

The Effect of *Bifidobacterium Bifidum* Supernatant and Cell Mass on the Proliferation Potential of Rat Bone Marrow-Derived Stromal Cells

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What's Known

- *Lactobacillus acidophilus* supernatant can promote angiogenesis and increase the proliferation potential of some cell lineages, such as macrophages and bone marrow stromal cells.
- MRS broth media can interfere with the treatment of bacterial agents.

What's New

- The effect of probiotics on the cell proliferation of individual low-proliferative stem cells has been confirmed. Common characteristics among probiotics have been demonstrated.
- The effect of both the supernatant and bacterial cell mass was investigated. The use of bacterial cell mass is shown to be slightly more effective than the supernatant.

Abstract

Background: Mesenchymal stem cells (MSCs) are widely used to treat various diseases, however, their proliferative potential reduces after a number of passages. It has been shown that some probiotics such as *Bifidobacterium bifidum* (*B. bifidum*) affect the proliferation of various cell lineages. The present study aimed to investigate the effect of *B. bifidum* on the proliferation of rat bone marrow stromal cells (rBMSCs) and to develop a method for compensating their proliferation reduction after some passages.

Methods: The present experimental study was conducted at Tehran University of Medical Sciences, Tehran, Iran, in 2017. The stromal cells were isolated from rBMSCs and their mesenchymal properties were confirmed by osteogenic and adipogenic differentiation media and staining. *B. bifidum* was cultured and the *B. bifidum* supernatant (BS) and bacterial cell mass (BCM) were extracted. The rBMSCs were treated with different concentrations of BS and BCM. The MTT assay was performed to measure the number of viable cells in the culture. Cell proliferation was analyzed using the paired-sample *t* test.

Results: Cell proliferation increased as the concentration of bacteria was increased logarithmically (0, 0.1, 0.3, 0.9, 3, 9, 30 μ L/mL). In comparison with BS, cells treated with BCM showed increased cell proliferation at lower concentrations. This effect was caused by removing the “de Man, Rogosa, and Sharpe” (MRS) broth medium from the BCM culture. The optimal concentration of bacteria with the most significant effect on rBMSCs proliferation was determined.

Conclusion: A significant increase in the proliferation of stromal cells was observed; confirming the stimulatory potential of probiotics (*B. bifidum*) on various cells. The use of products containing probiotic bacteria can increase the proliferation potential of BMSCs.

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Keywords • *Bifidobacterium bifidum* • Mesenchymal stromal cells • Cell proliferation • Probiotics

Introduction

Today, there has been a growing consumption of products containing probiotic bacteria to treat various diseases.¹ This group of bacteria has several benefits such as modulating

gastrointestinal microorganisms.² Studies have shown the health benefits of specific probiotic strains of *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Bacillus*, *Escherichia coli*.³ Although the activating mechanism of many probiotics has been identified, there are ongoing research studies to understand other mechanisms and their effects on various diseases. For instance, the effect of probiotics on diarrhea,⁴ cholesterol absorption,⁵ and lactose sensitivity⁶ has been demonstrated.

Several studies have addressed the effect of probiotics on diseases such as immune system disorders and cancer. A previous study has shown that oral administration of probiotics could be a mitogen for lymphocytes proliferation and increase serum levels of IgG, IgM, and secretory IgA.⁷ Studies have been conducted to investigate the effect of probiotics on the proliferation of various cell lineages. Some studies have shown the potential of probiotics on reducing the proliferation of cancer cells (especially gastrointestinal carcinoma),^{8, 9} and some others have used probiotic agents as adjuvants to control gastrointestinal cancer.¹⁰ A previous study showed that the supernatants of *Lactobacillus acidophilus* (LS) stimulated the proliferation of macrophages and lymphocytes by increasing chemotaxis and angiogenesis properties.¹¹ The effect of LS on embryonic stem cells and on the proliferation of rat bone marrow stromal cells (rBMSCs) has also been demonstrated.^{12, 13} Additionally, the role of probiotics in cell differentiation has been studied.¹⁴

Stem cells, particularly mesenchymal stem cells (MSCs), are widely used to treat various diseases such as cancer. Recent advancement in stem cell transplantation has resulted in increased effectiveness of such treatments.^{15, 16} However, one of the main limitations of MSCs is the reduction of their proliferative potential after numerous passages whereby cells tend to differentiate. Consequently, it is important to find methods to increase the proliferation rate of these cells.¹⁷ It has been shown that *Lactobacillus* stimulates the proliferation of macrophages and lymphocytes, and it has some common metabolic properties with *Bifidobacterium*.¹⁸⁻²⁰ Hence, the present study aimed to investigate the effect of *Bifidobacterium bifidum* (*B. bifidum*) on rBMSCs and to develop a method for compensating for their proliferation reduction after some passages.

Materials and Methods

The present experimental study was conducted at Tehran University of Medical Sciences,

Tehran, Iran.

Preparation of Bacterial Supernatant and Extract

B. bifidum strain (ATCC: 29521) was obtained from the Iran Research Organization for Science and Technology (IROST), Tehran, Iran, in 2017. The bacteria were cultured in MRS broth (pH value 5.5, Millipore-110661) with 0.05% L-cysteine (Sigma, 24850236) at 37 °C for 48 hours under microaerophilic conditions.^{11, 21} The suspension was centrifuged three times at 10,000 ×g at 4 °C until the bacterial count reached 2×10⁸ CFU, using the pour plate method.²² The supernatant was separated and filtered using a 0.2 μm microfilter. Then, the *B. bifidum* supernatant (BS) was frozen and stored for later use. To isolate the bacterial extract, the sediment mass was harvested after each centrifugation.¹²

Isolation of Rat Bone Marrow Stromal Cells

A male rat (6-8 weeks old, weighing 300 grams) was obtained from the Pasteur Institute, Tehran, Iran. In accordance with the University's code of ethics (number: IR.TUMS.REC.1394.533) the rat was sacrificed by CO₂ asphyxiation. The tibia was dissected under sterile conditions and the surrounding muscle tissue was completely removed. The tibia was cut at both ends and the bone marrow stromal tissue was flushed out with a mixture of Dulbecco's modified eagle's medium (DMEM), 2 mM Glutamax, 1 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, nystatin and amphotericin B, and 1% non-essential amino acids (all from Gibco, Carlsbad, CA). Finally, the cells were filtered through a 70 mm diameter filter (Hawach Scientific Co., China) to remove debris.¹³

Primary Stromal Cell Culture

Cell viability was determined using trypan blue dye (Merck, Millipore, 23850, Germany).²³ A Petri dish containing the complete culture medium, as mentioned above, was used to seed 25×10⁶ cells and was subsequently incubated under standard conditions with 5% CO₂ at 37 °C for 3 hours. Then, the unattached cells were removed and the medium was replaced with the fresh culture medium. After 8 hours, the medium was replaced again with 10 mL of fresh culture medium. This process was repeated every 8 hours for 72 hours, after which the remaining attached cells were washed with phosphate-buffered saline (PAA Laboratories GmbH, Austria); the medium was replaced every 3 days. After 4 weeks, when the culture

reached about 80% confluency, it was incubated for 2 minutes with 25% trypsin in 1 mM EDTA (L11-001, PAA Laboratories Inc, Austria). After trypsin neutralization, the cells were seeded in 25 cm² flasks.²⁴

Cell Differentiation into Osteogenic and Adipogenic Lineages

A total of 5×10⁴ cells were seeded in 6-well plates containing DMEM, 15% FBS, and 1% penicillin/streptomycin. The cultures were divided into three groups. The culture medium of the first group included rBMSCs without any *B. bifidum* contents. The second group included rBMSCs in the differentiation medium together with BS and the third group with *B. bifidum* bacterial cell mass (BCM). These cultures were incubated under standard conditions with 5% CO₂ at 37 °C for 7 days. The cells were then cultured in an osteogenic or an adipogenic differentiation defined medium and incubated for 21 days during which the medium was replaced every 3 days.²⁵

Each milliliter of the osteogenic culture medium composed of DMEM, 50 mg/mL ascorbic acid 2-phosphate, 10 nM dexamethasone, and 10 nM β-glycerol phosphate, all produced by Sigma Aldrich, US. Finally, the cells were fixed for 10 minutes in 10% formalin, stained with Alizarin red for 15 minutes at room temperature, and observed under a light microscope (Olympus, Germany).²⁶ Similarly, each milliliter of adipogenic culture medium composed of DMEM supplemented with 15% FBS, 50 mg/mL indomethacin, and 100 M dexamethasone. To determine adipocytes differentiation, the cells were stained with 0.5% Oil red-O for 15 minutes and then observed under a light microscope.²⁷

Cell Proliferation Assay

The cell viability was determined using the [3-(4,5 dimethylthiazol-2-yl)-1,5 diphenyltetrazolium bromide] MTT colorimetric assay. The principle of the MTT assay is the

ability of the living cells to convert tetrazulium to formazan.²⁸ In brief, 5×10³ cells were seeded in a 96-well microplate. The cells were then treated for 14 days with the medium from each of the two groups containing BS or BCM in different concentrations (0, 0.1, 0.3, 0.9, 3, 9, and 30 μL/mL of supplemented DMEM).¹³ During this period, the medium was replaced every 3 days. Besides, a control group (standard) was added, which included the cells with culture medium without BS and BCM.

The medium was replaced with 15% MTT solution (Atocel, Austria) in PBS (5 mg/ml) and incubated at 37 °C for 2 hours. The solution in each well was replaced with DMSO and shaken in a dark chamber for 15 minutes. Finally, the optical density for each well was measured using a plate reader at the wavelength of 570 nm and a reference wavelength of 630 nm. The number of cells was determined using a standard curve. The tests were repeated six times for each sample (n=6).

Statistical Analyses

Distribution of the data was evaluated using the Kolmogorov-Smirnov test (one-sample and pair-sample tests). The data were expressed as mean±SD and analyzed using the paired-sample *t* test. P<0.05 was considered statistically significant. The SigmaPlot software (version 12.0) was used for the graphical presentation of the data.

Results

Cell Differentiation into Osteogenic and Adipogenic Lineages

After the preparation of the primary cell culture, the cells were used in the second passage (figure 1a). The rBMSCs were cultured in osteogenic and adipogenic media, after which the cells differentiated into osteoblasts and adipocytes. Figure 1 (b, c) shows the cells subjected to osteogenic differentiation

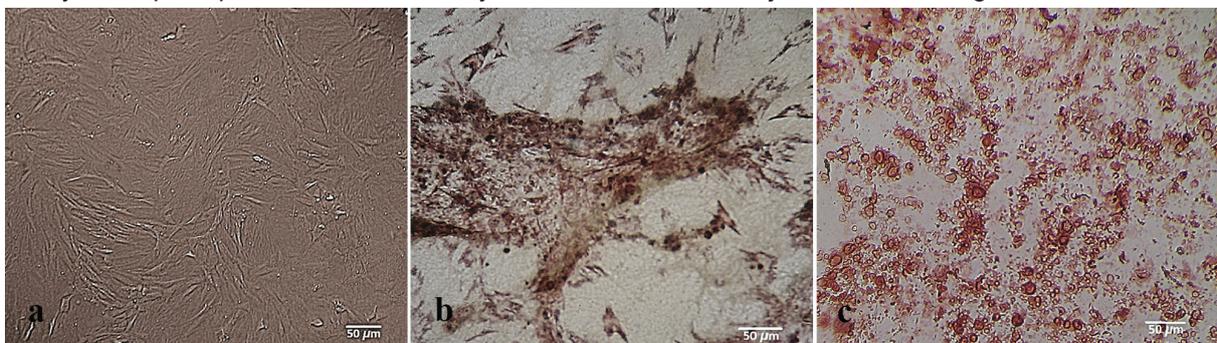


Figure 1: (a) The rat bone marrow stromal cells in the second passage (400× by inverted microscopic system). (b) The rat bone marrow stromal cells after 14 days in osteogenic media; stained with Alizarin red. (c) The rat bone marrow stromal cells after 14 days in adipogenic media; stained with Oil red-O.

for 14 days and then stained with Alizarin red. Calcium deposition was detected, indicating their differentiation potential (figure 1b). The rBMSCs cells cultured in an adipogenic differentiation medium (stained by Oil red-O) differentiated into adipocytes (figure 1c). This differentiation was considered a key feature of the rBMSCs, especially the MSCs. After 14 days of rBMSCs treatment with BS and BCM, using the exact same protocol, the staining process was performed with Alizarin red and Oil red-O. Figure 2 shows a comparison between the test sample and the negative control.

Cell Proliferation Assay

The viability of the cells was determined using the MTT assay after treating the rBMSCs with

different quantities of BS or BCM. The optical density for each well, measured with a plate reader, was converted to the number of cells using the standard curve (tables 1 and 2). In parallel, the viability of the cells was determined in cells treated with MRS broth medium with similar concentrations as BS and BCM. The results between the groups were analyzed and compared using the paired-sample *t* test. The results showed that changes in cell proliferation in the MRS broth medium were less than changes in BS and BCM. The cell proliferation at low concentrations of the MRS broth (0.1, 0.3, and 0.9 $\mu\text{L}/\text{mL}$) was similar to the control group. However, cell viability reduced at higher concentrations.

The results of the MTT assay showed

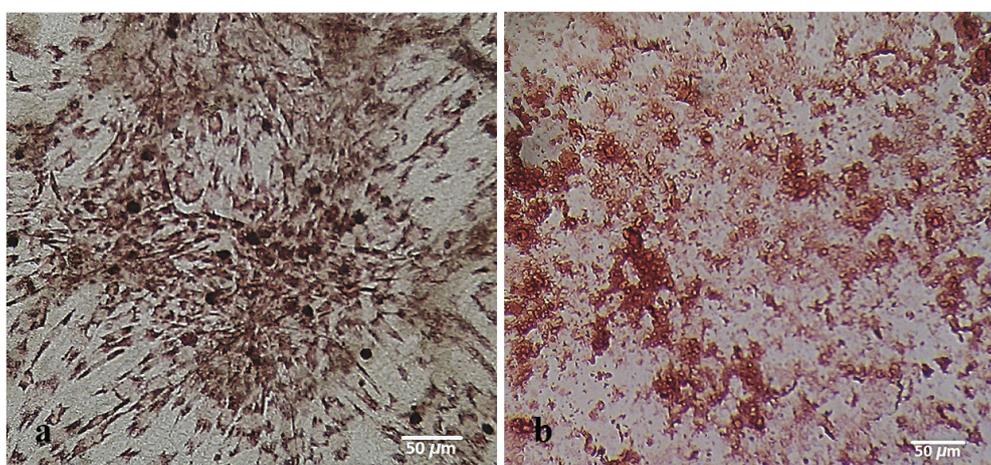


Figure 2: (a) The rat bone marrow stromal cells after 14 days of treatment with *Bifidobacterium bifidum* supernatant; stained with Alizarin red. (b) The rat bone marrow stromal cells after 14 days of treatment with *Bifidobacterium bifidum* supernatant; stained with Oil red-O (400 \times using the inverted microscopic system).

Table 1: The number of cells treated with *Bifidobacterium bifidum* supernatant in different concentrations

Bacterial supernatant concentration ($\mu\text{L}/\text{mL}$)	The number of cells						mean \pm SD	P value
0.1	12686	12309	12792	12786	12260	12680	12585.5 \pm 238.44	0.283
0.3	12663	12483	12852	12511	12917	12711	12689.5 \pm 175.40	0.271
0.9	12715	12905	12823	12688	12694	12992	12802.83 \pm 125.99	0.235
3.0	13336	13557	13244	13501	13218	13423	13379.83 \pm 137.47	0.186
9.0	14274	14841	14564	14510	14331	14404	14487.33 \pm 204.14	0.011
30.0	13780	14109	13914	14278	13899	13662	13940.33 \pm 222.73	0.046

Table 2: The number of cells treated with bacterial cell mass in different concentrations

Bacterial cell mass concentration ($\mu\text{L}/\text{mL}$)	The number of cells						mean \pm SD	P value
0.1	12686	12309	12792	12786	12260	12680	12585.5 \pm 238.44	0.283
0.3	12761	12585	12957	12611	12812	12611	12722.83 \pm 147.14	0.266
0.9	13105	12905	12823	12288	12914	12992	13004.5 \pm 285.65	0.249
3.0	13801	13757	14244	13901	14218	13723	13940.67 \pm 232.85	0.040
9.0	14450	14731	14812	14596	14646	14804	14673.17 \pm 138.73	0.036
30.0	12780	13109	12914	13278	12899	12662	12940.33 \pm 222.73	0.241

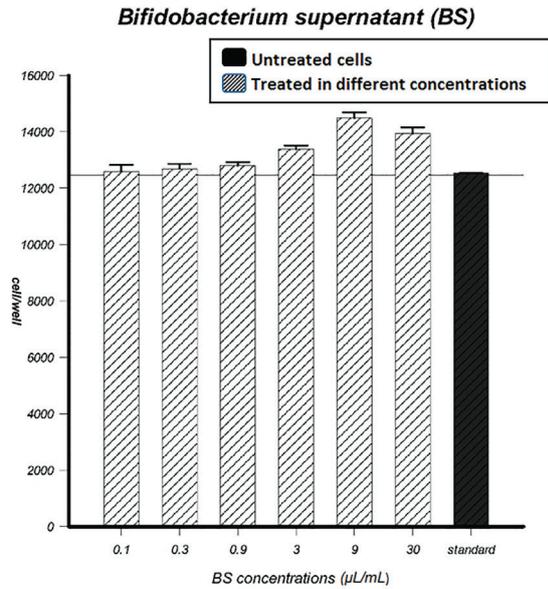


Figure 3: The effect of the logarithmic concentrations of *Bifidobacterium bifidum* supernatant on rat bone marrow stromal cells. The MTT assay showed that the highest proliferation occurred in 9 µL/mL concentration. The control group included cells with standard culture medium without *Bifidobacterium bifidum* supernatant and bacterial cell mass as a negative control.

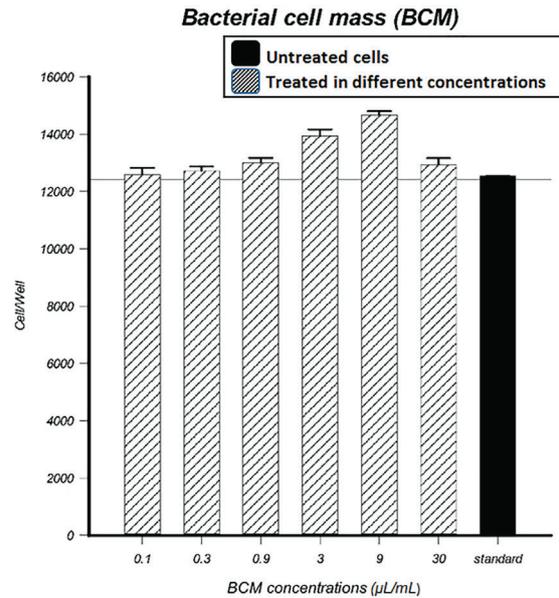


Figure 4: The effect of bacterial cell mass on rat bone marrow stromal cells in different logarithmic concentrations (MTT assay). The control group included cells and standard culture medium without *Bifidobacterium bifidum* supernatant and bacterial cell mass as a negative control.

that treatment with BS and BCM induced the proliferation of BMSCs (figures 3 and 4). An increase in cell proliferation with the BS treatment started at 0.9 µL/mL concentration and reached its peak at 9 µL/mL. It was also observed that cell proliferation reduced at higher concentrations (30 µL/mL). An increase in BMSCs proliferation with BCM treatment started at 0.3 µL/mL concentration and peaked at 9 µL/mL. The outcomes of the treatment were compared with the results of the negative control group.

The results obtained from the treatment of rBMSCs with *B. bifidum* showed that the maximum cell proliferation was at 9 µL/mL concentration. At this concentration, there was a significant increase in cells treated with BS (P=0.011) and BCM (P=0.036). It was observed that both the BS and BCM had the maximum effect at this concentration (tables 1 and 2). Comparing each concentration with the negative control showed that the highest level of significance was at 9 µL/mL concentration, but the effect in other quantities of BS or BCM was also significant. For instance, treatment with BS at 30 µL/mL concentration (P=0.046) and treatment with BCM at 3 µL/mL concentration (P=0.040).

Discussion

The results showed a significant increase

in the proliferation of stromal cells, which confirmed the stimulatory potential of probiotics (*Lactobacillus*¹³ and *Bifidobacterium*) on various cells. Several studies have shown the effect of probiotics in limiting the proliferation of neoplastic cells, especially in gastrointestinal tract cancers.²⁹ On the other hand, some other studies have confirmed the anti-apoptotic role of *Bifidobacterium* in the intestinal epithelial cells.³⁰ In contrast with the results of a previous study on the effects of *Lactobacillus*,¹³ we found a significant increase in cell proliferation with *B. bifidum* at 9 µL/mL concentration. The increase in cell proliferation in the BCM culture started at lower concentrations (0.3 µL/mL) while the onset of cell proliferation in the BS culture was at 0.9 µL/mL. The results also showed a decrease in cell proliferation in the BCM and BS cultures at higher concentrations (30 µL/mL); indicating increased effectiveness at lower concentrations.

To understand the mechanism responsible for increased cell proliferation, we used BCM as a supernatant while previous studies used probiotics.^{13, 31-33} In addition, *B. bifidum* was used in the present study to measure cell proliferation whereas previous studies used *Lactobacillus*. Nonetheless, the results are comparable since both bacteria exhibited similar cell proliferation characteristics. It was observed that both *B. bifidum* and *Lactobacillus* had the potential to increase stromal cell proliferation. Although *Lactobacillus*

was more effective at lower concentrations, the increase in both bacteria was somewhat similar. Therefore, we believe that these bacteria share common characteristics. Further research to confirm this finding is recommended.

The MRS broth was a suitable medium for the cultivation of *Lactobacillus* and *Bifidobacterium*.²¹ However, similar to a previous study,¹³ we observed an inhibitory effect on cell viability at high concentrations of MRS broth. Since the amount of MRS broth medium reached the lowest level in the BCM group, the inhibitory effect of the culture medium was reduced. If the bacterial culture medium is removed, it is anticipated that cell proliferation would be slightly higher than BCM. Therefore, using an alternative method (freeze-drying) may reduce the inhibitory effects of the bacterial medium. By comparing the results from cell treatment with BS and BCM, the most effective inhibitory role of the bacterial medium was in the use of supernatant. Note that during the process of BCM preparation, a maximum amount of MRS broth media was eliminated by centrifugation.

A previous study on *Lactobacillus* indicated that the substances secreted in the supernatant had primarily overcome the inhibitory effect of MRS broth.¹³ The results of the present study suggest that *B. bifidum* can also partially overcome the inhibitory effect of the bacterial medium. In addition, there was significant cell proliferation in some concentrations of BS. Therefore, it is most likely that there were common substances in *Lactobacillus* and *Bifidobacterium* supernatants that stimulated the proliferation of cells in multiple passages. In the absence of these substances, cell proliferation gradually decreased after some passages.³⁴ These findings confirm the presence of substances secreted by some probiotic bacteria that can be used as stimulants for the proliferation of stromal cells. Therefore, the use of *B. bifidum* supernatant in an optimum concentration can be beneficial and an effective factor in the proliferation of cells such as rBMSCs.

The main limitation of the present study was the fact that the MRS medium in high concentrations (>9 µL/mL) influenced cell proliferation. The complexity of the secreted substances in the bacterial supernatant was another constraint on the study. The use of magnetic-activated cell sorting (MACS) is recommended to isolate some specific cells such as MSCs.

Conclusion

The use of *B. bifidum* is a low-cost and accessible

method to increase the proliferation potential of BMSCs. *B. bifidum* can be easily cultivated, propagated, and is a suitable culture medium. Further studies are required to complement our initial findings and to provide detailed information on the effective substances causing cell proliferation by probiotic bacteria.

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Conflict of Interest: None declared.

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