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Protein engineering of recombinant human bone morphogenetic protein 2 with higher interaction with Ca phosphate based scaffold used for osteogenesis

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Abstract: The aim of the present study was to assess the recombinant bonemorphogenetic protein 2 (RHBMP-2) with higher substantively and solubility for use in calcium phosphate scaffolds for better release in differentiation of mesenchymal stem cells to osteoblast cells. Using bioinformatics tools, two mutations (p. L10D and p. S12E) were chosen and applied in BMP2 CDS sequence to increase interaction with calcium derived composite. The new recombinant mutated sequence ($BMP2^{mut}$) was synthesized and then subcloned to expression vector pBV220. Experimental data regarded functional protein expression in *E. coli*. Since no modification was made in the active sites of proteins namely β-sheets and α-helixes, not only was there any change in the specific activity occurred in the specific activity of the enzyme in comparison to its commercial counterpart, but also mesenchymal osteogenesis occurred more efficient on biphasic CaP scaffold model. As we hypothesized, use of negatively charged amino acids such as aspartate and glutamate in protein loops increased the interactions of BMP2-Ca^{2+} and resulted in its slower and more sustained released from CaP scaffolds compare to commercial RHBMP2. Our data suggested that new $BMP2^{mut}$ have greater osteoinductive capacity than RHBMP2 in the same time and dose than RHBMP2. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00B:000–000, 2017.

Key Words: recombinant human bone morphogenetic protein, protein engineering, osteogenesis

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INTRODUCTION

The BMP2 belongs to the family of transforming growth factors (TGFs).[1] It is a morphogenetic factor affecting osteogenesis and is used to enhance the healing of nonunion fractures. Due to the extensive clinical applications of BMP2, researchers are searching for carriers to deliver this factor to the wound site and enable its sustained release. BMP2 has osteogenesis and bone regeneration properties.[2] In the current study, we attempted to fabricate RHBMP2 with several influential properties for use in tissue engineering and biotechnology.

Considering the interactions of this protein as a bone morphogenetic factor with tricalcium phosphate (TCP) and other components of the extracellular matrix affecting its function, we attempted to increase the interactions of BMP2 with dicalcium phosphate anhydrous enhance the structural stability of RHBMP2 after loading on the scaffold.There is no report that calcium phosphate scaffolds have osteoinductive efficacy. Combination of BMP2 with CaP can result in a scaffold with greater osteoinductive capacity.

Calcium is the main component in the formulation of pulp capping agents and plays an integral role in mineralization.

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Increase in the concentration of calcium ions (Ca^{2+}) is associated with up-regulation and increased expression of osteopontin and osteocalcin.[3] The response of osteoprogenitor cells to calcium is very important in bone homeostasis and regenerative medicine. Human mesenchymal stem cells (hMSCs) can proliferate and differentiate in present of calcium ions hMSCs. Extracellular calcium induces the expression of BMP2 and subsequent activation of MEK1/2 signaling pathway, resulting in the consequent expression of genes involved in osteogenesis.[4] Several studies have investigated the role of calcium in bone tissue engineering and many are still ongoing to elucidate the efficacy of calcium derivatives for fabrication of new scaffolds.[5,6] Tetra calcium phosphate, hydroxyapatite (HAP), and dicalcium phosphate anhydrous are three biocompatible fillers; in the current study, dicalcium phosphate anhydrous was used as the basic scaffold for investigation interaction of BMP2 with Ca derived scaffold.

Protein engineering by the purpose of increasing the interaction of BMP2 and calcium, was evaluated in silico and two amino acids p. L10D, p. S12E were changed by considering preserving the second and third protein structures, three intra-strand disulfide bonds and one interstrand disulfide bond, optimizing the sequence of gene expression in *Escherichia coli* as a prokaryote, decreasing its hydrophobicity and increasing its solubility since BMP2 is a hydrophobic, noncollagenous glycoprotein, insoluble in water. However, it dissolves in high concentrations of urea and guanidine hydrochloride.[7]

In this regard, first BMP2 with PDB accession code 3bmp was exposed to different concentrations of Ca^{2+}in Gromac software. After assessment of protein structure, two amino acids with the greatest effect on Ca^{2+}interactions at lower concentrations were chosen. The afore-mentioned two amino acids also enhanced the water solubility of protein. Molecular dynamics simulation was performed and the RMSD of the two proteins was evaluated. Next, the nucleotide sequence was modified according to the protein sequence and the codons were optimized in the *E. coli* expression system using J-CAT. The functional protein sequence containing 114 amino acids was then determined. After gene synthesis, the respective nucleotide sequence was cloned in pBV220 expression vector and produced in the *E. coli* expression system. After purification of protein, it was injected onto the pores of scaffold like RHBMP2. The amount, duration of RHBMP2 and $BMP2^{mut}$release and MSC osteogenesis were then compared.

MATERIALS AND METHODS

In silico protein engineering

The three-dimensional crystallographic structure of BMP2 was obtained from the PDB database (PDB ID:3BMP).[8] The protein was exposed to $Ca^{2+}$with different concentrations in order to evaluate the complexes' stability, occurrence of possible translocations in BMP2, and the related receptors. Targeted molecular docking was performed on selected complexes. Parameters including RMSD, gyration diameter, total energy, and density were assessed over time. For final confirmation, molecular dynamics simulation was done using Gromacs 4.5.4 software for confirming interaction level between BMP2 and Ca^{++}[9]. For this purpose, hydrogen atoms were added and initial optimization of antigen–antibody complex was conducted using SPC model. The required amounts of Na^+ and Cl^- ions were utilized to neutralize the protein net charge. Using the steepest algorithm helps us to carry out the optimized drug–ligand complex. System reached equilibrium using NVT and NPT ensemble at 200 picosecond intervals over molecular dynamics simulation process[9] (data not shown).

Mutated RHBMP2 sequence development

After final modifications in the sequence (changing and optimizing the codons for expression in *E. coli*),[10] RHBMP2 sequence, $BMP2^{mut}$DNA sequence was synthesized by Trenzyme company.

SUBCLONING

The $BMP2^{mut}$ sequence was amplified by using mutft/mutrt primers. 25 μL PCR product was purified using the AmbiClean Kit (Vivantis Technologies, Malaysia) and subsequently digested using *Eco*RI and *Bam*HI restriction enzymes (Thermo Scientific, USA): 3 μL Tango buffer 10× (Thermo Scientific, USA) and 1 μL of each restriction enzyme was added to purify the PCR product. Then, the mixture was incubated at 37°C for 2 h. The pBV220 vector was also double digested using *Eco*RI and *Bam*HI restriction enzymes (Thermo Scientific, USA): 3 μL Tango buffer 10× (Thermo Scientific, USA) and 1 μL of each restriction enzyme was added to 250 ng plasmid, and the mixture was incubated at 37°C for 2 h. The digested pBV220 vector was electrophoresed in 1% agarose gel. The target band was cut and linearized pBV220 was extracted from agarose gel according to the instructions for the GF-1 gel DNA recovery kit (Vivantis Technologies, Malaysia). The recombinant pBV220 vector containing the $BMP2^{mut}$ gene was generated by mixing 60 ng $BMP2^{mut}$ PCR product, 30 ng linearized pBV220vector, 1 μL 10× ligation buffer, 2U of T4 DNA ligase (Thermo Scientific, USA), and water up to 10 μL. The ligation mixture was incubated overnight at 16°C.

For preparation competent cells, *E. coli* cells were grown overnight in 5 mL LB broth. The next day, 150 μL overnight culture (OD 600 = 1.2) was transferred to 10 mL LB broth and incubated at 37°C for 2 h with shaking (180 rpm). The bacterial cells (OD 600 = 0.6) were collected by centrifuging at 4500 rpm (4°C) for 15 min. The cell pellet was resuspended in 5 mL icecold 0.1*M* $CaCl_2$ and placed on ice for 30 min. The bacterial pellets were collected again by centrifugation and the process was repeated. Next, 5 μL ligation reaction was added to competent cells and placed on ice for 30 min. The recombinant pBV220vector was transformed into *E. coli* by heat shock protocol (2 min −42°C, then 5 min on ice). Subsequently, 800 μL LB broth was added to an Eppendorf tube and incubated at 37°C for 1 h. Finally, the cells were cultured on an LB agar contain 100 μg ampicillin and placed on incubator at 37°C overnight. Then single colonies subcultured in LB agar with ampicillin overnight.

Colonies plasmid was extracted for subclonig validation using a GF-1 plasmid extraction kit (Vivantis Technologies, Malaysia) according to the manufacturer's protocol.

PCR amplification with specific primers was performed to confirm the presence of the $BMP2^{mut}$ gene in the pBV220 vector. PCR mixture was prepared in a final volume of 30 μL containing 30 ng of recombinant pBV220 vector, 15 μL *Taq* DNA polymerase 2× Master Mix (Ampliqon, Denmark), 0.5 m*M* of each primer, and water up to 30 μL. The thermocycler program was the same as the BMP-2 amplification using cDNA. Products analyzed by running on a 1.5% agarose gel. The gel was run at 100 V for 50 min and stained with ethidium bromide solution.

RT-PCR

First-strand cDNA was synthesized using the Viva 2-stepRT-PCR kit (Vivantis Technologies, Malaysia). In the first step, a mixture of 10 μg RNA, 1 μL random hexamer primer (50 ng/μL), and 1 μL dNTP mix (10 m*M*) was prepared in 10 μL DEPC water and incubated at 65°C for 5 min and chilled on ice for 2 min. In the second step, a mixture of 2 μL 10× buffer M-MuLV and 100 U of M-MuLV reverse transcriptase was prepared in 10 μL DEPC water and added to the first mixture. This was then incubated 60 min at 42°C and subsequently 5 min at 85°C. Specific primers for the $RHBMP2^{mut}$ cDNA were designed using Gene Runner software by considering forward primer. Forward primer: *CGTGACAAAGAATCTTGCAAAC* and Reverse primer: *TTTTCGTCCAGGTACAGCATAGA* were used. The mutated codons are highlighted in forward primer. The primer pairs were synthesized by SinaClon BioScience Co., Iran.

A PCR amplification mixture was prepared in a final volume of 30 μL containing 5-μL cDNA, 3-μL 10× buffer, 1.5 m*M* $MgCl_2$, 0.8 m*M* dNTP, 0.5 μ*M* of each primer, 1 U *Taq* DNA polymerase, and water up to 30 μL. The thermal cycler (primus, Germany) was programmed for incubation at 94°C for 2 min, 35 cycles with denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min. To check the PCR reaction, 5-μL PCR product was mixed with 6× DNA loading dye and loaded on to 1.5% agarose gel. After the electrophoresis (100 V for 50 min), the gel was stained with ethidium bromide (0.5 μg/mL) and the amplicon was visualized using a UV trans-illuminator (Uvitec, UK). Protein purification was performed using His-Tag Purification Column.

Western blot

Protein lysates were prepared and subjected to standard SDS-PAGE and Western blot analysis, as described.[11] For primary and secondary antibody were used BMP21-C and 2040–05 (southern biotech) antibodies, respectively.

Fabrication of CaP scaffold

TTCP and DCPA with same molar concentration were mixed and then solved in DMEM with ratio 1:4.[12] The mixture was poured over the cell culture plate and incubated in 37°C for 7 days. 1 mg/mL of RHBMP2 or $BMP2^{mut}$ dissolved in 1 mL α-MEM were poured on plate in day 3. After day 7, if cement was prepared over the plate, MSc were seeded on it.[13]

Encapsulation efficiency and drug loading

The encapsulation efficiency (EE) and drug loading onto the scaffold were assessed using a spectrophotometer and the equation below:

$$\mathrm{EE\%} = (m_e/m_0)\times 100 \text{ and } \mathrm{Loading\%} = \left[(m_e/m_p)+m_e\right]\times 100$$

where m_e is the amount of encapsulated drug, m_0 is the primary amount of drug, and m_p is the CaP composite mass.

Scaffold porosity

We can calculate the porosity of scaffolds using a displacement liquid. We used water because it is capable of penetrating easily into the pores but does not make any changes in size shrinkage or swelling of the used material (as a main role for choosing a liquid in this method). The scaffold is placed in a cylinder with a premeasured volume of the displacement liquid. The open porosity can be computed using the following equation:[14]

$$\text{Porosity} = (v_1 - v_3)/(v_2 - v_3)$$

where V_1 = premeasured volume of liquid that is used to submerge the scaffold (but not a solvent for the scaffold), V_2 = volume of the liquid and liquid-impregnated scaffold, and V_3 = remaining liquid volume when the liquid-impregnated scaffold is removed.

Alkaline phosphatase (ALP) assay

Isolation and culture of human PDLSCs from reproductive were accomplished using the methods from our previous studies.[15]

After 21 days, MSc culture on scaffold, intracellular ALP activity was measured colorimetrically using an Alkaline Phosphatase Colorimetric Assay Kit (Abcam, Cambridge, UK) which uses *p*-nitrophenyl phosphate (pNPP) as a phosphatase substrate. Cell lysate was prepared using three cycles of freeze–thaw in deionized distilled water. 30 μL of the cell lysate was added to a 96 well plate with 50 μL assay buffer and 50 μL pNPP. The samples were shielded from direct light at room temperature for 1 h. After this, 20 μL stop solution (3*N* NaOH) was added to the wells and the plate was read at 405 nm in a microplate reader.

MINERALIZATION

The differentiated cells were evaluated for calcium production at 14 and 21 days by staining with 10% Alizarin Red solution. For this purpose, 3000 cells subcultured in 96 wells plate and set up for staining. Alizarin Red S binds to calcium salts. 100 μL of eluted Alizarin Red stain was added to 96 well plates and read at 550 nm using a spectrophotometer.

A standard curve was prepared using Alizarin Red stain. The calcium deposition was expressed as molar equivalent of calcium. One mole of Alizarin Red binds to two moles of calcium in an Alizarin Red S-calcium complex.

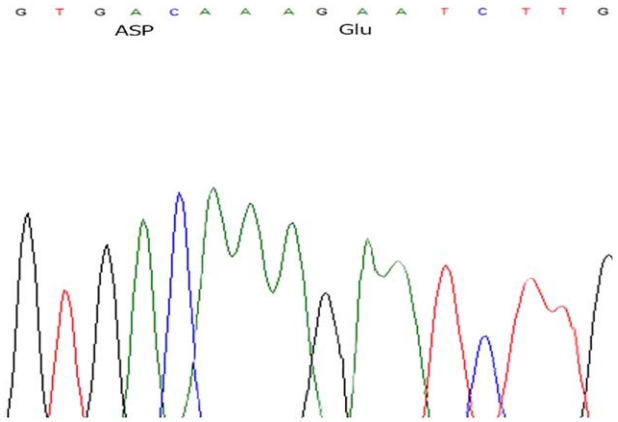

FIGURE 1. Finch TV sequence analysis. Asp and Glu acid codons were depicted in this graph.

Statistical analysis

Intracellular ALP and extracellular calcium deposition both normalized to control group (MSC culturing without any bmp2), were compared between groups for conditioned media and RHBMP2$^{-/+mut}$ studies separately with an one-way analysis of variance (ANOVA) followed by pairwise comparison (Tukey's HSD test). All analyses were performed with Graph pad prism 7.02 software. For all comparisons, the level of significance was $p \leq 0.0001$.

RESULTS

Protein engineering: molecular dynamics (MD)

After MD of RHBMP2 with $Ca^{2+,}$ performance of mutation in amino acids leucine and serine have the best effect on interaction of this protein with Ca^{2+} and subsequently its stability and solubility. In sequence Id No. 1 introduced RHBMP2 used in MD and in sequence Id No. 12 targeted mutated amino acids was shown. Sequence Id No. 3 shows relative DNA sequence of BMP2^{+mut} that codon optimized for expression in *E. coli* K12. For cloning of sequence Id No. 3 in pBV220 vector *Eco*RI and *Bam*HI restriction sites were put in 5′ and 3′ of sequence Id No. 3 and for expression in *E. coli* and secreting of it in extracellular space signal peptide sequence was put in 5′ of sequence ID No. 3 after *Eco*R1 restriction site sequence. Final sequence that was synthesized and we used it as BMP2^{mut} in this study was shown in sequence Id No. 4.

Subcloning and *E. coli* expression of BMP2^{+mut}

In order to expression of BMP2^{mut} in *E. coli* K12 strain, sequence ID No. 4 that was synthesized by Trenzyme subcloned in pBV220 vectors by EcoRI and BamHI. Cloning was checked by specific primers and the PCR product sequenced (PCR product agarose gel data not shown because in expression analysis right clone was approved) and the right clones were chosen for expression yield optimization.

DNA sequencing approved mutation codons in RT-PCR product

Sequenced was analyzed by Finch TV software and blast result showed correct mutation in sequence and also correct subcloning without changing in other codons (Fig. 1).

BMP2^{mut} expression analysis. The results of SDS PAGE, RT-PCR, and western blot showed correct expression of BMP2^{mut} in comparison with the commercial RHBMP2 (Fig. 2).

Characterization of human MSCs

Flow cytometer analysis for CD44, CD45, CD34, CD73, CD90, and CD105 confirmed MSC isolated from third molar tooth (method and data of isolation were not shown because there are not supporting our subject (Fig. 3).

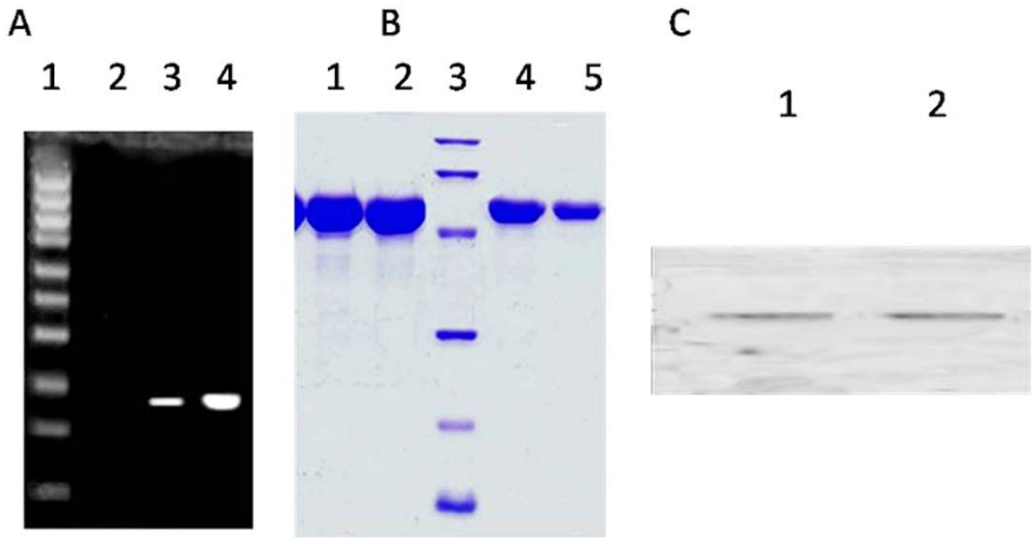

FIGURE 2. (A) Electrophoresis of BMP2mut with BMP2 specific primers. 1: 100 bp DNA ladder, 2: Negative control (H2o), 3 and 4: BMP2mut clones (B) SDS page for protein expression in optimizing conditions compare with commercial RHBMP2. 1 and 3 are Commercial RHBMP2, 2, and 5 are BMP2mut. (C) Western blot: 1: Commercial RHBMP2, 2: BMP2mut (molecular weight is 26 kDa).

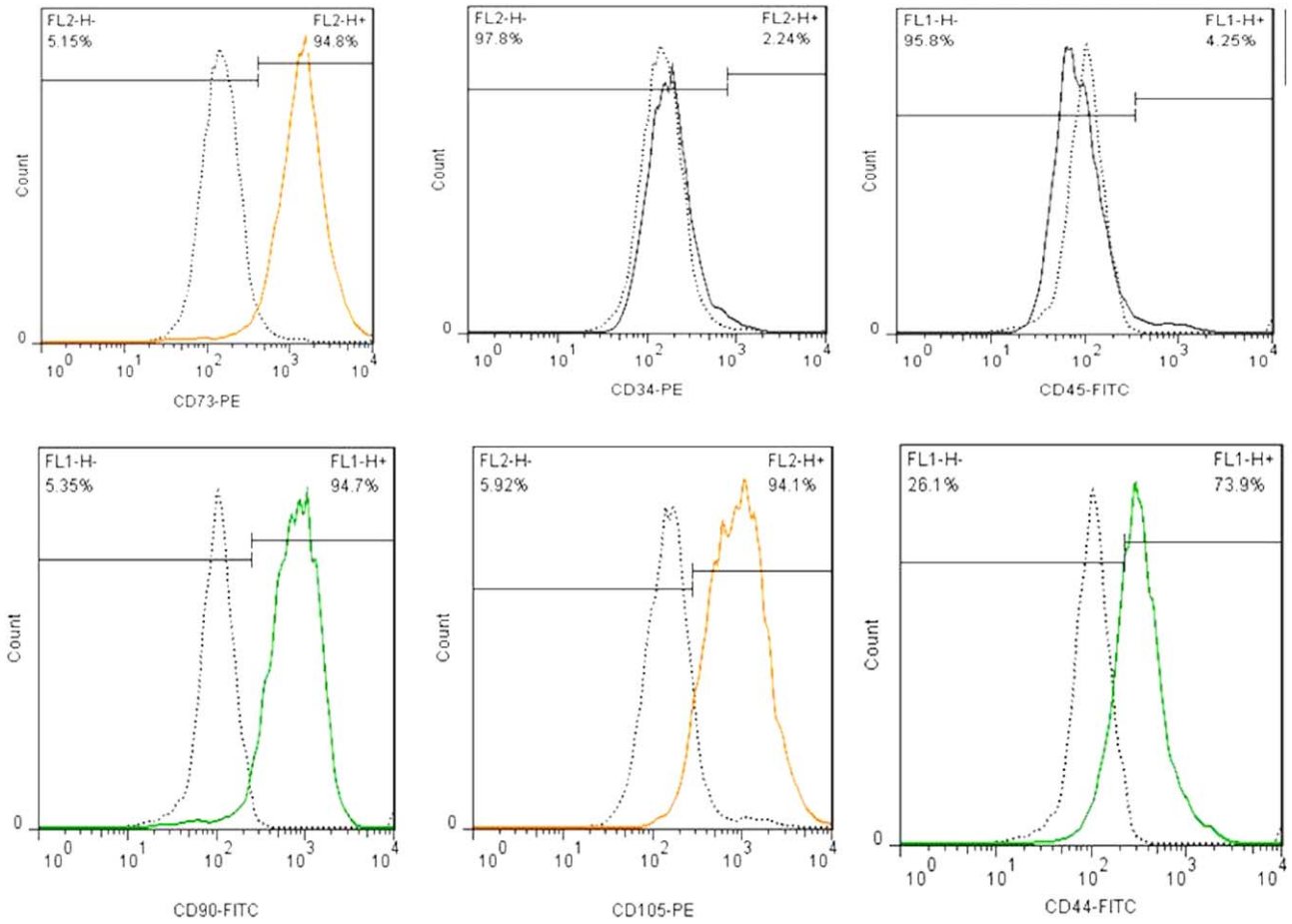

FIGURE 3. Flow cytometry analysis of MSc by Flowjo software. For CD34, CD73, and CD105 used PE and for CD44, CD45, and CD90 used FITC conjugating antibodies.

Osteogenesis gene expression

This result shows that $BMP2^{mut}$ could induce osteogenesis more that RHBMP2 because osteopontin and osteocalcin have higher expression that other groups. Osteocalcin and osteopontin expressions in RHBMP2 and BMP2 group was significantly different from the control group ($p < 0.0001$).

ALP activity and calcium deposition

The ALP activity by MSCs in RHBMP2 and BMP2 group was significantly different from the control group and was lower than the positive group ($p < 0.0001$) [Figs. 4 and 5(a)]. ALP activity by MSCs in the $BMP2^{mut}$ group was greater than that of the RHBMP2 group ($p < 0.0001$) [Fig. 5(a)]. Also the same results were obtained in cell calcium content.

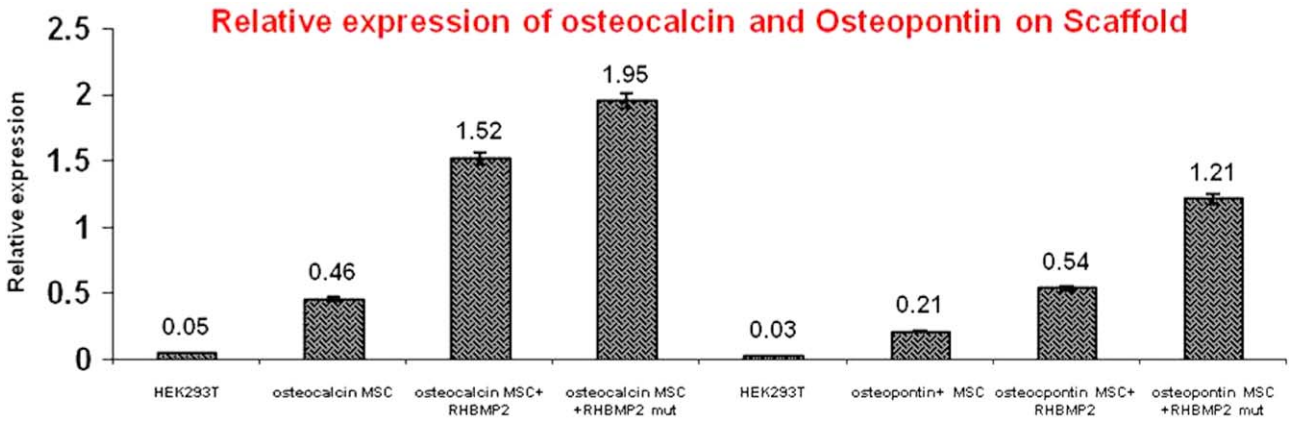

FIGURE 4. Real-time osteogenesis genes results analyzed by Livak method. HEK293T cells is a negative control for osteogenesis gene expression. Osteocalcin and osteopontin expressions level after differentiation of MSc on scaffold with or without BMP2mut was determined.

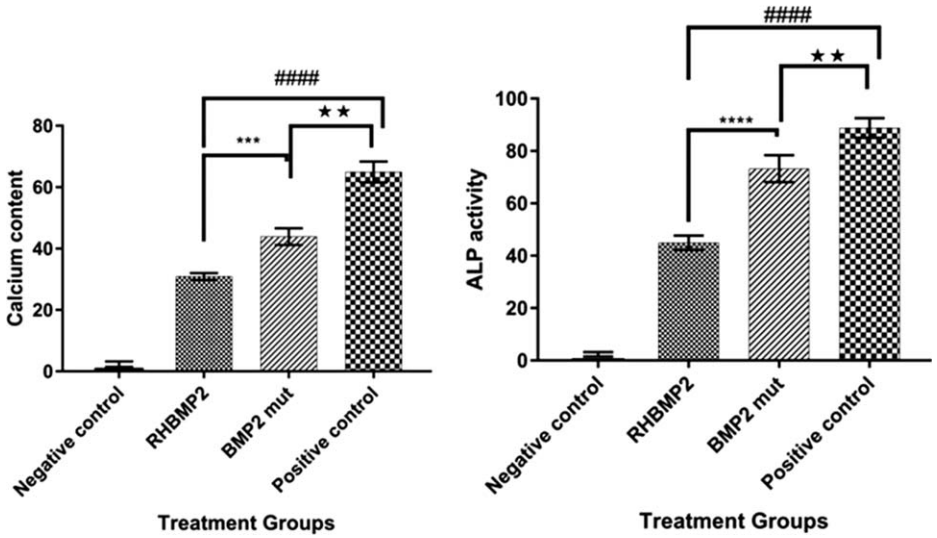

FIGURE 5. (A) ALP activity assay after MSc differentiation. (B) Calcium content assay after MSc differentiation. Negative control is MSc without BMP2 or inductive medium culturing. RHBMP2 and BMP2mut group means MSc that cultured in present of them. Positive control is MC3T3-E1 as an osteoblast positive marker.

RHBMP2 and BMP2 group were significantly different from the control. $BMP2^{mut}$ group was greater than the RHBMP2 group ($p < 0.0001$) [Fig. 5(b)]. It shows that mineralization occurred better in BMP2mut group in $BMP2^{mut}$ group.MC3T3-E1 as a positive group has the highest measure of ALP activity and calcium content between groups.

DISCUSSION

BMP2 as a signaling and inductive molecule is very important in tissue engineering. The yield of native BMPs purified from bone is extremely low, thus most commercially available BMP products are produced recombinant in either bacterial (*E. coli*) or mammalian cell cultures according to the purpose of consumption.[16] Yuvaraj et al. used BMP2 sequence from normal human ileum by direct PCR and is cloned in Topo pTris prokaryotic expression vector and transformed into *E. coli* K-12 strain and they showed large amounts of BMP-2 expressed by *E. coli*.[17] Some studies were done for evaluating BMP2 expression in mammalian cell because of *E. coli* end toxin and other advantage of recombinant protein production in CHO cell line,[16] but yield of recombinant RHBMP2 in mammalian cell culture systems is poor and low and they have tried to improve as a twofold increase in BMP-2 concentration. Sharapova and his colleagues expressed this protein in *E. coli* BL21 (DE3) and approved biological activity with *in vitro* induction of alkaline phosphatase synthesis in C2C12 and C3H10T1/2 cell culture and *in vivo* osteogenesis in site of injection.

Nowadays GMP Recombinant Human Bone Morphogenetic Protein 2 (BMP2–123 H) protein ref seq: NP-001191 is produced by *E. coli* expression system. This protein is expressed with sequence (Gln 283-Arg 396) of Human BMP2 by creative BioMart Company. Its bioactivity is <50 ng/mL. Its specific activity is 2.0×10^4 1 u/mg.

In this study, the aim was introducing a new BMP2 that can use in bone regeneration scaffolds that use CaP-derived compound. The mutations had no effect on the main activity of this protein but *in vitro* cell culture assay show the changing efficiency of it.

The simple method of generating a scaffold scaffold for evaluating BMP2 interaction with CaP showed that $BMP2^{mut}$ has the better interaction and has greater osteoinductive properties. The porosities on the surface of scaffold were suitable for BMP2 absorption. Spontaneous diffusion of solvent explains the irregular, unsmooth surface, and nonspherical shape of particles fabricated. The approach may find great utility in the use of BMP2 with CaP scaffolds. Drug release was at a rate that is comparable to the rate of MSc differentiation.[8,18]

Considering the presence of calcium in bone and tooth structure and its inductive role in differentiation of progenitor stem cells as well as its role in bone tissue engineering scaffolds, it is particularly important to increase the interactions of BMP2 with calcium.[19] Experimental data regarding protein expression and its analyses revealed that protein engineering in our study was successfully achieved *in silico*. *In silico* protein engineering decrease cost and time of protein study for generating investigational new drugs. Since no modification was made in the active sites of proteins namely β-SHEETs and α-helixes, no change occurred in the specific activity of the enzyme in comparison to its commercial counterpart. As we hypothesized, use of negatively charged amino acids such as aspartate and glutamate in loops increased the interactions of Ca^{2+} and resulted in its slower and more sustained release.[18]

This report presents a novel approach for fabrication of simple Cap scaffold. Simple change in BMP2 sequence for increasing efficiencies and simple controlled delivery of drugs like BMP2. In this approach, a drug is incorporated into an interconnected open-pore scaffold. Incorporation efficiencies were ranging between 21 and 36%. And both of

RHBMP2 and $BMP2^{mut}$was released from the pores within approximately 72 h but in real, gene expression, ALP activity and mineralization data were shown that $BMP2^{mut}$ is more effective in osteogenesis that RHBM2. The incorporated BMP2 remained intact.

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