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Bifidobacterium bifidum does not show cell toxicity on leukemic cells of AML patients but induced cell death on K562 cell line

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ARTICLE INFO	ABSTRACT
Article type: Original Article	***Background***: Leukemia is a kind of blood cancer diseases which are generally known as neoplasm. There are two types of blood cancer called acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). This type of leukemia targets plasma cells and prevents the production of antibodies in the body and makes the immune system vulnerable to infections.
	Methods: Blood samples from 10 people with AML and 10 normal individuals were collected who were defined as the case and control groups, respectively. PBMCs were isolated by Ficoll hypaque and cultured in order to the MTT assay. Then, the cell wall of *Bifidobacterium bifidum* bacterium was treated in five concentrations on these cells and the MTT test was performed at 24, 48 and 72hrs. Also, K562 leukemia cell was cultured then after; the MTT test was performed in the same protocol. The expression of BAX, Bcl2, β-actin genes were assessed by real-time PCR after 48 and 72hrs of treatment.
	Results: The results of MTT test could not show any significant differences in extracted PBMCs from individuals as compared to control group. But, K562 cell line in treatment with the cell wall of *Bifidobacterium bifidum* showed a significant cell toxicity effect at 48 and 72hrs versus non-treated K562 cells. BAX gene expression was significantly increased versus β-actin gene control only on K562 cell line, but not on AML derived leukemic cells.
	Conclusion: According to the obtained results, *Bifidobacterium bifidum, as a* probiotic bacterial species, can impact the growth and/or proliferation of cancer cells; however, it could depend on the type of cancer cells.

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Introduction

Cancer is caused by abnormal proliferation of normal cells and could be disseminated in other parts of the body through metastasis (1). The cancer term belongs to many types of the disease, and this disease disturbs the relationship between the cells and genes together. Oncogene, tumor inhibitor gene and DNA repair genes have important roles to administrate cancer cells. Four main types of leukemia are including acute lymphoblastic leukemia (2), acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL) and chronic myeloid leukemia (CML) (3, 4). Leukemia is a branch of cancer that is limited to some special tissues such as brain, bone marrow, and lymphoid system. Leukemia often is initiated from bone marrow and cause to form abnormality in white blood cells (WBCs) (5). Signs of this disease include bleeding problems, bruise, acute fatigue, fever and high risk of infection and also these signs are appeared because of lacking normal blood cells and blood assay or bone marrow biopsy are utilized for diagnosis of the disease (6). While etiology of leukemia is not well-known, but scientists have thought a mixture of genetic and peripheral factors have an important role to develop the disease. Some factors such as smoking, ion beam, some chemical materials (benzene), chemotherapy history, Down syndrome and the people who had leukemia history in their family provide underlying causes of leukemia (7, 8). Although, the current treatments of leukemia including chemotherapy, radiotherapy have a beneficial effects on the improvement of the patients, inevitable complications can be an important reason that the patients are suffering from these treatments. Therefore, other alternative or complementary treatments must be investigated to have a better outcome and less adversary effects on the patients (9, 10).

Probiotic is derived from pro and biotic and this term is belonged to live organisms which have suitable effects on the health of host body with consumption (11). Probiotics have many properties on the health of the body. These microorganisms could reinforce immunity with the production of effective factors in the immune system and cause to prevent diseases and infections. They also are able to enhance intestinal function via counteracting to pathogenic bacteria establishment in the gastrointestinal tract (12). Additionally, cholesterol reduction, lactose tolerance production, and decrease diseases or liver problems through the decrease of toxicity intake are other properties of probiotics. Also, probiotics intake could prevent gene mutations with their degradation by which absorbance of mutation materials was reduced in the gastrointestinal tract (13). The most common strains in probiotic production are related to acid lactic bacteria that are including the specific genus of *Bifidobacterium* and *Lactobacillus* bacteria. Most of these bacteria were found to be non-toxic and safe. *Lactobacillus* bacteria have bacillus and coccobacillus shape, positive gram, non-spoor, catalase, oxidase and indole negative and not able to regenerate nitrate (13, 14). Several studies showed that *Bifidobacterium* and *Lactobacillus* bacteria have beneficial effects on patients with cancer, especially colorectal cancer by many known mechanisms such as production of short fatty acid, alteration of the metabolic activities and physicochemical conditions in the colon environment (15). However, there are little findings about probiotics role in leukemia treatment. Therefore in the present study, the effect of *Bifidobacterium bifidum* cell wall extract on the leukemic blast of patients with leukemia and also K562 cell line were assessed.

Materials and Methods

Ethical certification
Ethical code of the present study is IR.TUMS.SPH.REC.1397.044 that approved by Ethics Committee of Tehran University of Medical Sciences.

Probiotic bacteria culture

Bifidobacterium bifidum standard strain was prepared from microbial collection of Organization of Industrial Researches of Iran (Tehran, Iran). Initially, lyophilized cells in MRS broth were cultured for 24hrs. The next, primary identification of bacteria was determined by gram staining, motility and nitrate reduction tests. Then the bacteria were propagated and then centrifuged at 4000 rpm/15min and then supernatant was discarded and the pellet was collected. Obtained pellet was solved in 10ml of PBS and in order to cell wall preparation, bacterial suspension was placed at -70°C and 37°C, respectively for 15min. These cycles repeated for 5 times. The protein concentration determined with Bradford and several concentrations as 1.36 up to 13600 µg/ml were prepared in order to cytotoxicity assays.

Blood samples collection

In the present study, 10cc blood sample was drawn from patients with AML voluntary (n=10) as well as ten healthy people as control group from Imam Khomeini Hospital (Tehran, Iran).

Inclusion criteria

Ten men and women with AML were included in this study and ten men and women were adopted as healthy control group. The control group did not have any inflammatory disease. Also, the patient group did not have any specific inflammatory disease. The adults over 30 years of age were involved into the present study.

Exclusion criteria

In the present study, individuals with acute flu or other viral inflammatory disease, autoimmune disorder, and smoking people were excluded from the study. And, some samples due to contamination and missing were ignored in the present study.

Peripheral blood mononuclear cells (PBMCs) separation

Heparinized blood samples were taken from patients with AML and healthy donors to separate PBMCs. So that, heparinized blood was diluted with 10ml of RPMI-1640 medium culture. The samples were added to Ficoll hypaque gently and were centrifuged at 4000rpm for 15min. Then PBMCs was collected and centrifuged at 2000rpm for 10min twice in order to remove Ficoll and platelet contamination. To determine cell viability in cell suspension, trypan blue was utilized. Then, the cells were counted by light microscopy and finally adjusted to 2×106 cells/ml in RPMI-1640 supplemented with 10 % FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 IU/ml penicillin.

K562 cell line

K562 cell line was purchased from Cell Bank of Pasteur Institute of Iran (Tehran, Iran) and cultured in RPMI-1640 medium containing 10% Fetal Bovine Serum with 1 mM non-essential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine, 100 µg/ml of streptomycin and 100 IU/ml of penicillin. After cell propagation, the cells were harvested and adjusted to 1×105 cell /ml and used for cell cytotoxicity assay.

Cytotoxicity assay

To evaluate the effect of *Bifidobacterium bifidum* cell wall cytotoxicity on the leukemic blasts, MTT assay was used. Initially, 100µl of cell suspension (containing 2×10^5 leukemic cells and normal PBMCs and/or 1×10^5 cells/ml for K562 cell line) were cultured in complete RPMI-1640 medium culture and treated with 100µl of different concentrations of bacterial cell wall. Un-stimulated wells considered as negative control. After 24, 48 and 72 hrs incubation at 37°C and 5% CO_2, 20µl of MTT solution were added to the wells and the plates were incubated for 4hrs. After that, supernatant was discarded and 100 µl of DMSO

were added to the wells. Then pipetting was done and absorbance was read at wavelength of 590nm for each plate. Then IC50 was calculated for optimum cytotoxicity dose of *Bifidobacterium* bacteria on K562 cell line for further studies.

Bax, Bcl2 Gene's expression in treated K562 cell line

K562 cell line (5×10^5 cells/4ml) were cultured in complete RPMI-1640 medium culture and treated with IC50 concentration of bacterial cell wall. After 48 and 72 hours of treatment, total cellular RNA was extracted according to the manufacturer's instruction (Yekta Tajhiz, Iran). The obtained RNA was transcribed to cDNA by Revert AidTM First Strand cDNA Synthesis Kit (Yekta Tajhiz, Iran). In briefly, 2 µL of 10x solution buffer, 10 of µL RNA, 1 µL of deoxynucleotide triphosphate mixture (dNTPs), 1 µL of random hexamer primer, 1 µL of RNase enzyme inhibitor, 1 µL of reverse transcriptase enzyme and 4 µL double-distilled water were used for synthesis. Then, mRNA level of Bax and Bcl2 was assessed by using Real-time PCR method (Qiagen, USA) at a temperature program as bellow: 95 °C for 1 min; 95 °C for 20 s; and 55 °C for 60 s. Primers for Bax and Bcl2 were used at 10 nM. The obtained ΔΔCT was used to analysis the gene expression and also β-actin was considered as normalize gene.

Statistical analysis

The mean of each duplicate was calculated and the result of each immunoassay is presented as Mean±SD. The data was analyzed using Graph pad prism V6.01 software. ANOVA method was applied to compare the statistical significance among the experimental doses. P-values of P< 0.05 were considered to represent statistically significant differences.

Results

Cytotoxicity assay on leukemic blasts

Results of cytotoxicity of *Bifidobacterium bifidum* cell wall on leukemic cells from patients with AML leukemia in all the concentrations (10 up to 160 µg/ml) after 24hrs showed that there was no significant difference between experimental groups as compared with each other and also there was no remarkable difference between treated and un-treated samples (Fig. 1a) (P>0.05). Results after 48 hrs treatment with *Bifidobacterium bifidum* cell wall demonstrated no significant differences between the experimental and un-treated samples (Fig. 1b) (P>0.05). As well as after 72hrs, the treatment with the probiotic bacterial cell wall did not show remarkable difference in the experimental group versus un-treated samples (Fig. 1c) (P>0.05).

Cytotoxicity assay on healthy samples

The results of cytotoxicity of *Bifidobacterium bifidum* cell wall on PBMCs of healthy people after 24hrs showed no significant difference as compared to untreated control (Fig. 2a-c) (P>0.05). After 48hrs treatment with *Bifidobacterium bifidum* cell wall, the results did not show any significant effect (P>0.05). As illustration shows, after 72hrs, the treatment with this probiotic bacteria could not show a remarkable difference in the treated cells versus un-treated samples (P>0.05).

Cytotoxicity assay on K562 cell line

The results of *Bifidobacterium bifidum* cytotoxicity on K562 cell line does not show a significant difference at 24hrs incubation time, but after 48hrs treatment a remarkable cytotoxicity is seen either at the concentration of 10 and 20 µg/ml (P<0.0002). This significantly difference was also observed after 72hrs incubation time at various concentrations of *Bifidobacterium bifidum* cell wall (10, 20, 40 µg/ml) (P<0.017). (Fig. 3a-c).

Bax and Bcl2 Genes expression

Results of genes expression after 48hrs treatment show that K562 cells treated with *Bifidobacterium bifidum* cell wall could significantly upregulate the mRNA level of Bax (Folds) as compared to untreated and β-actin gene expression (P<0.0057). However, our results could not show a significant different in Bcl2 expression versus β-actin gene expression (P> 0.05) (Fig. 4). Results of gene expression of Bax at 72hrs showed a significant increase as compared to anti-apoptotic Bcl2 gene (P=0.0003). Also, there was a significant increase in Bax gene expression as compared to β-actin (P=0.0008).

Discussion

As uncontrolled cell proliferation and its resistance to apoptosis are the main properties of cancer, therefore, some agents that are able to induce apoptosis in cancer cells could be considered as an anti-cancer element. The probiotics can modulate immune system in several levels such as enhancement cytokines and immunoglobulin levels, increase of mononuclear cells proliferation especially NK cells, activating macrophages and stimulation of the immune responses against pathogenic bacteria and protozoa. The positive effects on the immune system are produced by probiotics without any harmful inflammatory responses (16).

According to the obtained results from cytotoxicity assay in 24, 48 and 72hrs, it is founded that there was no cytotoxicity effect on leukemic cells of individuals with AML. Also, *Bifidobacterium bifidum* had no cytotoxicity effect in normal PBMCs. Lee at al. have reported that cytoplasmic extraction of *Lactobacillus* and *Bifidobacterium* have a direct effect on the prevention of cancer cell lines (17). Some studies showed that *Bifidobacterium bifidum* is able to inhibit colon carcinogenesis that reduction of PH level could be a reason for preventing

carcinogenesis (18). Some of in vitro studies demonstrated that *Bifidobacterium bifidum* has cytotoxicity effect on other cancer cell lines at 24, 48 and 72hrs. According to our findings it seems that anti-cancer properties are dependent on the type of cancer and depending on the dose and time. Along with, several studies showed that prescription of *Bifidobacterium bifidum* could potentiate cellular immunity and as is clear cellular immune responses are effective to prevent leukemia via enhancement of antibody responses and cellular immune responses (19-21).
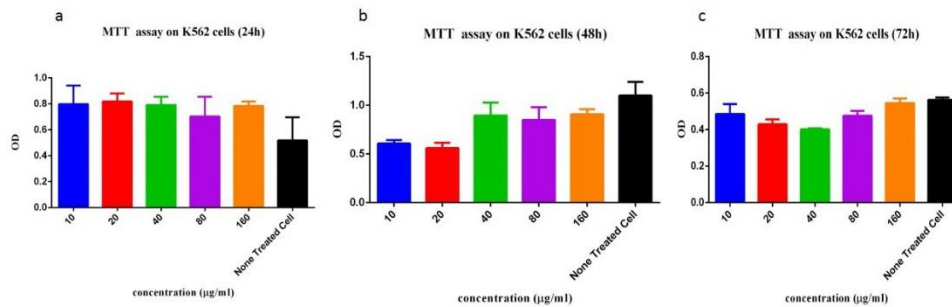
Cytotoxicity results of *Bifidobacterium bifidum* on K562 cells showed a reasonable capacity for killing the cancerous cells after 48hrs and 72hrs incubation time. These results represent that the cytotoxicity effect might be occurred by a time dependent manner. Because, after 72hrs treatment, more concentrations could exert the cytotoxicity effect in comparison to 48 hour-treatment. Saraki et al. indicated that probiotics as immune stimulators could improve antibody production by immune cells and increase the biological function of phagocytic cells (22). Actually, it seems that *Bifidobacterium bifidum* reinforce immune system with the indirect mechanism; also, different mechanisms are involved to kill the cancer cell such as cytokines secretion, antibody potentiation and attachment to the cancer cells and killing these cells through macrophages, complement and NK cells. In the present study, evaluation of *Bifidobacterium* cytotoxicity on K562 cell as another model for in vitro cytotoxicity could not show any significant cytotoxicity effect at 24hrs; however, cytotoxicity effect was remarkable at different concentrations, 48 and 72hrs. *Bifidobacterium bifidum* demonstrated the cytotoxicity effect on K562 which was derived CML leukemia. Therefore, the *Bifidobacterium bifidum* has the capability to eliminate wide range of cancer cells in in vitro situation. According to the obtained results from the evaluation of apoptotic gene expression.
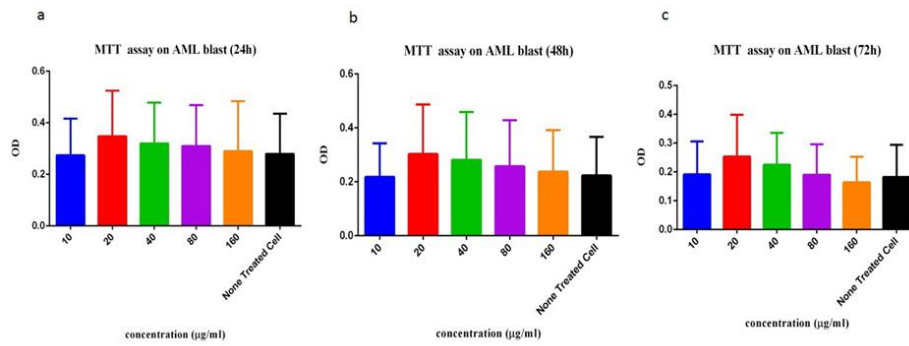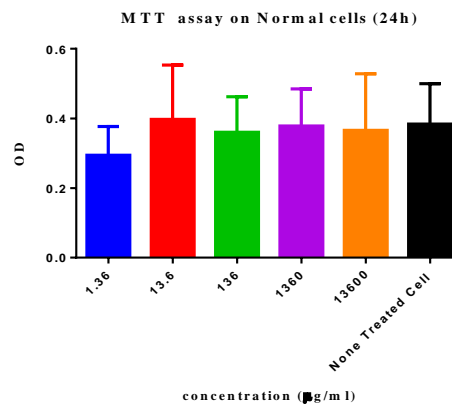
Figure 1.   Cytotoxicity of *Bifidobacterium bifidum* cell wall on leukemic cells from patients with AML leukemia after 24 (a), 48 (b), and 72 hours (c).
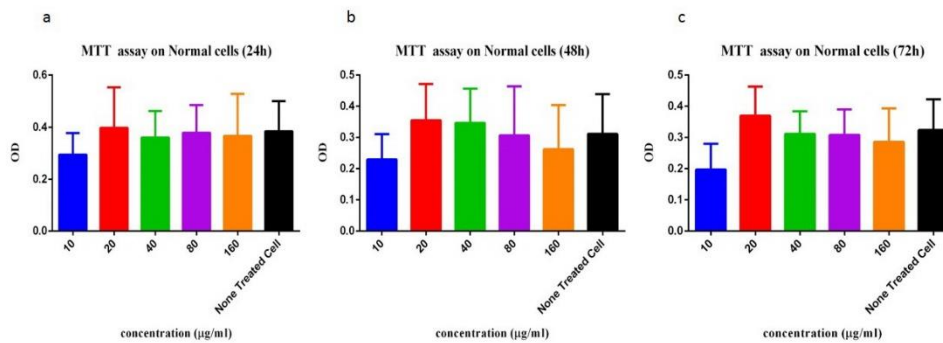
Figure 2.   Cytotoxicity of *Bifidobacterium bifidum* cell wall on PBMCs of healthy people after 24 (a), 48 (b), and 72 hours (c).

Figure 3. Cytotoxicity of *Bifidobacterium bifidum* cell wall on K562 cell line after 24 (a), 48 (b), and 72 hours (c).

Figure 4. Expression of *Bax* gene by K562 cells after treatment with *Bifidobacterium bifidum* cell wall.

Conclusion

In conclusion it seems that *Bifidobacterium bifidum* could eliminate tumor cells with reinforcement of immunity as indirect mechanism and induction of apoptosis as is involved to kill K562 cell line as direct mechanism. However may does not show toxicity effect on other types of cancer cells.

Acknowledgment

Conflict of interest

The authors declare no conflict of interest for this work.

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