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EXPRESSION OF BMP7 IN BONE TISSUE TREATED WITH ALOE VERA

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ABSTRACT: *Bone repair is a multistep process involving migration, proliferation, differentiation and activation of several cell types that leads to healing . Many biological activities associated with Aloe vera have been attributed to the chemical component contained in the gel of the leaves. The present study was designed to illustrate the effect of topical application of aloe vera on bone healing by histological examination and immunohistochemical evaluation of BMP7 .Fifteen male Swiss rats were subjected for femur bone defect , one in the left side represented the control(defect was left without treatment) ,while the right side represented the experimental(treated with 0.1 µl of aloe vera gel). The animals were sacrificed at periods 7, 14, and 28 day, as five rats for each period. Histological and immunohistochemical evaluation for expression of BMP7 were done for all study samples.In-vivo results showed aloe vera treated group had faster bone healing compared with untreated controls. A substantial findings for the growth of bone trabeculae was observed in treated group. The results revealed that A.vera significantly increased osteogenic cell proliferation, expression of bone morphogenetic protein7and maturation of newly formed bone,compared with the untreated group. Conclusion:These data suggest aloe vera could function as a bioactive molecule inducing bone formation by stimulating proliferation and differentiation of osteoblasts with high expression of BMP7.*

KEYWORDS: Aloe vera, bone healing, BMP7.

INTRODUCTION

Bone healing involves a complex process of many local and systemic regulatory and enhanced factors. The presence of several bone morphogenetic proteins and their receptors in the fracture healing process have been described **(Kloen *et al.,* 2003)**. During the early stages of bone healing, only a number of primitive cells are expressing bone morphogenetic proteins in the tissue of callus. As the process of endochondral ossification proceeds, the presence of bone morphogenetic proteins and their receptors increases dramatically (**Chim *et al.,* 2013**). Bone Morphogenetic Proteins 7 or **BMP7** is a protein that in humans is encoded by the BMP7 gene. Like other members of the bone morphogenetic protein family of proteins, it plays a key role in the transformation of mesenchymal cells into bone(**Chen *et al.,* 2012**), Moreover **Schiavi *et al.,* 2015** revealed that combining of BMP-7 with mesenchymal stem cells accelerate bone growth in vivo.

Aloe vera has been used as a popular herbal medicine since ancient times for many conditions including burns, wound healing, and in dental treatment it illustrates bone, cementum and periodontal ligament regeneration. Acemannan, an extracted product from Aloe vera shows to stimulate dental pulp cell proliferation, differentiation, mineralization, and dentin formation(**Jittapiromsak *et al.,* 2010; Chantarawaratit *et al.,* 2014**).The present study has been prepared to illustrate the biological effect of local application of *A. vera* in bone healing.

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MATERIALS AND METHODS

Animals

Fifteen male Swiss rats, weighting (0.25 – 0.30 kg), aged 4-5 months were used and maintained under control conditions of temperature, drinking and food consumption and all experimental procedures were carried out in accordance with the ethical principles of animal experimentation. In each rat femur 2mm bone defects were created ,one in the left side represented the control ,while the right side represented the experimental. The study groups include:

- Control group ,the bone defect was left without treatment .
- Experimental group ,the bone defect treated with 0.1 µl of aloe vera gel

Materials

-Aloe vera Gel 87.399% ,Phyto care company.
-Immunohistochemical Polyclonal Antibodies ,Bone morphogenic protein7 antibody (BMP7) from Abcam Company UK (ab56023).

Methods

Histopathological evaluation

After scarifying the animals at periods (7,14,28 days), five animals for each period .The specimens were fixed by 10% buffered formalin for 3 days.The samples then demineralized, dehydrated, and embedded in paraffin,5µm section was stained with hematoxylin and eosin (H&E). The histological examination was done under light microscope and examining four microscopical fields for each slide for counting the number of the osteocytes, osteoblasts, and osteoclasts.Mean readings were calculated and used in statistical analysis .

Scores for Intensity of inflammatory reaction (**Accorinte *et al.*, 2008**)

1. Absent or very few inflammatory cells; 2. mild: average number less than 10 inflammatory cells; 3.moderate: average number 10-25 inflammatory cells ; 4.severe: average number greater than 25 inflammatory cells

Immunohistochemical scoring of BMP7

Quantification method of Immuno-reactivity was estimated for positive cell that expressed BMP7 .It was assessed by identifying and scoring 100 cells in five fields (X40) along bone defect area of different sections according to **Suzuki *et al.*, 2010**, the scoring is: (Score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; score 4, >80%).

Statistic Analysis

A. Descriptive data analysis. B. Inferential data analysis.

RESULTS

Histological results

At 7 day period: deposition of osteoid tissue and primitive trabeculae
At 14 day period: it shows the formation of bone trabeculae surrounding by osteoblast and osteocyte trapped within bone matrix

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At 28 day period: it shows immature bone for control group while experimental group shows the formation of mature bone (figures 1,2).
Statistic analysis revealed that the treated group illustrated either mild or no inflammation, whereas the control group had various degrees of inflammatory score(table 1).
Bone cells count (osteoblast,osteocyte,osteoclast) in treated group at 14 day period shows high mean value with highly significant difference in comparison with control (tables 2,3)

Immunohistochemical results of BMP7

At 7,14 and 28 day periods: positivity for expression of BMP7 illustrated by osteoblasts, osteoclasts and bone marrow stromal cells ,experimental group records a strong expression in all periods (figures 3,4). Statistic analysis revealed that the treated group (at 7 and 14 day periods) illustrated high immunohistochemical score for BMP7 with highly significant differences in comparison with control (tables 4,5,6)

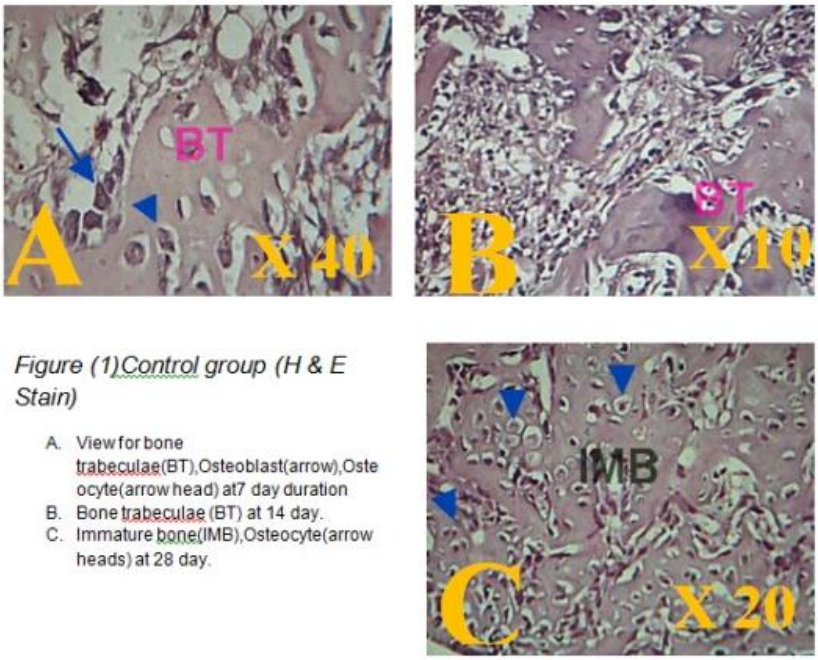

Figure (1)Control group (H & E Stain)

A. View for bone trabeculae(BT),Osteoblast(arrow),Osteocyte(arrow head) at7 day duration
B. Bone trabeculae (BT) at 14 day.
C. Immature bone(IMB),Osteocyte(arrow heads) at 28 day.

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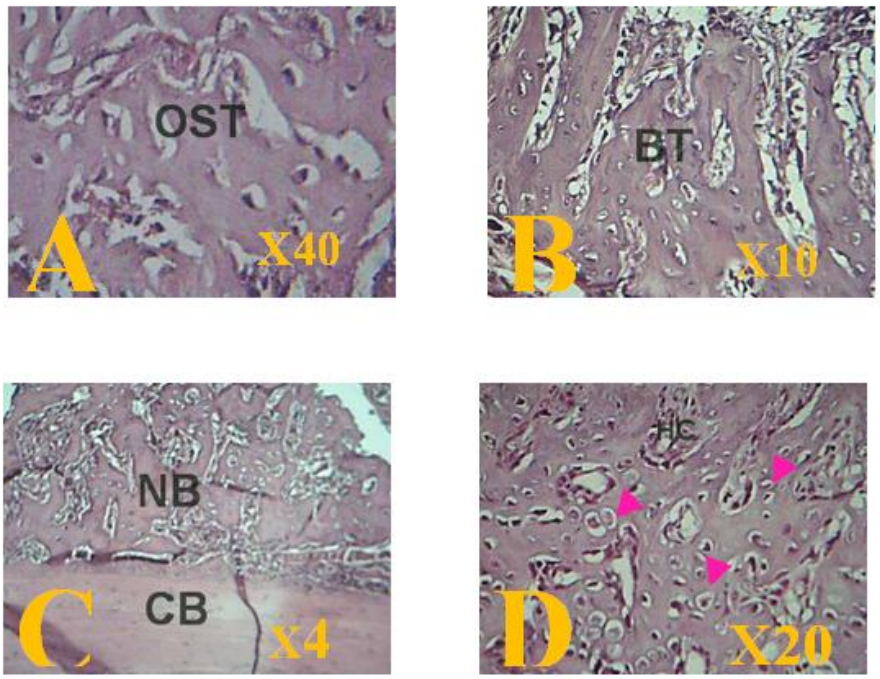


Figure (2) Experimental group (H & E Stain)

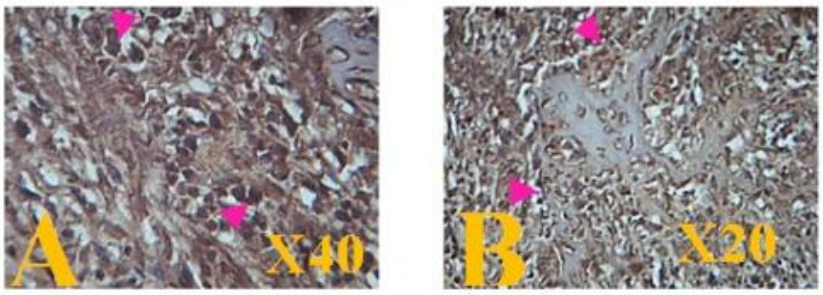
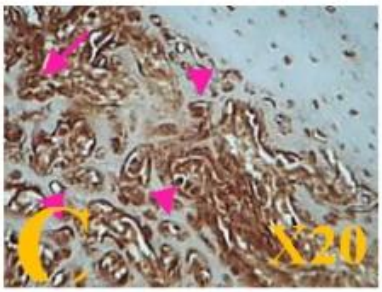


Figure (3) Control group with immunohistochemical expression of BMP7(DAPStain)

A. Bone marrow stromal cell(arrow heads)at7 day duration
B. Osteoblast(arrow heads)at 14 day.
C. Osteocyte(arrow heads),Osteoblast(arrow) at 28 day.



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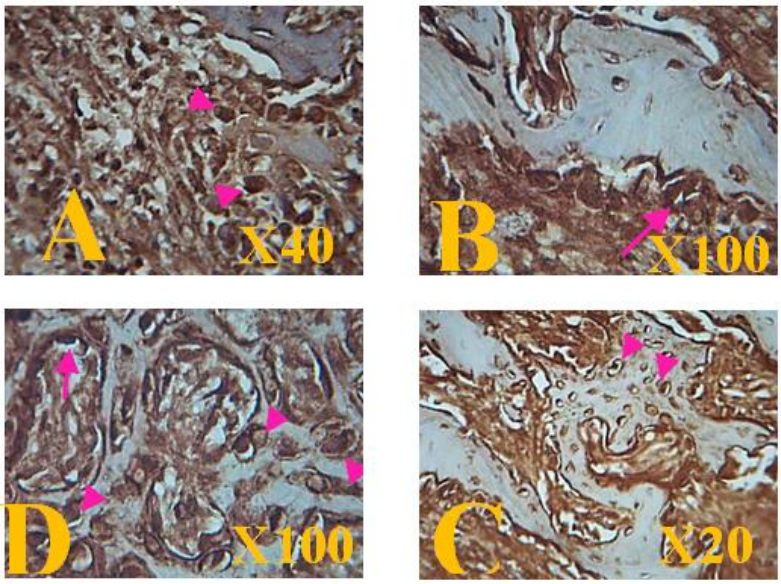

Figure (4) Experimental group with immunohistochemical expression of BMP7(DAPStain)

A. Bone marrow stromal cell(arrow heads)at7 day duration
B. Osteoclast(arrow)at 7 day.
C. Osteocyte(arrow heads) at 28 day.
D. Osteocyte(arrow heads),Osteoblast(arrow) at 28 day.

Table (1): Observed Frequencies of the Studied Inflammatory Scores in different groups by different (S.O.V.)

Inflammatory Score				
Groups	**Score**	**7Days**	**14Days**	**28Days**
Control	**Score - 1**	**0**	**0**	**3**
	Score - 2	**2**	**4**	**2**
	Score - 3	**3**	**1**	**0**
	Score - 4	**0**	**0**	**0**
Treated	**Score - 1**	**3**	**5**	**5**
	Score - 2	**2**	**0**	**0**
	Score - 3	**0**	**0**	**0**
	Score - 4	**0**	**0**	**0**

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Table (2): Summary statistics of the studied H&E Bone cells count Parameter in studied trials

Groups/ Bone Cells/periods	Sample No.	Mean	Std. Dev.	Std. Error	95% C. I. for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Control - Osteoblast - Days 14	5	8.97	1.03	0.42	7.58	9.75	8	10
Control - Osteoblast - Days 28	5	6.00	1.10	0.45	4.85	7.15	5	8
Control - Osteocyte - Days 14	5	10.67	3.88	1.58	6.59	14.74	7	18
Control - Osteocyte - Days 28	5	8.87	2.07	0.84	6.50	10.83	6	12
Control - Osteoclast - Days 14	5	1.00	0.63	0.26	0.34	1.66	0	2
Control - Osteoclast - Days 28	5	0.40	0.55	0.22	-	1.07	0	1
Treated-Osteoblast - Days 14	5	14.70	1.64	0.67	12.78	16.22	12	16
Treated-Osteoblast - Days 28	5	6.88	0.75	0.31	6.04	7.62	6	8
Treated-Osteocyte - Days 14	5	13.00	0.89	0.37	12.06	13.94	12	14
Treated-Osteocyte - Days 28	5	8.89	1.83	0.75	6.91	10.76	7	12
Treated-Osteoclast - Days 14	5	1.60	0.52	0.21	1.12	2.21	1	2
Exp.- Osteoclast - Days 28	5	0.53	0.75	0.31	0.04	1.62	0	2

Table (3): Multiple Comparisons by (LSD Method) of bone cells count According to different Groups in compact form

LSD test				
periods	groups	Mean Difference	Sig.(*)	C.S.
14 day	Control treated	-0.67	0.006	HS
28 day	Control treated	0.14	0.645	NS

(*) HS: Highly Sig. at P< 0.01; Non Sig. at P> 0.0

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Table (4): Observed Frequencies of the Studied immunohistochemical scoring of BMP7 in different groups by different (S.O.V.)

BMP7 Score				
Groups	**Score**	**7Days**	**14Days**	**28Days**
Control	**Score - 1**	**2**	**4**	**4**
	Score - 2	**2**	**1**	**1**
	Score - 3	**1**	**0**	**0**
	Score - 4	**0**	**0**	**0**
treated	**Score - 1**	**0**	**2**	**3**
	Score - 2	**3**	**2**	**1**
	Score - 3	**1**	**1**	**1**
	Score - 4	**1**	**0**	**0**

Table (5): Summary Statistics of the Studied No. of Positive Cells expressed BMP7 in the Studied Groups

Groups	**Positive cells**	**Mean**	**Std. Dev.**	**Std. E.**	**95% C. I. for Mean**		**Min.**	**Max.**
					L.B.	**U.B.**		
Control	**Stromal cells**	**23.8**	**1.6**	**0.7**	**22.2**	**25.5**	**22**	**26**
	Bone cells	**6.5**	**0.6**	**0.3**	**5.3**	**6.7**	**5**	**7**
treated	**Stromal cells**	**49.2**	**2.6**	**1.1**	**44.6**	**51.0**	**45**	**50**
	Bone cells	**16.9**	**1.0**	**0.4**	**15.6**	**17.8**	**16**	**18**

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Table (6): Multiple Comparisons by (LSD Method) of Positive cells count for BMP7 Parameter According to different Groups in compact form

LSD test				
periods	**groups**	**Mean Difference**	**Sig.(*)**	**C.S.**
7 day	**Control treated**	**-1.19**	**0.858**	**HS**
14 day	**Control treated**	**-0.97**	**0.918**	**HS**
28 day	**Control treated**	**-0.22**	**1.000**	**NS**

(*) HS: Highly Sig. at P< 0.01; Non Sig. at P> 0.05

DISCUSSION

At 7 day, in the treated group, either mild or no inflammation was found, whereas the control group had various degrees of inflammation .The study groups show osteoid tissue formation, as the periphery of the bone defect site, includes, stem cells differentiate into osteoblasts. As a result, bone deposits via intramembranous ossification 7–10 days after injury period, primarily as osteoid, a non-mineralized bone. At 14 day period the experimental group showed bone trabeculae filled a proximately the whole defect in comparison to histologic views for control,it may attributed to biological activities of *A.vera* include promotion of wound healing, by the presence of polysaccharide , that affects on BMSCs proliferation, differentiation, extracellular matrix synthesis, mineralization, and bone formation (**Hamman 2008; Boonyagul *et al.*, 2014**)

At 28 day period mature bone with osteon formation illustrates in experimental group only, while control shows immature form. This result could be explained as follows:
1. Polysaccharides in *A. vera* gel have therapeutic properties such as immunostimulation, anti-inflammatory effects, anti-viral, anti-fungal that stimulate of hematopoiesis and enhanced bone healing(**Talmadge *et al.*, 4004 ,Habeeb *et al.*, 2007**)
2. *A.vera* may stimulates bone formation by osteoblastic stimulation related to the presence of alkaline phosphatase in its chemical components (**Choi andChung2003, Boudreau and Beland 2006**) that increase bone formation earlier and to a larger extent in trabecular bone rather than compact bone
3. Presence of inorganic compounds Calcium, chlorine, chromium, copper, iron, magnesium, manganese,potassium, phosphorous, sodium and zinc in chemical composition of *A. vera* (. **Dagne *et al.*, 2000**) facilitates mineralization of new bone.

In the present study, *A.vera* significantly stimulated bone marrow stromal cell proliferation and expression of BMP7.The study has identified a high expression of BMP7 by infiltrated mesenchymal cells and osteoblasts during subsequent osteogenic response, suggesting the potential role of *A.vera* in modulating of healing response in comparison to control.

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Osteoprogenitor cells at the fracture site express bone morphogenetic proteins. These factors, along with inflammatory mediators, recruit mesenchymal stem cells and then guide their differentiation and proliferation.The present result shows strong positive expression of BMP7 by bone marrow stromal cells in treated group ,which related to enhancement of infiltration and proliferation of mesenchymal cells that occurs at the bone injury site, and some of these mesenchymal cells differentiate into osteoblasts (**Xian *et al.*, 2004**, **Jettanacheawchankit *et al.*, 2009**).

Zhou *et al.*, 2004 study reporting an upregulated expression of some cytokines or growth factors at a various phases of bony repair.
In study groups at the bone maturation stage, only BMP-7 expression was seen slightly upregulated and expressed in differentiated osteoblasts and bone marrow cells at the injury site.

CONCLUSION

The results suggest that aloe vera could be a candidate natural biomaterial function as a bioactive molecule inducing bone formation with high expression of BMP7 , and helps in bone regeneration.

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