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*Original Research*

# How does the supernatant of *Lactobacillus acidophilus* affect the proliferation and differentiation activities of rat bone marrow-derived stromal cells?

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**Abstract:** Low proliferation rate and unwanted differentiation of bone marrow-derived stromal cells (rBMSCs) during the frequent passages have limited the use of such cells in clinical cell therapy. Recently, the researchers have focused on the effects of the components produced by some bacteria on proliferation of the stem cells. In this study, we discussed the possible effects of the *Lactobacillus acidophilus* supernatant on proliferation and differentiation of the rBMSCs. For this aim, the cells were isolated from rat bone marrow, characterized by culturing on tissue specific differentiation media and stained. The cells (passage two) were treated with different concentrations of the *L. acidophilus* supernatant (0, 0.1, 0.3, 0.9, 3, 9 and 30 µl/ml) for 14 days. The proliferation and differentiation capacity of the cells were then determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT assay) and tissue specific staining. The results showed a positive effect of the supernatant on the cell proliferation in 3 and 9 µl/ml concentrations, while did not affect the differentiation capacity of the rBMSCs. The current study strongly suggests the *L. acidophilus* supernatant as an alternative material that could be added to the media with aim of improvement in the proliferation rate of the rBMSCs without affecting their differentiation capacity.

***Key words:*** Bone marrow-derived stromal cells, *Lactobacillus acidophilus*, Tissue engineering, proliferation, differentiation, Cell therapy.

## Introduction

Probiotic microorganisms are the most important bacteria associated with the human gastrointestinal tract (1, 2) where they can control diarrhea, decrease sensitivity to lactose and diminish absorption of cholesterol (3, 4). The exact mechanisms by which probiotics provide such beneficial effects to the host are not yet fully understood. However, it is well-established that these bacteria can utilize several mechanisms to exert their functions (5). For example, probiotic bacteria produce a variety of agents against harmful microorganisms which may stop their growth. They can produce lactic acid to decrease pH (6, 7). Importantly, some probiotic bacteria seem to promote immune system activity by increased cytokine production (8, 9). In addition, they are able to compete with pathogens for growth factors and nutrients, and eventually reduce their growth rates (10).

*Lactobacillus acidophilus* is one of the most important probiotic bacteria, residing in the gastrointestinal tract (11). Several studies have demonstrated that *L. acidophilus* plays an important role in human and animal health through increasing the activity of the immune system both *in vivo* and *in vitro* (8, 12). After treatment, *L. acidophilus* can affect the population and density of harmful microorganisms settled in the gastrointestinal tract and thereby prevent their overgrowth (13).

It has been reported that *L. acidophilus* is involved in indirect modulation of the immune system both *in vitro* and *in vivo*. Oral consumption of *L. acidophilus* was found to enhance mitogen-induced lymphocyte proliferation, as well as serum IgA, IgG and IgM levels secreted by mouse mucosal cells (14). Kitazawa *et al.* (15) showed that the macrophages isolated from mice secrete a high level of IFN-α/β, when exposed to heat-killed *L. acidophilus*. In a study conducted by Rangavajhyala and coworkers (16), it has been revealed that *L. acidophilus* leads to an induced production of TNF-α and TNF 1α in the murine macrophage cell line, RAW264.7, as well as TNF-α , IL-12 , IL-10 in the J774 A.1cell line. In this research line, Halper *et al.* (8) demonstrated the capability of the *L. acidophilus* supernatants (LS) in induction of angiogenesis and chemotactic property, *in vitro* proliferation of macrophages, lymphocytes, and *in vivo* proliferation of fibroblasts, endothelial cells and inflammatory cells. In several studies, increases in the number of neutrophils as well as blood vessel growth were detected after administration

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of *L.acidophilus* in young rodent ear lobes. In another study carried out by Li *et al.*(2), the effects of culture supernatant of *L.acidophilus* were investigated on a chicken embryo, a mouse neuroblasto, a cell line during the early stages of neuronal development and a cow embryo. In that study, they concluded that LS can increase the proliferation of a wide variety of cells, especially mesenchymal stem cells (MSCs). Stem cells are a population of primary cells with intrinsic capability of self-renewal and differentiation into various cell lineages under special conditions (17-19). Stem cells are generally known as colony-forming fibroblastic cells, mesenchymal progenitor cells and BM stromal cells (20-22). Two types of multipotent cells exist in adult bone marrow, hematopoietic and stromal cells (23, 24). Rat bone marrow-derived stromal cells (rBMSCs) are used as a potential source for cell therapy (25-29). The capability of rBMSCs to support hematopoiesis, bone reconstruction, and patients suffering from osteogenesis and myocardial defects have been well documented in the literatures (30, 31). Importantly, it is demonstrated that BMSCs immediately lose their multipotent characteristics, colony-forming capacity and proliferation ability through frequent passages (28, 32), and undergo to be differentiated into an unwanted cell type, depending on environmental conditions (33). After the third or fourth passages, the proliferation capacity gradually decreases to trace levels (34). This makes it difficult not only to prepare the cells required for clinical application, but also results in a decreased response to treatment (35).

In the present study, we sought to investigate whether the culture supernatant of *Lactobacillus acidophilus* could overcome the low-proliferative potential of the rBMSCs and delay their unwanted differentiation during the frequent passages, as a result of which they can acquire the ability to treat different diseases, especially bone lesions.

## Materials and Methods

### Preparation of culture Supernatant of L.acidophilus

The *L.acidophilus* strain, ATCC 4356, was purchased from Iranian Research Organization for Science and Technology, and cultured in Man, Rogosa and Sharpe broth medum (MRS broth) (pH:5.5, Merck Millipore,110661), supplemented with 0.05% L-cysteine (Sigma,24850236), for about 48 hours under microaerophilic conditions (8). When the bacterial concentration reached $25\times10^8$ CFU (by pour plate method (36)), the culture medium was centrifuged at 10000g for 15 minutes at 4°C (2). The resultant supernatant was filtered through a 0.2 µm nylon filter and stored at -20°C until used.

### Isolation and culture of Rat bone marrow-derived stromal cells (rBMSCs)

The *rBMSCs* were isolated and cultured according to the published protocol, with some modifications (37). Briefly, six-to eight-week-old Sprague-Dawley male rats (Pasteur Institute of Iran, Tehran) weighing 250-300 grams were sacrificed by $CO_2$ asphyxiation in according to ethical consideration. Their tibia and femur were then dissected, surrounding muscular tissues were removed and the naked bones were cut at both ends. Afterwards, the bone marrow cells were flushed into a 15 ml tube by a sterile syringe loaded with low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM Glutamax, 1 mM $_L$-glutamine, 1% penicillin/streptomycin, nystatin and amphotericin B, and 1% non essential amino acids (all from Gibco, Carlsbad, CA). All the cells were filtered through a 70 mm-diameter whatman filter (sigma, Z240060, USA). The cell viability was determined by trypan blue dye exclusion. A total of $25\times10^6$ cells were seeded in a Petri dish containing DMEM (PAA COMPANY, E15-806), as mentioned above, and cultured under standard conditions in a humidified 5% $CO_2$ incubator at 37°C for 3 hours. After the predetermined incubation time, the non-adherent cells were removed and replaced with the fresh media. After 8 hours, the media was again replaced with 10 ml of fresh medium; this was repeated every 8 hour for 72 hours. The remaining adherent cells were washed with phosphate buffered saline (PBS) and the media were changed every 3 days. After 4 weeks, when reached 70-80% confluence, the cells were washed with PBS, treated with 25% trypsin in 1mM EDTA (PAA COMPANY,L11-001) for 2 minutes at 37°C, and plated in 25 $cm^2$ flasks after trypsin neutralization (38, 39).

### Osteogenic and adipogenic differentiation of rBMSCs

Before exposure to *L. acidophilus* supernatant, the cells were treated with osteogenic and adipogenic differentiation-defined media after 14-day incubation with different concentrations of *L. acidophilus* supernatant and control samples (the cells cultured in *L. acidophilus* supernatant-free culture plate for 14 days). For determination of osteogenic differentiation capability, a density of $5 \times 10^4$ cells/ml were incubated in a 6-well plate containing DMEM supplemented with 15% FBS in the second passage. After 1 week, once the wells of the 12-well plate reached confluence, the media was replaced with osteogenic medium, consisting of DMEM, 50 mg/ml of ascorbic acid 2-phosphate, 10nM dexamethasone and 10nM of β-glycerol phosphate. The plates were cultured 21 days in a humidified atmosphere of 5% $CO_2$ at 37°C, with media replacement every 3 days. The cells were then fixed for 10 minutes in 10% formalin, stained by Alizarin red for 15 minutes at room temperature, and observed under a light microscope.

For differentiation into adipocytes, a total of $5 \times 10^4$ cells/ml were seeded in a 6-well plate containing DMEM supplemented with 15% FBS, 50Mg/ml indomethacin and 100 M dexamethasone. The plates were cultured 21 days in a humidified 5% $CO_2$ incubator at 37°C, with media replacement every 3 days. To determine the differentiation into adipocytes, the cells were stained by 0.5% Oil red for 15 minutes, and then observed under a light microscope.

### Cell proliferation

The cell viability was determined by the MTT colorimetric assay [3-(4,5 dimethylthiazol-2-yl)-1,5 diphenyltetrazolium bromide] (MTT). The ability of living cells to convert tetrazulium to fumarazin is the basic principle of the MTT assay. The MTT test was

performed by a procedure described in our recently published paper with some modifications (28). Briefly, a total of $5\times10^3$ cells were seeded in a 96-well microplate, treated with different concentrations of *L. acidophilus* supernatant (0, 0.1, 0.3, 0.9, 3, 9 and 30 µl of supernatant in 1 ml of supplemented DMEM) and incubated in the standard cell culture conditions for 14 days. The cells cultured in supernatant-free DMEM served as a negative control. The MTT test was also performed for the cells treated with the same concentrations of MRS broth (bacteria-free) to eliminate the interference arising from the components of bacterial culture media. The medium was changed each 3 days. After the predetermined incubation time, for the cell viability assay the culture medium was replaced with the 15% MTT solution (Atocel, Austria) in PBS (5mg/mlPBS), and incubated for 2 hours at 37°C. The solution in each well was replaced with DMSO and shaken in a dark chamber for 15 minutes. Finally, the optical density (OD) of each well was measured using a plate reader at a wavelength of 570 nm and a reference wavelength of 630 nm. The number of the cells was also determined by using a standard curve. The data obtained from each sample was normalized with those in the negative control, where the cell viability in control sample was considered as 100%. The test was repeated six times for each sample (n=6).

### Statistical analysis

Distribution of data was assessed by the Kolmogorov–Smirnov test. The data were expressed as means ± SD, and statistically analyzed by independent sample *t-tests*. A *P* value of $< 0.05$ was considered as the level of significance. The Sigma Plot 11.0 θ software was used for plotting graphs.

## Results

### Differentiation potential of the rBMSCs into osteogenic and adipogenic lineages

Figure 1(a) shows the isolated rBMSCs, the cells stained with Alizarin red (a) and Oil red (b) after treatment with osteogenic and adipogenic defined media, respectively, before treatment with supernatant. As expected, bone deposition was detected in the osteogenic medium, implying that the cells differentiated into osteocytes, which is considered as a key characteristic of the rBMSCs (Figure 1(b)). Figure 1(c) also showed

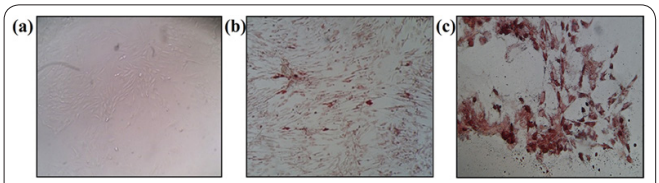


**Figure 1.** (a) The rat bone marrow mesenchymal stem cells in passage 2 (inverted microscope; magnification, ×100). (b) The cells stained with Alizarin red after 21-day incubation in osteogenesis defined media. Calcium deposition of osteocytes can be clearly observed near the cell margin, indicating the osteogenesis (inverted microscope; magnification, ×100). (c) The cells stained with Oil red after 21-day incubation in adipogenic defined media. The large-rounded cells with eccentric nuclei and large fat-filled vacuoles are visible, indicating adipogenesis (inverted microscope; magnification, ×100).

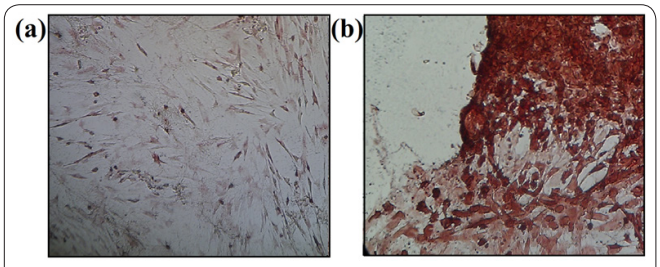


**Figure 2.** Alizarin red (a) and Oil red (b) staining of the cells cultured in supernatant-free Dulbecco's Modified Eagle Medium after 21-day culturing in the osteogenic and adipogenic differentiation medium, respectively. The cells were successfully differentiated into the bone and adipose lineages.

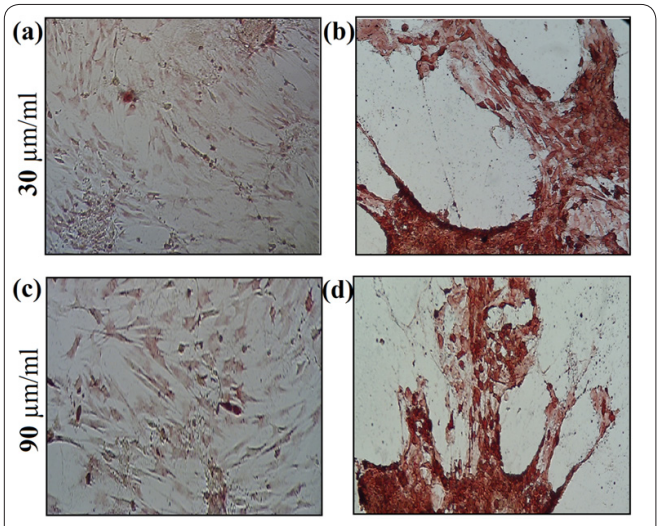


**Figure 3.** The rat bone marrow mesenchymal stem cells stained with Alizarin red (a,c) and Oil red (b,d). The cells were treated with 3 and 9 µl/ml of supernatant for 14 days, and then cultured in osteogenic (a,c) and adipogenic (b,d) differentiation medium for 21 days. The cells were successfully differentiated into the bone and adipose lineages.

that the cells were successfully differentiated into adipocyte cells.

After 14-day treatment with the supernatant, the cells cultured in supernatant-free DMEM (the negative control), 3 and 9 µl/ml supernatant were cultured in tissue specific differentiation-defined media and stained with Alizarin red and Oil red. The results showed that the cells retained their differentiation property after 14 day-treatment with both 3 and 9 µl/ml supernatants. Figures 2 and 3 show the control and 30 µl/ml sample, respectively.

### Cell proliferation

The viability of the rBMSCs was determined by MTT 14 days post treatment with different concentrations of the MRS broth media (Figure 4) as well as bacterial supernatant (Figure 4). The data obtained from the MTT showed that the MRS broth did not significantly affect the viability of the cells up to concentration of 9 µl/ml, while decreasing the viability in concentrations of 3 - 30 µl/ml (independent sample *t-test*, $p<0.05$) in comparison to the negative control. As clearly shown in Figure 4, the cell viability in the samples treated with supernatant 3 and 9 µl/ml was significantly higher than that in the negative control and MRS groups (independent sample *t-test*, $p<0.05$). In concentration of 30 µl/ml,

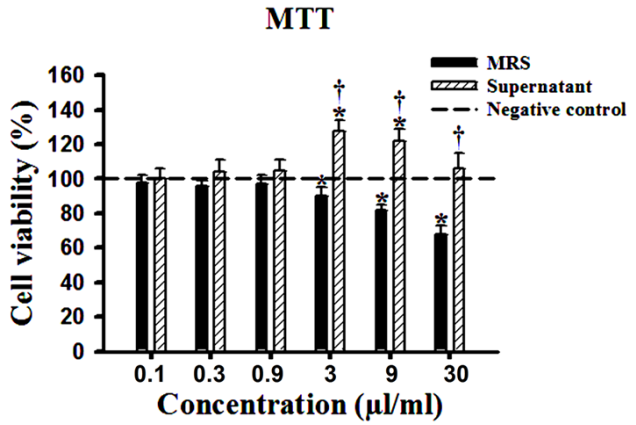


**Figure 4.** MTT results of the Man, Rogosa and Sharpe broth medum (MRS)- and supernatant-treated rat bone marrow mesenchymal stem cells after 14 day-incubation. * A significant difference with the negative control. † A Significant difference with MRS.

however, the viability of the cells showed an insignificant difference, as compared with the negative control (independent sample *t-test*, $p<0.05$), while significantly higher than MRS sample in same concentration.

## Discussion

Low proliferation capacities of bone marrow-derived stromal cells has remained challenging and limited the applications of such cells in clinic. It has been well-established that the rBMSCs lose their proliferation activity during the frequent passages. Several studies have been made to tackle such hurdle. For instance, using of some growth factors such as b-FGF, PDGF and TGF-β have a beneficial effects in proliferation of MSCs (40). However, these materials inflict high charge transpiration to health system and patients.

In the present study, we surveyed the possible effect of supernatant of *L. acidophilus* in proliferation and differentiation of the rBMSCs. To this end, the cells were isolated from the rats' bone marrow and treated with different concentrations of the supernatant obtained from the *L. acidophilus* culture medium for 14 days. The differentiation property of the cells before and after treatment with the supernatant was determined through culturing in the differentiation-defined media and cell lineage-specific staining. The effect of the extracted supernatant on the proliferation property of the cells was evaluated by MTT. The interesting results obtained from the present study were the significantly increased cell proliferation in supernatant treatment, especially in concentrations of 3 and 9 µl/ml in comparison with negative control (1.28-and 1.22-fold). In contrast, the MRS broth exhibited no significant effect on the cell proliferation at the same concentrations. In higher concentration (30 µl/ml), however, both supernatant and MRS broth treatment showed a significant decrease in cell proliferation in comparison to the negative control. A possible interpretation of this phenomenon could be the negative effects of the MRS broth present in the supernatant, although further examinations require to clarify such interference. In addition, growth prevention was more perceptible in more volumes of MRS broth cultures, which could be justified by more acidity of bacteria-free MRS broth medium. However, as the supernatant volume increases, the medium pH decreases and overcomes the optimal pH for MSC growth, leading to a reduction in the cell proliferation. Negative effects of the MRS on the cell proliferation were clearly detected in the MTT results. In fact, in concentrations of 3 µl/ml MRS, the proliferation rate clearly decreased, although there was no significant difference. With the increase of the MRS concentration to 9 µl/ml or higher, the proliferation level of the rBMSCs in MRS-treated group showed a significant decrease, when compared to the control.

In several studies regarding the effect of *L.acidophilus* on the cell proliferation, significant increases were also found in the embryonic stem cell proliferation (2, 8). It was also found that the cell proliferation in the MRS broth was less than that in *L. acidophilus* supernatant. This results from the importance of secreted substances from *L. acidophilus* governing the stromal cell proliferation and growth (41). It is important to investigate whether such substances could retain stemness of the rBMSCs and thereby prevent their unwanted differentiations *in vitro*. For this purpose, the differentiation property of the cells was also determined for those of supernatant concentrations (3 and 9 µl/ml) displaying positive effects on the cell proliferation. According to the results obtained from this study, rBMSCs showed a high differentiation property even after 14-day incubation with the tested concentrations of the supernatant. These findings were consistent with other studies in this research line (2, 8).All the results in the present study strongly suggested the potential application of the *L. acidophilus* supernatant in cell therapy, such as materials positively increased the proliferation of the rBMSCs without affecting their differentiation property. Further examinations are required to extract and purify the *L. acidophilus* supernatant from MSR broth medium to eliminate the negative effects of the bacterial culture medium on the stromal cells.

Low proliferation property of the bone marrow-derived stromal cells has limited their applications in clinic. Therefore, development of a method for enhancing the proliferation rate of such cells without affecting their differentiation activity is worthy. In the current study, we found that the supernatant derived from *Lactobacillus acidophilus* could positively affect the proliferation activity of such cells in a certain concentration. Further investigations require to purification the supernatant from bacterial culture medium (MRS) and optimization the appropriate concentration of the pure supernatant in which the cells show highest response to the treatment.

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