J. Food Sci. Nutr.
Vol. 5, No. 4, p. 225~229 (2000)

Effect of Kefir Extract on the Growth of Serum-Free Mouse Embryo (SFME) Cells

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Abstract

The antioxidative and protective activities of kefir, low-fat kefir, non-fat dry milk (NFDM) extract and fractions on SFME cells in serum-free medium were investigated. Kefir and low-fat kefir and NFDM extract were made by solubilizing the freeze dried powder forms in deionized water, filtering through glass prefilter, 12 μm and 2 μm membrane, and demineraling with chelating resin. Kefir, low-fat kefir and NFDM extract were fractionated into dialyzate and retentate by dialysis with membrane tube having the molecular cut-off of 3,500 Dalton. An antioxidative activity was analyzed by the *in vitro* model system using a linoleic acid. In the case of kefir an antioxidative activity was detected only in the retentate of kefir extract. On the other hand NFDM showed an antioxidative acivity in extract, demineralized extract, dialyzate and retentate. The retentate of kefir extract had the higher antioxidative activity than that of NFDM extract. Kefir showed the protective effect of SFME cells in serum-free medium in extract, demineralized extract and retentate, but low-fat kefir didn't. NFDM had the similar protective effect on SFME cells as extract, demineralized extract and retentate of kefir.

Key words: kefir, antioxidative activity, protective effect, SFME cells

INTRODUCTION

Kefir is a traditional fermented milk which has been consumed for thousands of years. It originated from the Caucasus mountains in the former Soviet Union where the drink was fermented naturally in bags made of animal hides. It is now becoming popular in western Europe, particularly in Finland, Norway and Sweden (1), and has recently been introduced in west Germany, United States and Japan. Kefir is made by the fermentative activity of kefir grains containing species of lactic acid bacteria and yeasts in a protein-polysaccharide matrix (2).

Kefir is a refreshing slightly carbonated and acidic fermented milk. The distinctive organoleptic properties differ from yogurt in that small amounts of CO_2, alcohol and aromatic molecules are produced as a result of a dual fermentation of lactic acid bacteria and yeasts.

Kefir has been known to have various biological activities. Several studies have investigated the antitumor activity of kefir (3-5) and of kefir grains (6,7). Immune system stimulation with kefir (5) and with sphingomyelin isolated from the lipid of kefir (8) have been demonstrated in both *in vitro* and *in vivo* studies. Kefir (9) possesses antimicrobial activity *in vitro* against a wide variety of gram-positive and gram-negative bacteria, and against some fungi (3). Zacconi et al. (9) reported the antagonistic effects of kefir against *Salmonella kedougou*. De Vrese et al. (10) demonstrated that fresh disintegrated kefir grains suspended in kefir directly enhanced intestinal lactose digestion in minipigs. This effect was attributable to microbial β-galactosidase activity of kefir.

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The purpose of this study was to evaluate the antioxidative activity and the protective effect of kefir extract on serum-free mouse embryo (SFME) cells in a serum-free medium.

MATERIALS AND METHODS

Materials

Regular kefir and low-fat kefir were obtained as freeze dried powders, produced by a Japanese milk company. Non-fat dry milk (NFDM) used as a control was bought from Nestle Food (CA, USA). Thiobarbituric acid, $FeSO_4$, linoleic acid, Tween 60, vitamin E, bovine insulin, bovine fibronectin, human transferrin, sodium selenite, trypsin and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, USA). BCA protein assay kit was bought from Pierce Chemical Co. (Rockford, Il, USA). Mouse epidermal growth factor (EGF) was obtained from Upstate Biotechnologies (New York, NY, USA). High density lipoprotein (HDL) was prepared by KBr ultracentrifugation and filter-sterilized after dialysis. Glass prefilter, 12 mm and 0.2 mm membrane filters were purchased from Micron Separations Inc. (Westboro, MA, USA). Chelex-100 for demineralization was from Bio-Rad (Hercules, CA, USA). Dialysis tube (M.W cut off 3,500 Dalton) for dialysis from Spectrum Company (Houston, TX, USA) was used. The reagents used were of guaranteed grade unless otherwise stated and water was prepared by distillation and deionization.

Preparation of kefir extract and fractionation

Five grams of kefir was dissolved in 40 mL deionized water, stirred for 30 min at room temperature and centrifuged at

18,000 rpm for 30 min at 10°C. Supernatant was filtered through a glass prefilter, with a 12 μm and, subsequeally, 0.2 μm membrane, and stored at 4°C until use. To remove any kinds of metal ions from the kefir extract, 0.25 g of Chelex-100 was added to 5 mL of kefir extract, stirred mildly for one hour at room temperature and filtered through a 0.2 μm membrane. Demineralized kefir extracts were fractionated by dialyzing against Spectrapor membrane tubing having a molecular weight cut off of 3,500 Dalton into dialyzate and retentate according to molecular size. Retentates containing molecules over 3,500 Dalton were filtered through a 0.2 μm membrane for sterilization and stored at 4°C. Dialyzates were concentrated to 2 mL with a rotary evaporator at 60°C and filtered through a 0.2 μm membrane for sterilization.

The concentrations of protein in the kefir extract, dialyzate and retentate were measured according to micro BCA protein assay using micro BCA protein assay kit from Pierce Chemical Co..

Measurement of antioxidative activity using *in vitro* model system

Half mL of linoleic acid was emulsified with 50 mL deionized water containing 0.25 mL of Tween 60 (40% w/v) (11,12). The emulsion was neutralized with 0.1 N KOH. 6.7 mL of neutralized emulsion was resuspended in 33.3 mL of 0.05 M potassium phosphate buffer (pH 7.0), and made up in a final volume of 50 mL with deionized water. 15 mL of linoleic acid emulsion was incubated at 37°C for one hour with the test sample, and a peroxidation was initiated by adding 20 mL of $FeSO_4$ to a final concentration of 50 mM Fe^{+2}. After two hours, 5 mL of peroxidized linoleic acid emulsion tampered for 10 min at 4°C was added to a screw-caped test tube containing 2.5 mL of 6 M phosphoric acid and TBA aqueous solution mixture (5:1). 6 M phosphoric acid and TBA aqueous solution were mixed just before use. The mixture of linoleic acid emulsion and TBA reagent was mixed well and heated for 15 min in a boiling water bath. After cooling with running tap water, 7.5 mL of n-butanol was added and mixed vigorously by vortexing. The n-butanol phase was separated by centrifugation at 3,000 rpm for 30 min at 10°C and absorbance was measured at 535 nm. The antioxidative activity was expressed as IC_{50} which represents the concentration of sample which inhibits 50% of linoleic acid peroxidation under experimental conditions.

SFME cells culture

The basal nutrient medium was a one to one mixture of Dulbecco's modified Eagles medium containing 4.5 g/L glucose and Hams F12 supplemented with 15 mM Hepes, pH 7.4, 1.2 g/L sodium bicarbonate, sodium selenite (10 mM), penicillin (200 U/mL), streptomycin (299 mg/mL), and ampicillin (25 mg/mL) (F12:DME). SFME cells which were harvested from 16~18 days old female mice and kept frozen in liquid nitrogen in serum-free medium with 10% dimethyl sulphoxide (DMSO) were used (13-16). Cells were cultured in F12:DME supplemented with insulin (10 mg/mL), transferrin (10 mg/mL), EGF (50 mg/mL), human high density lipoprotein (HDL, 10 mg/mL) and selenium (Se, 10 nM) in 75 cm^2 tissue culture flask pre-coated with bovine fibronectin (10 mg/mL). Cells cultured continuously in the complete serum-free medium were detached from stock flasks by trypsinization, diluted into trypsin inhibitor solution, centrifuged, resuspended, counted using Coulter counter, and plated at 2×10^4 cells per 35 mm-diameter dish. After cells were incubated in serum-free medium without HDL and Se for two days, they were changed into a serum-free medium containing different concentrations of kefir extract. When most of cells in the medium without HDL and Se died, cells in dishes treated differently were detached by trypsinzation, diluted into trypsin inhibitor solution and counted using a Coulter counter to confirm the effect of kefir extract on the growth of SFME cells in serum-free medium.

RESULTS AND DISCUSSION

Antioxidative activity of kefir extracts

Antioxidative activities of kefir, low-fat kefir and NFDM are shown in Table 1. Kefir and low-fat kefir extracts did not

Table 1. Antioxidative activity of kefir extracts and fractions by *in vitro* model system

Sample		Protein concentration (mg/mL)	Antioxidative activity	
			IC_{50} (μL)[1)]	IC_{50} (mg)[2)]
Kefir	Extract	9.06	Not detected	Not detected
	Demineralized	8.63	Not detected	Not detected
	Dialyzate	8.55	Not detected	Not detected
	Retentate	0.99	628	0.62
Low-fat kefir	Extract	10.17	Not detected	Not detected
	Demineralized	9.50	Not detected	Not detected
	Dialyzate	7.81	Not detected	Not detected
	Retentate	1.16	Not detected	Not detected
NFDM	Extract	22.00	1,208	26.58
	Demineralized	20.60	382	7.87
	Dialyzate	9.81	304	2.98
	Retentate	7.44	817	6.08

[1),2)] IC_{50} means the amount of sample to be required to prevent the peroxidation of linoleic acid by 50%.

show antioxidative activity but NFDM extract had an antioxidative activity.

The demineralization of NFDM extract increased an antioxidative activity about 3.4 times from 26.58 mg to 7.87 mg while demineralization of kefir and low-fat kefir extract did not have any effect. Demineralization was performed to eliminate metal ions which could be used as a catalyst in peroxidation of linoleic acid. As it was expected, a demineralization increased the antioxidative activity of NFDM extract, indicating that NFDM contained some metal ions to catalyze the peroxidation of linoleic acid.

Each extract was divided into dialyzate having molecules less than 3,500 Dalton and retentate having molecules bigger than 3,500 Dalton by dialysis using a membrane tube (M.W. cut-off 3,500 Dalton). The dialyzate of the kefir extract and low-fat kefir did not show antioxidative activity, but that of the NFDM extract showed higher antioxidative activity than the demineralized one. The retentate of the kefir extract had a higher antioxidative activity than that of NFDM extract, suggesting that the antioxidative activity of kefir extract might be attributable to peptides larger than 3,500 Dalton which were produced from milk proteins by the dual fermentation of lactic acid bacteria and yeasts. But in low-fat kefir extract an antioxidative activity was not detected.

Bodana and Rao (17) observed the antioxidative activity in milk fermented by lactic cultures, which might influence the action of carcinogens. Lee et al. (18) reported that the reactive oxygen scavenging activity of skim milk was increased by the fermentation of lactobacilli. The increase of reactive oxygen scavenging activity might be exerted by peptides or amino acids from milk proteins by the action of lactobacilli.

The protective effect of kefir extract on SFME cells

To find out the replacing effect of each extract for HDL and SE which are essential for the growth of SFME cells in serum-free medium by suppressing the toxicity of fatty acid peroxidation, SFME cells were incubated in serum-free medium without HDL and Se for two days and then in a serum-free medium with different concentrations of kefir, low-fat kefir and NFDM extract until most of cells in medium without those were dead. After seven days most SFME cells died due to the toxicity of fatty acid peroxidation. As a control, SFME cells incubated in medium without HDL and Se for two days were incubated in medium containing HDL and Se.

The number of SFME cells increased in proportion to the concentration of NFDM extract (Fig. 1) and demineralized samples (data not shown). The retentate of demineralized NFDM extract showed a protective effect on the growth of SFME cells as the concentration was increased (Fig. 1). However, the dialyzate did not protect SFME cells from the toxicity of fatty acid peroxidation. Even if SFME cells were incubated in serum-free medium containing HDL and Se, the number of those decreased in proportion to the concentration of dialyzate, suggesting the existence of a toxic substance to SFME cells in the dialyzate of NFDM extract.

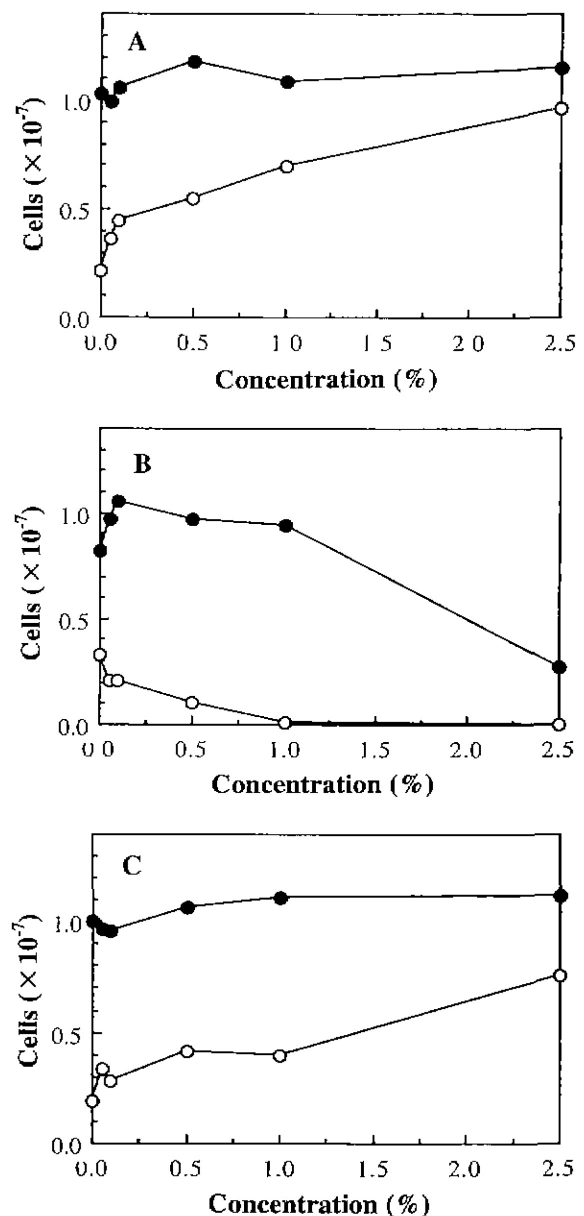

Fig. 1. The effect of NFDM extract, dialyzate and retentate of NFDM extract on the growth of SFME cells in serum-free medium. A, NFDM extract; B, dialyzate of NFDM extract; C, retentate of NFDM extract. ●, with HDL and Se; ○, without HDL and Se.

The number of SFME cells increased proportionally with the concentration of kefir extract (Fig. 2). In case of demineralized kefir extract, a similar trend was observed (data not shown). The dialyzate of kefir extract showed the reverse effect of kefir extract and the retentate increased the number of SFME cells in the proportion to the concentration. It suggests that some components in kefir extract which have a protective effect on SFME cells in serum-free medium without HDL and Se were transferred into the retentate.

The low-fat kefir extract and the retentate didn't affect the growth of SFME cells (Fig. 3). Like the dialyzate of kefir ex-

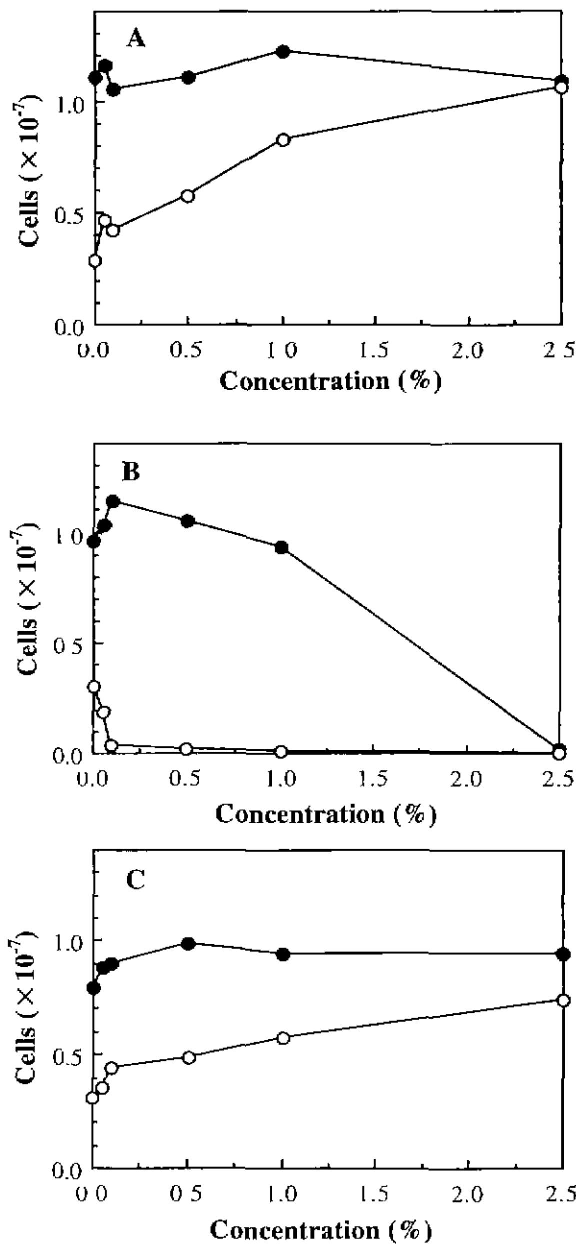

Fig. 2. The effect of kefir extract, dialyzate and retentate of kefir extract on the growth of SFME cells in serum-free medium. A, kefir extract; B, dialyzate of kefir extract; C, retentate of kefir extract. ●, with HDL and Se; ○, without HDL and Se.

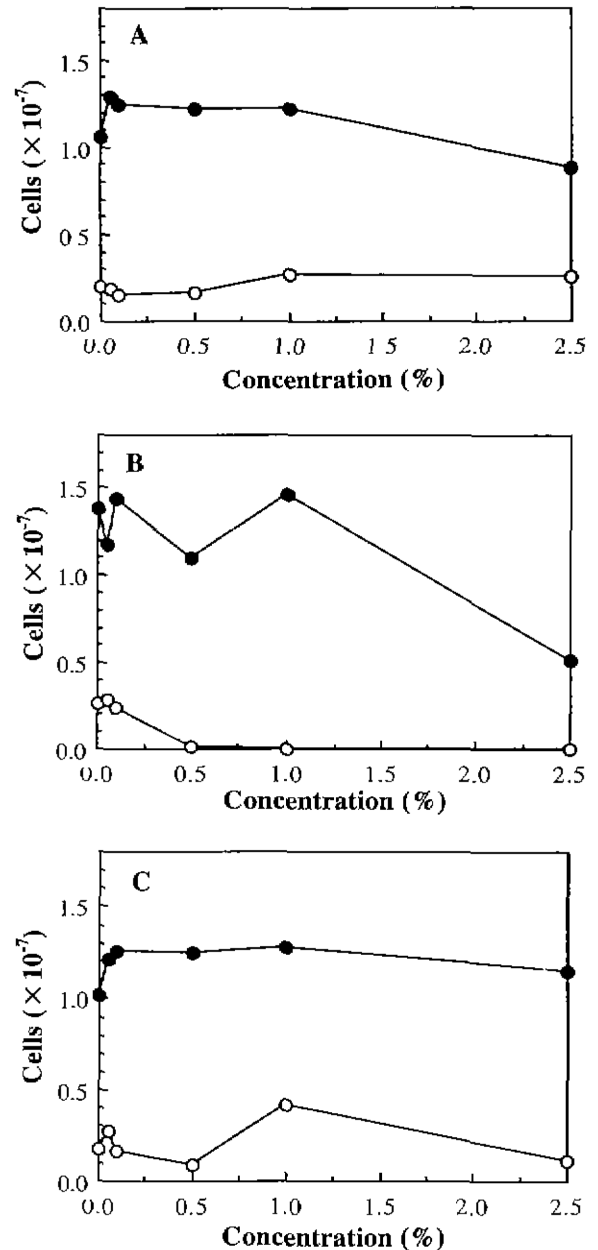

Fig. 3. The effect of low-fat kefir extract, dialyzate and retentate of low-fat kefir extract on the growth of SFME cells in serum-free medium. A, low-fat kefir extract; B, dialyzate of low-fat kefir extract; C, retentate of low-fat kefir extract. ●, with HDL and Se; ○, without HDL and Se.

tract, that of low-fat kefir extract showed a negative effect on the growth of SFME cells in serum-free medium.

HDL is known to be a growth stimulatory factor for many types of cells including SFME cells. Delipidated (apo) HDL did not exhibit any stimulatory activity on SFME cells. Loo et al. (13) proposed that the lipid composition of HDL might be an important factor determining stimulatory activity. Iio et al. (14) found that HDL protected SFME cells in serum-free medium from the toxicity of lipid peroxidation. They suggested their HDL preparations might contain vitamin E which suppresses the peroxidation of fatty acid.

Seleniun is a key structure component of the enzyme glutathione peroxidase, which plays an essential role in the neutralization of metabolically generated peroxides by lipid peroxidation (15). Therefore, Se could contribute to the antioxidative activity of serum-free medium like vitamin E of HDL preparation.

The protective effect of tested samples on SFME cells in serum-free medium without HDL and Se appeared to be related closely to their antioxidative activities except the dialyzate of demineralized NFDM extract which showed no protective effect. Some peptides or proteins larger than 3,500 Dal-

ton contained in NFDM extract might be responsible for the antioxidative activity and the protective effect on SFME cells. In kefir, lactic acid bacteria and yeasts could break down some proteins into small peptides which have a higher antioxidative activity than unbroken proteins and a protective effect on SFME cells in serum-free medium.

ACKNOWLEDGEMENTS

This research was supported from Research Fund provided by Korea Research Foundation, Support for faculty research abroad.

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(Received October 4, 2000)