Evaluation of *Lactobacillus rhamnosus* Antioxidant Effects on Survival of Human Mesenchymal Stem Cells

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Abstract

**Introduction**: Multipotent mesenchymal stromal cells (MSCs) have novel therapeutic potential to treat a wide variety of diseases but they have poor survival in oxidative stress conditions. Probiotics are one of the most effective antioxidant substances in the improvement of cell resistance to oxidative environments. The main purpose of this study was to evaluate the protective effects of *Lactobacillus rhamnosus* on MSCs viability in stressful conditions.

**Materials and Methods**: The MSCs were exposed to live or killed *L. rhamnosus* in oxidative-stress conditions to evaluate the expression of antioxidant genes; heme oxygenase (HO1), metallothionein 1 (MT1), superoxide dismutase 1 (SOD1) and 2 (SOD2). Also, the antioxidant activity was assessed.

**Results**: In harmful conditions (H$_2$O$_2$) and serum deprivation, killed *L. rhamnosus* not only increased the expression of HO-1, MT1, SOD1 and SOD2 genes of the MSCs (P<0.001) but also enhanced the antioxidant activity of MSCs (P<0.001), leading to a better survival under oxidative stress conditions. The synergism effect of killed *L. rhamnosus* increased the antioxidant potential of MSCs to resist oxidative stress conditions.

**Conclusions**: The killed *L. rhamnosus* has protective effects on the survival of MSCs in stress conditions.

**Keywords**: Mesenchymal Stem Cells, *Lactobacillus*, Antioxidant


Introduction

Mesenchymal stem cells (MSCs) are multipotent cells capable of proliferation and self-renewal and can also differentiate into several cell types, including osteoblasts, chondrocytes, myocytes, adipocytes, endothelial cells, hepatocytes, nerve and pancreatic cells.¹,² Several studies have shown that MSCs have great potential in the stimulation of innate immune cells via induction of a wide range of cytokines and immunomodulatory mediators.³,⁴ In addition, MSCs secrete a large number of diverse bioactive compounds that have been involved in anti-inflammatory, antimicrobial, chemotactic, and antiapoptotic effects.⁵,⁶ Several studies have highlighted that a wide variety of MSC are used to treat diseases such as severe osteogenesis imperfecta, Hurler syndrome, metachromatic leukodystrophy, chronically injured hearts and acute graft versus host disease.⁷,⁸ The primary limitation in MSC-based therapy is poor viability due to apoptosis and/or necrosis at the administration site.⁹,¹⁰

Probiotics have shown an effective alternative therapy for preventing and treating inflammatory or allergic diseases by regulating immune responses.¹¹,¹² The main groups of probiotics in the food and drug industries are lactic acid bacteria (LAB).¹³ Several studies have shown that LAB has the anti-inflammatory and immunomodulatory activities, which is used to treat ulcerative colitis and metabolic diseases.¹⁴,¹⁵ Moreover, *L. rhamnosus* (LA) and *L. lactis* have a positive effect on the growth, immune system, and oxidative status of sea bream, *Pagrus major*.¹⁶,¹⁷ It has been documented that the antioxidative ability of *L. plantarum* is associated with inhibiting oxidation of unsaturated fatty acids, scavenging reactive oxygen species (ROS), resisting hydrogen peroxide and chelating the metal ions.²⁰,²¹ In fish, LAB strains prevent numerous diseases related to oxidative stress by limiting excessive amounts of reactive radicals.²² The LA applied strong antioxidant activity in an oxidative stress situation, suppressed the effects of ROS and raised antioxidant levels.²³ However, to the best of our knowledge, there are no studies on the effects of LA on the antioxidant activity and viability of MSCs. Considering the limitations of using genetic manipulation to increase survival and antioxidant properties
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of MSCs, it seems reasonable to use alternative methods such as probiotic. The present study aimed to investigate whether live or killed LA can increase MSC viability by inducing antioxidant gene expression or not.

Material and Methods

Bacterial Strains
In the current experimental study, L. rhamnosus was purchased from the Pasteur Institute (Tehran, Iran). De Man, Rogosa and Sharpe (MRS) medium (Merck, Germany) was used for the culture of LA. Bacterial cultures were grown in MRS broth medium for 72 hours to an optical density (OD) of 1, which was equivalent to 10^6 colony forming units (CFU)/mL. Cultures were centrifuged, washed with phosphate buffered saline (PBS) for 3 times, and re-suspended in PBS to the desired CFU/mL doses for in vitro experiments. Also, in another experiment, 1mM of phenylmethanesulfonyl fluoride (PMSF; Sigma, USA) was added. The bacterial cells were sonicated 3 times at 130 kHz with 10-second intervals, each time for 30 seconds (Hielscher Ultrasound Technology, Germany). The suspension was then centrifuged to remove cell wall debris. Then dialysis was performed in phosphate buffered saline (PBS; Sigma, USA) 1x solution. To confirm the bacterial killing procedure, the suspension was plated on MSA medium.

Mesenchymal Stem Cells Preparation

Human MSCs were obtained from Bon Yakh (Tehran, Iran). The cells were in accordance with the criteria for the classification of MSCs are fully compliant with the standards of the International Society of Cellular Therapy. In addition, the cells were negative for CD45 and CD19 as confirmed by immunofluorescence. The MSCs were cultured in DMEM Low Glucose with 1% penicillin/streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco) and 10% fetal bovine serum (FBS, Gibco) in the presence of 5% CO\textsubscript{2} and 37°C. When the cells reached 70%–80% confluency, the culture supernatant was removed and replaced by an addition of DMEM Low Glucose media without FBS and incubated at 37°C in 5% CO\textsubscript{2} humidified incubator. After passage 4, the cell was used for in vitro assays.

Cell Viability

The determination of cell viability was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, as described previously. Briefly, 1 × 10^4 cells/mL was cultured in 96-well plates (Nunc; Naperville, IL) and treated with 1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 80 and 100 µL of live or killed LA for 48 hours. Then, the cells were subsequently treated with MTT solution (Sigma-Aldrich; Merck Millipore) at 37°C in a 5% CO\textsubscript{2} incubator for 4 hours. In the next procedure, the culture medium was removed and 200 µL of DMSO was added. The absorbance was measured at 570 nm by ELISA microplate reader. The percentage of cell viability was calculated via: Cell viability (%) = [1 – (absorbance of experimental well/absorbance of negative control well)] × 100.

Preconditioning of MSCs With FBS and H\textsubscript{2}O\textsubscript{2}

The killed LA-treated MSCs were cultured in 96-well plates and incubated with FBS and H\textsubscript{2}O\textsubscript{2}. The Cell viability was evaluated with MTT as previously described.

Real-Time Polymerase Chain Reaction

The mRNA expression of antioxidant genes including heme oxygenase (HO-1), metallothionein 1 (MT1), superoxide dismutase (SOD1) and SOD2 (primer sequence motioned in Table 1) were measured by SYBR Green real-time polymerase chain reaction (PCR) analysis using specific oligonucleotide primers. Then, the total RNA of treated and untreated cells were extracted by using a TRIZOL reagent (Invitrogen, USA), which was used for cDNA synthesis by implementing a RevertAid\textsuperscript{TM} first stranded cDNA Synthesis Kit (Fermentas, Lithuania). The β-actin gene was utilized as an endogenous control. The PCR reaction mixture included 12.5 µL of SYBR Green Master Mix, 2 µL of template DNA, forward and reverse primers (0.25 µM each), and 11 µL of nuclease-free water. Finally, RT-PCR was cycled at a 95°C denaturation step for 5 minutes, between 95°C for 15 seconds and 60°C for 1 minute for 40 cycles.

Assessment of Antioxidant Ability

Antioxidant Assay Kit (Sigma-Aldrich) was used to evaluate the antioxidant capacity of treated MSCs with killed LA against oxidative stress. In the ABTS method, ferryl myoglobin radical is formed from myoglobin and hydrogen peroxide, which ultimately oxidizes ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to produce ABTS+. The absorbance of this green radical cation was measured at 405 nm.

Statistical Analysis

Statistical analyses were performed by IBM SPSS Statistics for Windows, version 21.0 (IBM Corp. Released 2012, Armonk, NY: IBM Corp.) and GraphPad Prism version 6.00 for Windows (La Jolla California USA). Normally distributed data were analyzed by ANOVA with the Tukey multiple comparison test. Nonparametric data were analyzed by Kruskal-Wallis ANOVA followed by multiple comparison test. Results were expressed as the mean ± standard error of the mean (SEM), and a P value of < 0.05 was considered as statistically significant.

<table>
<thead>
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<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>B actin</td>
<td>Forward: 5'-TTCTCAATAGGCTGGTGG-3'</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTGGTGAAGCTCTCAAAAC-3'</td>
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<tr>
<td>HO-1</td>
<td>Forward: 5'-TCTGGAATGGAAGGACGTCGGA-3'</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCGGAAAGACTGATGTCAGTA-3'</td>
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<td>MT-1</td>
<td>Forward: 5'-ACCAGCCTTCTGCGTGGC-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse: 5'-CACCACCCTGACATGCGTCC-3'</td>
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<tr>
<td>SOD-1</td>
<td>Forward: 5'-ACCCAGTACTCTGGGACGAGCT-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGTCTAAATCCATGGCRAACC-3'</td>
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Table 1. Sequence of Primers Used for Real-Time PCR Assay

http://www.biotechrep.ir
Results
The Cytotoxic Activity of LA
The cytotoxic activity of live or killed LA against MSCs was determined by the MTT assay. As displayed in Figure 1B, the cytotoxicity activity of live LA against MSCs at all volumes was significantly higher than that of untreated-cells (P < 0.01). As shown in Figure 1A, live LA significantly decreased MSCs viability in a volume-dependent manner. Live LA at the volume 15 µL showed the highest cytotoxicity activity against live LA, with the cell viability of 3%, which was significantly higher than another amount of live LA (P < 0.05). In addition, the cell viability in 10 µL of probiotic was significantly lower than 5, 2.5 and 1 µL of live LA (P < 0.05). Also, 5 and 2.5 µL of live LA significantly reduced the cell viability to 19 and 21%, respectively compared to 1 µL of live LA and controls (P < 0.05). The cell viability in 1 µL of live LA was significantly lower than the control (P < 0.05).

As shown in Figure 1B, in the presence of 30, 20 and 15 µL of killed LA (bacterial lysis), the cell viability of MSCs was 21%, 42% and 63%, respectively. There was a significant difference between 30, 20 and 15 µL of killed LA in the reduction of the cell viability (P < 0.05). The cell viability in the presence of 10, 5 and 2.5 µL of killed LA (bacterial lysis) was slightly reduced compared to the untreated-cells; however, there was no significant difference between MSC viability treated with 10, 5 and 2.5 µL of killed LA with that of untreated-cells (Figure 1B, P > 0.05). In fact, data determined that 10, 5 and 2.5 µL of the volume killed LA (bacterial lysis) did not have a cytotoxicity effect on MSCs and also the cell viability of MSCs was similar to untreated-cells. Furthermore, no significant differences was observed regarding the cytotoxic activity of killed LA between 24 and 48 hours treatments.

Killed LA Increased the Cell Viability of MSCs in the Presence of FBS and H₂O₂
The effects of killed LA on the cell viability of MSCs in presence of FBS was determined by the MTT assay. As shown in Figure 2A, in the presence of FBS 10%, the cell viability of preconditioned MSCs with different amounts of killed LA was significantly higher than other groups. However, there was no significant difference between the treated MSCs with different amounts of killed LA (P > 0.05). In the presence of FBS 5%, the highest cell viability was observed in treated MSCs with 10 µL of killed LA, which was significantly higher than the other volumes (Figure 2A, P < 0.05). In the presence of FBS 5%, the cell viability of treated MSCs with 50 µL of killed LA was significantly lower than the other volumes (P < 0.05). In the presence of FBS 5 or 10%, the cell viability of treated MSCs with different volumes of killed LA was significantly higher than the cell viability of treated MSCs without FBS (Figure 2A, P < 0.05). In the absence of FBS, there was no significant difference between treated MSCs with different volumes of killed LA and untreated-cells (P > 0.05) (Figure 2A).

Moreover, in the presence of 2 and 4 mM of H₂O₂, the highest cell viability was observed in treated MSCs with 10 µL of killed LA, which was significantly higher than other volumes (Figure 2B, P > 0.05). In the presence of H₂O₂ 2 mM, the cell viability treated MSCs with 5 and 15 µL of killed LA was 65.2 and 96.1%, respectively, which was significantly higher than untreated-cells (P < 0.05). In the presence of H₂O₂ 4 mM, there was a significant difference between the cell viability of MSCs treated with 15 and 5 µL of killed LA (Figure 2B, P > 0.05). In the presence of H₂O₂ 4 mM, the cell viability of treated MSCs with 5 µL killed LA was 21.8%, which was significantly higher than the untreated-cells (Figure 2B, P > 0.05).

Probiotic Increased the Antioxidant Genes Expression of MSCs
In order to evaluate the possible antioxidant effect of killed LA, HO-1, MT1, SOD1 and SOD2 mRNA expression of MSCs were first evaluated by using RT-PCR. Then, the relative expression of the genes was determined by dividing its expression amount to that of the β-actin gene. As shown in Figure 3A-D, in the presence of H₂O₂ 2mM, the expression of HO-1, MT1, SOD1 and SOD2 genes treated MSCs with 10 µL of killed LA was significantly increased compared to the untreated MSCs (Figure 3A-D, P < 0.05). In the presence of FBS 5%, the expression of HO-1, MT1, SOD1 and SOD2 genes treated MSCs with 10 µL of killed LA was significantly increased compared to the untreated MSCs (P < 0.05). In the absence of H₂O₂ and FBS, there was no statistically significant difference between treated MSCs with 10 µL of killed LA and untreated MSCs in the expression of HO-1, MT1, SOD1 and SOD2 genes (P > 0.05, Figure 3A-B).

Figure 1. The Comparative Analysis of Cytotoxic Effects of Killed or Live LA on MSCs. MSCs were incubated with different amounts of live (A) and killed (B) (0-30 µL). The result was shown that live LA did not increase survival of MSCs but killed LA could be cytoprotective effect on MSCs. Data represent the mean ± SEM of three independent experiments. *P < 0.05 and **P < 0.01 indicate the groups which were significantly different.
Probiotic Increased Antioxidant Properties

For the estimation of antioxidant effects of killed LA on MSCs, treated cells were exposed to H$_2$O$_2$ and serum deprivation. The antioxidant activity in cell culture medium of the MSCs-killed LA was increased, but low levels of antioxidant activity was observed in MSC cell (untreated cell) (Figure 4). When the release of oxidative stress was lower, the color absorbance was lower. In other words, it is probable that killed LA inhibits the formation free radicals of oxidative stress.

**Discussion**

The goal of this study was to evaluate the effects of killed or live LA on the antioxidant activity and viability of MSCs in vitro. The MSCs have self-renewal potential and an ability to differentiate into various cell types; therefore, they are a major source for therapeutic studies of stem cells. The MSCs have shown regenerative properties in damaged tissues comprise cell fusion, differentiation, and paracrine effects, such as anti-apoptotic and immunomodulatory effects and the induction of local progenitor stem cells. Several reports from in vitro and in vivo preclinical studies and clinical trials have revealed that MSC based therapy is effective and safe. However, the MSC based therapy is weak for transplantation. Because for harmful stress cause decrease survival rate of MSCs after transplantation. Several approaches involving hypoxic preconditioning, pretreatment with cytokines or growth
induced high and low levels of IL-8 from Caco-2 cells, respectively, which suggests that heat-killed probiotics have the potential to prevent intestinal inflammation without an adverse pro-inflammatory response.\textsuperscript{41} In another study, heat-killed lactobacilli isolated from human improved the Th1/Th2 balance and inhibited IgE production, which indicates the fact that dead probiotic cells can act as biological response modifiers for allergic diseases.\textsuperscript{42,44} Additionally, the results demonstrated that the killed LA are capable to influence the growth and metabolism of MSCs, which are the most suitable attributes of an adjunct therapy and this potential should be considered for more drug development.

In the next procedure, the expression of anti-oxidant genes HO-1, MT1, SOD1 and SOD2 in killed LA-preconditioned MSCs in oxidative stress condition was investigated by using RT-PCR. The results showed that the treatment of MSCs with killed LA in oxidative stress condition lead to an increase in the expression of the HO-1, MT1, SOD1 and SOD2 in order to protect the cells. The SOD, as an important antioxidant defense against oxidative stress, have three forms of SOD; namely, cytoplasmic superoxide dismutase (SOD1), mitochondrial superoxide dismutase (SOD2), and extracellular superoxide dismutase (SOD3), which catalyzes the dismutation of superoxide (O2\textsuperscript{-}) into oxygen and hydrogen peroxide.\textsuperscript{45} It has been shown that in most tissues in cellular stress conditions such as hypoxia, ischemia, hyperoxia, radiation or inflammation preserve antioxidant/oxidant homeostasis HO-1 induce to maintain antioxidant/oxidant homeostasis and in the inhibition against vascular injury.\textsuperscript{46} Metallothioneins (MTs) belong to the group of intracellular cysteine-rich, metal-binding proteins that have an important role in the detoxification of heavy metals and also in maintaining essential metal ion homeostasis.\textsuperscript{47,48} Several studies have shown that the expression of MTs dramatically increased in oxidative stress conditions to protect the cells.\textsuperscript{49-51} Also, the expression of MT in tumor tissues is principally related to the proliferative ability of tumor cells by the regulation of apoptosis.\textsuperscript{52} The supplementation of MSCs supplemented with Hibiscus sabdariffa L. (roselle) increased SOD and MSCs survival and protected against H\textsubscript{2}O\textsubscript{2}-induced DNA damage.\textsuperscript{53} Moreover, L. rhamnosus increased the HO-1 expression in gut-associated lymphoid tissue and the enhancement of Foxp3+ regulatory T cells, induced a tolerogenic environment.\textsuperscript{54} In addition, Halabian et al showed that treatment of BMSCs with lipocalin-2, a natural cytoprotective factor, increased SOD gene expression, proliferation and enhanced cellular adhesion to culture media upon H\textsubscript{2}O\textsubscript{2} exposure, in comparison to untreated cells.\textsuperscript{55} In light of these findings, it seems rational that the presence of killed LA has increased the potential of MSCs by increasing HO-1, MT1, SOD1 and SOD2 genes to improve the survival under oxidative stress conditions. Regarding the results, the synergism effect obtained from the treatment of pluripotent mesenchymal cells with killed LA can increase the antioxidant effect of these cells, so that these cells can be used in therapeutic fields.
Conclusions
In conclusion, killed LA qualifies as an antioxidant option against oxidative stress conditions because it increases HO-1, MT1, SOD1 and SOD2 genes expression, which leads to an improvement of the MSCs survival. Based on these initial results, future goals are to evaluate the efficacy of killed LA-treated MSCs for being used as an alternative to increase cell viability in the mouse model. Purportedly, probiotic may work in combination with the current MSCs treatment, as an alternative approach towards effective cell therapy.

Authors’ Contributions
NA performed experiments and wrote the paper. HMH designed & performed experiments and analyzed data. RH supervised the research, designed experiments and co-wrote the paper.

Conflict of Interest Disclosures
The authors declare they have no conflicts of interest.

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