The Antiproliferative Effect of Kefir Cell-Free Fraction on HuT-102 Malignant T Lymphocytes

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Abstract

Kefir is produced by adding kefir grains (a mass of proteins, polysaccharides, bacteria, and yeast) to pasteurized milk; it has been shown to control several cellular types of cancer, such as Sarcoma 180 in mice, Lewis lung carcinoma, and human mammary cancer. Human T-cell lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia, which is a fatal disease with no effective treatment. The current study aims at investigating the effect of a cell-free fraction of kefir on HuT-102 cells, which are HTLV-1–positive malignant T-lymphocytes. Cells were incubated with different kefir concentrations: the cytotoxicity of the compound was evaluated by determining the percentage viability of cells. The effect of all the noncytotoxic concentrations of kefir cell-free fraction on the proliferation of HuT-102 cells was then assessed. The levels of transforming growth factor (TGF)-α mRNA upon kefir treatment were then analyzed using reverse transcriptase polymerase chain reaction. Finally, the growth inhibitory effects of kefir on cell cycle progression and/or apoptosis were assessed by flow cytometry. The maximum cytotoxicity recorded at 80 µg/µL for 48 hours was only 43%. The percent reduction in proliferation was very significant, dose and time dependent, and reached 98% upon 60-µg/µL treatment for 24 hours. Kefir cell-free fraction caused the downregulation of TGF-α, which is a cytokine that induces the proliferation and replication of cells. Finally, a marked increase in cell cycle distribution was noted in the pre-G_1 phase. In conclusion, kefir is effective in inhibiting proliferation and inducing apoptosis of HTLV-1–positive malignant T-lymphocytes. Therefore, further in vivo investigation is highly recommended.

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Introduction

Some bacteria have been shown to have many health benefits and to confer resistance to disease, whether alone or through the changes which occur as a result of fermentation. In recent years, the interest in such probiotic species has increased noticeably. Kefir is a viscous, slightly carbonated dairy beverage containing small quantities of alcohol.[1] It is believed to contain many functional substances to which the numerous positive effects of kefir on health might be attributed. In fact, it has been postulated that the longevity of Bulgarian peasants is in part attributed to consumption of this fermented milk.[2] Kefir is produced by adding kefir grains, which are similar to small cauliflower florets, to pasteurized milk.[1,3] It differs from other milk products like yogurt, for example, in that it is fermented with a mixture of bacteria and yeast confined to a matrix of discrete kefir grains that are recovered after fermentation.[2,4]

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Both bacteria and yeasts in kefir grains are surrounded by a polysaccharide matrix, which contains a water-soluble branched glucogalactan,[5] produced by lactobacilli within the grains. The polysaccharide, named kefiran, is composed of a hexasaccharide repeating unit[1] and has been reported to possess many health benefits.[5]

Bacteria and yeasts are found in kefir grains living in symbiosis, even though this special symbiotic nature has made it difficult to identify and study the constituent microorganisms within kefir grains. It has been reported that the bacteria and yeasts of kefir, when taken apart as pure cultures, either were not able to grow in milk or showed a lower biochemical activity, and this also makes it more complicated to study the microbial population of kefir grains.[1] Some of the bacteria that were identified in kefir include *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus kefir*,[2] *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus parakefir*,[6] *Lactobacillus acidophilus*,[1] *Lactococcus lactis* subsp *lactis*, *Lactococcus lactis* subsp *cremoris*,[1] *Streptococcus thermophilus*,[1] *Enterococci durans*,[1] *Leuconostocs mesenteroides*,[1] *Bacillus subtilis*, *Micrococcus* sp, and *Escherechia coli*. Metabolites produced by the yeasts add to the taste and mouthfeel of kefir and provide a milieu for the growth of kefir bacteria.[1] Some of the yeasts that were identified in kefir include *Saccharomyces* sp,[1] *Saccharomyces cerevisiae*,[6] *Saccharomyces unisporus*, *Saccharomyces exiguus*, *Saccharomyces turicen-*

sis,[1] *Saccharomyces delbrueckii*,[6] *Saccharomyces dairensis*,[1] *Saccharomyces unisporus*, *Saccharomyces lipolytica*,[6] *Candida pseudotropicalis*, *Candida friedrichii*, *Candida tenuis*, *Candida maris*, *Candida inconspicua*, *Candida lambica*, *Candida tannotelerans*, *Candida valida*, *Candida holmii*,[1] *Candida kefir*, *Torulaspora delbrueckii*,[1] *Brettanomyces anomalus*,[1] *Issatchenkia occidentalis*,1 *Pichia fermentans*,[1] and *Kluyveromyces marxianus*.[1] The health benefits of kefir in Eastern European countries have been identified since early times, where it is linked with general well-being.[1] Kefir has been found to stimulate the immune system[2,7] and enhance the ability to digest lactose for lactose maldigestors.[1,8] Kefir possesses antimicrobial activity in vitro against a wide variety of Gram-positive and Gram-negative bacteria, as well as some fungi.[1,9] Kefir also seems to be a promising compound in terms of enhancement of mucosal resistance to gastrointestinal pathogen infection.[1,10,11] In addition, kefir was found to have antiallergic and antimutagenic effects.[5,10,12] Kefir as a complex fermented milk and its cell-free fraction were both able to exert beneficial effects on the immune system and prevent several types of cancer[13]: Ehrlich carcinoma or Sarcoma 180, Lewis lung carcinoma, E-ascites carcinoma, and human mammary cancer (MCF-7).[2,4,14,15]

The first retrovirus to be identified in humans is the human T-cell lymphotropic/leukemia virus (HTLV), which was discovered in 1980 in the blood of a patient with cutaneous T-cell lymphoma.[16,17] Approximately 5%-10% of infected individuals develop adult T-cell leukemia (ATL) or HTLV-1–associated myelopathy/tropical spastic paraparesis, whereas the rest remain asymptomatic carriers.[18] The latency period of this retrovirus is very long and can go up to 50 years. The average age of onset of ATL is 55 years, and the male:female patient ratio is 1.4:1.[19] There are 4 subtypes of ATL: acute, chronic, smoldering, and lymphoma, a classification based on the number of abnormal T cells in peripheral blood, serum lactic acid dehydrogenase levels, tumor lesions in various organs, and clinical course.[20] The clinical manifestations of ATL include malaise, fever, cough, drowsiness, lymphoadenopathy, hepatosplenomegaly, hypercalcemia, and jaundice. Lymphocytes have a soluble type of the interleukin-2 receptor α.[18,19] The virus usually transforms $CD4^+$ lymphocytes,[21] with a small percentage occurring within $CD8^+$ lymphocytes.[22] Transformed lymphocytes look like flowers. The treatment of the aggressive forms of ATL remains disappointing.[23] The current study aims to test the cytotoxic and antiproliferative effect of kefir cell-free fraction on a virus-infected human leukemia cell line.

Materials and Methods

Cell Line and Cell Culture

One leukemia cell line was used: HuT-102, which is an HTLV-1–positive cell line. HuT-102 cells are ATL-derived, HTLV-1–infected $CD4^+$ lymphocytes that constitutively express the retrovirus upon replication.

The cells were suspended in RPMI 1640 medium containing 25 mM HEPES and L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 µg/mL of streptomycin, and 100 U/mL of penicillin and grown in a humidified incubator (37°C) at 95% air and 5% CO_2. Cells were split every 2 days at a ratio of 1:4. Before seeding, the cell viability was tested by trypan blue dye exclusion method: 10 µL of cells were mixed with 10 µL of trypan blue and the cells were counted using a hemocytometer. For all experiments, cells were seeded at a density of 1×10^5. The test compound was added after 24 hours of seeding.

Primary Fresh Cultures of Human Lymphocytes

Human peripheral blood lymphocytes from healthy individuals were isolated using a Ficoll-isopaque gradient (1.077) for immediate growth (courtesy of the American University of Beirut Medical Center). These lymphocytes were grown in the presence or absence of kefir in complete growth medium consisting of RPMI 1640 supplemented with 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin for 24 hours. These lymphocytes were used as a negative control, and the effect of kefir cell-free fraction on cytotoxicity and proliferation of the lymphocytes was determined using the methods described below, and the percentage change was determined in comparison with the control.

Preparation of Cell-Free Fraction of Kefir

Sterilized pasteurized skimmed milk (150 mL) was inoculated with kefir grains (50 g). All inoculated milk samples were incubated at 20°C for 24 hours in a glass-sealed container. At the end of fermentation, the milk was strained to remove the kefir grains.

The yeast and bacteria in the samples were removed by centrifugation and filtration. The kefir samples were centrifuged (35,000 rpm, 10 minutes, 4°C), and the supernatant was filtered through a 0.22-µm Millipore filter. The kefir cell-free fraction filtrates were then solidified using a Centrivap Concentrator and stored at –20°C until required.

On the day of treatment, the solidified cell-free fraction of kefir was redissolved in media (RPMI 1640) and applied on the seeded cells.

Cytotoxicity

Cells were seeded into 96-well plates at a density of 1×10^5. After 24 hours of seeding, cells were treated with different concentrations of kefir cell-free fraction or media alone for the controls. Cytotoxicity of kefir on HuT-102 cells was assayed at 6, 24, and 48 hours using two methods:

1) Cytotoxicity Detection Kit (LDH; Roche, Mannheim, Germany) according to instructions of manufacturer: At the end of the treatment period, cells were centrifuged and the supernatant of each well was transferred to a new 96-well plate. The reaction mixture supplied with the kit was then added to the wells and kept in the dark at room temperature for 30 minutes. This method measures the amount of lactate dehydrogenase (LDH) released by dead cells. The conversion of tetrazolium salt into a red formazan dye product was measured colorimetrically at 492 nm using an enzyme-linked immunosorbent assay microplate reader.
2) Trypan blue exclusion method: 10 µL of cells were mixed with 10 µL of trypan blue. Dead cells appear blue, whereas living cells exclude trypan blue and appear bright. The percent viability was determined by visually counting the dark cells compared with the live bright cells.

Proliferation

Cells were seeded into 96-well plates at a density of 1×10^5. After 24 hours of seeding, cells were treated with different concentrations of kefir cell-free fraction or media alone for the controls. Proliferation was measured at 6, 24, and 48 hours using two kits:

Table 1 Primer Sequences Used in Reverse Transcriptase Polymerase Chain Reaction

Gene	Size	Sequence
TGF-α	373 bp	5'ATGTTGTTCCCTGCAAGTCC3' 3'ACTATGGAGAGGGGTCGCTT5'
β-Actin	550 bp	3'TCGCCCTTTAGCCACGCACTG5' 5'CGCCTGCGCCTGGTCGTCGACA3'

1) Cell Titer96™ Nonradioactive Cell Proliferation Kit (Roche, Mannheim, Germany) according to the instructions of the manufacturer: at the end of the treatment period, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to all wells of the plate and kept in a humidified incubator (37°C) at 95% air and 5% CO_2 for 4 hours, after which a solubilization buffer was added and kept overnight. This method is an MTT-based method that measures the ability of the mitochondria of metabolically active cells to convert tetrazolium salt into a blue formazan product that is measured colorimetrically at 595 nm.
2) Cell Proliferation Reagent (WST-1; Roche, Mannheim, Germany). At the end of the treatment period, water-soluble tetrazolium salt (WST-1) was added to the cells and kept in a humidified incubator (37°C) at 95% air and 5% CO_2 for 4 hours. WST-1 is a tetrazolium salt that, when in contact with metabolically active cells, gets cleaved to formazan by mitochondrial dehydrogenases. The formazan dye was then measured colorimetrically at 450 nm. The results were expressed as percent of control.

Reverse Transcriptase Polymerase Chain Reaction for Analysis of Transforming Growth Factor

Cells were grown in 3 mL of RPMI 1640 complete growth medium at a cell density of 1×10^5 cells/mL and were treated with various noncytotoxic concentrations (20, 40, and 60 µg/µL) of kefir cell-free fraction for 24 hours. Cells were collected and stored at –80°C, and the total RNA was extracted using an RNA extraction kit: NucleoSpin RNA II kit (MACHEREY-NAGEL GmbH & Co. KG.) according to the manufacturer's instructions.

Reverse transcriptase polymerase chain reaction (RT-PCR; ReddyMix Reverse-iT One-Step Kit, ABgene) was used to amplify RNA of transforming growth factor (TGF). Two hundred nanograms of the RNA of TGF-α was converted to cDNA by RT-PCR using (Reverse-iT Blend) reverse transcriptase.

Polymerase chain reaction was conducted in 25 µL volume using oligonucleotide primers designed to detect the cDNA according to the subsequent mentioned conditions. See Table 1 for the primer's sequence. To amplify the product, the RNA template was added to 0.5 µL of each primer (forward and reverse; 10 µM), 0.5 µL of reverse transcriptase (50 units/µL) was added to 12.5 µL 2× ReddyMix RT-PCR Master Mix supplied with the kit containing 1.25 U/50 µL of thermoprine plus DNA polymerase, dNTP mix with 0.2 mM of each nucleotide, and 1.5 mM of $MgCl_2$ and 10 µM of each primer. This 14-µL mixture was added to the RNA sample, and the volume was adjusted to 25 µL with RNase-free double-distilled water. The PCR program for TGF-α RNA was as follows: first strand synthesis at 47°C for 60 minutes, initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation for 45 seconds at 94°C, annealing at 51°C for 45 seconds and elongation for 45 seconds at 72°C, and the final step at 72°C for 5 minutes. Fifteen microliters of the PCR products was run on 0.8% agarose gels stained with 4% ethidium bromide at 100 V for 30 minutes. The resulting bands were visualized by ultraviolet light and photographed. β-actin was used as the loading control.

Flow Cytometry

Cells were seeded at a density of 1×10^5 cells/mL in 3 mL of complete growth medium and after 24 hours treated with various noncytotoxic concentrations of kefir cell-free fraction for 24 hours. Cells were centrifuged at 1200 rpm at 4°C for 5 minutes. The pellet was then washed by resuspending it in 1 mL of ice-cold 1× phosphate buffered saline (PBS). Cells were centrifuged again under the same conditions as before and resuspended in 1 mL of 70% ethanol and stored at –20°C for a few days. Later, cells were thawed and centrifuged at 1200 rpm for 5 minutes, washed with 1 mL of ice-cold 1× PBS, centrifuged again, and then treated with 50 µL of RNase and incubated for an hour and 15 minutes at 37°C. The cells were then pelleted at 1200 rpm for 5 minutes, and the pellets were washed with 1 mL of 1× PBS, centrifuged, and stained with 30 µL of propidium iodide. Cells were analyzed using a FACScan (Becton-Dickinson; San Jose, CA), which indicated the distribution of the cells into their respective cell cycle phases based on their DNA content. G_0/G_1 cells were 2n; S-phase cells were > 2n but < 4n while G_2/M cells were 4n. Cell DNA content was determined by CellQuest software. An increase in cells in the pre-G_1 phase is indicative of an increase in apoptosis. The ratio of cells in the pre-G_1 phase was compared with those of the control.

Statistical Analyses

All experiments were carried out in triplicates. Results were reported as the mean value ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher least significant differences at $P \leq .05$ (Fisher PLSD). An effect was considered significant when the value (±) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

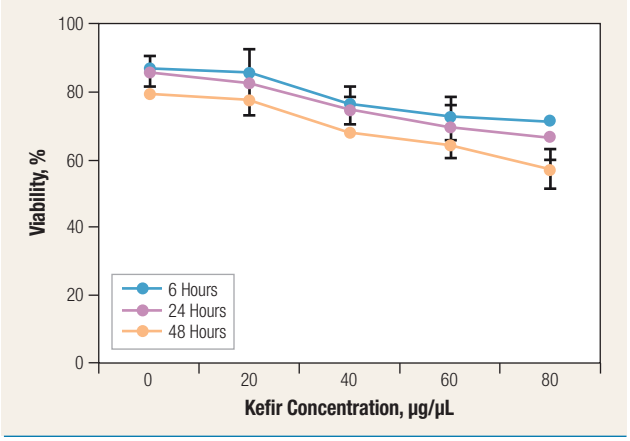
Results

Cytotoxicity

The cytotoxicity of kefir cell-free fraction was determined on HuT-102 cells using the trypan blue exclusion method. The maximum cytotoxic value reached was 43% in the 48 hours treatment of 80 µg/µL, and the IC_{50} (concentration at which 50% of the cells die in culture) was not reached (Figure 1). Hence, all concentrations were used in subsequent experiments to assess the effect on proliferation.

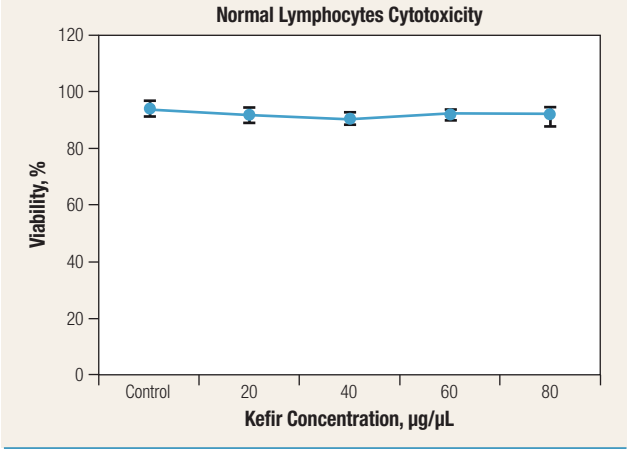
The Cytotoxicity Detection Kit (LDH) was also used to determine the cytotoxicity of kefir cell-free fraction. However, the results obtained were not reported, as kefir had a high interference with the production of the red formazan dye, thus giving invalid values for the cytotoxicity. Figure 2 shows that the cell-free fraction of kefir, at all experimental doses used earlier, is not toxic to normal T lymphocytes taken from the peripheral blood of healthy individuals ($P > .05$).

Figure 1 Cytotoxicity of Kefir on HuT-102 Cells



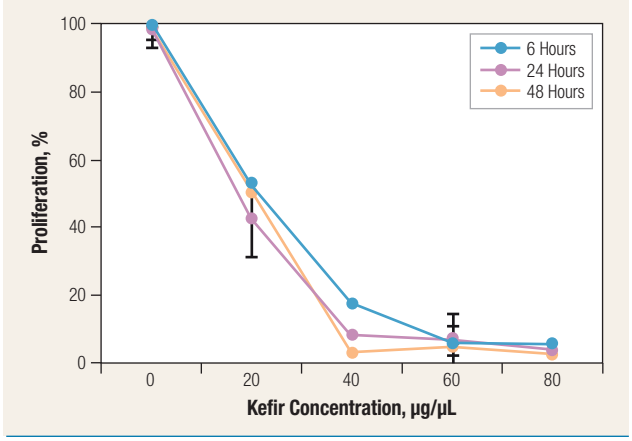
Bars represent the standard error of the results.

Figure 2 Effect of Kefir on the Viability of Normal Lymphocytes From the Blood of Healthy Individuals



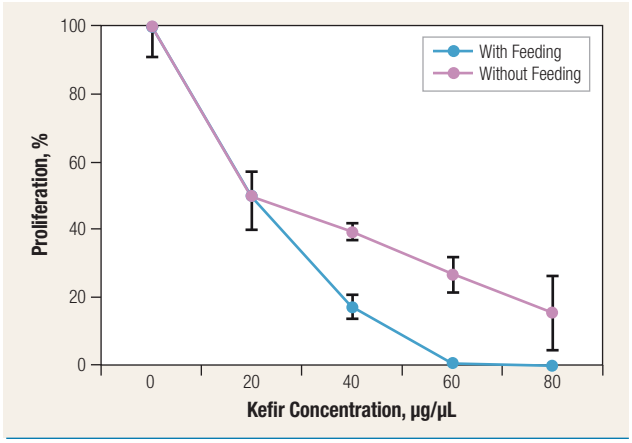
Bars represent the standard errors. Kefir does not result in significant reduction in the viability of normal lymphocytes.

Figure 3 Effect of Kefir on the Proliferation of HuT-102 Cells



Bars represent the standard error of the results. Kefir causes a dose-dependent reduction in proliferation.

Figure 4 Effect of Kefir on the Proliferation HuT-102 Cells Upon Treatment for 48 Hours Without Feeding and With Feeding



Bars represent the standard error of the results. Kefir causes a dose-dependent reduction in proliferation.

Proliferation

The cell-free fraction of kefir was found to decrease the proliferation of the cell line in a dose- and time-dependent manner ($P < .05$). The percent reduction in proliferation upon 60-µg/µL treatment was found to be around 82%, 96%, and 98% at 6, 24, and 48 hours, respectively (Figure 3). In all subsequent experiments, the treatment was done by using 20, 40, and 60 µg/µL concentration of kefir for 24 hours. The cell line was also treated with kefir for 48 hours with and without feeding: in feeding experiments, proliferation assays were performed as described previously but by adding kefir cell-free fraction twice, once at the beginning of the experiment and the second time after 24 hours to determine whether an additional treatment will cause further inhibition in proliferation. The results showed no major change upon additional treatment in the feeding experiments (Figure 4). To test the effect of the cell-free fraction of kefir on normal cells, T lymphocytes obtained from healthy donors were treated with various concentrations of kefir, and then their proliferation levels were tested. The cell-free fraction of kefir showed no significant reduction on the proliferation of normal T lymphocytes ($P > .05$). After treatment with kefir cell-free fraction for 24 hours, the maximum level in decrease of proliferation recorded was only 8%.

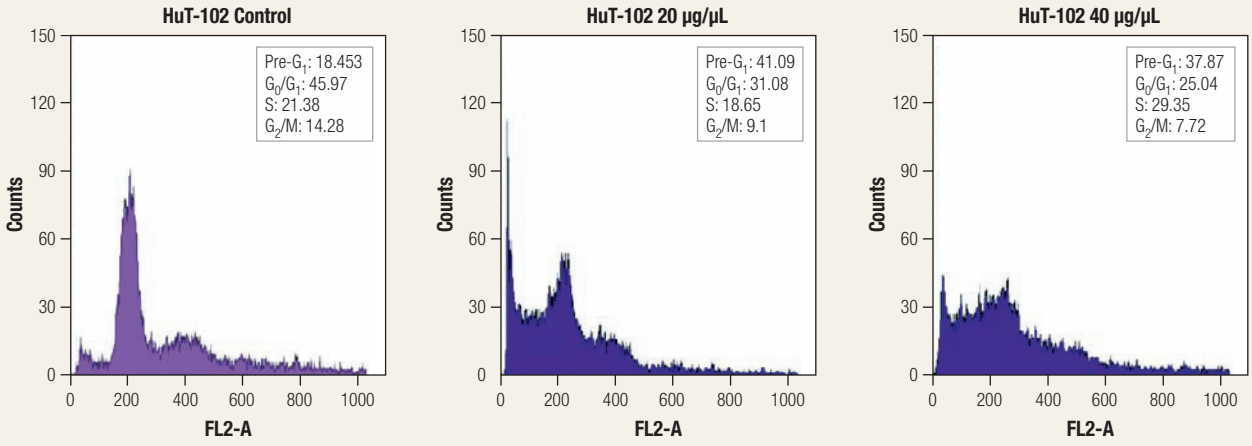
Flow Cytometry

The effect of the cell-free fraction of kefir on proliferation and cell cycle progression of the cell line was tested using flow cytometry analysis. Kefir cell-free fraction was found to induce a G_0/G_1 arrest and an increase in cells in the pre-G_1 phase indicative of a decrease in proliferation. The cell-free fraction of kefir induced a 2-fold increase in cells in the pre-G_1 phase as portrayed in Figure 5.

Effect of Kefir Cell-Free Fraction on Transcriptional Levels of Transforming Growth Factor

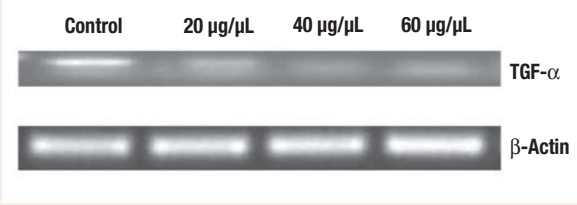
The transcriptional levels of TGF-α was assayed using RT-PCR. TGF-α is a cytokine that induces the proliferation and replication of cells through binding to epidermal growth factor receptor (EGFR).[24]

Figure 5 Effect of Kefir on the Cell Cycle and Apoptosis by Flow Cytometry



Results indicate that kefir causes a G_0/G_1 arrest as evidenced by the increase in the cells in the pre-G_1 phase. FL2-A is the channel of the flow cytometry machine

Figure 6 Effect of Kefir on the Transcriptional Level of TGF-α in HuT-102 Cells



β-Actin was used as the loading control.

Treatment with 20, 40, and 60 µg/µL of kefir cell-free fraction was found to decrease the transcriptional levels of TGF-α in the cell line tested in a dose-dependent manner. Results are shown in Figure 6.

Discussion

The results obtained in this study indicate that the cell-free fraction of kefir exhibits an antiproliferative effect in malignant T lymphocytes infected with the HTLV-1 virus. This is consistent with the previously reported antitumor effect of kefir on many types of cancer, such as slowing down Sarcoma 180 growth in mice by introducing kefir both orally and intraperitonially,[14] inhibition of pulmonary metastasis in Lewis lung carcinoma by orally administered polysaccharide fraction from kefir grains,[15] reduction of proliferation, and apoptosis induction in Sarcoma 180,[2] as well as the regression of human mammary cancer demonstrated by Chen et al.[4] However, the antitumor effect of kefir on leukemic cancers has not been previously reported. Moreover, most of the previous studies demonstrated antitumor activity of kefir by stimulating macrophages and immune responses. The aim of the present investigation was to demonstrate the direct inhibitory effect of a kefir cell-free fraction on tumor cell growth in vitro, even though the active components remain to be elucidated.

In the current study, the cell-free fraction of kefir treatment caused a significant inhibition in the proliferation of cancerous cells at the various concentrations used (20, 40, 60, and 80 µg/µL). The cytotoxicity of the compound was analyzed by determining the percent of viable cells remaining after 6, 24, and 48 hours of incubation in order to use these concentrations as noncytotoxic. The IC_{50} (concentration at which 50% of the cells remain viable) was not determined because kefir cell-free fraction caused a substantial reduction in the proliferation of HuT-102 cells, reaching 97%, at doses that exhibited < 50% cytotoxicity; because kefir is a viscous compound, it was preferable not to use higher concentrations in order to avoid color interference in the absorbance results.

In order to elucidate the therapeutic effect of kefir in cancer, kefir should not be toxic to normal cells, so its effect on cytotoxicity and proliferation of normal human lymphocytes was also studied. No toxic effect on the normal cells was observed, as the viability remained above 90% at 80 µg/µL, which is the highest concentration of kefir cell-free fraction used in the current study. In addition, the proliferation of normal lymphocytes was only reduced by about 8%, which is not a significant effect. These results are comparable to what was reported by Chen et al, where the kefir extract did not suppress the growth of normal human mammary cells,[4] which indicates that kefir contains bioactive ingredients that exert a growth suppressive effect that is specific to cancer cells.

Analysis of the cell cycle distribution at which the inhibition was taking place revealed that the cell cycle inhibition occurred at the G_0/G_1 phase, which appears in the pre-G_1 increase. This G_0/G_1 arrest might indicate that the cell-free fraction of kefir could induce apoptosis in HTLV-1–infected and malignant cell lines.

To further investigate the effect of the cell-free fraction of kefir on the proliferation of HuT-102 cells, the transcriptional level of TGF-α was analyzed. Kefir cell-free fraction, at all noncytotoxic concentrations used, caused the downregulation of TGF-α expression. Transforming growth factors are a large family of cytokines that have various effects on cells: TGF-α is a cytokine that induces the proliferation and replication of cells through binding to EGFR.[24] Binding to EGFR induces autophosphorylation of the receptor and thus triggers a phosphorylation cascade with some targets binding

Src and JAK-2. Another consequence of EGFR binding is the activation of the proto-oncogene *RAS*. It is now established that *RAS* overexpression might lead to the development of skin papillomas in mice as well as many types of cancer.[25] Moreover, TGF-α was shown to inhibit apoptosis in gastric mucosal cells via stimulation of nuclear factor-κB pathway, which resulted in the upregulation of BCL-2 antiapoptotic proteins.[26]

The transcription levels of other transforming growth factors such as the proapoptotic TGF-β1 and TGF-β2 could be examined in further subsequent studies. Also, testing the activity of the gelatinases matrix metalloproteinases MMP-9 and MMP-2 could be useful in studying the effect of kefir cell-free fraction on the invasion potential of leukemic cells.

The current study demonstrates the beneficial effects of kefir cell-free fraction in one malignant cell line infected with the HTLV-1 virus. However, the effect of kefir on other HTLV-1–negative and –positive leukemic cell lines should be examined in vitro before evaluating its effect in vivo and in clinical trials.

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Disclosures

The authors report no relevant financial conflicts of interest.

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