European Review for Medical and Pharmacological Sciences 2016; 20: 3507-3513

Leptin improves osteoblast differentiation of human bone marrow stroma stem cells

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Abstract. – OBJECTIVE: To study the effects of leptin (LEP) on the osteogenic differentiation of human bone marrow stromal cells (hBMSCs) and to explore the mechanism controlling the process.

MATERIALS AND METHODS: Respectively cultivated the third-generation hBMSCs with 100 ng/ml bone morphogenetic protein (BMP) culture media containing 320, 160, 80 and 40 ng/mL LEP, and regular medium. We administered alkaline phosphatase (ALP) dye (on the 7th day) and mineralized nodules alizarin red (on the 21st day) and tested the ALP activity as well as osteocalcin (OCN) level on 7th, 14th, 21st day in each group to establish the best inducing concentration of LEP. 7 days later, we tested bone differentiation related genes expression in the control, 160 ng/mL LEP and 100 ng/mL BMP groups using RT-PCR.

RESULTS: The activity of ALP and OCN in the 160 ng/mL LEP group after 7, 14 and 21 days was lower than that of the BMP group but higher than that of other groups. However, LEP significantly promoted the expression of bone differentiation related genes, namely, Cbfal, ALP, COL-I and OCN.

CONCLUSIONS: LEP promoted the bone differentiation in hBMSCs by promoting the expression of genes related to bone differentiation.

Key Words
Bone tissue engineering, Marrow stromal stem cells, Osteoblasts, *In vitro* culture.

Introduction

Bone marrow stromal cells (BMSCs), also called mesenchymal stem cells, have self-updating and multi-directional differentiation potentials, and they can differentiate towards bone, cartilage, fat, fibroblasts, cardiac muscle and nerve cells under appropriate inducing conditions[1–4]. Leptin (LEP) is an important cell factor which plays a regulatory role in bone metabolism. It is unclear whether it has any influence on hBMSCs bone differentiation and if it does, what is the mechanism involved in the process. In order to provide evidences for future study, we used *in vitro* culture model of hBMSCs to study the effects of LEP on the Gegenbaur cells specific markers of, ALP, OCN and the expressions of bone differentiation related genes (Cbfal, ALP, COL-I, OCN).

Materials and Methods

Main reagents and equipment

For this study we used the following materials and equipments: DMEM culture medium (Hyclone, South Logan, UT, USA); fetal bovine serum (FBS); Trizol (Invitrogen, Carlsbad, CA, USA); LEP (ProSpec, Ness-Ziona, Israel); ALP test kits (Nanjing Built Biological Reagent Company, China); NBT/BCIP alkaline phosphatase dye liquor (Beyotime, China); alizarin red (Amresco Co., Solon, OH, USA); osteocalcin radioimmunoassay kit (Radioimmunoassay Institute, Institute of Technology and Development Center, PLA General Hospital); rhBMP-2 (Peprotech, Hill, NJ, USA); DNA Marker (Qiagen, Hilden, Germany); Type SN-682 gamma counter (Shanghai Nuclear Fu Photoelectric Instrument Co., Ltd., Shanghai, P.R. China); ELX 800 enzyme standard (BioTek, Shanghai, P.R. China); PCR amplificator (Biometra, Göttingen, Germany); DYY-2 stabilized electrophoresis apparatus (Liuyi Instrument Plant, Beijing, China); gel imaging system (Bio-Rad, Hercules, CA, USA).

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Methods

The whole bone marrow method was used to culture hBMSCs and, after the passage, the media was changed routinely. The third generation cells in good growth state were used for the experiments. We divided cell culture media into 6 experimental groups. We added, respectively, 320; 160; 80 and 40 ng/ml of LEP to groups A to D. The 5th group or the BMP group received 100 ng/ml of BMP and the 6th group only had cell culture media and was called the normal medium group or the control group.

Observation index

ALP dyeing (NBT/BCIP)

Cells were seeded into 6 holes plates to mount the slides and to prepare cell cultures. After the adherence, 2 ml of each group (A, B, C, D, BMP and control) were added for intervention. Original culture media were discarded on the 7th day and each hole was rinsed twice using phosphate buffer (PBS). Paraformaldehyde was added to fix the cells for 10 minutes followed by rinsing with distilled water (3 times). ALP dye solution was added next and samples were incubated for 30 minutes at 37°C. Extra dye was discarded and samples were rinsed 3 times using distilled water, slides were then dried, and sealed.

Mineralized nodule staining

Cell processing method was the same as above. Original culture media was discarded on the 21st day, followed by washing with PBS 2 twice. At, 4°C, 95% ethanol was added to fix the samples for 10 minutes and samples were rinsed with distilled water (3 times). Alizarin red (0.1%) was added to dye the samples for 30 minutes at 37 C followed by rinsing with distilled water.

ALP quantity test

Cells were added into 24 holes plates (10^4 cell/hole), using the same protocol explained before. ALP activity level was examined in each group on 7th, 14th, 21st day. After rinsing and splitting the cells, absorbance value (A value) of each hole was measured at 562 nm wavelength using ALP test kit and repeated the measurements 3 times and calculating ALP activity level (King unit/100 ml) using the following formula: [King unit/100 ml = sample tube absorbance value × standard tube phenol activity (0.005 mg) × 100 ml/standard tube absorbance value × 0.05 ml].

OCN quantity test

OCN levels on the 7th, 14th, 21st day were verified. After rinsing and splitting the cells, 100 µl supernatant was taken, and 100 µl OCN was added followed by 100 µl of OCN I^{125} marked antibody and incubated at 4°C for 24 hours. We then added 0.5 ml of separating agent into each tube and left them at room temperature for 20 minutes followed by centrifugation at 35,000 rpm at 4°C for 25 minutes. Supernatant was discarded and the radiation dose was measured in pellets. The related parameters, the standard curves and the sample concentrations were directly produced by the software programmed in the automatic gamma counter.

Osteogenic related gene expression test

160 ng/ml LEP group and 100 ng/ml BMP group were added to culture medium group, and after culturing the cells from each group for seven days, we used Trizol Two-step protocol to extract total RNA from the cells. Two µg RNA was used to conduct reverse transcription and amplification was done according to the protocol of One Step RNA PCR Kit. The PCR conditions were as follows: (i) 95°C for 5 min,

Table I. The primers of osteoblastic related gene (cbfa1, ALP, COL-I, OCN).

Gene	Annealing temperature (°C)	Upstream primer (5′-3′)	Downstream primer (5′-3′)	Product length
Cbfa1	56	GAGTAGGTGTCCCGCCT CAGAACCC	TCTGAAGCACCTGC CTGGCTCTTCT	325bp
ALP	58	TGGAGCTTCAGAAGCTC AACACCA	ATCTCGTTGTCTGAGTACC AGTCC	454bp
OCN	60	AGGTGCGAAGCCCAGCGGTGCA	CCTGGAGAGGAGCAGAACTGGG	292bp
COL-I	56	CTGGCAAAGAAGGCGGCAAA	CTCACCACGATCACCACTCT	501bp
GAPDH	55	AATCCCATCACCA TCTTCCA	CCTGCTTCACCACCTTCTTG	580bp

94°C for 30 s, the annealing temperatures are in Table 1 and the annealing time was 30s; (ii) 72°C for 40 sec, 35 cycles; (iii) 72°C for 7 min. Primers were synthetized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). 4 μl of PCR product was put into 2% agarose gel for electrophoresis, and Bio-Rad gel imaging system was used for imaging.

Statistical Analysis

We used SPSS 13.0 statistic software (SPSS Inc., Chicago, IL, USA) for our statistical analyses and mapping. Measurement data were expressed with $\bar{\chi}\pm s$. Comparison between each group of time series data was conducted with the variance analysis of repeated measuring data. We used LSD method to compare multiple times between different levels of time factor major effects. We used the single factor variance analysis method to compare data for each group. $p<0.05$ was considered statistically significant.

Results

Influences that LEP has on hBMSCs osteogenic differentiation

hBMSCs morphologic observation

P3 generation hBMSCs with good growth state, under the microscope, had clear cell outlines with uniform sizes and appearance, long spindle and fiber-shaped, fusion into pieces, aggregated growth, showing a spiral arrangement in some areas (Figure 1 A). After cells had been added into conditional culture medium and LEP conditional culture medium of different concentration, some cells died. The shape of cells gradually changed into square, circle, triangle and polygon and lost the spiral growth appearance. The limits between cells became vague and gathered together, and partial cells demonstrated multi-layer growth, which had the similar shape and growth features as Gegenbaur cells. Culture media with different concentration had different numbers of small granular substance, and well-differentiated ones had maturely-growing mineralized nodules (Figure 1 B).

ALP dyeing observation

Cells from each group culture ALP Group A, B, C, D and BMP cells had clear outlines, and the cytoplasm in each group was blue with positive staining. The control group had weak positive staining (Figure 2).

Mineralized nodule dyeing observation

Cells in groups A, B, C, D and BMP were successfully stained with alizarin red dye while in the control group cells did not show any color (Figure 3).

ALP and OCN quantitative detection

On the 7th, 14th and 21st day, the differences between the ALP expression in group B and that of other groups were statistically significant (n=6, $p<0.05$) (Figure 4-A). Meanwhile, OCN expression level in group B was higher than that of the control group and lower than that of BMP group on the 7th and 14th day (n=6, $p<0.05$). On the 21st day, the differences between the OCN expression level in group B and other groups were statistically significant (n=6, $p<0.05$) (Figure 4-B).

Osteogenic cell differentiation related gene expression test

Compared with control group, expression of osteoblast-related genes was enhanced in LEP group, although still lower than BMP group (Figure 5).

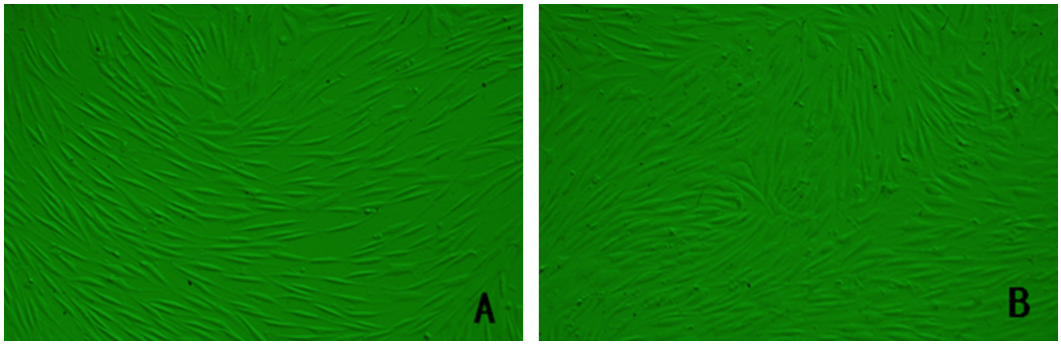

Figure 1. hBMSCs morphological observation under the microscope (inverted phase contrast microscope 100X) (**A**) The 3rd-generation hBMSCs grew colony-like and radially (**B**) The inductive hBMSCs was cell body well-stacked and having the morphology and growth characteristics similar to those of the osteoblasts.

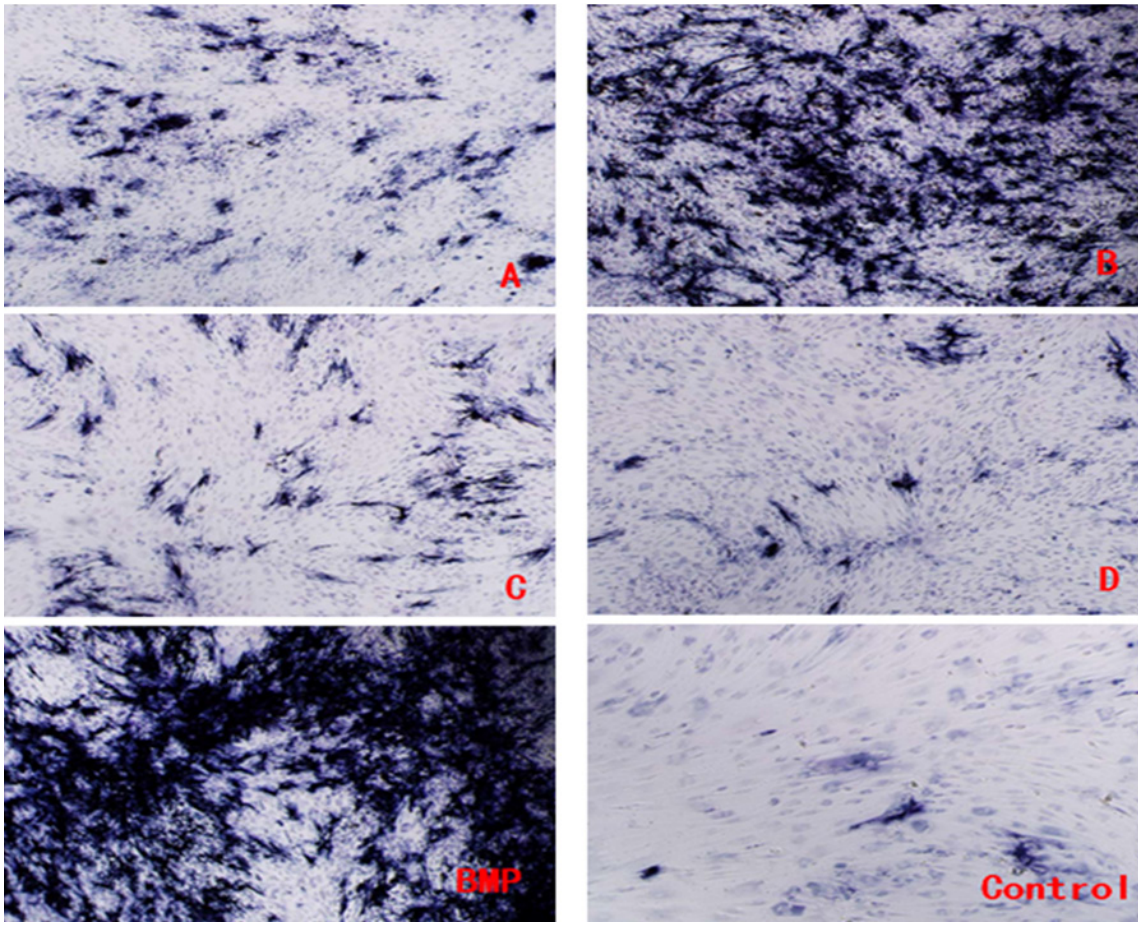

Figure 2. Staining of ALP in hBMSCs at 21st day (NBT/BCIP stain, inverted phase contrast microscope 100×). Positive staining in A, B, C, D and BMP groups, lower levels of positive staining in the control group.

Discussion

The potential for the application of multipotent hBMSCs cells, in clinical settings had a profound effect on the future of bone tissue engineering and osteoporosis therapeutics[5-7]. Seeking the factor that regulates the differentiation of these cells towards Gegenbaur cells has always been a hot topic in the bone biology field. When the obesity gene, LEP, was found in 1994[8], it was thought that its function was limited to regulating food intake and energy balance[9,10]. However, LEP can also regulate bone metabolism through two pathways namely, central and periphera[11-14]. Although currently we have some understanding of the function of LEP in bone metabolism, our knowledge is still limited.

Prior studies[15] showed that hBMSCs were LEP's target cells. Thomas et al[16] studied human bone marrow stromal cell line hMS2-12, and found out that LEP did not influence the proliferation of hMS2-12 cells and the Cbfa1 expression level. Nevertheless, it increased the ALP, COL-I and OCN expression levels which enhanced the mineralized matrix of the long-term cultured hMS2-12 cell by 59%. These results suggested that LEP had some effects on hBMSCs differentiation towards Gegenbaur cells and inhibited its differentiation into the adipose cell at the same time. Cuizhu and Qiang[17] showed that LEP inhibited the BMSCs *in vitro* proliferation but promoted the expression of early osteoblast-related genes such as Cbfa1 and Cbfb. Currently, there is still no definite answer about how LEP can influence the mechanism hBMSCs activity and its relation with the formation of bones.

Generally hBMSCs growth, differentiation and gene expression are completed in several stages: (i) Differentiation and proliferation stage, with DNA replication and protein synthesis; (ii)

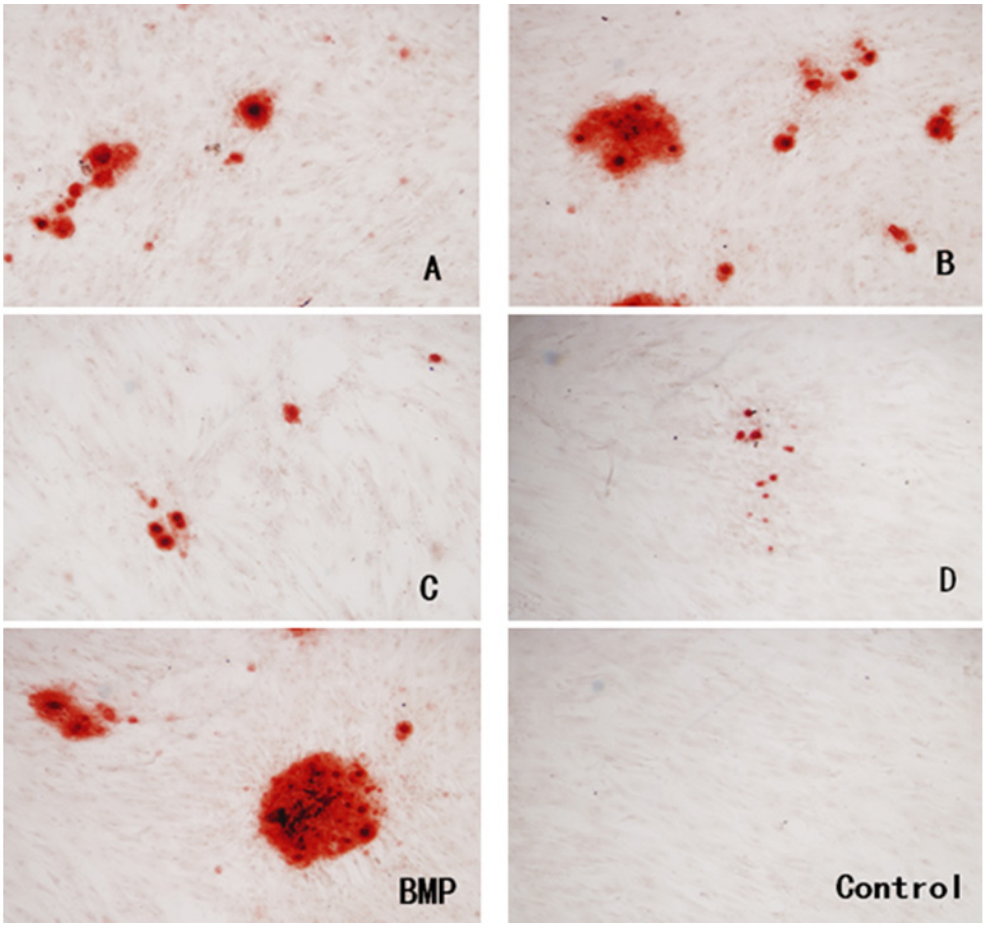

Figure 3. Observation on mineralized nodules of hBMSCs at 21th day (chinalizarin stain, inverted phase contrast microscope 100×). Red-stained mineralized nodules in **A**, **B**, **C**, **D** and BMP groups and negative staining in the control group.

Matrix formation and maturation stage, with the increase of ALP activity and the secretion of COL- I; (iii) Matrix calcification stage, with OCN secretion and calcium deposition and continuous increase of ALP activity[18]. It is generally believed that ALP is the early index and functional marker of osteoblast differentiation, which plays a crucial part in calcification *in vitro*. Its activity is not only related to the number of hBMSCs, but also the degree of self osteogenic differentiation, which means ALP activity reflects the general inducing effect. OCN is a polypeptide secreted by maturely-differentiated osteoblast cells, which can be regarded as the medium-term indicator of osteoblast differentiation and the marker of osteoblast differentiation and maturation[19-21]. Cbfa1 is an indispensable key factor in the process of hBMSCs osteogenic differentiation and bone formation function. Cbfa1 belongs to the Runt domain family, and is also known as Runx2 (Runt related gene 2). It is a common signal molecule that many types of bone inducing factors promote osteogenesis, and it is an early marker of osteoblast differentiation, which activates and starts the transcription of a series of downstream osteoblast-related genes including COL-I, ALP and OCN, to achieve the biological function of osteoblasts[21-23]. The activity of ALP and OCN reflects the state of osteoblast differentiation. The increase in ALP and OCN activities indicates a higher degree of osteoblast differentiation.

The present work showed that the effective *in vitro* level of LEP was 7 to 100 ng/ml and this concentration was in the same range as the physiological range of LEP human body[24]. To establish the best LEP concentration, our study was carried out by this grouping method (making BMP and normal culture medium as positive and negative comparison respectively). Results showed that the activity of ALP and OCN in 160 ng/ml LEP

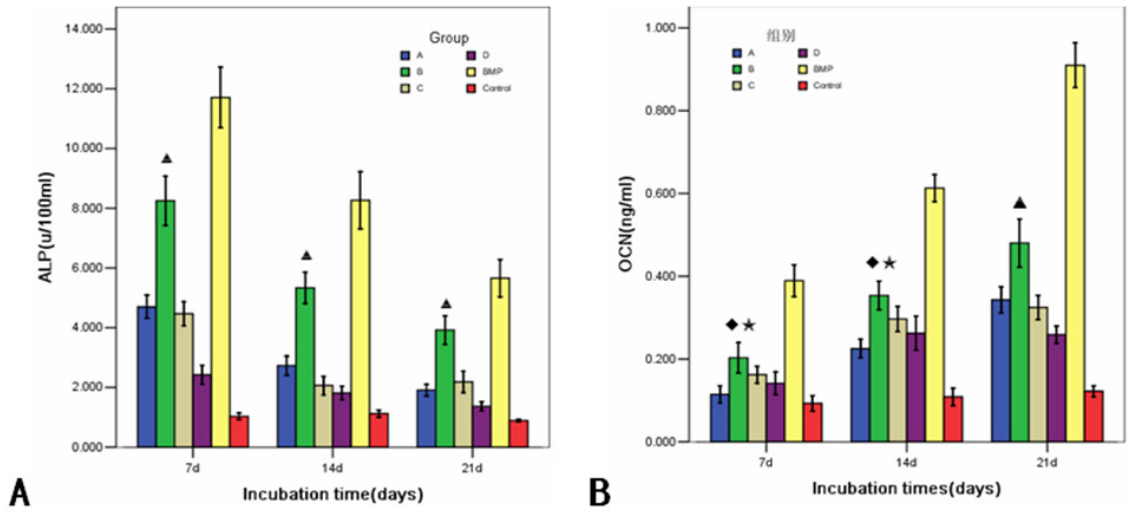

Figure 4. ALP (**A**) and OCN (**B**) activities of hBMSCs in all groups at 7th, 14th, 21st day) ▲Compared with the other groups, $p<0.05$; ◆Compared with the BMP group, $p<0.05$; ★Compared with the Control group $p<0.05$ (n=6).

group is lower than BMP group, but higher than other groups; Besides, Cbfa1, ALP, COL-I and OCN mRNA expression could be detected on the 7th day of culture in hBMSCs pretreated with 160 ng/ml LEP.

Conclusions

LEP could promote the osteoblast differentiation in hBMSCs and its mechanism may improve the expression of Cbfal mRNA. As for the cause

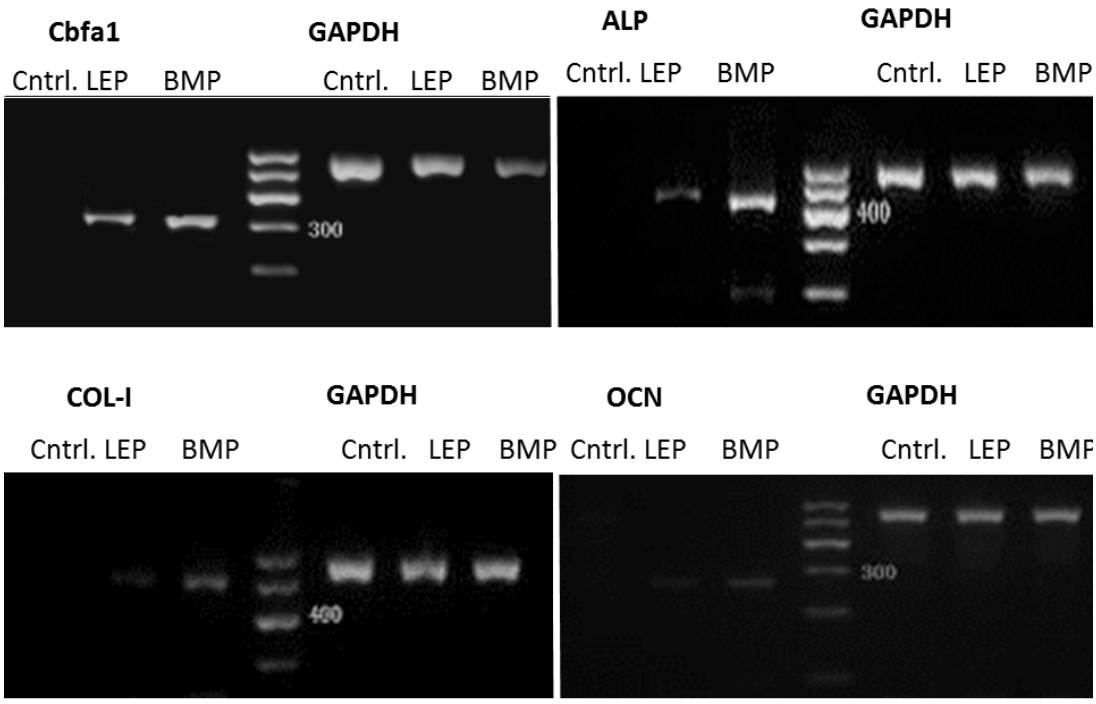

Figure 5. RT-PCR products (Cbfal, ALP, COL-I, OCN) electrophoretogram. There were no Cbfal, ALP, COL-I, OCN mRNA expression in the control group and a moderate expression in the LEP group, whereas there was a strong expression in the BMP group.

of LEP regulating the expression of hBMSCs Cbfa1, the conduction path that participates in regulating is still an open question now, which requires further research.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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