

## Comparative Evaluation of Cytotoxic Effects of Milk from Various Species on Leukemia Cell Lines

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### ABSTRACT

**Objective:** Previous studies have shown milk to contain cancer inhibitors. In this context, this study was conducted to screen the potential cytotoxic properties of four different types of milk, namely cow's milk, goat's milk, mare's milk and human milk. **Methods:** In evaluating the cytotoxic properties of milk, two different human leukemia cell lines namely, Raji and CEM-SS were used. The treated and untreated cells of milk were cultured at 37°C in 5% CO<sub>2</sub> for 5 days according to standard guidelines. The CellTiter 96® Aqueous (MTS) assay was carried out on the first, third and fifth days to measure cell viability. The percentage of cell viability was determined by comparing the optical density of the treated cells against the untreated controls. One-way ANOVA at p<0.05, Duncan's Multiple Range test and Independent T-test were carried out to determine the mean count differences of cell viability between and within cow's milk, goat's milk, mare's milk and human milk. **Results:** Interestingly, only mare's milk was found to cause statistically significant cytotoxicity on both the Raji (p<0.05) and CEM-SS (p<0.05) cells at 10% dilution. The cells treated with cow's milk, goat's milk and human milk showed no significant difference in cell viability when compared to the untreated controls where cell growth effects were observed instead. In addition, the cells treated with mare's milk underwent morphological changes that were indicative of apoptosis. **Conclusion:** The findings of this study show that among the four types of milk, mare's milk appears to possess cytotoxic properties against leukemia cell lines.

**Keywords:** Human cell lines, milk, leukemia, cytotoxic

### INTRODUCTION

Milk is a physiological fluid with a high nutritional value as it is naturally rich in energy, proteins, vitamins and minerals.<sup>[1]</sup> In addition, milk has been shown to have many unique properties that help regulate cells of the immune system, which in turn help fight against microbes.<sup>[2]</sup>

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Cow's milk is the most popular milk that is consumed worldwide. It has high biological value averaging 84.5% for whole cow's milk and 79.7% for milk protein casein. Whole cow's milk contains good amounts of vitamin A, carotene, vitamin B, C (11 IU/I), D,E (1025 IU/I) and selenium (5-50 µg/L). Furthermore, it has been known for decades that cow's milk contains three times more protein than human milk and that cow's milk fat contains a number of cancer inhibitors.<sup>[3]</sup>

In a series of studies on bovine milk proteins and their biological functions, it was found that lactoferricin (Lfcin-B), a peptide derived from a bovine lactoferrin (LF-B) has direct cytotoxicity to THP-1 tumor cells.<sup>[4]</sup> It was the first study to show that Lfcin-B induces apoptosis in human leukemia cells. This apoptosis inducing activity is associated with the production of intracellular reactive oxygen species (ROS) and activation of (Ca<sup>+2</sup>/Mg<sup>+2</sup>) dependent endonucleases. These findings suggest that Lfcin-B is applicable to the development of an anticancer agent against tumor cells. In addition, milk fat from cow's milk has also been shown to be the richest natural source of conjugated linoleic acid which is a potential anticarcinogen.<sup>[5]</sup>

Unlike cow's milk, the fat globules in goat's milk are much smaller and remain suspended in solution. Goat's milk can sometimes be used as an alternative for individuals with sensitivity to cow's milk. Goat's milk is reportedly a good source of calcium. In recent studies, this important mineral has been shown to help protect colon cells from cancer-causing chemicals.<sup>[6]</sup>

Biologically, human milk is a highly unique secretion, very different from milk of other species. Breast milk contains various nutrients such as vitamin C (43 IU/L), Vitamin E (1898 IU/L), selenium (13-50 µg/L) and calcium (340 mg/L) which are beneficial for the growth of infants.<sup>[7]</sup> It also contains protein components that regulate cells of the immune system and protects infants from various diseases.<sup>[1]</sup> Unique components in the protein fraction of human milk include host defense factors such as immunoglobulins, lysozyme and lactoferrin, digestive enzymes, specific binding proteins and growth factors.<sup>[2]</sup> The whey protein component in human milk has been reported to exhibit antitumor activity<sup>[8]</sup> and was shown to induce apoptosis in certain cancer cells.<sup>[9]</sup>

A type of milk that is rarely consumed is mare's milk. The composition of mare's milk is similar to human milk and consists of high polyunsaturated fatty acids with a low cholesterol content although it has some peculiarities in its fats, carbohydrates and protein contents.<sup>[10]</sup> Thus, mare's milk is suitable for human consumption and has been claimed to have medicinal value. For example, it has been shown to be useful in chronic hepatitis and peptic ulcer patients.<sup>[11]</sup>

To our knowledge, there has been no scientific research documented on the effects of mare's milk on cancer cell growth. This study therefore included a comparison of the cytotoxic effects of the different types of milk on leukemia cell lines.

## MATERIALS AND METHODS

### *Cell Lines*

Two leukemia cell lines were used in this study. They were the human T-cell acute lymphoblastic leukemia (CEM-SS), formerly known as PN6, and acute myelogenous monocytic leukemia (Raji) cell lines obtained from the Riken Cell Bank, Japan. The cells were grown in suspension cultures in RPMI-1640 media (Sigma, USA) supplemented with 10% (v/v) Fetal Calf Serum and maintained at 37°C in a 5% CO<sub>2</sub> incubator. The growth media contained penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Flow Lab, Australia). Cell stocks were periodically prepared according to standard practice.

### *Materials*

The various samples of animal milk were obtained from reliable milk suppliers. Essentially, goat's milk was obtained from a farm in Negeri Sembilan, cow's milk from a dairy farm in Bangi, Selangor while mare's milk was supplied by Setia Brothers Sdn. Bhd. in Kuala Lumpur. A sample of human milk was obtained from a healthy donor from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor. The materials used in this experiment also included the CellTiter 96® Aqueous (MTS) kit<sup>[12]</sup> (Promega, USA), RPMI-1640 media (Sigma, USA), Fetal Calf Serum (PAA Laboratories, Australia), sterile distilled water and absolute ethanol (MERCK, Germany).

### *Sample Preparation*

The milk samples were heat-activated at 56°C for 45 minutes to remove allergens and kept in the refrigerator at 4°C. Following this, 35 ml of milk was centrifuged (Beckman, UK) at a speed of 150,000 x g for 1.5 hours. The supernatant was then filter-sterilised using a 0.22 µm filter. Finally, 10 ml aliquots were placed into sterile serum vials and frozen at 70° C until further use.

### *Preparation of Milk Dilution*

The supernatant of the mare's milk, goat's milk and human milk was diluted using a ten-fold serial dilution gradient. The stock was prepared by diluting 25 µl of the original sample into 225 µl of sterile RPMI-1640 media giving a 10% dilution. Additional dilutions were at 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup>.

### *Cell Culture, Plating and Treatments*

CEM-SS and Raji cell lines were maintained in 25 cm<sup>2</sup> culture flasks. The cell concentration was determined using an improved Neubauer hemocytometer. The cells were then plated in 24-well cell culture plates, at a density of 2.50 x 10<sup>4</sup> cells/450µl/well for both Raji and CEM-SS, in RPMI 1640 medium containing 10% fetal calf serum (FCS). An aliquot of 50µl of each of the samples was appropriately added into the wells, at the different dilutions described

earlier and incubated for 1-5 days. For the untreated controls, 500 µl of the cell suspension was added at the same cell densities.

#### *Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay*

The Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay, a colorimetric method used for determining the number of viable cells in proliferation or chemosensitivity assays, was performed according to the manufacturer's instructions. The conversion of MTS into aqueous formazan was accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of the formazan product was measured at 490 nm. The absorbance is directly proportional to the number of living cells in culture.<sup>[13]</sup>

#### *MTS Assay*

A volume of 100 µl of treated and untreated cells was removed from the 24-well plated cell culture and transferred into 96-well plates pre-labelled 1, 3 and 5, in order to conduct the Cell Titer 96® MTS assay and have the absorbance read. Subsequently, 20 µl of 5 mg MTS labeling solution (Sigma, USA) per ml of PBS was added to each well. The microtiter plate was then incubated at 37°C in 5% CO<sub>2</sub> incubator for another 4 hours. The solubilised formazan product was spectrophotometrically quantified using an ELISA reader (Dynex MRX II) at 490 nm. Cell viability (% cytotoxicity) was obtained by dividing values obtained from the OD readings of the sample with the OD readings of the control and multiplied by a hundred.

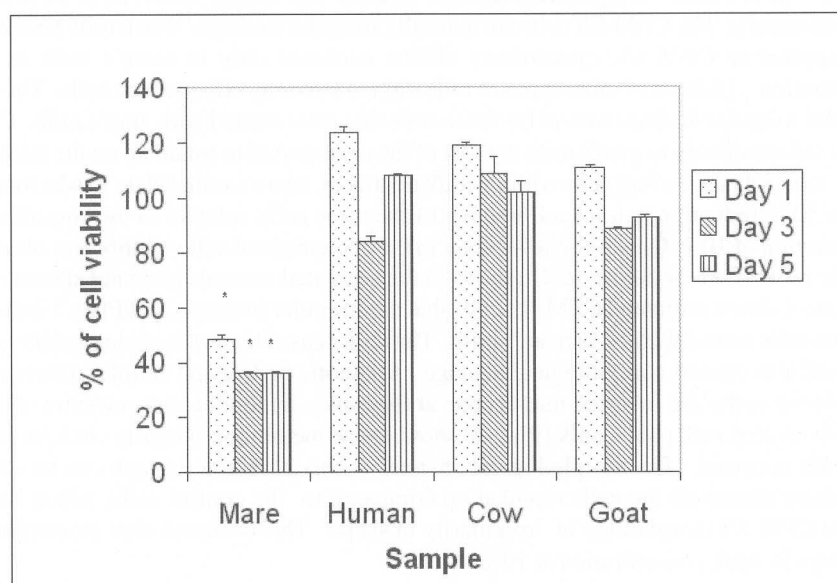
#### *Data Analysis*

Data was analysed using the SPSS software for Windows (Version 12.0). Descriptive analysis was performed and one-way ANOVA was used to compare the percentage of cell viability in the cancer cell lines within and between the treatments of the different milk dilutions. Duncan's Multiple Range Test was used to identify milk samples with significant difference in terms of mean cell viability.

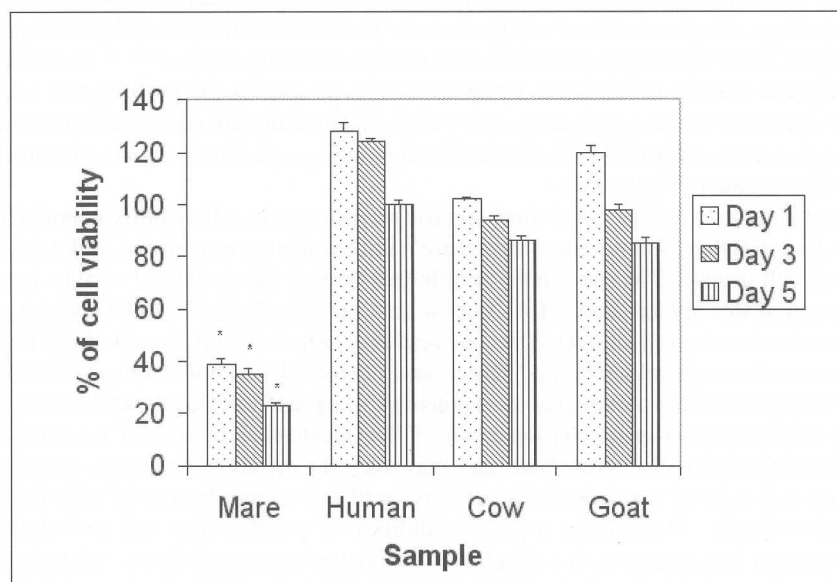
## **RESULTS**

The histograms (*Figs. 1 & 2*) show the percentage of cell viability for each of the different samples (from day 1 until day 5) treated with 10% milk dilution on both Raji and CEM-SS cell lines. Overall, it was observed that the cells treated with mare's milk showed the lowest cell viability at the different milk dilutions from day one until day five. Only mare's milk was found to be statistically significant at the 10% dilution ( $p < 0.05$ ).

Plate 1 (control) shows Raji cells without milk treatment. Mare's milk (Plate 2) was found to cause a 50 % reduction in total cell number of the same cells, relative to the control at a concentration of 10%. Only a slight decrease in the percentage of cell viability was observed with the milk dilutions below 10% relative to the untreated controls (data not shown).



**Figure 1.** Percentage of cell viability vs sample from day 1 to day 5 at 10% concentration (Raji cells). Values are means  $\pm$  SD. \* $p < 0.05$  vs other milk samples.



**Figure 2.** Percentage of cell viability on day 1 until day 5 vs sample at 10% milk concentration (CEM-SS). Values are means  $\pm$  SD. \* $p < 0.05$  vs other milk samples.

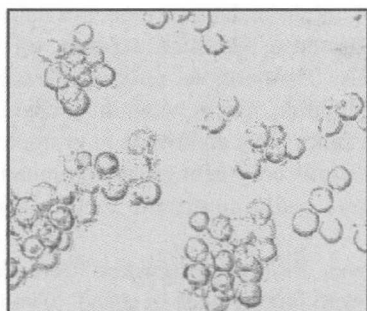
The percentage of viable cells decreased by 39% (Plate 3) in the Raji cell line treated with 10% goat's milk. A significant number of cells was clearly observed to be affected morphologically. The CEM-SS cells are naturally irregular in shape. When milk treatments were applied to CEM-SS, cytotoxicity effects occurred only in mare's milk at 10% concentration. Addition of other types of milk did not have any effect on the cells. The cells remained irregular in shape except for those cells that were treated with goat's milk. CEM-SS reacted sensitively to goat's milk as most of the cells started to round up on the third day. Plate 1 (control) shows Raji cells without milk treatment. Mare's milk (Plate 2) was found to cause a 50 % reduction in total cell number of the same cells, relative to the control at the concentration of 10%. Only a slight decrease in the percentage of cell viability was observed with the milk dilutions below 10% relative to the untreated controls (data not shown).

Plate 4 shows untreated CEM-SS cells that are irregular in shape, and Plate 5 indicates the same cells treated with 10 % mare's milk. The milk caused the death of almost 60% of the cells and also caused morphological damage. Apoptotic bodies and cellular debris could be observed under the inverted microscope at the milk's treatment concentration of 10%. The cells treated with goat's milk (Plate 6) shows some membrane-integrity changes but no cell death occurred. The morphological changes in this photomicrograph can be clearly seen where almost all the cells rounded up compared to the control cells, which had an original CEM-SS morphology of irregularity in shape. This occurred after incubation for three days in milk concentration at 10%.

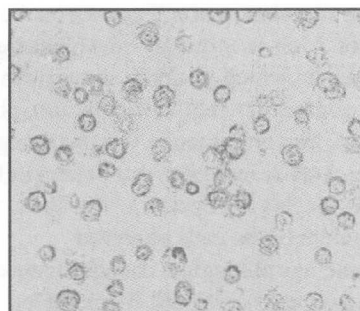
## DISCUSSION

The Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assays offers a colorimetric method where a large number of samples can be handled simultaneously by determining the number of cell viability in proliferation or chemosensitivity assays.<sup>[12,13,14]</sup> In this study, the assay was used to evaluate the cytotoxic effects as well as the proliferation of cells treated with mare's milk, goat's milk, cow's milk, and human milk on two different human tumor cells lines namely CEM-SS (human T-cell Acute Lymphoblastic Leukemia) and Raji (Acute Myelogenous Leukemia).

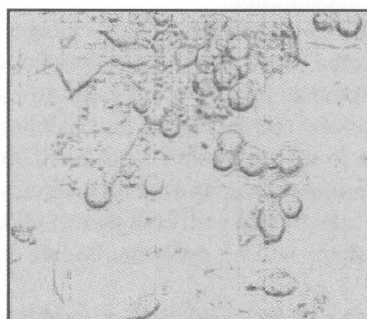
The histograms in *Figs 1 & 2* show significant decrease in cell viability in both CEM-SS and Raji cell lines when treated with mare's milk while the other three milk tended to stimulate cell growth. The substances in milk that impede the growth of various cancers include conjugated linoleic acid (CLA), butyric acid and ether lipids.<sup>[5]</sup> Milk fat which is the richest natural source of conjugated linoleic acid may help to inhibit growth of cell lines of malignant melanoma, colorectal and breast cancer cells. CLA is found almost exclusively in animal products and is among the most potent of all naturally occurring anti-carcinogens even in extremely low dietary concentrations.<sup>[15]</sup> The mechanism by which CLA influences carcinogenesis is largely unresolved, and may vary for different sites, age, duration of exposure and stage of carcinogenesis. Butyric acid is a unique feature of milk fat from ruminant animals. Butyrate is a potent inhibitor of proliferation and an inducer of differentiation and apoptosis in a number of colorectal cancer cell lines. Alkylglycerol, alkylglycerolphospholipid and ether lipid exhibited potential antineoplastic properties that inhibit growth, show antimetastatic activity and induced differentiation and apoptosis in human leukemia cancer cell lines.<sup>[16,17]</sup>



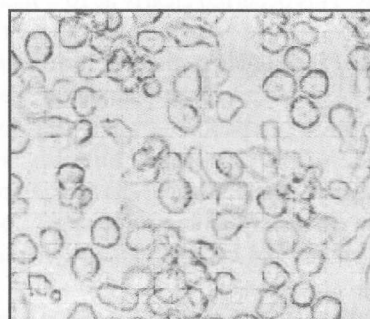
**Plate 1.** Inverted microscopy examination of untreated Raji cell line (magnification X200).



**Plate 2.** Inverted microscopy examination of Raji cell line treated with 10% dilution of mare's milk (magnification X200).



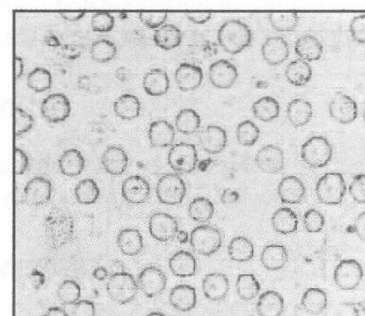
**Plate 3.** Inverted microscopy examination of Raji cell line treated with 10% dilution of goat's milk (magnification X200).



**Plate 4.** Inverted microscopy examination of untreated CEM-SS cell line (magnification X200).



**Plate 5.** Inverted microscopy examination of CEM-SS cell line treated with 10% dilution of mare's milk (magnification X200). Arrows indicate some classical apoptotic features of crescent chromatin condensation and nuclear fragmentation.



**Plate 6.** Inverted microscopy examination of CEM-SS cell line treated with 10% dilution of goat's milk (magnification X200).



The stimulation of cell growth on the other hand, might be due to presence of nutrients, enzymes such as alkaline phosphatase, lipase, protease, xanthine oxidase, or other components, which promote the growth of the cells. Furthermore, milk is an excellent natural source of riboflavin, calcium, phosphorus, thiamin, niacin, vitamin A, vitamin E, selenium, iron and vitamin C, which might also be factors that contribute to promote cell growth. A study indicated that human milk and cow's milk are potent growth promoters for several species of bacteria.<sup>[18]</sup> Thus, these factors could contribute to the induced proliferation of the cells observed.

Some morphological changes were also observed. Raji cells originated from a *homo sapien*, an eleven-year old Black male. The cells were lymphoblast in shape. Cytotoxic effects were not seen in the cells when human, cow and goat's milk were applied except for those cells, which were treated with mare's milk. When the effects of cytotoxicity occurred, some changes in the cells could be observed such as damage to the cell membrane and nucleus. This occurred clearly at 10% mare's milk concentration.

The cells that were treated with goat's milk also showed morphological changes with the actual lymphoblast shape of Raji cells being altered (Plate 3) and the cells adhering to the surface of the cell culture flask. Certain compounds in milk are thought to induce interferon in lymphoblastoid cells and to affect various morphological and biochemical properties of cultured cells. Milk has been shown to induce erythroid differentiation in immature erythroleukemic cells and hemoglobin synthesis in human erythroleukemic cells. There were no morphological changes observed in cells treated with both human milk and cow's milk. The cells remained lymphoblast in shape and no apoptotic bodies or cell damage were seen.

Although much research has been done on human milk and cow's milk on its anticancer components such as linoleic acid, sphingolipid fraction in cow's milk and casein fraction in human milk<sup>[4,9]</sup>, no significant effects were observed in this study. The possible reasons for the insensitivity of the human and cow's milk on the two cell lines studied could probably be due to the use of unfractionated milk, and that a higher concentration of greater than 10% may be needed to show significant results.

## CONCLUSION

The findings of this study showed that among the four types of milk tested, mare's milk appears to be the best milk in terms of cytotoxic properties.

## ACKNOWLEDGMENTS

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