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ORIGINAL ARTICLE

Potential of *Lactobacillus acidophilus* to modulate cytokine production by peripheral blood monocytes in patients with endometriosis

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ABSTRACT

Background and Objectives: Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity. Peripheral blood monocytes cells (PBMCs) may have altered function to some extent in women with endometriosis. *Lactobacillus acidophilus* is a probiotic bacterium within the human body with the ability of alleviating many inflammatory diseases. Here, we examined the effect of *L. acidophilus* on PBMCs of endometriosis patients.

Materials and Methods: In this study, peripheral blood samples were obtained from endometriosis patients (n=11) and non-endometriosis individuals (n=11). After isolation of peripheral blood mononuclear cells with Ficoll, cells were cultured in the presence and absence of phytohemagglutinin. Also, these cells were co-cultured with 1×10^6 CFU/ml of *L. acidophilus.* IL-6 and IL-1 cytokines were measured by ELISA method and the two groups were evaluated and compared.

Results: The results showed that in endometriosis patients, the production of pro-inflammatory cytokines, including IL-1 and IL-6, by PBMC was increased compared to non-endometriosis subjects, and stimuli such as PHA intensified this elevation. Also, *L. acidophilus* increased the levels of pro-inflammatory cytokines including IL-1 and IL-6. However, the production of these cytokines decreased due to the modulatory properties of bacterial cells after 48 h.

Conclusion: According to the results of the current study, IL-1 and IL-6 production was significantly increased in PMBCs of endometriosis patients compared to that of the healthy controls. Also, *Lactobacillus acidophilus* was considered as an antigenic compound and induced IL-1 and IL-6 production. According to these results, probiotics can be further used for the treatment of endometriosis patients and more investigations are needed to confirm these results.

Keywords: Probiotic; Endometriosis; *Lactibacillus acidophilus*; Inflammatory cytokines

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INTRODUCTION

Endometriosis is a chronic disease causing severe pelvic pain and subsequent subfertility (1). The rate of endometriosis in female populations is unknown as the only decisive detection method for this disorder is laparoscopy (2). However, based on symptoms, endometriosis may affect 10% of all and 30%-50% of symptomatic premenopausal women, representing 176 million cases globally (3).

Multiple studies have shown the association of endometriosis with alterations in cell- and antibody-mediated immunity (4). Nevertheless, the exact role that the immune system plays in the pathogenesis of endometriosis and the particular immunologic mechanisms that underlie this role are not fully elucidated (5).

Even though the lesions that have been described in endometriosis are mostly limited to the peritoneal cavity and pelvic structures, the systemic immunologic alterations are involved in the development of this disease. According to several previous studies, endometriosis should be viewed as a systemic condition that has local manifestations. Of several cell types within the immune system, perhaps the most essential cells for maintaining normal homeostasis are the mononuclear phagocytes (5, 6). These cells, typically referred to as monocytes and macrophages, are the most functionally diverse cells of the immune system involved in no antigen-specific inflammatory responses and serving as a bridge to antigen-specific immune responses (6).

Peripheral blood monocytes cells (PBMCs) are considered as the main source for the inflammatory peritoneal macrophages, each of which may have altered function to some extent in women with endometriosis (7, 8). Lesions and activated macrophages, which are abundant in the peritoneal fluid of women with endometriosis can also secrete pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) (9). Therefore, secretory products of monocytes and macrophages can induce or suppress the function of other immune cells, thereby contributing to immunologic responses associated with endometriosis (10). Endometriosis is an inflammatory disease with several implications for females and the application of probiotics appears to suggest a novel aspect for its treatment (11).

Lactic acid bacteria (LAB) are well known for their wide usage and some LAB strains exert beneficial effects on the host and are therefore called probiotics (11). Several probiotic strains have been studied for their immunomodulatory activities. A selection of 152 species within the *Lactobacillus* genus have been identified to date, implying the unusually great phylogenetic and functional variety of these microorganisms (12). Also, it is known that each strain can exert a distinct immunomodulatory function. A proposed effect of probiotics is the down-regulation of Th2 cytokine production either by inducing Th1 cytokines or the regulatory cytokine IL-10, produced by antigen-presenting cells such as monocytes (13). LAB can up-regulate Treg cells, trigger the secretion of regulatory cytokines and control the balance between Th1 and Th2 immune cells (14).

So far, very few studies have investigated the effect of probiotics on endometriosis. The aim of this study was to evaluate the immune balance in PBMCs of endometriosis patients after LAB treatment. The immunomodulatory properties of the selected LAB were assessed by measuring innate and adaptive cytokine production. *Lactobacillus acidophilus* showed an overall stimulatory effect on IL-1 and IL-6, while decreasing the production of prototypical Th2 and differentially stimulating Th1 production.

MATERIALS AND METHODS

Patients. Blood was collected from 11 endometriosis patients and 11 healthy blood donors (HBD). The state of disease or non-disease of the subjects was confirmed by a specialist. Samples were taken from patients with severe endometriosis (stage 3.4) who did not take antibiotics two months prior to sampling and did not suffer from cancer or autoimmune diseases. The age of the subjects was about 20-35 years old. The study was performed in January 2019 at Rasoul Akram Hospital in Tehran, Iran. About 5 ml of peripheral blood was collected and poured into EDTA-containing tubes and transferred to a laboratory for the isolation of mononuclear cells.

Preparation of bacteria. According to the standard protocols, we transferred the lyophilized *L. acidophilus* (PTCC CS/F/721/01/01) purchased from the Scientific and Industrial Research Organization of Iran to 20 ml of Man Rogosa Sharpe (MRS) (Ibersco, Iran) broth culture medium. The pH of the medium

was adjusted to 6.2-6.4 and incubated at 37°C for 24 h under microaerophilic conditions.

After an additional 48 h of incubation at 37°C, bacterial cells were pelleted by centrifugation at 1000 g, washed with phosphate-buffered saline (PBS) (Sigma Aldrich, Milan, Italy) two times, and re-suspended in PBS. For viable bacterial counts, serial dilution was carried out and bacterial cells were plated on MRS agar (Ibersco, Iran). Bacterial cells used for the immune-stimulation assays were killed with heat (15 min at 115°C) and stored at -40°C until further use (11).

Isolation, culture and induction of PBMCs. For the isolation of mononuclear cells from peripheral blood, 5 ml of blood was diluted with PBS (1:1) then transferred on the gradient medium of Ficoll (Sigma Aldrich, Milan, Italy) with a density of 1/077 mg. Blood sample was gently mixed and layered over a prepared Ficoll gradient, and subjected to centrifugation at 600 g for 20 min. All procedures were carried out at 4°C. After centrifugation, different cell layers could be removed by gentle suction with a syringe and needle. In fact, four layers were separated including red blood cells, Ficoll, PBMCs and plasma, respectively (bottom-up) (15).

For the screening experiment one buffy coat was used and cells from the interphase were harvested, washed and cultured in 24-well plates at 1×10^6 cells per well in RPMI 1640 (Roswell Park Memorial Institute) medium containing 10% fetal calf serum, 2mM glutamine and 1% penicillin–streptomycin (all from Gibco-BRL, Paisley, Scotland) (16).

Total cell count and viability. The counting mononuclear cells were performed manually using a haemocytometer, and cell viability is determined by the trypan blue exclusion method by most investigators

Counting mononuclear cells. First we put the glass cover on the Neubauer chamber central area. Then released the plunger slowly close to the glass cover edge, right at the centre of the Neubauer chamber. We Placed the Neubauer chamber on the microscope stage and counted the number of cells in all four outer squares. Then we calculated the number of cells through the following formula:

Concentration = Number of Cells × 10.000 / Number of square × dilution

Cell viability. Trypan blue exclusion test PBMC viability after isolation was determined using the standard trypan blue exclusion test (Sigma-Aldrich, St. Louis, MO). The total number of viable and nonviable cells was counted using a Burker counting chamber, and the percentage of viable cells was calculated accordingly (17).

Stimulation experiments. For the experiment, the heat-killed bacteria were thawed, suspended in the appropriate culture medium and added directly to the PBMC culture. Except for a pilot experiment to identify the most suitable ratio of bacteria to the PBMC, a 1: 1 ratio (1×10^6 CFU/ml of *L. acidophilus* was added to 1×10^6 PBMCs per well) was used throughout this study. The PBMCs were stimulated with PHA (5 µg/ml) or left unstimulated. Medium with PBMC, but without bacteria, was used as a negative (medium) control. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. Cultured cells and culture supernatants were harvested after 24 h and 48 h of culture and these supernatants were stored at -20°C and overnight transferred to -80°C before analysis (17).

Cytokine analysis. The ability of IL-1 and IL-6 production by PBMCs was tested in cell supernatants obtained after 24 h and 48 h. Cytokine production was detected with enzyme-linked immunosorbent assay (ELISA), using commercially available ELISA kits from Bioscource (Cytosets, Biosource Europe SA, Nivelles, Belgium). Values are expressed as $pgmL^{-1}$ (average of strains within the same species_ SEM) deduced from the OD of the standard curve. The sensitivity limits for all tested cytokines were below $3pgmL^{-1}$ (18).

Statistical analysis method. To analyze the results of cytokine production in the tested groups, data were statistically analyzed by SPSS software using the Independent- Sample T Test method. Diagrams were drawn with EXCEL software. The results were presented as the mean standard error of SE and p values were used as the minimum level of significance of the difference between the means. Non-parametric methods were employed to compare the means in the absence of normally-distributed data.

Ethical statement. This project was done based on the ethical guidelines as previously approved by the Iran University of Medical Science, Tehran, Iran

(project no: IR.IUNS.FMD.REC1398.108).

RESULTS

Viability of PBMCs after isolation. Peripheral blood mononuclear cells include B lymphocytes, T lymphocytes, tumor killer cells, and monocytes, which were isolated using Ficoll and used for various experiments. Here, we took 5 ml of peripheral blood of the patients and healthy subjects. After staining and counting the number of alive and dead cells under an inverted microscope, we reported the viability of cells. Viability of harvested PBMCs was >80% for all the study groups.

Cytokine analysis. Evaluation of IL-1 and IL-6 production by PBMCs derived from endometriosis patients and HBD subjects. After 24 and 48 h cytokine levels (IL1 and IL6) were evaluated from endometriosis patients and HBD subjects. A significant increased level of IL-1 and IL-6 was observed after 24 h in the patient group compared with the HBD group ($P<0.05$) (Figs. 1 and 3). The results showed that after 48 h, the induction of cytokines by PBMCs derived from the patient cells was higher than that of PBMCs of the healthy subjects. This could be due to the increased induction of cytokines by patient-derived cells (Figs. 2 and 4).

Evaluation of IL-1 and IL-6 by PBMCs derived from endometriosis patients and HBD subjects after co-culture with *Lactobacillus acidophilus*. The PBMCs of endometriosis and HBD groups were cultured with 1×10^6 CFU/ml of bacterial cells (in a 1:1 ratio) in the presence and absence of PHA (5 µg/ml). After 24 h and 48 h, the concentration of IL1 and IL-6 in the supernatant was measured. After co-culture of *L. acidophilus* with PBMCs, an increased IL-1 and IL-6 production was observed in the endometriosis patients and HBD group, however, this increase was not statistically significant ($P>0.05$). Elevated production could be due to cell differences. However, after 48 h, a significant reduction in IL1 and IL-6 production was observed due to the modulatory properties of the bacterial cells. (Figs. 2 and 4).

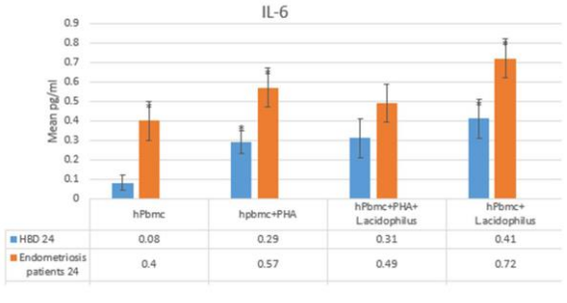

Fig. 1. Evaluation of IL- 6 production by PBMCs in HBD group and endometriosis patients after 24 h

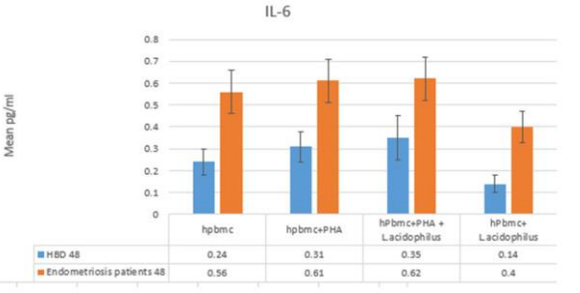

Fig. 2. Evaluation of IL- 6 production by PBMCs in HBD group and endometriosis patients after 48 h

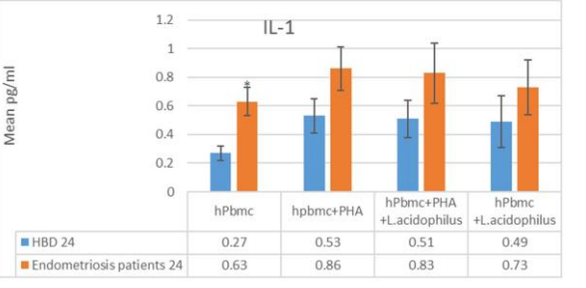

Fig. 3. Evaluation of IL-1 production by PBMCs in HBD group and endometriosis patients after 24 h

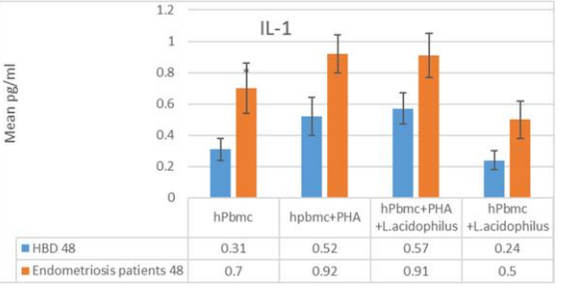

Fig. 4. Evaluation of IL-1 production by PBMCs in HBD group and endometriosis patients after 48 h

Effects of PHA on IL-1 and IL-6 production by PBMCs of endometriosis patients and HBD subjects. The results of ELISA test showed that PHA significantly increased IL1 and IL-6 production by

PBMCs after 24 h (P <0.05). Accordingly, PHA can stimulate PBMCs; however, no significant alterations was observed in IL-6 levels at 48 h. (Figs. 2 and 4).

PHA + *L. acidophilus* increased IL1 and IL-6 production by PBMCs compared to PHA alone after 24 h, which was not statistically significant (P >0.05). PHA + *L. acidophilus* did not significantly change IL-1 production by PBMCs when compared to PHA alone (P >0.05).

IL-6 production following treatment of PBMCs with PHA + *L. acidophilus* was not statistically different from that of the PHA treatment alone (P >0.05).

Also, the combination of PHA and *L. acidophilus* reduced the production of IL-1 by PBMCs after 24 h compared to PHA alone (P>0.05) (Figs. 1 and 3). As PHA alone induced IL-1 production, the presence of bacteria could be the cause of the decreased IL-1 production. On the other hand, the combination of PHA and *L. acidophilus* increased IL-1 production by PBMCs after 48 h (Figs. 2 and 4).

DISCUSSION

Endometriosis is one of the most common diseases in women, the cause of which is still unknown. One of the most widely accepted theories for endometriosis is the reversal of menstrual blood theory, developed by Sampson. Menstrual blood returns to the outside of the uterine cavity. It is a phenomenon that occurs in most women, however only 5-10% of the subjects develop endometriosis (19).

It is very important to pay attention to the immune system in the course of this disease. Also, immunological therapies are crucial in controlling the duration of the disease. Given the importance of the immune systems in the pathogenesis of endometriosis, the exact evaluation of the agents involved can be crucial in disease prevention and control (20).

The exact mechanism of developing endometriosis is not yet fully understood. The hypothesis of menstrual blood returning to the abdominal cavity and sometimes attaching to the peritoneal surface and eventually causing lesions is still reliable and valuable. Also, disturbance of the balance of cellular immunity prevents the clearance of menstrual remnants from the peritoneal cavity and as a result, causes the implantation of endometrial cells elsewhere, ultimately complicating endometriosis. It is not yet clear why only a few women develop endometriosis. The answer to this question is probably related to the state of the immune system, which plays an important role in the onset and progression of the disease. Also, the ratio of helper T cells to inhibitory T cells as well as the number of peritoneal macrophages increase abnormally in the peripheral blood and peritoneal fluid of women with endometriosis, which ultimately increases inflammatory cytokines in these patients (8).

In this study, the induction of IL-1 and IL-6 cytokines by PBMCs was examined. One of the aims of this study was to measure two important immune factors in patients with endometriosis, namely IL-1 and IL-6. Consistent with previous studies, the results of our study showed that the production of cytokines by peripheral blood mononuclear cells of patients was significantly higher than the healthy control group.

Another goal of this- study was to assess whether *L. acidophilus* can balance immune markers in cases with endometriosis *in vitro.* Co-culture of *L. acidophilus* with PBMCs for 1 day showed a statistically significant induction of IL-6. On the other hand, although IL-1 production increased, this elevation was not statistically significant.

Co-culture of *L. acidophilus* with PBMCs for 1 day showed a clear induction of IL-1 and IL-6 production, confirming the widely observed pro-inflammatory cytokine response induced by LAB. This response is presumably induced by monocytes as it occurs rapidly after encountering bacteria or bacterial compounds by pattern recognition-mediated interaction (6).

However, after 48 h, we observed the inflammatory cytokine levels. The precise mechanism by which probiotic LAB modulate the host immune responses is unknown. Bacterial cell surface macromolecules (such as long surface appendages, extracellular polysaccharides and teichoic acids) are in direct interaction with different immune cell types. The structure of the main cell wall macromolecules is strongly preserved; however, various modifications, such as glycosylation and quantitative deviations, provide the strain-specific attributes of probiotics (6, 21). Little information is available on the specific bacterial components that induce cytokine production. genome sequencing and transcriptomic analyses can provide more data on specific immunomodulatory properties of bacterial cells (21). High cytokine levels are hardly found in the blood of normal subjects; however, high levels of these molecules can be de-

tected in pathological disorders such as septic shock, cerebral malaria and purpura fulminant (22).

Measuring the ability of immunocompetent cells in cytokine production by polyclonal activators such as LPS, PHA, CON-A is necessary. These activators can stimulate harvested PBMCs. The isolation procedure eliminates polymorphonuclear cells that might be involved in the intercellular interactions that modulate cytokine secretion. In this study, we used PHA for the stimulation of cytokine production. Mostly, a large number of candidate probiotic strains are tested for their immunomodulatory properties in in vitro models to choose those with the best characteristics. In these *in vitro* studies, the effects of heat-killed bacteria may not be directly extrapolated to the effects of viable bacteria. Recent literature demonstrates similar effects of live bacteria with heat-killed bacteria and even with components of various bacteria (23). Very limited information is available regarding the *in vivo* molecular responses to probiotic bacteria in human mucosal tissues; however, a later study of Van Baarlen et al. demonstrated a noticeable overlap between *in vivo* response to heat-killed and *L. plantarum*, and these bacteria were in the same phase of growth (24). Systematic studies on linking *in vitro* data to *in vivo* effects have been rarely carried out thus far and the results have also not been consistent (25). Considering the limitations of the *in vivo* models, extrapolations of *in vitro* effects to *in vivo* results must be considered with caution. The present study showed that LAB can modulate cytokine induction by PBMCs derived from endometriosis subjects *in vitro.*

Probiotics contribute to the modulation of T cell subsets, improve humoral immunity and interactions with dendritic cells and macrophages. Understanding the molecular basis of intrinsic signaling in response to probiotics and the interaction between LAB and host is a challenge and requires further investigations.

CONCLUSION

According to the results of the current study, IL-1 production was significantly increased in PMBCs of endometriosis patients compared to that of the healthy controls. Also, PHA induced IL-1 production after 24 and 48 h. Also, *Lactobacillus acidophilus* was considered as an antigenic compound and induced IL-1 production. In addition, IL-6 production was significantly higher in PMBCs of endometriosis patients compared to the healthy controls and PHA significantly increased IL-6 production after 24 h. In addition, IL-6 production was significantly elevated following *L. acidophilus* treatment after 24 h; however, after 48 h, a reduced level of this cytokine was observed. According to these results, probiotics can be further used for the treatment of endometriosis patients and more investigations are needed to confirm these results.

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