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Expression of VEGF and BMP-2 in Osteoblast cells exposed to a combination of polymethylmethacrylate (PMMA) and hydroxyapatite (HAp)

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ABSTRACT

Objectives: Polymethylmethacrylate (PMMA) has been widely used, but it has several fallback properties in its interaction with bone tissue, so the addition of hydroxyapatite (HAp) material aims to improve the biocompatibility, regeneration process, and osteointegration of bone implants. The HAp material can be sourced from bovine bone and processed through Good Manufacturing Practice from Tissue Bank (HApGMP), and from limestone (CaCO3) processed by Balai Besar Keramik (HApBBK).
This study was to observe the expression of vascular endothelial growth factor (VEGF) and Bone morphogenetic protein-2 (BMP2) in cultured osteoblasts exposed to PMMA-HApGMP and PMMA-HApBBK as implant candidate materials.
Methods: Sample of PMMA and HAp materials with a mixture of PMMA and HApBBK in the first group and a mixture of PMMA and HApGMP in the second group. Twenty-four fetal rat calvarie osteoblast cell cultures were randomly divided into 6 groups: 7- and 14-day control group, 7 and 14 days PMMA-HApGMP group, 7 and 14 days PMMA-HApBBK group. The expression of VEGF and BMP-2 was seen by immunocytochemical examination.
Results: The one-way ANOVA with a significance value of 0.000 ($p < 0.05$). BMP-2 and VEGF expression was increased in the 7- and 14-days groups after exposure to PMMA-HApGMP and PMMA-HApBBK.
Conclusion: The application of PMMA-HApGMP and PMMA-HApBBK showed an increase in the expression of VEGF and BMP-2 in osteoblast cell cultures which indicates a potential increase in the accelerated angiogenesis and osteogenesis in the bone regeneration process of bone implants.

1. Introduction

One of most common disease in oral cavity is a periodontal disease. Periodontitis, or periodontal tissue damage, occurs when gingivitis is left untreated leading to bone loss and destruction of the periodontal ligament which can cause tooth loss.[1] Moreover, aging is also non negligible factor associated with an increase in the number of missing teeth.[2]

The condition of tooth loss if not treated can result in impaired chewing function, esthetics and phonetics that will affect quality of life. The replacement of missing teeth in dentistry can be achieved with a variety of treatments and materials. The available treatments could include the installation of fixed dentures, partial or full dentures, and replacement with dental implants. Dental implants have made a major contribution to the world of dentistry because of their breakthrough in replacing missing teeth with a high success rate.[3] Besides that, oral implants provide good retention of dentures, have natural teeth-like characteristics, and are aesthetically pleasing and comfortable. For edentulous patients, dental implants would also improve their mental status and quality of life.[4]

The material choice for a particular implant application will be a general consideration to fill a lot of different functions that are

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specifically required in the fabrication of an implant design. Despite the diversity of needs for the creation of an implant, one aspect that is always a major concern is the tissue response around the implant area as a result of its contact with the implant materials. This aspect holds a key role because at the beginning of the installation, the implant will be identified as foreign material by the tissue.[5]

A biocompatible material is needed in the manufacture of implants. The role of biomaterials as implant material needs to be understood regarding the development of implants in the future because of their biocompatibility properties. The definition of a biomaterial itself is a material that is compatible with living tissue. The physical property of the material, its surface, tissue induction, and its potential to induce an inflammatory response or tissue rejection are important factors in the interaction process between the material and the tissue. Biomaterials are divided into two groups, based on their chemical composition (eg. metals, ceramics), and their biological activity (biological responses of tissues to the materials: bio-tolerant, bioinert, bioactive).[6]

One of the materials that have been widely used as a substitute for bone implants is Polymethylmethacrylate (PMMA). PMMA is a very well-known ceramic material in the medical field as a bone replacement material.[7] PMMA has several properties that are less than optimal in its interaction with bone tissue, so the addition of other ingredients such as hydroxyapatite (HAp) aims to improve biocompatibility, regeneration process, and osteointegration as a bone implant. Thus, it is expected that PMMA with a mixture of HAp can be used as an implant candidate. The HAp material can be sourced from bovine bone and processed through Good Manufacturing Practice from bovine bone processed by tissue bank (HApGMP), and from limestone (CaCO3) processed by Balai Besar Keramik (HApBBK).[8]

The addendum of HAp to conventional PMMA-based bone cement can produce reinforced bio-ceramic polymers with better biological and mechanical properties. The polymeric phase of PMMA/HAp composites can also provide a means of chemically binding other bioactive molecules that have been shown to stimulate the function of osteoblast and promote the formation of bone.[9]

Moreover, the HAp is a porous bio-ceramic that allows the growth of capillaries and other blood vessels in the outer pore of HAp, and so provides a better osteogenesis because vascularization and oxygen permeability are easier to developed throughout the process. The molecule of HAp can repair and regenerate hard tissue because its chemical composition and structure are similar compared to the natural bone.[10,11] Meanwhile, direct attachment of HAp to the bone will prevent fibrous tissue formation and lead to bone formation. This will allow the invasion by connective tissue from the surrounding bone to HAp, which will later harden (ossify) and retain its original characteristics.[12]

Osseointegration is a process of a contact between the implant and bone. It is one of the most important factors that contribute to the success of dental implants.[13] This situation demands an understanding of the interaction between the material and the tissue as important in developing optimal strategies to control osseointegration.[14] Osseointegration has also become a standard in the application of implant materials and its clinical effectiveness, which in the process it requires activation of regulatory pathways that can affect osteoblastogenesis, promote osteoblast differentiation and maturation, as well as repair or regeneration of the bone.[15] In addition, osseointegration relies on the basic principles of bone regeneration and osteoconductivity of the biomaterials used.[16]

The calcium ions from HAp would be responded by the transmembrane Calcium Sensing Receptors (CaSR). These transmembrane receptors convey information from the extracellular matrix to the intracellular compartment by involving the Extracellular Signal-Regulated Kinase (ERK) pathway via Protein Kinase C (PKC) and Phospholipase C (PLC) pathways. The ERK signaling pathway allows the transcription of Runx2 and then increases the expression of Bone Morphogenetic Protein-2 (BMP-2) which is an important factor in osteoblastogenesis.[17,18]

The BMP-2 is an inducer of osteogenesis, as it can induce cellular responses resulting in bone formation. BMP play an important role in the process of differentiation, proliferation, growth inhibition, and regulation of osteoblast cells. Besides that, BMP-2 is an important protein in the bone healing process with its ability to induce bone formation and osteoblast cell proliferation.[19]

The process begins with the interaction between HAp (extracellular matrix) and osteoblast cells through several trans membrane receptors, one of which is bone morphogenic protein receptor (BMP-R), vascular endothelial growth factor receptor (VEGF-R) and CaSR. BMP-R is a receptor that responds to stimuli and involves the MAPK pathway which can then stimulate the expression of osteoblast-specific genes. The VEGF–VEGFR interaction activates the MAPK/ERK pathway, which ultimately leads to cell proliferation and expression of bFGF and BMP-2-affected genes in the nucleus.[17]

The activated and phosphorylated BMP-BMPR interactions of Smad 1, 5 and 8. Activation of R-Smads can transform into heteromeric complexes with Smad 4 in the cytoplasm. This heteromeric complexes are translocated to the nucleus and participate in modulating the expression of target genes, including VEGF, Runx2, and bFGF. The biological effects of BMP-2, VEGF, and bFGF are modulated by each other and the complex interactions among growth factors in turn will alter gene expression and cell function. The bFGF and VEGF can also increase the expression of BMP-2 receptors on surface cells, which have a higher binding capacity by the addition of BMP-2. [20,21]

According to research, the use of PMMA-HAp can increase bone repair response.[22] The potential of PMMA-HApGMP and PMMA-HApBBK as implant candidate materials can be seen by observing the expression of BMP-2 and VEGF on osteoblast cells in vitro with ICC.[23]

So far, a few is known about the osteoblasts cellular response to PMMA/HAp materials.[24] Based on this background, this study aimed to observe the expression of BMP-2 and VEGF markers in osteoblast cell cultures exposed to PMMA-HApBBK and PMMA-HApGMP materials.

2. Methods

Ethical approval

The research protocol was approved by ethical clearances No.608/HRECC.FODM/XI/2021 (Date approval: September 30, 2021) from the Faculty of Dentistry Research Ethics Commission, Airlangga University, Surabaya, Indonesia.

2.1. Study design

The study was an experimental randomized post-test only control group design. The study was conducted on 24 osteoblast cell cultures from fetal rat calvarie exposed to PMMA-HApBBK and PMMA-HApGMP at CDAST University of Jember which were divided into sex groups, including the control group on 7 and 14 days, the 7th-day and 14th-day treatment group PMMA-HApBBK, and 7th-day and 14th-day treatment group PMMA-HApGMP. Immunocytochemistry staining was carried out on the preparations using anti-VEGF monoclonal antibodies and anti-BMP2 monoclonal antibodies.

2.2. Preparations and sterilization of PMMA-HA

PMMA was mixed with HAp with a planetarium mixer in a ratio of 80: 20 for 3 h. For each mixture of 0.1gr PMMA/HAp, 0.016 ml of monomer is added so that the ratio of PMMA: HAP becomes 83.8: 16.2. The mixture of PMMA and HAp is stirred using a cement spatula on a dappen glass for 1–1.5 min until it reaches a dough stage consistency. The mixture of PMMA and HAp was molded using a nylon mold with a height of 2 mm and a diameter of 1 mm, waiting for setting time for 5–10 min, then removed from the mold, and the prints were uniformed

with a weight of 0.003 mg. Samples were washed with Phosphate buffered saline (PBS) for three repetitions, followed by immersion in 70% ethanol for 2 h, followed by radiation with UV light for 2 h.[25]

2.3. Osteoblast cells culture

Osteoblasts isolated from a 19-day-old fetal rat calvarie. Pretreatment of calvariae with type II collagenase for 10 min, at 37 °C. Osteoblasts were released from the calvariae twice for 10 min and then continued twice for 20 min. Osteoblasts were left overnight until 25,000 cells/cm2 reached on culture plates. Osteoblasts were removed, using 0.05% trypsin and 0.53 mm EDTA in buffered saline solution. Osteoblasts were grown at 36,000 cells/cm2 density. Osteoblasts grown in alpha-Minimum Essential Medium were added with 10% fetal bovine serum, 0.1% gentamicin, 0.5% Fungizone, 1% L-glutamine, and 0.5% non-essential amino acids. Every 3 days, the media was supplemented with b-glycerophosphate (3 mm) and ascorbic acid (50 mg/ml).[26]

2.4. Testing and immunochemistry

Synthetic materials PMMA-HApBBK and PMMA-HApGMP have soaked overnight in Osteoblast Growth Medium and FBS-specific media; osteoblast cell culture was applied to both groups and incubated at 37 °C and 5% CO2. On days 7 and 14 of the incubation period, cells washed with PBS, applied 3% H2O2 (15 min), washed under running water 3 times (5 s), add primary antibody 2 μg/100 μL (2 h), secondary antibodies (1 h), washed under running water 3 x (5 s), Strep Avidin Horse Predise (40 s), then washed with PBS and aquadest, application of Diamino Benzoade (10 s) with a ratio of buffer: substrate = 20:1 until brownish color acquired, rinsed using distilled water 3 times (5 s), washing and drying, add 1–2 drops of entelan, and after its dry, cover with a cover glass then proceed to reading under the microscope.

2.5. Results analysis with image J

Quantitative observations of VEGF and BMP-2 expression were carried out using immunocytochemistry technique under a light microscope with 400x magnification and 5 fields of view, then the results were calculated using a tool image (image J) and then documented. This technique is a technique for determining the location of antigens (target proteins) in tissues or cells using the principle of the antigen-antibody reaction.

2.6. Statistical analysis

This study was an experimental randomized post-test only control group design. The results of the study were calculated with the mean and standard deviation by using the SPSS version 25. The Shapiro-Wilk Test was conducted to determine the distribution of the data followed by homogeneity test using Levene's Test. To test the differences the one-way ANNOVA test with a significance level of 0.05 was used. If there is a difference, it is continued with the Tukey HSD test to determine the difference between each group ($p < 0.05$).

3. Results

Based on the test results on the 6 treatment groups, the results on BMP-2 expression are showed in Tables 1 and 2. Based on the mean of PMMA-HApGMP and PMMA-HApBBK groups resulted was showed higher BMP-2 expression than the control group (Fig. 1). The highest mean was found in the PMMA-HApGMP group on day 14 (Table 1), (Fig. 2).

Based on the results of the ANOVA test showed that there are significant differences in the expression of BMP-2 on the 7th day of the control and PMMA-HApBBK groups as well as the control and HApGMP groups, the difference in values is not significant in the PMMA-HApGMP and PMMA-HApBBK groups on the 7th day. While On day 14 there was a significantly difference in expression between the control, PMMA-HApBBK and PMMA-HApGMP groups (Table 2).

Based on the test results on the 6 treatment groups, the results on VEGF expression are showed in Tables 3 and 4. The mean of PMMA-HApGMP and PMMA-HApBBK groups resulted showed higher VEGF expression than the control group (Fig. 3). The highest mean was found in the PMMA-HApGMP group on day 14 (Table 3), (Fig. 4).

Based on the results of the ANOVA test showed that there were significant differences in VEGF expression on day 7 and 14 between the control, PMMA-HApBBK and the PMMA-HApGMP groups (Table 4).

4. Discussion

This study was conducted on osteoblast cells cultured from fetal rat calvarie with the aim of comparing the effect of PMMA-HApGMP and PMMA-HApBBK exposure on the expression of BMP-2 and VEGF in osteoblast cells. The results of immunocytochemistry observations by Image J gave readings in the form of arbitrary unit (au) values in each group. In this study, osteoblast cell culture from fetal rat calvarie was used because fetal rat calvarie osteoblast cells have biological aspects as human osteoblasts, can proliferate faster and easier to handle by experimental analyst.[27]

PMMA is a polymer with good mechanical properties but lacks in its bioactivity ability. In order to overcome this weakness, a mixture of biomaterials such HAp is used. HAp is a porous bio-ceramic that allows the growth of capillaries and other blood vessels in the outer pore of HAp, and so provides a better osteogenesis because vascularization and oxygen permeability are easier to developed throughout the process. The molecule of HAp can repair and regenerate hard tissue because its chemical composition and structure are similar compared to the natural bone.[10,11]

In general, the result of the study shows that PMMA-HApBBK and PMMA-HApGMP groups was significantly expressing BMP-2 and VEGF scores higher than the control group. Meanwhile, the PMMA-HApGMP group achieved the highest mean score of BMP-2 expression, with or without significant differences compared to other groups. The increasing score in PMMA-HApGMP can be caused by various factors.[19]

Besides that, BMP-2 is an important protein in the bone healing process with its ability to induce bone formation and osteoblast cell proliferation. Under normal conditions, the peak of BMP-2 expression occurs on day 14.[28] This is aligned with the results of this study showing that in the HApGMP and HApBBK groups, osteoblasts expressed higher BMP-2 than the control group with highest mean was found in the PMMA-HApGMP group on day 14.

Another feasible factor is the strong influence of blood supply or

Table 1
The Mean and standard deviation of BMP-2 expression.

Group	Day 7		Day 14	
	Mean	Standard Deviation	Mean	Standard Deviation
Control	183,6948	±8,306538	198,119	±2,084021
HApBBK	227,4528	±20,841726	237,4447	±7,034285
HApGMP	247,8913	±7,243836	266,6894	±7,892438

Table 2
The ANOVA test of BMP-2 expression.

		Mean Difference	p-value
C7	**HApBBK7**	43,758009*	0,001
C7	**HApGMP7**	64,196473*	0,001
HApGMP7	**HApBBK7**	20,438464	0,071
HApBBK14	**C14**	39,325729*	0,001
HApGMP14	**C14**	68,570410*	0,001
HApGMP14	**HApBBK14**	29,244681*	0,001

*$p < 0.05$ = there is a significant difference.

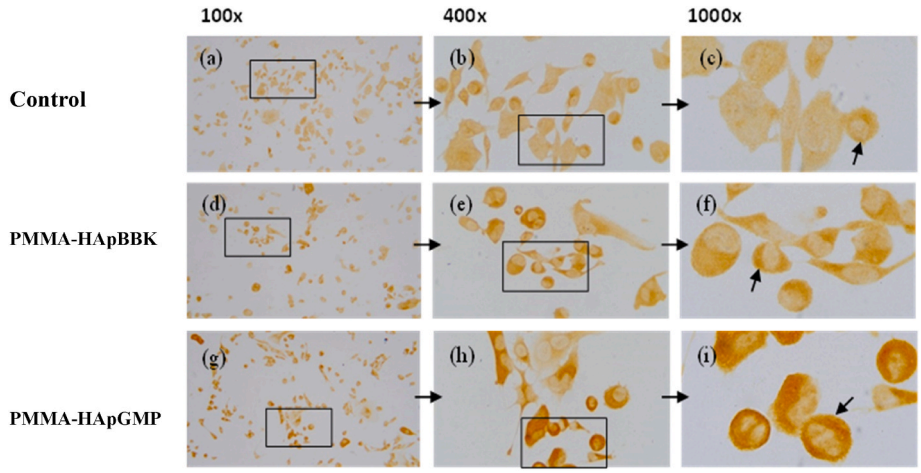

Fig. 1. Immunocytochemistry staining under a light microscope with 100x, 400x, and 1000x magnifications of BMP-2 for control, PMMA-HApBBK, and PMMA-HApGMP groups on day 7.

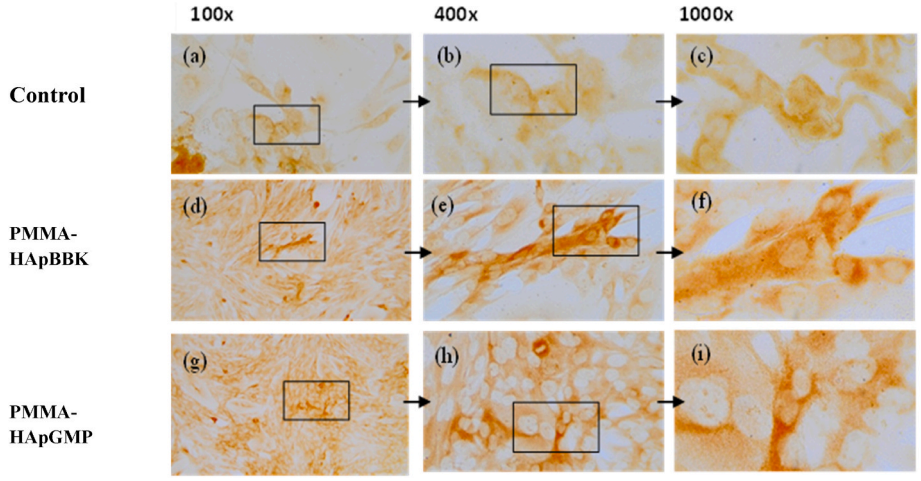

Fig. 2. Immunocytochemistry staining under a light microscope with 100x, 400x, and 1000x magnifications of BMP-2 for control, PMMA-HApBBK, and PMMA-HApGMP groups on day 14.

Table 3
The Mean and standard deviation of VEGF expression.

Group	Day 7		Day 14	
	Mean	Standard Deviation	Mean	Standard Deviation
Control	118,8449	±11,930117	124,5067	±8,306538
HApBBK	215,3717	±1,920571	236,3265	±20,841726
HApGMP	255,9525	±9,470246	267,0961	±7,243836

Table 4
The ANOVA test of VEGF expression.

		Mean Difference	p-value
C7	**HApBBK7**	96,526829*	0,001
C7	**HApGMP7**	137,107514*	0,001
HApGMP7	**HApBBK7**	40,580684*	0,001
HApBBK14	**C14**	111,819783*	0,001
HApGMP14	**C14**	142,589383*	0,001
HApGMP14	**HApBBK14**	30,769600*	0,01

*$p < 0.05$ = there is a significant difference.

angiogenesis factors, especially within the process of bone remodeling because bone is a tissue that depends on vascularization to maintain its being.[9] A good angiogenesis process will facilitate the process of bone remodeling and so accelerate achieving osseointegration. VEGF plays a role in mediating the processes of osteogenesis and angiogenesis, as well as controlling the function and differentiation of osteoblast cells.[29]

An increase in VEGF expression can be seen as equal with an increase of angiogenesis, which plays an important role in bone repair process. In bone regeneration, VEGF expression increases initially and reaches a peak at day 5 till 10. [30] This is aligned with the results of this study showing that in the HApGMP and HApBBK groups, osteoblasts expressed higher VEGF than the control group both on day-7 and day-14. This can be explaining because HAp is a bio-ceramic material with a chemical structure like natural bone, so it can increase the proliferation of osteoblasts cell, which in this study is indicated by the increase in VEGF expression compared to the control group.

5. Limitation of the study

In this study, the interaction of biological potential with the mechanical strength of PMMA-HApBBK and PMMA-HApGMP materials and the biocompatibility of those materiel to living tissue for undesirable effects on implant properties has not been carried out, considering

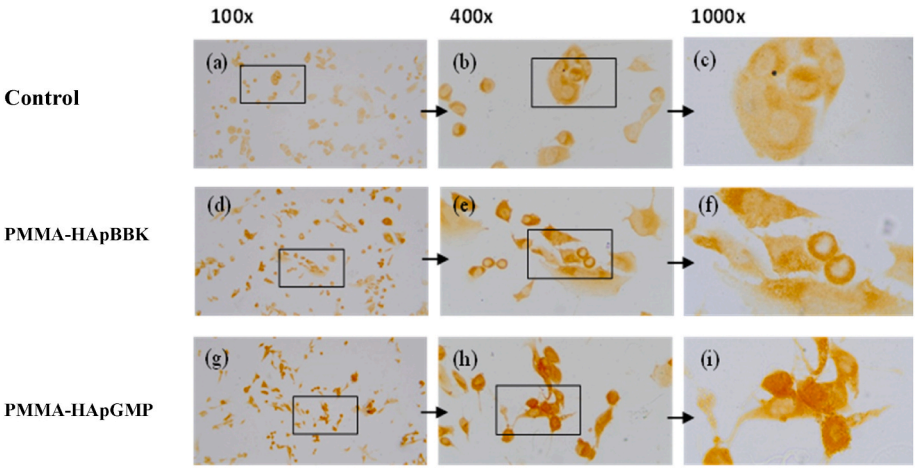

Fig. 3. Immunocytochemistry staining under a light microscope with 100x, 400x, and 1000x magnifications of VEGF for control, PMMA-HApBBK, and PMMA-HApGMP groups on day 7.

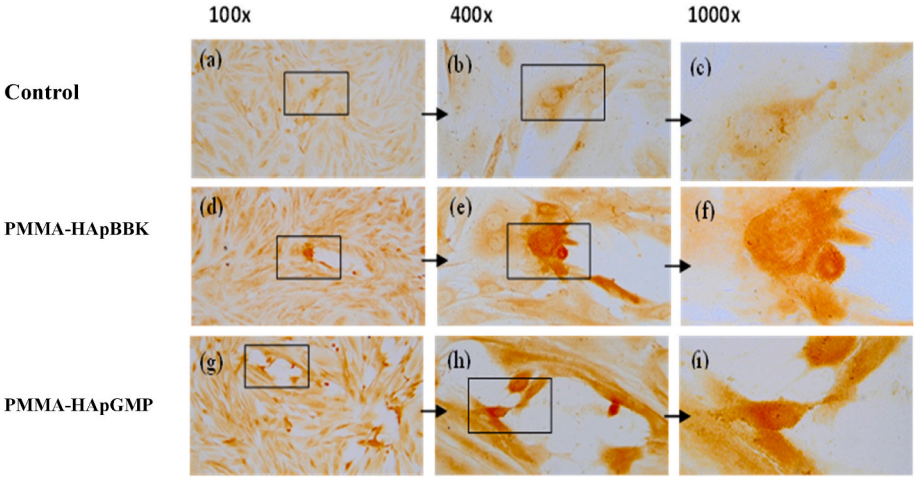

Fig. 4. Immunocytochemistry staining under a light microscope with 100x, 400x, and 1000x magnifications of VEGF for control, PMMA-HApBBK, and PMMA-HApGMP groups on day 14.

the need to understand various aspects in these areas to create an implant material that is suitable for various clinical applications. Further research is also needed on the potential of PMMA-HApBBK and PMMA-HApGMP as a graft or implants materials in many forms (scaffold, powder graft, abutments, etc.).

6. Conclusion

In this study it can be concluded both the PMMA-HApBBK and PMMA-HApGMP groups could increase the VEGF and BMP-2 expression value in osteoblast cells culture. The PMMA-HApGMP group showed a better increase in VEGF and BMP-2 expression when compared to PMMA-HApBBK but both are giving higher expression value of VEGF and BMP-2 compared to control group. The increase in the expression of these markers indicates a potential increase of osseointegration by PMMA-HApBBK and PMMA-HApGMP materials. Thus, these two materials have the potential to be developed as an alternative implant material.

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Declaration of competing interest

No conflicts of interests.

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