Iraqi Journal of Cancer and Medical Genetics

Effects of Breast Milk Treatment on the Growth of Two Transformed Cell Lines

Haider S. Abid *

* Department of Biology, College of Science for Women, University of Baghdad.

Abstract :

Both epidemiological studies and in vitro experiments suggested a potential activity of breast milk as anti-cancer biological fluid, which provides a promising area that needs more research. In this study, the effects of human whole milk samples (M1, M2, and M3) taken from three donors on the cell growth of REF and Hep-2 cell lines are tested. The cells were exposed to concentrations of 50, 67 and 75% of each milk sample for 72h. The results demonstrated that only treatment with M1 50%, M1 67% and M2 75% caused a significant decrease in cell growth of REF cells compared to the control. Hep-2 cells showed no significant growth response to the concentrations of the human milk samples used. When the results were analyzed concentration-wise regardless of the donor from whom the sample was taken, 50% milk caused significant growth inhibiting activity for REF cells only, while 75% milk exerted such activity on both REF and Hep-2 cell lines compared to control. When a comparison was made between the responses of the two cell lines to milk treatment, REF cells showed significantly decreased growth percentage compared to Hep-2 cells, but only with concentration of 50% milk. This study suggests that the cell growth inhibiting activity of whole breast milk is both sample- and concentration-dependent. However, milk samples in the present study might have lost some high-molecular weight biologically active constituents during their preparation to the cell growth assay. The use of paired transformed/non-transformed cell lines derived from the same cell type is recommended since such model provides more comparable results for the effects of human milk on cancer cells.

Key Words:

Breast Milk, Growth Inhibition, Cancer Cells.

Introduction:

During the last four decades, research on human milk provided evidence on the multiple and essential roles played by this biological fluid in infant and adult lives. The high nutritional value of human milk plays key role in the growth and development of the breastfed infants, leading the WHO to recommend that breast milk should be the exclusive food for the infant during the first six months of life (1). However, breast milk has other highly important roles played by its biologically-active constituents such as the proteins, sugars and lipids (2). Several milk components were described to provide the mucosal surfaces of the infant's gastrointestinal and respiratory tracts with mucosal immunity against different infections (3). For example, in vitro studies showed that components like fatty acids, lactoferrins, lysozyme, antibodies and leukocytes in breast milk provide several types of protection against microbial infectious pathogens (4). Furthermore, epidemiological studies suggested that breast feeding associate with lower risks of several immune-related diseases in children or adults, including inflammatory bowel disease (5), types1 and 2 diabetes mellitus (6,7), acute respiratory infections, diarrhea, otitis media (2), leukemia and lymphoma (8, 9). Besides the cell growth promoters that were identified in milk, it also contains growth inhibitors that were suggested to provide protection for the infant's tissues from cancer (10).

However, only few studies have investigated the effects of breast milk on malignant or transformed cell lines and the need to cover the various cell models is obvious. Despite that, these few studies showed powerful tumoricidal activities of milk or its purified constituents in different cell lines. Hakansson and colleagues demonstrated that treatment of A549 (human lung carcinoma cell line) with whole breast milk reduced their growth to 0-4%. Furthermore, they found that the active compound responsible for this effect was a human multimeric α-lactalbumin (MAL) which, when purified,

Corresponding Address:
Haider S. Abid
Department of Biology, College of Science for Women, University of Baghdad.
Tel: 07902533810
E-mail: haider.abid@gmail.com

reduced the growth of A549 cells to 0%. In the same study, MAL exerted the same activity against several malignant cell lines (A549, NCI, A-498, J 82, Caco-2, HT-29, HTB9, GMK, Vero, MDCK). Interestingly, MAL did not show this growth reduction activity against the non-transformed cell lines (11). Also, human milk fractions were found to have high inhibitory activity to the growth of MDCK and Caco-2 cells, while bovine milk lacked such effect (10).

In fact, the beneficial properties of human milk listed above will provide a promising area of research, ranging from risk assessment studies that correlate breastfeeding with different diseases to the studies at cellular and molecular levels using various cellular models. Therefore, the present study evaluated the growth behavior of REF and Hep-2 cell lines, which were not studied before in this respect, in response to treatment with unprocessed human milk.

Materials and Methods:

Milk Samples

Three breast milk samples, designated here as M1, M2 and M3, were kindly provided by 3 volunteer breastfeeding mothers who have signed a written consent. All of donors were multiparous and mothers of full-term infants, with mean age of 26.33 ± 4.04 years. Using a manual breast pump, left- breast, transitional, foremilk samples (8-10 ml) were collected in polypropylene tubes after breakfast. The samples were immediately stored at -20°C until used. Upon time of the experiments, milk samples were brought to 37°C and mixed to restore their homogenous form. Three dilutions, 1:1, 2:1 and 3:1, were made for each sample using distilled water, providing concentrations of 50, 67 and 75%, respectively. All samples were filtered using 0.2µm membrane filters in order to remove the microbial contaminants that might affect the results of this study.

Cell Lines and Cultures

Rat Embryo Fibroblast (REF) and Human Laryngeal Epidermoid Carcinoma (Hep-2) cell lines as well as all media and materials were purchased from the Iraqi Center for Cancer and Medical Genetics Research, Baghdad, where the experiments were also performed. Hep-2 is a malignant cell line taken from a 57 years old man with larynx carcinoma whereas REF is a spontaneously transformed cell line of rat embryo fibroblasts (12,13). Cell culturing was performed according to Freshney (14) as described below. REF cell line was maintained with MEM supplemented with 10% FCS, while Hep-2 cell line was maintained in RPMI-1649 supplemented with 10% FCS.

Milk Treatment and Cell Growth Assay

Cell suspension was prepared by treatment of the cell layer with trypsin-ferricin solution. Then, 20ml of the culture media containing 10% fetal calf serum was added and the cells were mixed well. Using automatic micropipette, 0.2ml aliquots of the cell suspension were dispensed into the well of the calibration plate. After 12-18h incubation at 37°C, the medium was removed from the wells and 0.2ml of milk samples with the concentrations described above were added, with three duplicates for each concentration. For the control cells, 3 duplicates were made. Then the cells were incubated at 37°C for an exposure time of 72h. The cells were washed with PBS, detached with trypsin and harvested.

Cell viability was determined using the MTT stain (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described by Mosmann (15). Cells in the plates were read using ELIZA reader at a wave length of 620nm. Cell viability was calculated according to the following equation:

Viability % = (Absorbance of the sample/Absorbance of the Control) X 100

Statistical Analysis

Results were analyzed by the SPSS 10.0 for Windows software using Student's t-test. The results were expressed as Mean ± standard deviation. The differences were considered significant at $P \leq 0.05$.

Results:

Effects of Milk Concentration per Sample

Since viability provides direct indicator of cell growth, the results of the present study were expressed in terms of percentage of viable treated cells as compared to that of control which was considered as 100%.

As for REF cells, treatment with sample 1 of breast milk (M1) showed significant decreases in mean cell growth using the concentrations of 50% and 67% of milk (68.84 ± 11.16 % and 67.41 ± 14.40 %, respectively), while the decrease using 75% milk was not significant (81.14 ± 15.55 %) as compared to the control. In contrast, treatment with sample 2 (M2) significantly reduced the mean percentage of viable cells only with using 75% milk (81.17 ± 8.85 %), whereas treatment with 50% and 67% milk showed no significant decrease (82.64 ± 12.63 % and 87.32 ± 17.08 %, respectively) as compared to the control. However, treatment with all the three concentrations of sample 3 (M3) did not cause any significant decrease (86.36 ± 13.49 %, 81.77 ± 19.43 % and 90.37 ± 12.34 %, respectively) as shown in table 1.

Regarding Hep-2 cells, no significant differences in the mean percentage of viable cells were observed using all the concentrations of the three milk samples as compared to the control cells. However, cells treated with 50% or 67% of M1 sample showed an increase in their growth (108.56 ± 8.46 % and 128.38 ± 42.32 %, respectively), but still not significant as compared to the control cells. However,

treatment with 75% of M1 showed insignificant decrease (91.30 ± 7.03 %). Similarly, insignificant increase was observed using 50% of M2 sample (101.80 ± 20.53 %) as compared to the control, whereas the dilutions of 67% and 75% resulted in insignificant decrease (82.95 ± 12.72 % and 89.34 ± 7.68 %, respectively). Also, the three concentrations of M3 sample did not cause significant decrease in cell growth as compared with the control (98.34 ± 17.29 %, 96.75 ± 2.78 % and 90.90 ± 8.48 %, respectively), as indicated in table 1.

Table 1. Viability (%) of REF and Hep-2 cell lines in response to the treatment with 50, 67 and 75% concentrations of breast whole milk samples (M1, M2, M3) taken from three mothers.

Cell Type	Milk Sample									Control
	M1			M2			M3			
	50%	67%	75%	50%	67%	75%	50%	67%	75%	
REF	73.43	66.4	89.06	82.03	101.56	86.71	96.09	92.18	97.65	100.00
	56.12	53.54	63.22	70.32	68.38	70.96	70.96	59.35	76.12	100.00
	76.99	82.3	91.15	95.57	92.03	85.84	92.03	93.8	97.34	100.00
Mean	**68.84***	**67.41***	**81.14**	**82.64**	**87.32**	**81.17***	**86.36**	**81.77**	**90.37**	**100.00**
S.D.	**11.16**	**14.40**	**15.55**	**12.63**	**17.08**	**8.853**	**13.49**	**19.43**	**12.34**	**0.00**
Hep-2	108.77	118.42	99.12	124.56	94.73	80.7	117.54	98.24	100.00	100.00
	100.00	174.8	89.31	96.18	69.46	95.41	83.96	98.47	83.2	100.00
	116.93	91.93	85.48	84.67	84.67	91.93	93.54	93.54	89.51	100.00
Mean	**108.56**	**128.38**	**91.30**	**101.80**	**82.95**	**89.34**	**98.34**	**96.75**	**90.90**	**100.00**
S.D.	**8.46**	**42.32**	**7.03**	**20.53**	**12.72**	**7.68**	**17.29**	**2.78**	**8.486**	**0.00**

**Significant difference at P≤0.05.*

Effects of Collective Milk Concentrations

The results were also analyzed in a concentration-wise, regardless of the mother from whom the sample was collected (Table 2). As for REF cells, milk samples with concentration of 50% taken from all of the three mothers caused a significant decrease in cell viability (79.28 ± 13.43) as compared to the control. Although the decrease caused by 67% milk was relatively high (78.83 ± 17.27) but it was statistically insignificant, while 75% milk resulted in a significant decrease (84.22 ± 11.80). As related to Hep-2 cells, neither 50% nor 67% milk showed significant differences, although the trend was toward increase rather than decrease of cell viability (102.90 ± 14.77 and 102.69 ± 29.95, respectively). In contrast, a significant decrease (90.51 ± 6.77) was recorded using 75% milk.

Comparisons between REF and Hep-2 cells in terms of growth percentage in response to the same concentrations of milk are also demonstrated in table 2. Treatment with 50% milk showed highly significant decrease in the growth of REF cells as compared to Hep-2 cells. However, treatment with 67% and 75% milk did not exert any significant difference.

Table 2. Collective effects of the concentrations of 50, 67 and 75% of breast milk on the viability (%) of REF and Hep-2 cell lines.

Cell Type	Milk Concentration			Control
	50%	67%	75%	
REF	73.43	66.4	89.06	100
	56.12	53.54	63.22	100
	76.99	82.3	91.15	100
	82.03	101.56	86.71	
	70.32	68.38	70.96	
	95.57	92.03	85.84	

	96.09	92.18	97.65	
	70.96	59.35	76.12	
	92.03	93.8	97.34	
Mean ± S.D.	**79.28 ± 13.43 *†**	**78.83 ± 17.27**	**84.22 ± 11.80 ***	**100.00 ± 0.00**
Hep-2	108.77	118.42	99.12	100
	100.00	174.8	89.31	100
	116.93	91.93	85.48	100
	124.56	94.73	80.7	
	96.18	69.46	95.41	
	84.67	84.67	91.93	
	117.54	98.24	100.00	
	83.96	98.47	83.2	
	93.54	93.54	89.51	
Mean ± S.D.	**102.90 ± 14.77 †**	**102.69 ± 29.95**	**90.51 ± 6.77***	**100.00 ± 0.00**

**Significant against control at P<0.05.*
†Significant against the other cell line treated with the same concentration.

Discussion:

Human milk composition is affected by several factors including infant's gestational age, lactation stage, parity, time in the day and mother's diet (16). The present study considered these factors when collecting milk samples from the three donors. However, milk composition has yet not fully explained individual variations among mothers. Therefore, the results presented here were based on two assumptions; first, that the strict control measures in sampling were sufficient to ensure homogenous samples. Thus, the results were analyzed depending on the concentration and regardless from which mother milk was collected. Second, that there were still variations in milk composition in the three samples due to individual differences among mothers. Thus, the results were analyzed individually for each mother (Table 1).

Generally, the results of this study clearly indicate that milk samples taken from each of the three donors were able, in few cases, to cause a reduction in cell growth of the transformed (REF) but not the malignant (Hep-2) cell line. The significant effects were only present using 50 and 67% milk of sample M1 and 75% of M2 on REF cells, while M3 did not cause significant effects. However, some individual replicate readings indicated tumoricidal activity that reduced the viability of REF cells down to 53%, as shown by one replicate of 67% M1.

The variable effects of milk samples from different mothers shown in this study are highly consistent with the results of Pocoví and colleagues, who also referred to sample-dependent manner of such effects on MDCK and Caco-2 cells (10). Although it is well established that human milk can promote cell growth through its own growth factors such as the epidermal growth factor (EGF) (17) and insulin-dependent growth factors I and II (IGF-1, IGF-2) (18), it also has potential growth inhibiting activity through factors such as the mammary-derived growth inhibitor (MDGI) (18) and lactoferrin (19). The most promising inhibiting factor for cancer cells is a casein derived alpha lactalbumin (α-la) known as HAMLET (Human α-la Made Lethal to Tumor cells). Hakansson and colleagues found that this protein has potent anti-growth activity against several lines of transformed cells (11). These researchers showed that calcium ion elevation and apoptosis induction were the mechanisms behind such cytotoxic activity of α-la. Although the studies of Povoci and Hakansson and their colleagues didn't include REF and Hep-2 cell lines, the growth inhibiting activity caused by certain concentrations of human milk samples in the present study might be due to the activities of the above mentioned cytotoxic agents.

From another perspective, the present study showed

that 75% milk had a significant growth inhibiting effect for both REF and Hep-2 cells, regardless of the source of the sample. While, 50% milk showed significant effects only on REF cells and 67% milk didn't exert significant differences. The concentration dependent effects of human and bovine milk on embryo fibroblasts were also referred to by several previous studies (10,19) and they can be attributed to altered ligand-receptor interaction capacity between the growth factors in the milk and their receptors on the surfaces of the target cells. Also, the present study demonstrated insignificant difference between the response of REF and Hep-2 cells to treatment with breast milk, except when 50% milk was used.

The present work aimed as far as possible to measure the effects of whole milk on cell growth without any prior processing for milk samples, since the epidemiological studies referring to possible anti-cancer effects of breast milk were actually testing the activity of direct breastfeeding. In other words, the samples here were not subjected to heat treatment, as this might denaturate certain important growth controlling proteins as suggested earlier (19). Also, no ultracentrifugation was performed to remove fat globules from milk because this might eliminate certain fat molecules, such as glycolipids and MDGI, which were shown to have potential growth inhibition activity to REF cells (18,20). Nevertheless, milk samples might have lost some high molecular weight and important growth controlling factors, whether proteins, lipids, or sugars, since these samples had to be membrane-filtered before treatment of cell lines.

Overall, the present study demonstrated that individual milk samples have mild growth inhibiting effects on REF but not Hep-2 cell lines. These effects were sample- and concentration- dependent. Also, 75% milk was the only concentration that exerted significant growth inhibition for both cell lines when the effects were analyzed regardless of the milk sample source. It seems that whole milk does not have the same powerful growth inhibiting activity on cancer cells as that showed by its fractions in previous studies. However, it is recommended that whole milk effects be studied using a methodology that ensures keeping its high molecular weight molecules present and active. Also, if available, hybrid cell lines that include normal and transformed cells derived from the same kind of tissue would certainly provide better insight for the specific effects of breast milk on these different systems.

Acknowledgements:

The author would like to thank the staff of the Iraqi Center of for Cancer and Medical Genetics Research, specially Dr. Zaid Al-Hilly and Ms Ma'ida Hussain, for their help in performing the cell growth tests. Also, many thanks to Dr. Abdul Hameed Al-Jawady from the University of Technology for his advice with the statistical analysis.

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تأثير المعاملة بحليب الثدي في نمو نوعين من الخلايا المتحولة

حيدر صاحب عبد *

* *علوم الحياة، كلية العلوم للبنات، جامعة بغداد*

الخلاصة:

لقد اقترحت كل من الدراسات الوبائية والتجارب في الزجاج وجود فعالية محتملة لحليب الأنسان كسائل بايولوجي مضاد للسرطان، مما يوفر مجالاً واعداً يحتاج للمزيد من البحث. في هذه الدراسة اختبرت تأثيرات ثلاثة عينات من حليب الانسان (M1،M2،M3) مأخوذة من ثلاث امهات واهبات على نمو الخطوط الخلوية REF و2-Hep. عرضت الخلايا الى التراكيز 50% و 67% و 75% من كل عينة حليب لمدة 72 ساعة. أظهرت النتائج ان المعاملة بالعينات M1 50% و M1 67% و M2 75% فقط أدت الى انخفاض معنوي في نمو خلايا REFبالمقارنة مع السيطرة. لم تظهر خلايا Hep-2 أية استجابة نمو معنوية للتراكيز المستخدمة من عينات حليب الانسان. عندما حللت النتائج وفقاً للتركيز وبغض النظر عن الأم التي اخذت منها العينة ظهر بأن الحليب بتركيز 50% امتلك فعالية معنوية مثبطة للنمو لخلايا REF فقط، بينما أظهر تركيز 75% مثل هذه الفعالية ضد النوعين كليهما من الخلايا مقارنة بالسيطرة. عندما قورنت استجابة نوعي الخلايا مع بعضهما للمعاملة بحليب الانسان أظهرت خلايا REF نسبة مئوية للنمو منخفضة معنوياً مقارنةً مع خلايا Hep-2 وذلك فقط عند استخدام تركيز 50%. تقترح هذه الدراسة ان الفعالية المثبطة لنمو الخلايا التي اظهرها حليب الانسان هي معتمدة على كل من العينة والتركيز. على كل حال، قد تكون عينات الحليب في هذه الدراسة قد فقدت بعض مكوناتها الفعالة حيوياً ذات الوزن الجزيئي العالي خلال تحظيرها لأختبار نمو الخلايا. توصي الدراسة باستخدام خطوط خلايا مزدوجة متحولة/ غير متحولة مشتقة من النوع ذاته من الخلايا لكون مثل هذا النموذج يوفر نتائج قابلة للمقارنة بشكل أكبر لتوضيح تأثير حليب الانسان في الخلايا السرطانية.