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Original Article

EFFECT OF VITAMIN C ON mRNA EXPRESSION OF BMPs DURING FRACTURE HEALING IN THE FEMUR CALLUS OF *SPRAGUE-DAWLEY* FEMALE RATS

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ABSTRACT

Fracture reparation is a complex physiological process. During fracture healing no scar is left but a new bone tissue is created. In addition to mechanical stabilization, inflammatory cytokines, growth factors, vitamins and trace elements are needed for better and faster fracture healing. Though vitamin C and BMPs are implicated in fracture healing, the interactions between them are not known.

Objective: The present study was performed to delineate the role of vitamin C in the regulation of BMPs mRNA expression in the callus femur.

Methods: In this regard, unilateral fracture was induced in the femur of Sprague-*Dawley* female rats and treated with vitamin C (200 mg/kbwt/ip on alternate days for 21 days).

Results: The mRNA expression of BMP 2, BMP 4 and BMP 6 was assessed by RT-PCR from the harvested fracture callus on days 7, 14 and 21. The mRNA expression of BMP-2 and BMP-6 was significantly increased in the callus femur on all points of study. However, BMP-4 mRNA was increased on days 7 and 14 only.

Conclusion: Thus the results clearly indicated that vitamin C directs the fracture healing by up regulating the expression of BMPs during 7, 14 and 21 days. Future studies are essential to quantify the levels of these BMPs. Thus it is concluded that vitamin C places a pivotal role in increased expression of BMPs during fracture healing.

Keywords: Bone fracture, Callus, Fracture repair, Vitamin C, Ascorbic acid, Bone Morphogenetic Protein, mRNA expression.

INTRODUCTION

Bone is a dynamic biological tissue composed of metabolically active cells (10%) that are integrated within a collagen matrix (90%) **[1]**. Bone has a substantial capacity for repair and regeneration in response to injury or surgical treatment. Both processes involve a complex integration of cells, growth factors, and the extracellular matrix. Approximately, 5-10% of the 6.2 million fractures occurring annually in the United States are associated with impaired healing including delayed healing or non-union **[2]**. Studies on fracture healing in rats have shown several dietary components such as protein, calcium, magnesium, zinc, copper, iron, fluoride and vitamin A, D, E and K are required for normal bone metabolism **[3]**. Among those, vitamin C (Ascorbic acid) has the ability to induce differentiation of multipotent progenitor cells to osteoblasts, chondrocytes, cardiac myocytes, and dopaminergic neurons suggested that vitamin C regulates cell differentiation at early stages and subsequent bone formation besides its well studied action to promote collagen synthesis, maturation and matrix protein interaction**[4,5]**.

Various animal studies and clinical trials in humans have been performed and demonstrated the potential use of several biological factors described in bone regeneration and skeletal repair, with that Bone Morphogenetic Proteins(BMPs) to be the most promising **[6]**. BMPs have the potentials to promote fracture healing and bone regeneration, and they are now clinically available for treating fracture non-union and augmenting bone loss due to fracture**[7]**. BMPs may provoke a sequential multistep cascade of events, i.e., migration of progenitor cells, proliferation of mesenchymal cells, differentiation to chondrogenic or osteogenic cells, vascular invasion, and remodeling of bone **[6, 8]**. There are atleast 20 structurally and functionally related BMPs, most of which play roles in embryogenesis and morphogenesis of various tissues and organs **[9]**. In the early stages of fracture healing BMP-2, -4, -6 and -7 were strongly induced in the thickened periosteum near the fracture ends [10]. As a BMP family member, BMP-2 is necessary for postnatal bone repair and is genetically associated with the maintenance of normal bone mass **[11, 12]**.BMP-4, a stimulator of chondrogenesis, both in vitro and in vivo, is a potential therapeutic agent for cartilage regeneration. BMP-6 was localized in the hypertrophied cartilage during endochondral ossification and it has a regulatory role in the terminal differentiation of chondrocytes **[13]**.

Fracture repair involves proliferation and differentiation of several tissue types in a sequence, followed by remodeling. The benefits of various minerals and vitamins on fracture healing have been demonstrated in animal models. Among that vitamin C is an essential substance that facilitates fracture healing either alone or in combination with other substances like plant extract, vitamins and growth factors. But the molecular mechanism involved in the healing process has not yet been studied. Although vitamin C and BMPs were implicated as positive regulators of fracture healing, there is little evidence for their interactions in this process.

In view of this, the present study was performed to delineate the role of vitamin C in the regulation of BMPs mRNA expression in the callus femur. In this regard, unilateral fracture was induced in the femur of Sprague-*Dawley* female rats and treated with vitamin C.

MATERIALS AND METHODS

Chemicals

Vitamin-C, Chloroform, Isopropanol, Agarose, Tris, Trizol, ethanol and primers for β-actin, BMP-2, BMP-4 and BMP-6 were purchased from Sigma, St. Louis, USA. cDNA Synthesis Kit and PCR kit was purchased from Bio-Rad Laboratories, Canada 94547. The 6X gel loading dye and 100 bp DNA ladder were purchased from Bangalore Genei, Bangalore, India.

Experimental animals

Animals were maintained as per national guidelines and protocols, approved by our Institutional Animal Ethical Committee. Healthy adult female *Sprague-Dawley* rats, weighing about 220-250 g (120 days old) were used. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal

house with constant 12 h light and 12 h dark schedule. The animals were feed with standard rat pellet diet (Lipton India Ltd., Mumbai, India) and clean drinking water was made available *ad -libitum*.

Fracture induction

Unilateral fractures in experimental animals (Left femur of Sprague-Dawley female rats) were induced by Bonnarens and Einhorn, 1984 **[14]**. An experimental unilateral fracture was made in the femora of all the rats. Fracture inducing apparatus was constructed for fracture induction **[14]**. Before two weeks of fracture induction, all rats were undergone for to subjected to intramedullary pinning of their left femurs. The rats were anesthesied with an intraperitoneal injection of ketamine 80 mg/kg body weight (ketamine hydrochloride 500, Neon laboratories Ltd, Mumbai, India) and 10mg/kg body weight xylazine (Indian Immunologicals Ltd, Hydrabad, India) providing approximately 20 min for deep anesthesia. All animals were prepared for surgery before that shaving and cleansing were made in the left leg.

The knee joints were exposed through an external lateral incision. A 1 mm hole was drilled into the metaphysis of the distal femoral condyles. The canal was reamed with a 22 gauge needle inserted between the condyles (Fig 1a). A 0.45 mm Steinmann pin was introduced into the canal and driven in a retrograde fashion up the shaft exiting through the greater trochanter. Distally, the pin was cut flush with the cortex of the patella femoral groove and buried beneath the bone so as not to interfere with knee motion. The knee joint were closed, after the intramedullary pinning procedure, the rats were kept in their cages. After the wounds were closed, a radiograph was taken to confirm pin placement (Fig 1b). The middiaphysis of the pinned femur was fractured by a force generated by dropping a 500-g weight from a height of 35cm to produce a transverse fracture of the left femur (Fig 1c) and the fracture induction were confirmed (Fig 1d) by a dental X-ray machine (Dental Trophy 70 CCX, Iri Paris, France: exposure 0.10 s, 70 Kv, 80 mA). When the fracture was not transverse the animal was removed from the experiment.

Fig.1: Fracture induction.(a) Intramedullary pinning of left femur; (b) Radiograph of a femur taken immediately after pinning; (c) Fracture induction on middiaphysis of the pinned femur by fracture apparatus; (d)Transverse fracture of the femoral diaphysis.

After the fracture was made, the rats were divided into two groups, **Group I**: Fracture induced rats with vehicle-treated [ip]. **Group II**:Fracture induced rats with Vitamin-C 200mg/kg body weight/ip). Treatment is given on alternative days for 3 weeks through intraperitonial injection. A set of six animals was killed at post fracture (PF) day 7 (Fig 2a), 14 (Fig 2b) and 21 (Fig 2c) the callus formed in fractured femurs was dissected cleansed from adherent tissues and stored in liquid nitrogen till use for further experiment.

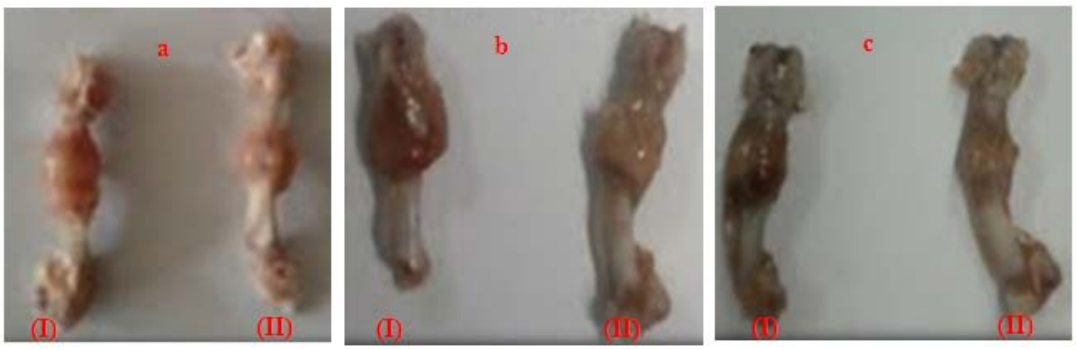

Fig.2: Callus formation of 7, 14 and 21 days. a(I)= callus formation in control rat after 7 days of post fracture; a(II)= callus formation in vitamin C treated rat after 7 days of post fracture; b(I)= callus formation in control rat after 14 days of post fracture; b(II)= callus formation in vitamin C treated rat after 14 days of post fracture; c(I)= callus formation in control rat after 21 days of post fracture; c(II)= callus formation in vitamin C treated rat after 21 days of post fracture.

Analysis of mRNA expression

Total RNA was extracted from control and vitamin C treated Callus using the Trizol reagent (Sigma) according to manufacturer's instructions. The concentration and purity of total RNA was determined by absorbance at 260/280 nm in a UV-spectrophotometer. If the ratio of A260/280 is 1.8–2.0, then 2µg of total RNA was used for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis.

RT-PCR was carried out using one-step RT-PCR kit in a total reaction volume of 20 µl. The first strand synthesis was carried out at 50 °C for 30 min using gene-specific oligonucleotide primers for BMP-2, BMP-4 and BMP-6 followed by the initial PCR activation at 95 °C for 15 min. The primer sequences used in the present study are listed in Table 1. The three step PCR cycles consisted of denaturation at 95 °C for 3min, annealing at 53^0C - 58^0C for 20 sec and extension at 72 °C for 5 min. The PCR amplification was carried out up to 35 cycles and

to ensure that the products are extended completely, a final extension at 72 °C for 5min was carried out. Gene-specific oligonucleotide primers for the house-keeping gene β-Actin was added to the same PCR reaction vial and co-amplified. RT-PCR product (5 µl) was taken from each reaction tube, mixed with gel loading dye and resolved in a standard 2% agarose gel containing ethidium bromide (0.5 µg/ml) under an electrical field (60 mA and 80 V) for 2.5 h. Molecular weight DNA marker (100 bp ladder) was simultaneously resolved in the first lane.

After electrophoresis, the gel was subjected to densitometric scanning and the band intensity of cDNA fragment of each gene of interest was normalized against the band intensity of cDNA fragment of the house-keeping gene using β-Actin quantity one software (Bio-Rad, USA).

Table 1: A list of primers for RT-PCR

Genes primers	Product size	Annealing temperature and no. of cycles
β-ACTIN Sense 5′-TCC ACC CGC GAT ACA ACC TTC-3′ Antisense 5′-GGG CCA CAC GCA GCT CAT TGT A -3′	358 bp	58°C, 35
BMP-2 Sense 5′-AAG GCA CCC TTT GTA TGT GG -3′ Antisense 5′-CAT GCC TTA GGG ATT TTG GA -3′	189 bp	53°C, 35
BMP-4 Sense 5`-TGC CGT CGC CAT TCG CTC TA -3` Antisense 5`-AGG TGG TCC GCC AGT GGA AA-3`	125 bp	58°C, 35
BMP-6 Sense 5`-TCG CAC CCA AAG GCT ACG CT -3` Antisense 5`- GGT TGG TGC GCA GCA TGG TT-3`	152 bp	58°C, 35

Statistical analysis

Statistical analysis was performed using a two tailed student's *t*-test, with equal variance.

*$p < 0.05$ were considered significant.

RESULT AND DISCUSSION

In the present study, on day 7 of post fracture BMP-2 (Fig 3a), BMP-4 (Fig 3b) and BMP-6 (Fig 3c) mRNA expression was significantly increased in vitamin C treated groups when compared to fracture control groups. Antioxidant vitamins directly stimulate the expression of antioxidant response element (ARE) containing genes, leading to osteoblast differentiation and bone formation**[15]**. They also found that Ascorbic acid induced acute osterix expression during bone marrow stromal (BMS) cell differentiation via stimulating interaction of Nrf1 with ARE in the promoter region. Similarly, the increase in BMP-2, -4 and -6 mRNA expressions may be due to activation of Nrf1 by vitamin C in the callus on day 7 of fracture healing.

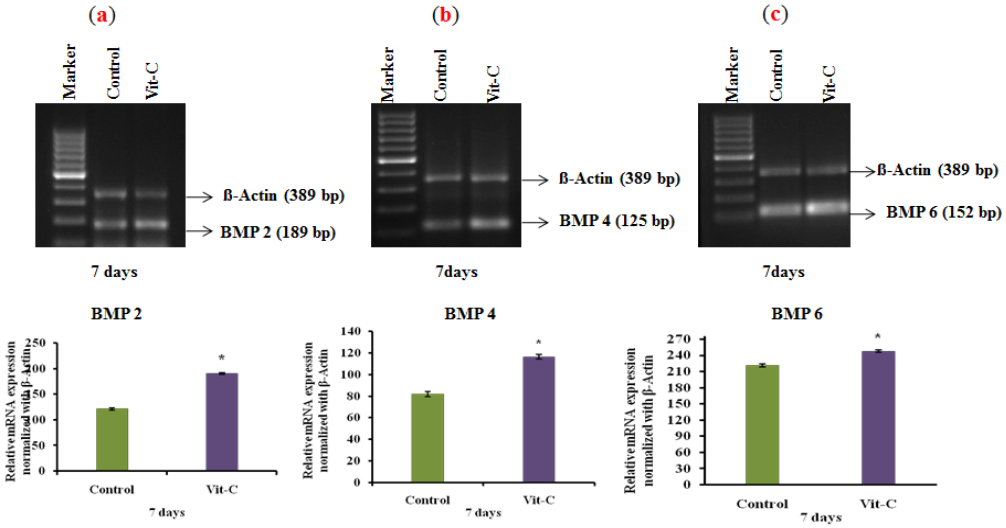

Fig. 3: **BMPs mRNA expression on day 7 after fracture in the femur callus. Each bar represents Mean ± SEM of 3 observations.* denotes statistical significance at p<0.05 level**

In the present study, on day 14 of post fracture, when compared to fracture control rats there was a significant increase in the mRNA expression levels of BMP-2 (Fig 4a), BMP-4 (Fig 4b) and BMP-6 (Fig 4c) in the vitamin C treated rats. *Invitro* studies have showed that BMP-4 may play an important role in early chondrogenesis**[16]** most probably by inducing Runx2 to promote cellular condensation together with BMP-2 **[17]**. Various *in vivo* studies, **[6] [18]** reported that BMP-4 delivery can improve the healing process of an articular cartilage defects by stimulating the synthesis of cartilage matrix constituents of type II collagen and suppressing the production of collagen type X and aggregan.

In an *in vitro* study, it was reported that vitamin C is a critical factor in the processes of cartilage and bone development. Vitamin C can promote the differentiation of ATDC5 cells (ATDC5 cell line exhibits the multistep chondrogenic differentiation observed during endochondral bone formation) by promoting the formation of collagenous matrix **[19]**. The addition of vitamin C to ATDC5 cultures shortened the pre-chondrogenic proliferation phase, produced earlier chondrogenic differentiation, heightened gene expression and robust hypertrophic differentiation **[20]**. Thus vitamin C promotes endochondral ossification by increasing mRNA expression of BMP-2, -4 and -6.

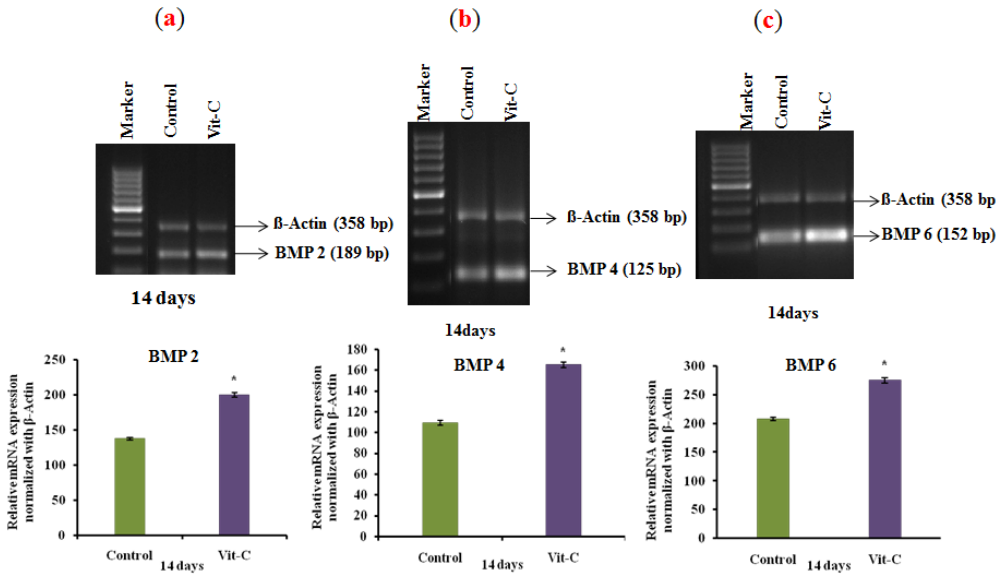

Fig. 4: BMPs mRNA expression on day 14 after fracture in the femur callus. Each bar represents Mean ± SEM of 3 observations.* denotes statistical significance at p<0.05 level.

On the day 21, Fracture healing is preceded by chondrocyte hypertrophy and cartilage matrix calcification. A decrease in the major cartilage protein collagen type II and an increase in collagen type X as the effect of a marked increase in alkaline phosphatase levels during the mineralization process which is known to be dependent on the presence of vitamin C **[21]**.

In the present study, on day 21 of post fracture, the BMP-2 (Fig 5a) and BMP-6 (Fig 5c) mRNA expression was significantly increased in vitamin C treated rats when compared to fracture control rats. But there is no effect in BMP-4(Fig 5b) mRNA expression. *In situ* hybridization analysis of the developing bone showed that BMP-2 and BMP-6 were exclusively expressed in hypertrophic chondrocytes and marginally in the osteoblasts. Therefore, it is possible that BMP-2 and -6 might function more effectively when stored in the collagen-containing extracellular matrix that forms in response to vitamin C.

A specific CRE in the BMP-2 promoter which is responsible for CREB trans activation of the BMP-2 gene in osteoblasts has been identified **[22]**. Previous studies have indicated that the BMP-2 gene regulation during limb morphogenesis and osteoblast differentiation may involve multiple mechanisms and signaling pathways, such as ER, prostaglandin E2, retinoic acid, Hoxa13, Gli2/3, interferon, and interleukins **[23, 24, 25]**. Gli2 is a powerful activator of BMP-2 gene expression and Gli2 regulates target genes in a similar manner to that of Gli3 **[25]**. The promoter region of the BMP-6 gene has rich Cp Gislands **[26]**. The major BMP-4 promoter in bone (1A promoter) is induced by Retinoic Acid (RA). In addition to this, a minor promoter (1B) and a novel promoter in intron2 (i2) are repressed by RA. The i2 promoter is highly expressed in developing inner ears, whereas the 1A promoter is not. Probably, vitamin C engages any of these promoters through its downstream molecules to promote the expression of BMP-4.

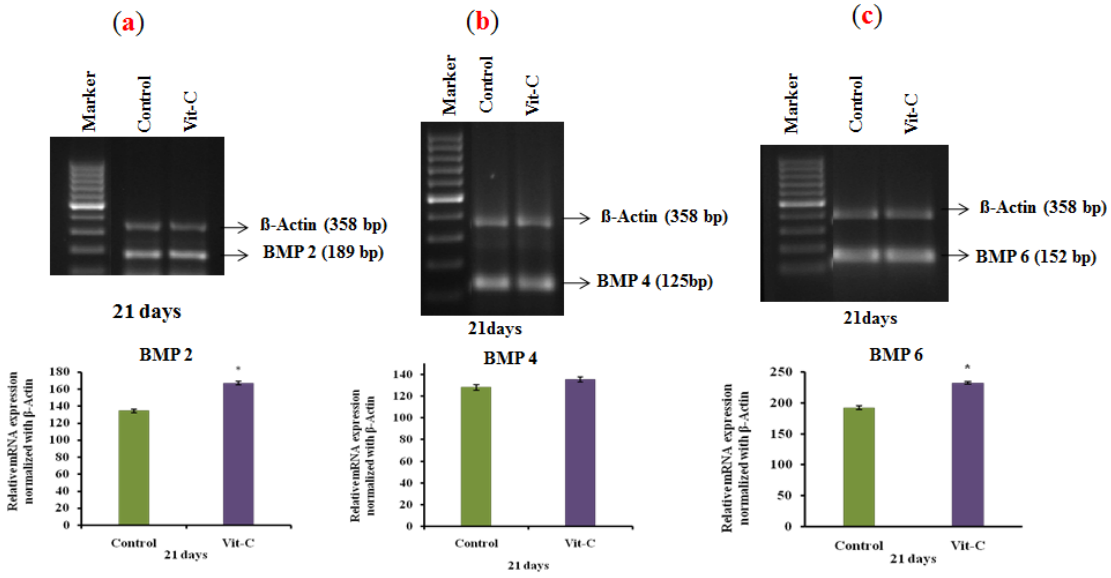

Fig. 5: BMPs mRNA expression on day 21 after fracture in the femur callus. Each bar represents Mean ± SEM of 3 observations.* denotes statistical significance at p<0.05 level

CONCLUSION

In conclusion, we state that vitamin C has a vital role on BMPs expression in fracture healing.To the best of our knowledge, this is the first report to show that vitamin C important for BMPs expression in *in vivo* bone formation.

CONFLICT OF INTERESTS

Declared None

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