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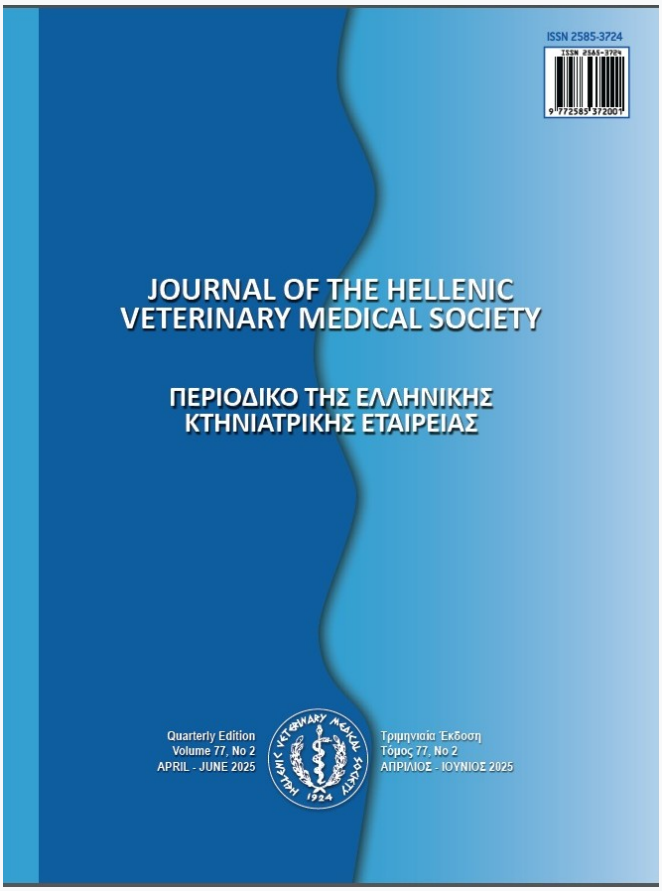

Impacts of pasteurized buffalo milk on pr oliferation of Hela cells: Role of Caspase-3, Caspase-9, and P53 genes

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Impacts of pasteurized buffalo milk on proliferation of Hela cells: Role of *Caspase-3, Caspase-9, and P53* genes

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ABSTRACT: Buffalo milk is characterized by its high nutritional value, and there is a significant association between excessive consumption of commercially available buffalo milk and the development of various cancer types. The current study was designed to assess how buffalo milk affects the growth and viability of Hela cells, a cervical cancer cell line, as well as to investigate the molecular mechanism behind these effects. Pasteurized-buffalo milk was added to cells at 0% (negative control), 0.00025%, 0.0005%, 0.001%, 0.0015, 0.002%, 0.0025%, 0.005%, and positive control (H2O2) for 24 hours. Results indicated that the growth of Hela cells was notably boosted as the concentration of buffalo milk increased. The buffalo milk reduced the apoptosis of Hela cells and increased the viability of cells. Moreover, Caspase-3 and Caspase-9 activities were stimulated in treated Hela cells, and significantly increased with 0.001% of buffalo milk, compared to positive control cells. In contrast, buffalo milk treatment for 24 hours showed substantial reduction in P53 gene expression within Hela cells. In conclusion, buffalo milk increases the proliferation and viability of Hela cells by decreasing the apoptosis and cell viability of examined cells. Furthermore, it induces upregulation in Caspase-3 and Caspase-9 expressions while deactivating the expression of P53 gene.

Keyword: Buffalo milk; Hela cells; Apoptosis; p53; Caspases.

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INTRODUCTION

Cancer a non-communicable disease and is the cause for most human deaths (Torre et al. 2016). Cervical cancer remains a prevalent form of cancer among women globally, ranking fourth in frequency following breast, colorectal, and lung cancer. Globally, around 604,000 fresh instances of cervical cancer were documented each year, resulting in a total of 342,000 deaths. Most of these cases and deaths are concentrated in countries of low- and middle-income, where cervical cancer ranks as the third most prevalent form of cancer that affects women. These statistics are based on data from GLOBOCAN 2020 (Bhatla et al. 2021).

The onset of cancer is linked to various risk factors, some of which are genetically determined and cannot be altered. However, certain factors such as lifestyle choices can be avoided to minimize the incidence of cancer (Kerr et al. 2017; Winters et al. 2017; Wiseman et al. 2007). Consuming a nutritious diet (by minimizing the intake of calorie-rich, high-fat diet, and increasing the consumption of vegetables and fruits) along with participating in consistent physical activity has been related to a decrease in developing breast cancer (Swisher et al. 2015). Epidemiological evidence had consistently showed that diet play essential role in either preventing or promoting cancer development (Aragón et al. 2014).

Previous reports has indicated that buffalo milk has been found to exhibit a higher nutritional value than cow milk, containing elevated quantities of lactose, fat, protein, total solids, and nonfat solids, and demonstrating a higher buffer capacity (Khedkar et al. 2016; Yang et al. 2013). Individuals who consume higher amounts of milk have an elevated risk of cancer developing relative to lower milk intake levels (Maliou et al. 2018). Increased consumption of commercially available buffalo milk has been strongly associated with various types of cancer (Melnik 2017; Shin et al. 2002). Dairy products play a significant role as valuable sources of diverse nutrients that can potentially beneficially impact cancer risk. These include calcium, vitamin D, butyrate, linoleic acid, and various other phytochemicals and nutrients. However, dairy products may contain compounds like IGF-1 (insulin-like growth factor-1) and other growth hormones that could potentially have a negative impact on the risk of cancer (Bolland et al. 2011; Jacobs et al. 2016; McCann et al. 2017). The *p53* gene functions as a suppressor of tumor formation, playing a critical role in halting growth of some tumors. It regulates genes associated with cellular responses (cell cycle arrest and apoptosis) (Fischer 2017).

Caspases are a class of cysteine proteases that are broadly divided into two categories: those involved in apoptosis (Caspase-3, 6, 8, 9) and those involved in inflammation (Caspase-1, 4, 5, 12). In tumor tissues, *caspase*-3 and *caspase*-9 protein levels were notably elevated compared to the levels in nearby normal tissues (Liu et al. 2017). Current study assessed the impact of buffalo milk on the viability and growth of Hela cells, a type of cervical cancer, as well as to investigate the underlying molecular mechanism involved in such regulation.

MATERIALS AND METHODS

Retrospective study

Data about the cancer outbreaks which occurred in 2020 all over the world were collected from the Global cancer observatory website (Sung et al. 2021). The prevalence and mortality rates of different cancer types were calculated. The data were modified as presented by Gearing et al (Gearing et al. 2006) that carried out to monitor cancer outbreaks which occurred in 2020 all over the world and were collected from the Global cancer observatory website based number of cases, sex and deaths. Then the prevalence and mortality rates of different cancer types. All were calculated and graphed using Excel software.

Milk preparation

The pasteurized buffalo milk was collected from healthy animals on a buffalo farm in Hubei Jinniu Co., Ltd., Hubei, China. Different concentrations of pasteurized buffalo milk were prepared at 0, 25, 50, 100, 150, 200, 250, and 500 µl of pasteurized buffalo milk in 100 ml of serum-free media to give the following concentrations: 0% (negative control), 0.00025%, 0.0005%, 0.001%, 0.0015, 0.002%, 0.0025%, and 0.005%.

The fresh pasteurized buffalo milk was prepared as following 3 steps:

Step 1; Place the raw milk in the top part of a double boiler. Gradually raise the temperature of the milk to 74°C (165°F) or hotter and keep it at this temperature for at least 15 seconds. Stir often to keep all the milk at the same temperature. Step 2; Cool the milk quickly by putting the top part of the double boiler in an ice water bath. Stir often to help it cool faster until it reaches 20°C (68°F) or colder.

Step 3; Pour the cooled milk into the sanitized bottles. Promptly put them in a refrigerator to further cool the milk to 4°C (40°F) or colder. Under ideal conditions, home pasteurized milk can keep in the refrigerator for up to 2 weeks (Mejares et al. 2022).

Cancer Cell line

The Hela cell line, obtained from the Cell Bank of Shanghai, China, was derived from human cervical cancer. A 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin supplemented DMEM media was used to cultivate the cells. The cell cultures were maintained at 37 °C in a CO_2 incubator.

Cell viability and proliferation assay

Performing cell counts

The cells were detached using trypsin, neutralized with fetal bovine serum, and rinsed with phosphate-buffered saline. To assess the cells viability, cells were mixed with trypan blue and were counted using an automated cell counter (Bio-Rad TC®).

Cell viability by CCK-8 assay

To check the possible cytotoxic effects of buffalo milk on Hela cells, the CCK-8 assay was employed. Hela cells were introduced into 96-well plates at a concentration of 10^4 cells per well and were given time to adhere overnight prior to the commencement of treatment. The pasteurized buffalo milk was prepared as mentioned in serum-free media and added to the cells at 0% (negative control), 0.00025%, 0.0005%, 0.001%, 0.0015, 0.002%, 0.0025%, and 0.005%. Positive control wells (Hela cell line treated with H_2O_2 anticancer drug) were prepared. The cells were treated and then maintained in a CO2 incubator for 24 hours. Each well of the plate was thereafter filled with 10 l of CCK-8 solution and stored in the dark for 4 hours. Microplate absorbance was read at 450 nm on a Bio-Rad (Tokyo, Japan) micro plate reader. Cell viability was calculated and graphed.

Cell apoptosis assay

Hela cells were cultivated in 6-well plates at a density of 1×10^6 cells per well and incubated for 24 hrs to facilitate cell adhesion appropriate. After that, the cells spent 24 hours in media containing 0% (negative control), 0.00025% (positive control), 0.0005% (negative control), 0.001% (positive control), 0.002% (positive control), and 0.005% (positive control) of pasteurized buffalo milk. After the end of treatment periods, cells were washed 3 times with phosphate-buffer saline. The Annexin V-FITC Apoptosis detection kit (Abcam, catalog# ab14085, Xiamen City Fujian Province 350028, China) was used to prepare the samples according to the manufacturer's instructions. After that, the Epics Altra II flow cytometer was used to look at the ready cells. The apoptosis rate was calculated by taking the mean of the cells that died at the early and late apoptotic cells based on the flow cytometry machine and software inserted, more details are listed here (Hingorani et al. 2011) .

In short, apoptosis, or programmed cell death, is a normal physiologic process for removal of unwanted cells. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface. Annexin V, a Ca2+-dependent phospholipid-binding protein, has high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposed PS using flow cytometry. The BD Pharmingen™ Annexin V FITC apoptosis detection kit provides a set of reagents for the detection of apoptosis stages using flow cytometry. The BD FACSVerse™ system includes the cytometer, BD FACSuite™ software for acquisition and analysis, and BD FACSuite research assays for use with specific reagent kits. Based on the Annexin V FITC apoptosis detection kit, the Annexin V FITC assay in BD FACSuite software provides acquisition, analysis, and reporting functions for generating reliable and consistent data using the BD FACSVerse system. This application note describes proof-of-principle experiments for the detection of camptothecin-induced apoptosis in Jurkat cells and stimulated peripheral blood mononuclear cells (PBMCs) using the Annexin V FITC apoptosis detection kit on the BD FACSVerse system (Hingorani et al. 2011). The experiments were carried out in triplicate.

Quantitative real time PCR analysis (qRT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) was employed to assess the expression of examined genes. Total RNA from Hela cell lines was extracted and treated with pasteurized buffalo milk, as well as untreated cells, using the E.Z.N.A. reagent from OMEGA Bio-Tek. RNA integrity was confirmed by running the extracted RNA on a 1.5% ethidium bromide-stained agarose gel in a 1x Tris-acetate-EDTA (pH 8.0) buffer (Sigma, Germany). Gel images were captured using a UV transilluminator (Azure c200). Samples with OD A260/A280 ratios between 1.8 and 2.0 were

deemed suitable for complementary DNA (cDNA) synthesis, which was performed using reagents from Fermentas (Waltham, MA, USA) according to the manufacturer's protocol.

Subsequently, cDNA was synthesized from the RNA as described in Superscript II reverse transcriptase kit. Primers for the target genes and β-actin (housekeeping gene) were designed on the primer bank website. AUGCT DNA-SYN Biotechnology Synthesis Lab, China was used to design primers reported in Table (1).

Quantitative real-time PCR (qRT-PCR) was performed using the Stratagene MX300P system to amplify and analyze gene expression levels.

The qPCR analysis was done as follows: a denaturation step at 95 °C for 30 seconds, followed by 40 cycles of an denaturation at 94°C for 15 seconds, at 59.5 °C for 30 seconds for annealing, and final extension at 72 °C for 15 seconds. Subsequently, a melting step was conducted with three stages: 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds.

The SYBR Green method was applied to quantify gene expression, utilizing the TOPreal™ preMIX SYBR Green qPCR master mix (Enzynomics, cat. RT 500) and an RT-PCR system from Agilent Technologies, USA. Relative gene expression changes were calculated using threshold cycle (Ct) values, which were normalized to beta actin and calibrated against the control sample using the $^{2-\Delta\Delta}$Ct method **(Pfaffl 2001).** Three replications of each reaction were performed.

Statistical analysis

The data were analyzed by SPSS program version 20. One-way-analysis of variance (ANOVA) was performed to establish statistical significance, and subsequently, Tukey's test was applied with a significant threshold set at $p < 0.05$.

RESULTS

Retrospective study

The retrospective study revealed that the prevalence of cervix uteri cancer was ranked the 4th cancer disease in females all over the world in 2020 (Figure 1A) and was ranked the sixth cancer disease mortality rate all over the world in 2020 (Figure 1B). More-

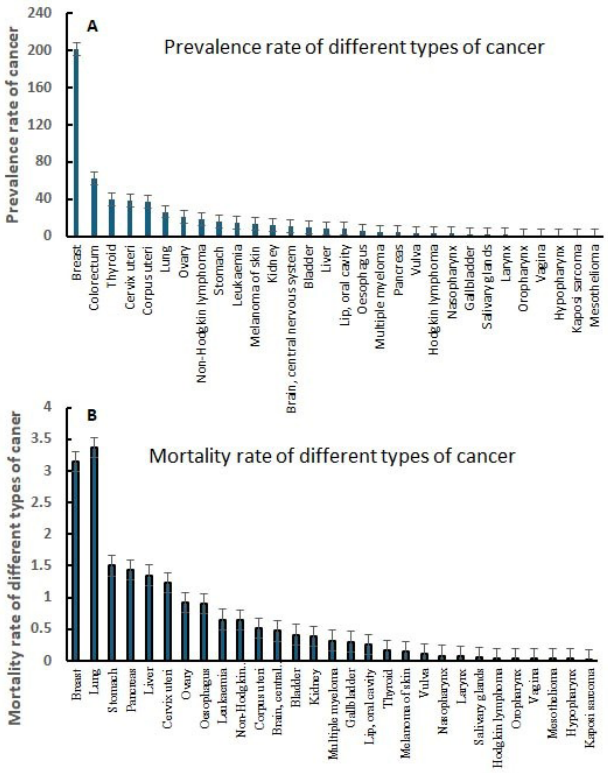

Figure 1. Prevalence (A) and mortality (B) rates of cancer in females all over the world in 2020.

Table 1. Primers for real-time quantitative PCR analysis.

Gene	Primer	Sequence
β-actin	Forward	5′-CACCATTGGCAATGAGCGGTTC-3′
	Reverse	5′-AGGTCTTTGCGGATGTCCACGT-3′
P53	Forward	5′-CCTCAGCATCTTATCCGAGTGG-3′
	Reverse	5′-TGGATGGTGGTACAGTCAGAGC-3′
Caspase3	Forward	5′-GGAAGCGAATCAATGGACTCTGG-3′
	Reverse	5′-GCATCGACATCTGTACCAGACC-3′
Caspase 9	Forward	5′-GTTTGAGGACCTTCGACCAGCT-3′
	Reverse	5′-CAACGTACCAGGAGCCACTCTT-3′

over, approximately 90% and 85% of new cases and deaths of this type occur in middle- and low-income countries, respectively. Cancer is the third among the woman affected in these countries.

Cell viability and proliferation assay

The proliferation of cells in Hela cell lines was significantly increased with buffalo milk culture (Table 2). This result reflected a strong positive effect of buffalo milk on the proliferation and survival of the Hela cell line.

Apoptosis-induction in Hela cells by buffalo milk

The findings presented in Figure (2) demonstrate the flow cytometric assessment of apoptosis induced in Hela cells following treatment with varying concentrations of buffalo milk and H_2O_2. Next, cells were stained with Annexin V-FITC and then flow-cytometrically analyzed. Each quarter (Q) represented types of cells. The Q4 was viable cells, Q3 was designed as early apoptotic cells, Q2 expressed as late apoptotic cells and Q1 was reported as dead cells. Exposure to H_2O_2 induced a rise in the proportion of late apoptotic and decreased cells, along with a decrease in the fraction of viable cells. Conversely, the diverse concentrations of buffalo milk exhibited a noteworthy rise in the proportion of viable cells, accompanied by a decrease in the percentages of early and late apoptotic cells, as well as deceased cells. The proportion of viable cells was increased with increase of buffalo milk concentration. The viable cells showed normal morphological cells with spindle and adhered under the TC20.

Table 2. Effects of buffalo milk at different concentrations on cell viability of Hela cell line after 24 h incubation by using CCK-8 assay.

		Mean ± SE
Control	Negative control	3.52± 0.00840 ^{bcd}
	Positive control	0.6465 ± 0.0231^{a}
Milk concentrations	0.00025%	3.45 ± 0.0231^{b}
	0.0005%	3.48 ± 0.00810^{b}
	0.001%	3.49 ± 0.00834^{b}
	0.0015%	3.50 ± 0.00181^{b}
	0.002%	3.55 ± 0.0183^{cde}
	0.0025%	3.56 ± 0.00932^{de}
	0.005%	3.59 ± 0.0195^{e}

Superscript with different letters in the same column indicates significant different at level $P \leq 0.05$.

Caspase-3, Caspase-9 and P53 genes activity

Figure 3 displayed the impact of various concentrations of pasteurized buffalo milk on *Caspase-3* and *Caspase-9* expression in the Hela cell line after 24 hours of culture. The results indicated an elevation ($p< 0.05$) in the *Caspase-3* and *Caspase-9* activity in Hela cells treated with buffalo milk. *Caspase-3* and *Caspase-9* activity was moderately increased in Hela cells treated with 0.001 % of buffalo milk in relation to the control (untreated Hela cells). Regarding the *p53* activity, it decreased significantly at 0.0005 % concentration of buffalo milk for 24 hours. In contrast, the H_2O_2 treated Hela cells showed decrease in *Caspase-3* and *Caspase-9* activity with an increase in *p53* activity compared to the untreated

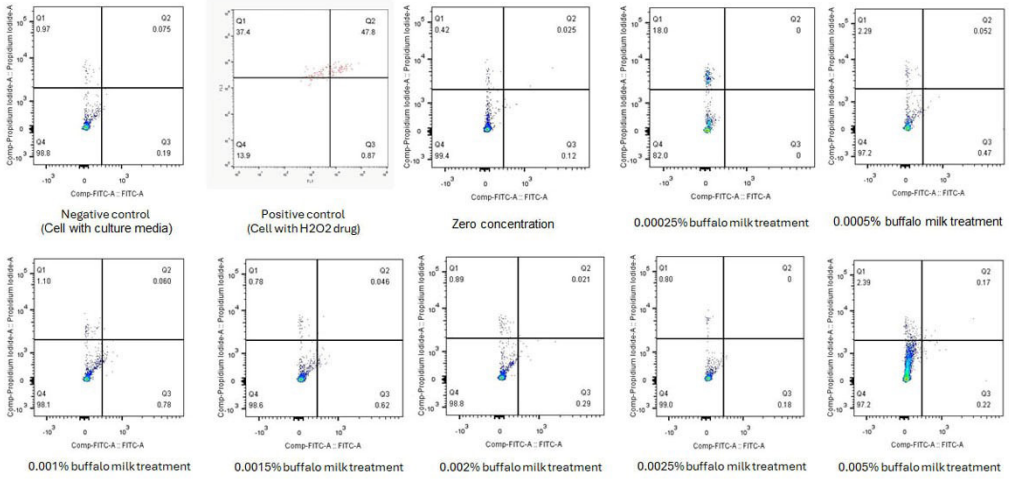

Figure 2. Flow cytometric analysis of apoptosis induction in Hela cells treated with different concentrations of pasteurized buffalo milk.

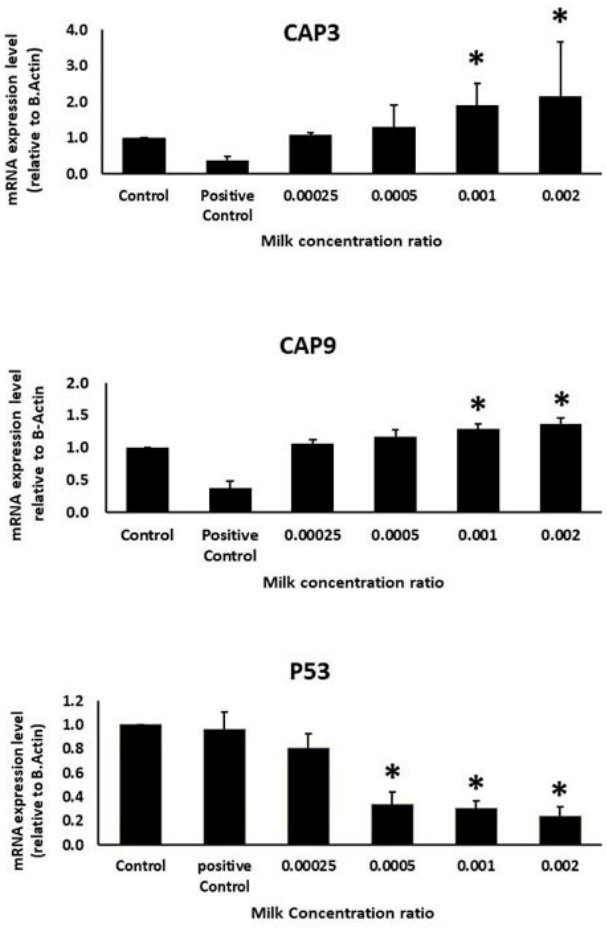

Figure 3. Effect of different concentrations of pasteurized buffalo milk on the gene expression of Caspase-3 and Caspase-9 and P53 activities in Hela cell line after 24 hrs.

Hela cells (control), as seen in figure 3. It clearly seen that the degree of caspases activation is not so high as suspected possibly due to cell proliferation reported in high doses of pasteurized buffalo milk.

DISCUSSION

Cancer is a leading cause of death worldwide, defined by uncontrolled cell growth within the body. The naming of cancer is based on the specific body part where it originates, regardless of whether it later spreads to other parts. Cancer development is influenced by a multitude of risk factors, including but not limited to tobacco use, alcohol consumption, an improper diet, physical inactivity, and air pollution exposure (Ferlay et al. 2021). The retrospective analyses revealed that the prevalence of cervix uteri cancer was the 4th most frequent cancer in women and was ranked the 6th cancer disease mortality rate all over the world in 2020. Approximately 90% of these instances happen in countries with lower and middle-income economies (Bhatla et al. 2021; Sung et al. 2021). Earlier case-control epidemiological investigations have documented a positive correlation between regular milk consumption and different types of cancers, indicating that certain dairy products might have an impact on the risk of cancer in humans (McCann et al. 2017; Perez-Cornago 2020). The association between the daily milk- intake and the observation of different kinds of cancer are different but the risk of many types of cancer increases with increase the dairy milk-intake (Chagas et al. 2012; Zang et al. 2015). Dairy products are valuable providers of various nutrients that have the potential to positively influence cancer incidence. However, dairy products also contain substances, such as growth hormones and IGF-1, which could potentially have an unfavorable impact on cancer risk (Jacobs et al. 2016; McCann et al. 2017).

Our study reported that the viability of Hela cell lines was significantly increased when cultured for 24 hours with different concentrations of pasteurized buffalo milk. The cells significantly proliferate and grow after buffalo milk treatment. Buffalo milk possesses a higher nutritional value compared to cow milk, containing elevated levels of protein, lactose, fat, nonfat solids, and total solids, and exhibiting a high buffer capacity (Khedkar et al. 2016; Yang et al. 2013). However, increased consumption of dairy milk has been linked to a higher risk of cancer (Fraser et al. 2020; McCann et al. 2017). Due to the use of growth hormones for enhancing milk production, the levels of IGF-1 in liquid milk are comparatively elevated (Cifelli et al. 2016).

In addition, milk contains several pollutants and compounds that may be hazardous to human health, including estrogen, which has been associated with increased replication abnormalities of DNA and mitotic activity, and insulin-like growth factor I (IGF-I), which promotes breast cancer cellular proliferation (Zang et al. 2015). Previous study suggested that IGF-1, a protein found in both cow milk and human, could be probably relate between milk intake and cancer risk (Outwater et al. 1997). It has been shown that IGF-I promotes cancer cell growth [42]. Furthermore, malignant transformation caused by acellular or viral oncogene can be prevented by removing or obstructing of IGF-I receptors from the cellular membrane, thus IGF-1 plays an important role in cellular transformation (Bell et al.

2013). According to these researchers, dairy cows are regularly given bovine growth hormone in order to produce more milk, thus increases the amounts of IGF-I that is produced in the milk (Prosser et al. 1989). Outwater et al. concluded that since IGF-I is not eliminated during pasteurization, it is possible it will not be broken down during digestion in the gastrointestinal system (Outwater et al. 1997).

There were significant interaction between IGF-1 and the ER (estrogen receptor), there is a positive connections between cancer and dairy products (Qin et al. 2009). Studies have demonstrated that consuming milk protein can lead to increased post-meal hyperinsulinemia, potentially promoting cell growth and proliferation (Jacobs et al. 2016; Qin et al. 2009).

Our study demonstrated that buffalo milk significantly affects the proliferation and the viability of Hela cell lines. Apoptosis, a natural process within cells, involves the regulated death of cells and is triggered by a range of external and internal signals and stimuli. It plays a crucial role in numerous disease mechanisms (Vecchione and Croce 2010). The apoptosis of cancer cell lines is a tight process regulated under the control of different signaling pathways (Herr and Debatin 2001; Thornberry and Lazebnik 1998). The buffalo milk reduced the induction of apoptotic Hela cell lines and increased the viability of the cells. The findings of our study indicated a significant increase ($p< 0.05$) in the percentage of viable cells and a decrease in the proportions of early and late apoptotic cells, as well as dead cells, with the application of various concentrations of buffalo milk compared to H_2O_2 treated cells. These data referred to the buffalo milk treated Hela cells amplified the viable cells.

Once cells activated, it increased caspase-9 cleavage and activates downstream effector caspases-3 and -7, thus resulting in apoptosis. Caspase-3, the executioner caspase, can directly degrade multiple substrates including structural and regulatory proteins. In current study, there were an increase in cell proliferation that are associated with caspases activations to initiate apoptosis (Ho et al. 2009; Lüthi and Martin 2007; Soung et al. 2003; Wen et al. 2012).

Apoptosis is initiated by activating caspase-3, which necessitates the activation of initiator caspases, such caspase-8 or -9, in response to proapoptotic signals (Lowe and Lin 2000). Induction of apoptosis with formation of ROS by cancer chemoprotective drugs, such as doxorubicin, not only produces cancer cell death but also causes DNA damage and genomic instability (Tsang et al. 2003; Zhivotovsky and Kroemer 2004). Thus, the development of new chemo-preventive agents able to inhibit cell proliferation and induce apoptosis in cancer cells but with less or no side effects is important and anticipated. Taken together, it would suggest that induction of ROS in response to milk triggers caspase-3 activation which is a direct effect and is not caused by a decrease in the levels of Bcl-2, a protein possesses antioxidant function and blocks ROS production (Hockenbery et al. 1993).

On the other hand, *p53* gene is a tumor suppressor gene that plays a vital impact in inhibiting the development of tumors. *P53* gene acts as a transcriptional factor that regulates some genes involved in DNA repair, cell growth, and apoptosis (Gupta et al. 2001; Khazaei et al. 2017; Matsui et al. 2001; Plasay et al. 2016). In the present study, we observed a significant down-expression of the *P53* gene in Hela cells incubated with buffalo milk for 24 hrs compared to untreated cells. In cervical cancer, the *P53* gene was inactivated due to interaction with the cellular or viral proteins (Boregowda et al. 2018; Chopra et al. 2018; Hietanen et al. 2000). The activity of the *P53* gene was decreased significantly at 0.0005 % buffalo milk treated Hela cells for 24 hrs. The mutation and inactivation of *P53* gene caused inactivation of P53 protein, so cell growth and proliferation cannot be controlled. Finally, the buffalo milk suppressed P53 activity in Hela cell lines, which inhibited the apoptosis and death of cancer cells.

Loss-of-function mutations of p53 are associated with a multitude of human cancers including prostate cancer (Dean and Knudsen 2013). It has been shown that a persistent down-regulation of p53 activity via p53 targeting milk-derived miRNAs may thus enhance cancer progression (Michaëlsson et al. 2014). Moreover, milk consumption is associated with increased serum levels IL-6 (Michaëlsson et al. 2014). Intriguingly, p53 was identified as a key suppressor of IL-6 and plays a pivotal role in suppressing inflammation and oxidative stress (Liu and Xu 2011; Zhang et al. 2016) and this is in agreement of our finding as pasteurized buffalo milk decreased p53 in dose effect. As an increase in IL-6 expression has been detected in some types of cancer and has been related to initiation of cancer (Culig 2014).

CONCLUSIONS

Pasteurized buffalo milk increased the proliferation and viability of the Hela cells and decreased the apoptotic and dead cells. It activated the expression

of caspase genes, that were represented by Caspase-3 and Caspase-9 genes with inactivation of the expression of the p53 gene. Therefore, it is recommended that women's who are at high cervical cancer risk should decrease their daily intake of buffalo milk. Future investigations must be carried out and to confirm such effect on other types of cancer. The collective impacts of pasteurized buffalo milk are illustrated in figure 4.

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FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical Committee of Benha University, Egypt approved the study under number BUFVTM 05-11-22.

CONFLICT OF INTEREST

No conflict was reported for the current study.

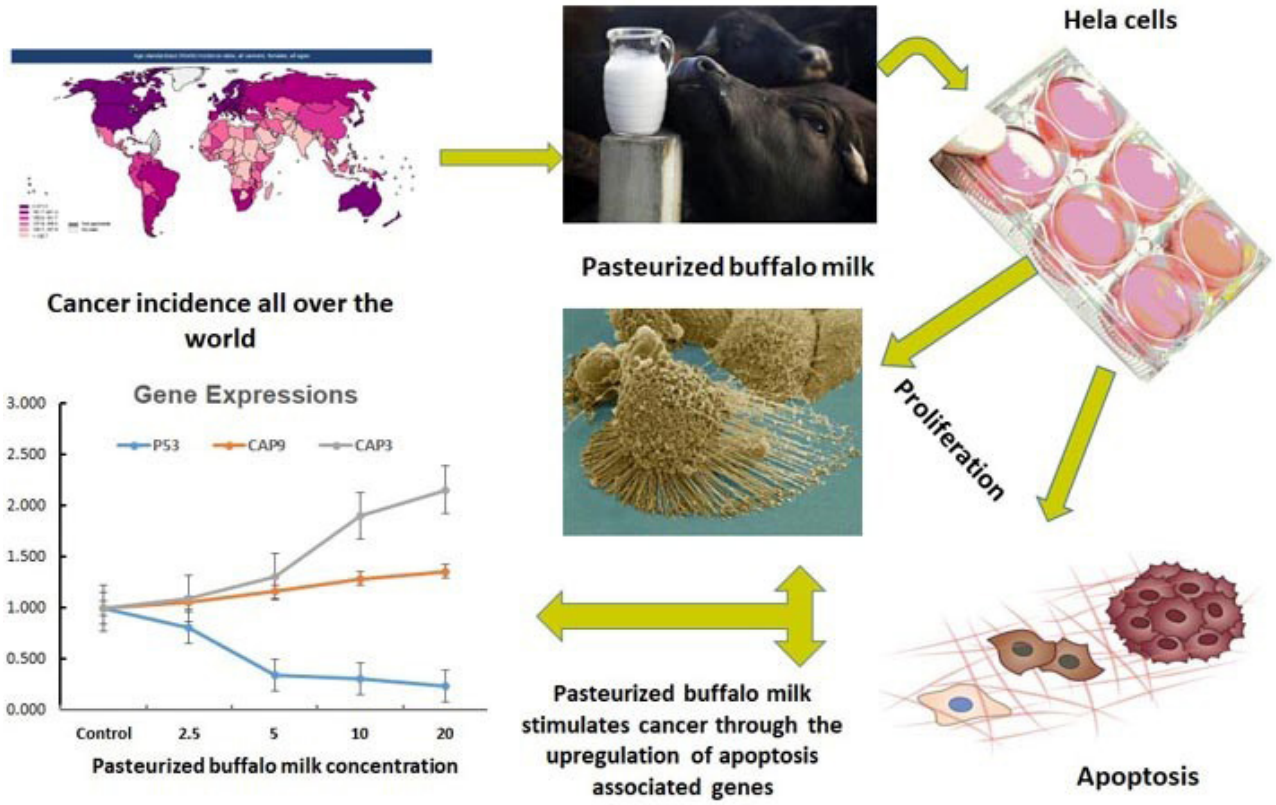

Figure 3. Collective graph about the impacts of pasteurized buffalo milk against Hela Cells.

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