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Effect of Bone Morphogenic Protein on Bone Healing Process in the Horse: Histomorphological Study

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Abstract: Bone Morphogenic Protein (BMP) can cause molecular and cellular impulses and induce rapid bone healing process. Therefore, it is good for cases of delayed or non-union fractures as well as filling up of intervertebral gaps by osteogenic activity. The bovine long bone were collected and processed through defating, grinding, decalcification, degreasing, protein extraction, dialyzing, purification and lyophilizatioan. The extracted BMP was kept in the refrigerator. Ten adult horses of both sex were used and divided randomely into 2 equal groups. The right metetarsus was prepared and anesthesia was induced by xylazin, diazepam and ketamin Hcl and maintained with halothane 2%. All horses were handled according to Shiraz University regulation for animal rights. Under general anesthesia a window defect was made through midmetatarsal region by electric osteotom in all horses. The horses of the first group received BMP that was injected at the window site of metatarsal bone. The wound was closed, bandaged and splinted in both groups. They were monitored clinically for 12 weeks and histomorphometric evaluation were performed after 12 weeks. Clinically the horses in BMP group were using their legs slightly from 28th and completely on 42th postoperative day but the horses in control group did not use their legs even on 42th postoperative day. Histomorphometric data showed better thickness of cortical and trabecular bone in treatment group and periosteum was peresent and intact at the window site in patients of treatment group compared to control group.It can be concluded that the extracted protein was BMP and it had accelerated the bone healing process by osteogenic activities.

Key words: Bone morphogenetic protein, bone healing, histomorphological BMP

INTRODUCTION

In bone grafting, autogenous bone is considered the golden standard, to which other methods are compared. (Kawack, 2000) The amount of autograft bone is limited and its harvesting causes secondary morbidity at the donor site (Johnson *et al*., 2000). Allograft bone is widely used instead. Main disadvantages of using the allografts are: The lower healing capacity as compared with autografts and the risk for certain diseases, such as hepatitis and HIV. Occasionally incomplete healing is seen in spite of proper grafting procedures (Itoh and Nishimura, 1998). Autogenous Cancellous Bone grafts (ACB) in horses serve several functions, including enhancement of fracture repair, joint arthrodeses and healing of bone defects. Although large amounts of ACB are available from several sites in horses, problems can arise from their use. These include donor site morbidity (wound dehiscence, pain and catastrophic fracture); increased surgery time; occasional need for a second surgical team; and reduced graft viability associated with prolonged storage. Problems with ACB have led to the development of substitute compounds, including allografts, xenografts, synthetic compounds and growth factors. Limited research on the use of these substitutes in horses exists. A readily available, equally functional substitute would simplify the clinical use of bone grafts.Thus other methods have been searched. Synthetic biomaterials can only be used as filling material without any biological activity in initiating bone regeneration (Wang *et al*., 2003). Stimulation of the regeneration of bone is a challenging idea, which would solve many problems in cases with bone defects. The pioneering work of Urist in 1965 aroused the interest in agents able to induce bone. He demonstrated the ability of Demineralized Bone Matrix (DBM) to induce bone in an ectopic place, when implanted in rabbits and rats intramuscularly (Kirker, 1993). The importance of this work lies in the carefully controlled demonstration that new bone can be induced independently of the surrounding bone tissue. Later, it was shown that low- molecular weight proteins extracted from demineralized bone matrix

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had more osteogenic activity (Lovedo *et al*., 1996; Sykaras *et al*., 2001). It was called Bone Morphogenic Proteins (BMPs). There are however, many unanswered questions concerning such matters as the carrier materials for BMPs, the risks of gene vectors and the basic mechanisms by which BMPs exert their effect in humans and the animals. The aim of BMP studies is to answer these questions and to develop BMPs to be used in clinically different bone defects, such as bone tumor treatment, joint prosthesis surgery, maxillocranio-facial surgery and fracture treatment (Sykaras *et al*., 2001). Bone morphogenic proteins are dimeric molecules with 2 chains held together by one disulphide bond. Each monomer consist of about 120 amino acids with seven canonical cysteine residues (Wozeny *et al*., 1995). The fracture of locomotory system in the horse exhibits the greatest economical loss for the horse owners. Use of BMP in equine surgery has been limited. BMP placed in muscle pouches can induce local mesenchymal cells to differentiate (Kirker, 1993). Results from these studies confirm a particle size effect on osteoinduction and also describe different responses to intramuscular and subcutaneous implantation (Kirker, 1993). Intermediate formation of cartilage is also much less marked in horses than it is in rats implanted with BMP. No reports yet describe the reaction of BMP in orthotopic sites in horses. The present study focuses on the effect of bovine bone morphogenic protein in the treatment of bone defects in the horse.

MATERIALS AND METHODS

Ten normal mix breed adult (4±0.7 years old) horses with an average body weight of 350±50 kg were used in.

Extraction of Bone Morphogenic Protein (BMP): The bone morphogenic protein was extracted from bovine diaphyseal bone in shiraz University-Iran. Frozen fragmented bone was ground to particles of 1mm in size. The bone matrix was extracted in 4M GUHCL at 4°C for 72 h after the pulverized bone had been demineralized in 6.6N Hcl the extracted solution was passed through a millipore filter (pore size, 0.6m, Millipone Corporation MI, USA). The filtered solution was dialysed against deionzed water and the water- insoluble precipitate was re-disolved in 4M GUHCL. Gelatine peptides were removed by dialysis against 0.52 M citrate buffer and the precipitate was centrifuged and lyophilized.The water insoluble bovine BMP was collected (Gao *et al*., 1993).

Surgical procedures: Horses were given xylazine (1.1 mg kg^{-1}) as premedication, anesthesia was Induced with Ketamine (2.0 mg kg^{-1}) and diazepam (0.5 mg kg^{-1}) following endotracheal intubation maintained with 2% halothane in combination with oxygen.All horses were heavily padded during the surgical operation to minimize muscular damage. The right metetarsus was clipped free of hair before the horse was anesthetized and on Esmarch bandage and a tourniquet was applied to the limb. A 8-cm skin incision was made over the mid metetarsal region in the medial site and metatarsus III was exposed by blunt dissection. A rectangular-shape defect 30 mm in length, 20 mm in wide and 10mm depth was created by electrical osteotome (Stanley Works Inc, New Britain, CT) on midshaft of right metatarsal bone of each animal (Fig. 1). An iron template which was striled in autoclave was used to measured defect dimensions in all horses. The subcutaneus tissue was sutured with syntethic absorbable suture material (no 2 vicryl®) and the skin was sutured with no 2 Nylon suture material in horizontal mattress pattern. All horses were handled according to Shiraz University regulation for animal rights Horses were recived flunexin meglumin (2.2 mg kg^{-1}) and pencillin 3000000+3 g streptomicine (40000 u kg^{-1}) after surgery. In five horses the defects were filled with 9 mg of liquid bone morphogenic protein (1.50 mg mL^{-1} was used to fill the 20×30×10 mm defect) and five with normal saline, 4 days after operation.The liquid BMP was injected into the defect site (that was filled with blood clot) transcutaneusely. The operated limbs were supported by external cast for 10 days after injection of BMP. Three months after surgery, the horses were humanely euthanised by over dosage of nesdonal® (20 mg kg^{-1}) and the operated part of bone was harvested. The harvested pieces were decalcified in 10% formic acid with constant agitation over ion resin exchange, dehydrated in alcohol, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Areas of fibrous tissue, cartilage and bone were identified by their histological characteristics. Bone was further characterized as woven or lamellar and as viable or nonviable by the presence or absence of osteocytes. The percentage of osteogenic

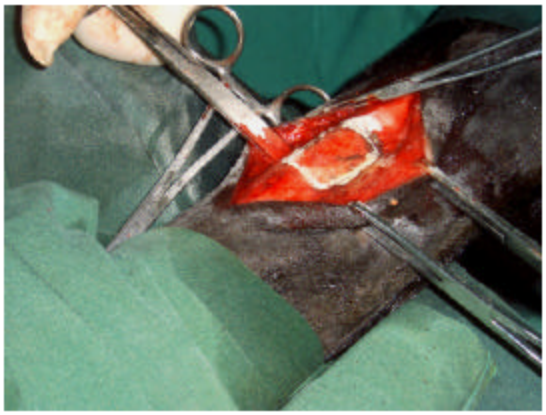

Fig. 1: A rectangular-shape defect on midshaft of right metatarsal bone

tissue, thickness of trabecular and cortical bone and presence of priosteum was measured as an indicator of osteogenesis. For explorative statistical analysis, the Mann-Whitney test was used (SPSS software, Chicago, IL, USA). A p-value <0.05 was considered as significant level. The extracted material from bovine bone were sent to laboratory for electrophoresis

RESULTS

In both control and treatment groups new bone formation was identified. There was no evidence of immature mesenchymal cells and cartilage formation in both groups. Cell morphology was identical and normal in control and treatment cases. However, new bone formation including trabeclar and cortical bone was seen in both groups. The trabeclar and cortical bone were thinner in control group compare to treatment cases ($p = 0.017$), (Table 1) (Fig. 2 and 3). preosteal formation identified in bmp treated cases but incomplete preosteal formation was seen in only one case of control group.

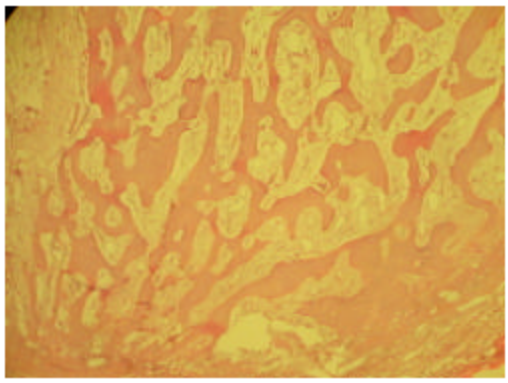

Fig. 2: Thin trabeclar bone formation in control group (H and E stain×400)

Fig 3: Thick trabeclar bone formation in BMP group (H and E stain×400)

Table 1: Comparative histomorphological data in both groups. (*N=Normal)

	Control				BMP			
	A	B	C	D	A	B	C	D
A- Periosteum								
1) Present	×				×	×	×	×
2) absent		×	×	×				
3) Intact	NO				×	NO	×	×
4) Thickness	N*					N	N	N
5) Cell morph	N					N	N	N
B- Cortical bone								
1) Present	×	×	×	×	×	×	×	×
2) absent								
3) Thickness	80%	70%	50%	80%	100%	90%	100%	100%
4) Cell morph	N	N	N	N	N	N	N	N
C- Trabeclar bone								
1) Present	×	×	×	×	×	×	×	×
2) absent								
3) Thickness	100%	80%	50%	80%	100%	90%	100%	100%
4) Cell morph	N	N	N	N	N	N	N	N
D- Cartilage								
1) Present								
2) absent	×	×	×	×	×	×	×	×
3) Type								
4) Where								
E- Immature mesenchymal								
1) Present								
2) absent	×	×	×	×	×	×	×	×
3) Type								
4) Where								

DISCUSSION

Bone Morphogenetic Proteins (BMPs) form a unique group of proteins within the Transforming Growth Factor beta (TGF-b) superfamily. BMPs were first identified by Urist (1965) when demineralized bone matrix implanted in nonskeletal sites in rats was found to induce bone formation. Today, there is extensive evidence in support of their role as regulators of bone induction, maintenance and repair (Reddi, 1993; Ripamonti *et al*., 1992; Wozney, 1992). BMPs are critical determinants of proliferation and differentiation of a wide variety of cells (Oksanen, 1998). Bone marrow stromal cells and perivascular mesenchymal cells form an important source of pluripotential progenitors that are capable of differentiating into both osteoblasts and chondroblasts under the appropriate conditions (Ahrens *et al*., 1993; Vukicevic *et al*., 1989; Wozney, 1992). Segmental long bone defects have been used as models for bone reconstruction to evaluate different transplant materials as well as the efficacy of BMP. This model is valid in studying osteoconductive agents when the defect (large enough) does not heal spontaneously (Einhorn *et al*., 1984). Animal studies with bone defects treated with bone substitute materials or BMP include (dog radius) (Heckman *et al*., 1999), dog femur, dog fibula, sheep tibia, rabbit ulna, rabbit radius and rat femur and dog ulna (Tapio, 2001). In evaluating the results, various

Table 2: Summary of the methods of analysis used in segmental bone defect models treated with BMP (Tapio, T., 2001).

Implant/carrier	Species	Bone	Defect size	Analysis methods	Authors and year
bBMP	Rat	Femur	1.0 cm	Radiography, histology	Tagaki and Urist 1982
bBMP	Dog	Ulna	2.5 cm	Radiography, histomorphometry	Nilsson *et al*., 1986
bBMP/PLA dBMP/PLA	Dog	Radius	0.3 cm	Radiography, histomorphometry	Heckman *et al*., 1991
rhBMP-2/DBM	Rat	Femur	0.5 cm	Radiography, torsion test, histology, radio-isotope boneimaging	Yasko *et al*., 1992
rhOP-1/collagen	Rabbit	Ulna	1.5 cm	Radiography, torsion test, histology	Cook *et al*., 1994
rhOP-1 /collagen	Dog	Ulna	2.5 cm	Radiography, torsion test, histology	Cook *et al*., 1994
rhOP-1 /collagen	Green monkey	Ulna and tibia	2.0 cm	Radiography, torsion test, histology	Cook *et al*., 1995
rhBMP 2/PGA	Rabbit	Ulna	2.0 cm	Radiography, torsion test, histology	Boström *et al*., 1996
sBMP/TCP	Sheep	Tibia	1.6 cm	Radiography, torsion test, histology	Gao *et al*., 1996
mBMP/coral	Sheep	Tibia	1.6 cm	Radiography, torsion test, histology	Gao *et al*., 1997
bBMP/DBM	Dog	Radius	2.5 cm	Radiography, torsion test, histology	Sciadini *et al*., 1997
bBMP/coral	Dog	Radius	2.5 cm	Radiography, torsion test, histology	Sciadini *et al*., 1997
rhBMP-2 /PLA	Rabbit	Radius	2 cm	Radiomorphometry, histomorphometry	Zegzula *et al*., 1997
rhBMP-2/PLA	Rabbit	Radius	1.0 cm	Radiography	Zellin and Linde 1997
rhBMP-2 /PDLLA	Dog	Ulna	2 cm	Radiography, histomorphometry	Itoh *et al*., 1998
rhOP-1 /collagen	Dog	Ulna	2.5 cm	Radiography, torsion test, histology	Cook *et al*., 1998
rhBMP-2 /PDLLA/PGA	Sheep	Femur	2.5 cm	Radiography, histology	Kirker-Head *et al*., 1998
rhBMP-2 /PDLLA/PGA	Rat	Femur	0.5 cm	Radiography, torsion test,	Lane *et al*., 1998
rhBMP-2/PLA/PGA	Rabbit	Radius	2.0 cm	Radiography	Texeira and Urist 1998
rhBMP-2/PLA	Rabbit	Radius	2.0 cm	Radiomorphometry, torsion test	Wheeler *et al*., 1998
cBMP/PLA	Dog	Radius	0.3 cm	Radiography, histomorphometry	Heckman *et al*., 1999
rhBMP-2 /PLA/PGA	Rat	Femur	0.5 cm	Radiography, histology	Isobe *et al*., 1999
rhBMP-2/TCP-MCPM	Rat	Femur	0.5 cm	Radiography, torsion test	Ohura *et al*., 1999
rhBMP-2/collagen	Dog	Radius	2.5 cm	Radiography, histology, biomechanical testing	Sciadini and Johnson 2000

methods of analysis have been used, the principal methods being radiography, histology and torsion testing (Table 2) (Tapio, 2001).

Over view the literature, reveal that no body has worked on the effect of bmp on bone healing in horse. This study evaluate the effect of probable available bmp in demineralized bone matrix, which we extract from bovine long bone on third metatarsal defect in horse. Metatars III is an easily accessible bone in horse, so we could easily expose it without further complications that may alter the result of our study. Bovine long bone is the most available material for BMP extraction as there is no slaughter for horse in our country. There is also no literature available about extraction of BMP and the probable extraction method from equine long bone, so we used bovine long bone to extract the protein material for bone induction. Early studies indicated that the molecular weight of bovine BMP was in the range of 12-30 kD, with strong evidence for a BMP of 17-18 kD (Urist *et al*., 1982, Mizutani and Urist, 1982). SDS gel electrophoresis identified a series of low molecular weight protein (15-50 KD) in the extracted materials. Also injection of 1 mg of liquid extracted material to rabbit muscle result in a radioopaque density at the injection site in the radiograph that can be a evidence for presences of BMP in extracted material. Small bone defects and nonunions can generally be managed by stable fixation and autogenous cancellous bone grafts (Bauer and Urist, 1981; Bentz *et al*., 1989). However, because large defects may require harvesting substantial autogenous corticocancellous graft and increase donor site morbidity, other treatment approaches are desirable. Bone Morphogenic Proteins (BMPs) are small proteins that induce bone formation but are quickly cleared from the wound 3 Therefore, use of a biodegradable matrix that permits controlled release of the BMP is necessary to achieve local bone induction. (Canalis *et al*., 1988). In this study, we did not use any carrier but we inject a paste form of BMP at the defect site in 5 days after surgery that the blood clot formation is present at the fracture site. The clot has the ability to save the paste form of BMP at the defect site. The histological demonstrated bone formation in both groups at the end of study but the amount and the quality of bone was significant in the BMP group ($p = 0.017$). Current studies are directed at characterizing the time-course of BMP to formation of bone in an orthopedics site with use of a similar experimental model in other animals. The effects of BMP on responsive populations of cells and the interactions of BMP with other endogenous growth and differentiation factors, are not completely understood and are the subject of ongoing investigations studies. Our results suggest that this extracted protein including material may be useful as a therapeutic adjuvant for horse in clinical situations when local formation of bone is needed in shorter period.

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