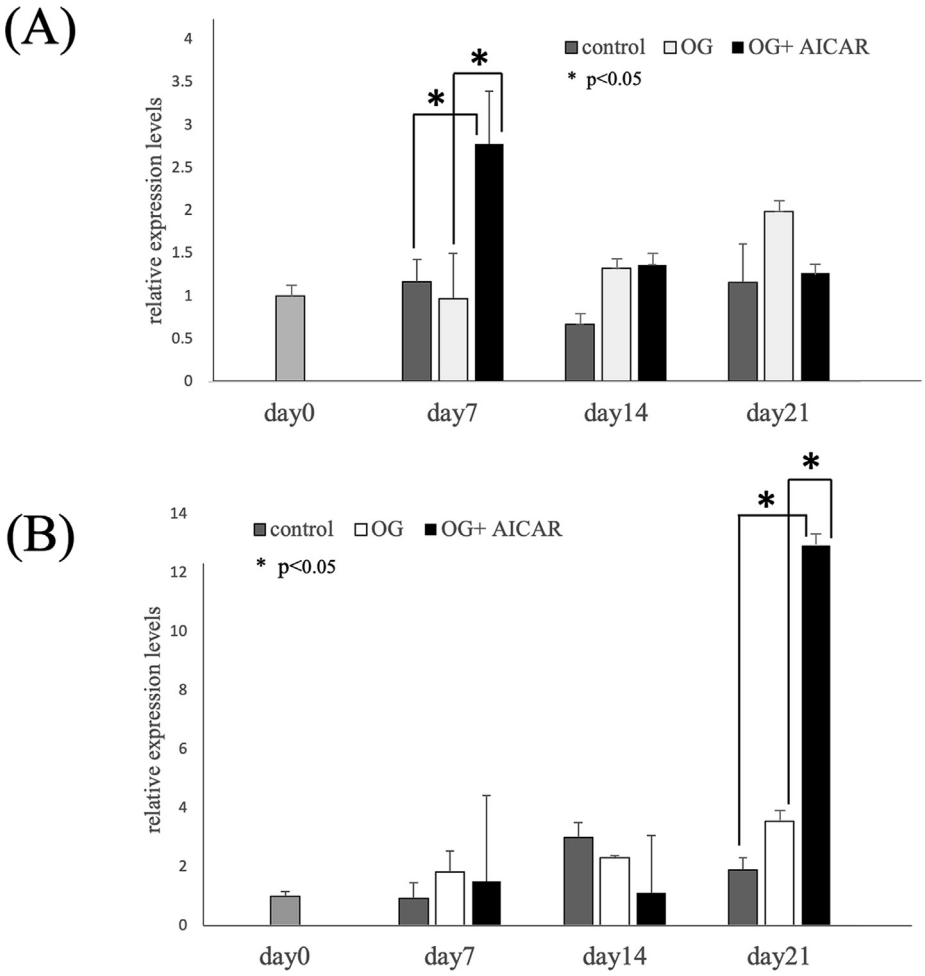

Figure 2 Evaluation of mRNA expression levels of ALP; Alkaline phosphatase and Osterix of MC3T3-E1 cells cultured on titanium plate was performed by real-time PCR at day 7, 14, and 21 after experiments in control group, OG group and OG + AICAR group. (A) ALP. (B) Osterix. (*p < 0.05, compared to the control group).

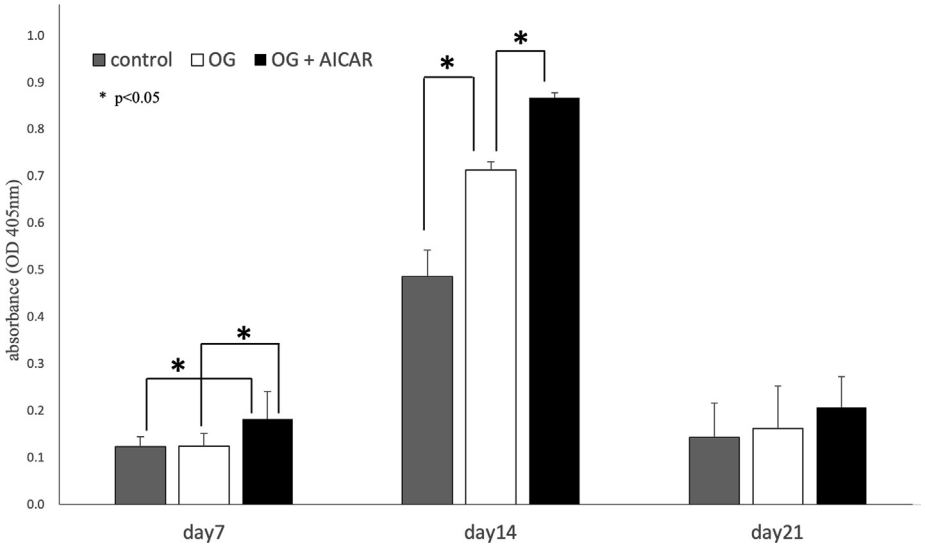

Figure 3 Evaluation of ALP activity of MC3T3-E1 cells cultured on titanium plate was performed using ALP Assay kit measuring 405um absorbance. Measurement was performed at day 7, 14, and 21 after experiments in control group, OG group and OG + AICAR group. (*$p < 0.05$, compared to the control group).

21, a significant increase in the degree of calcification was observed in the OG + AICAR group compared to the control and OG groups (Fig. 4, B).

Discussion

In this study, no significant difference was found in the cell growth number among any of the control, OG, and OG + AICAR groups (Fig. 1). Additionally, number of cells in three groups was decreased at day 21. That indicated cell proliferation of osteoblasts on titanium plate used in this study reached plateau at day 7, 14 and gradually decreasing afterwards. It was therefore learned that AICAR has no adverse effects on cell growth. In addition, the results of real-time PCR showed that the ALP expression level was significantly higher in the OG + AICAR group than in the other 2 groups at Day 7 (Fig. 2, A). Moreover, ALP activity, as well, was significantly increased in the OG + AICAR group compared to the other groups at Days 7 and 14 (Fig. 3). These results therefore suggested that AICAR-induced AMPK activation induces not only an increase in the expression of ALP, which is a marker of the initial stages of osteoblast differentiation, but also an increase in bioactivity. Real-time PCR found that Osterix expression was also significantly increased in the OG + AICAR group compared to the other 2 groups at Day 21 (Fig. 2, B). The calcification potential was significantly increased in the OG + AICAR group compared to the other 2 groups at Day 21, as well (Fig. 4, B). Because significant increases were observed in both the expression levels of a gene that is a late-stage marker of osteoblast differentiation and also the calcification potential, this suggests that AICAR-induced AMPK activation promotes the differentiation and maturation of osteoblasts in the implant periphery, and the activation of calcification although no crucial effect on cell proliferation. This in turn suggests that AICAR-based drug treatments could have an important function in maintaining the stability of the bone tissue of the implant. However, we need further exploration of precise mechanism of results from this study through analyzing undergo cell signaling pathway.

Multiple study reported activation of AMPK/mTOR signaling pathway facilitated osteoblast differentiation.[25,26] Won Gu et al. reported that AICAR-induced AMPK activation promoted the differentiation of osteoblasts (MC3T3-E1) via Smad1/5/8 phosphorylation.[16] Takeno et al. reported that BMP-2 phosphorylates AMPK and then Smadl1/5, promoting the induction of osteoblasts (MC3T3-E1) differentiation.[27] On contrary, Shar et al. reported that AICAR and metformin act to cause the phosphorylation of the Thr-172 residue of AMPK, resulting in activation of osteoblast (ROS 17/2.8: rat osteoblast-like cells) differentiation and maturation.[28] On the basis of these reports, activation of cell signaling pathway could be considered has variety depends on type of an AMPK-stimulating substance and cells used in experiments. Thus, further experiments comparatively investigate various AMPK-stimulating substances and/or other cell lines is needed. Moreover, we should also evaluate effect of AMPK inhibitor or silencing of target receptor in our further study.

Previous study has reported that AMPK activation results in autophagy.[17,28,29] Among them, Veldhuis-Vlug et al. reported AMPK activation facilitated autophagy and provoked osteobalstogenesis of the mesenchymal stem cells derived from bone marrow niche.[28] Zhou et al. reported activation of AMPK/mTOR signaling pathway induced differentiation of MC3T3-E1 cells.[29] AICAR-induced AMPK activation resulting in autophagy and osteoblast differentiation and maturation is therefore considered a powerful candidate as the mechanism that led to the results that were obtained in this study. However, because no research has been done on osteoblast culture on titanium plates in which autophagy caused by AMPK activation has been assessed, further experiments are needed.

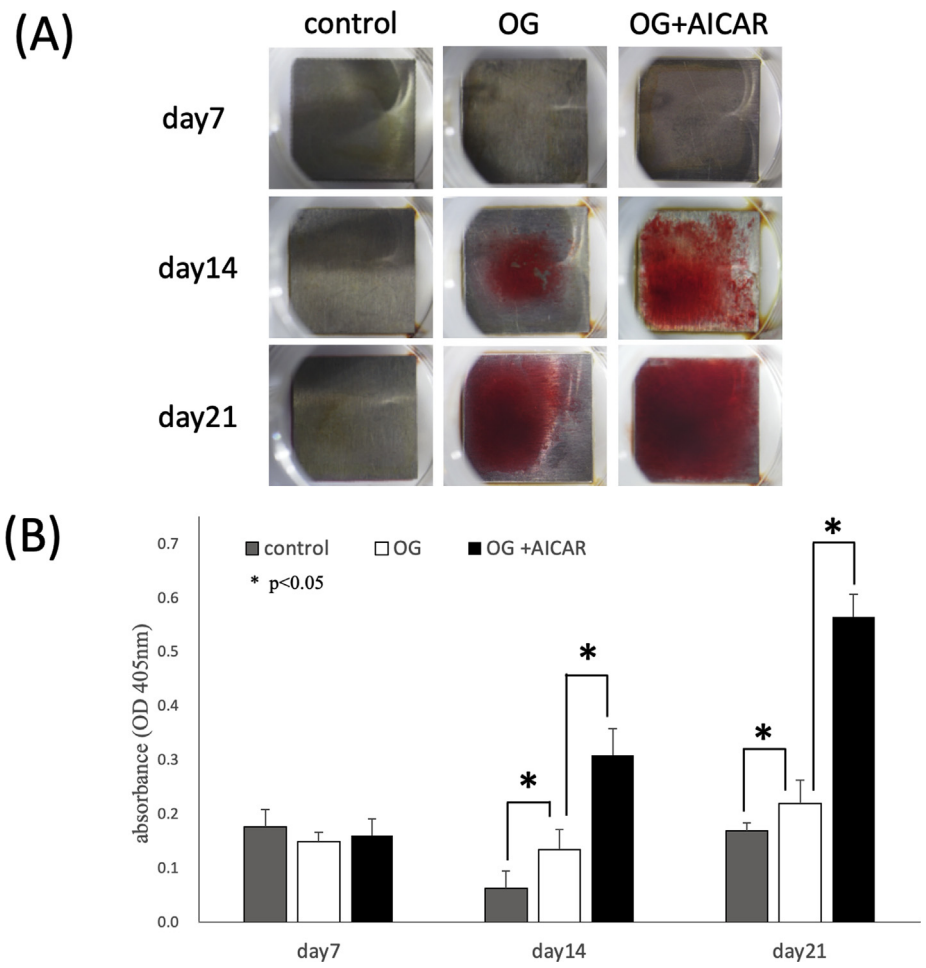

Figure 4 Mineralization of MC3T3-E1 cells cultured on titanium plate was evaluated using Alizarin red staining kit. (A) Representative image of MC3T3-E1 cells cultured on titanium plate stained by Alizarin red at day 7, 14, and 21 after experiments in control group, OG group and OG + AICAR group. Original magnification × 40.The height and width of titanium plate placed in center is 10 mm × 10 mm as mentioned above. (B) Evaluation of mineralization was performed measuring 405um absorbance at day 7, 14, and 21 after experiments in control group, OG group and OG + AICAR group. (*p < 0.05, compared to the control group).

It was hence suggested that AMPK activation promotes osteoblast differentiation and maturation in the implant periphery, and activates calcification from results in this study. This means AMPK activation could afford good implant stability, but further research is required to elucidate this mechanism in more detail.

Declaration of competing interest

The authors declare no conflict of interest in this study.

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