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In vitro cytotoxic and genotoxic effects of donkey milk on lung cancer and normal cells lines

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Abstract: *In vitro* cytotoxic and genotoxic effects of donkey milk on cancer (A549) and normal (BEAS-2B) lung cell lines were investigated. The XTT and WST-1 tests as well as clonogenic assays were used to evaluate cytotoxicity. The comet assay and micronucleus test were used as genotoxicity endpoints. Donkey milk showed lower cytotoxic effects against normal lung cell line BEAS-2B in comparison to the tumor cell line A549. Genotoxicity experiments revealed dose dependent increases in the frequencies of micronuclei and single stranded DNA breaks in A549 cells whereas no significant damage was observed in BEAS-2B cells. The results indicate that donkey milk has anti-proliferative and genotoxic effects on lung cancer cells at concentrations which are non-toxic to normal lung cells.

Keywords: anti-cancer; cytotoxicity; jenny milk; genotoxicity; *in vitro*

In recent years, there has been a growing scientific and commercial interest in donkey's milk due to its unique nutritional and biochemical properties. It is considered as a substitute for cow's milk, which causes allergy in the 2–7% of infant population due to its protein content (Host 2002). Recent studies also indicated that donkey milk is the best substitute for human milk as their characteristics are quite similar (Choifalo *et al*. 2011).

Aside from being an excellent nutrition source, milk proteins can exert different physiological and biological activities. For instance, it is suggested that milk proteins may play an important role in cancer therapy (Parodi 2007; Lopez-Exposito & Recio 2008; Sah *et al*. 2015). In fact, milk from different mammalians such as cow (Gill & Cross 2000; Praveesh *et al*. 2011), goat (Anandhini & Palaniswamy 2013) and camel (Quita Salwa & Kurdi Lina 2010; Korashy *et al*. 2012) have been evaluated for their potential effects on cancer cells. However, donkey milk has certain characteristics which make it different from other types of mammalian milk. It has been suggested that high lysozyme, lactoferrin and specific protein content of donkey milk is responsible for its antimicrobial and anti-inflammatory, anti-ageing, antioxidant and anti-proliferative effects (Mao *et al*. 2009; Amati *et al*. 2010; Lionetti 2012).

Although it has been traditionally claimed that donkey milk has medicinal benefits for cancer patients, these claims have not been scientifically evaluated until recently. In a study performed by Mao *et al*. (2009) it was shown that donkey milk treatment reduced the viability of A549 human lung cancer cells and increased the production of cytokines such as IL-2, IFN-γ, TNF-α in murine lymphocytes and macrophages. The authors suggested that the protein content of donkey milk might have potential as an antitumor agent in the treatment of lung cancer. However, there are no data on the genotoxicity of donkey milk on cancer as well as healthy cells.

Thus, in the present study we aimed to evaluate comparatively the *in vitro* cytotoxic and genotoxic

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effects of donkey milk on lung cancer cells (A549) and on their normal (non-malignant) counterpart BEAS-2B lung cell line. The clonogenic assay, XTT and WST-1 tests were used to evaluate anti-proliferative and cytotoxic effects. The micronucleus test, as indicators of chromosomal damage and the comet assay, an indicator of DNA strand breaks were used to evaluate genotoxicity. To the best of our knowledge this is the first study focused on the genotoxicity of donkey milk on healthy and cancer cells.

MATERIAL AND METHODS

Donkey milk. Milk from Miranda donkeys located in Miranda, southwest Portugal, was used for this study. Lyophilized and pasteurized donkey milk samples were obtained from Naturasin-Livestock asinine Ltd. (Portugal). Hydrogen peroxide (H_2O_2) was used as positive control at a single concentration of 150 µM. Sterile distilled water was used as a solvent control.

Cell culture. Normal human bronchial epithelial cell line (Beas-2B) and human non-small-cell lung cancer (A549) cell line were kindly provided by (Uludag University). The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), penicillin–streptomycin (50 µg/ml), 2 mM L-glutamine, and 1% sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and grown in 75 cm^2 flasks.

Cytotoxicity assessments. WST-1 test, XTT test and clonogenic assays were used to evaluate the cytotoxic effects of donkey milk on BEAS-2B and A549 cells. To determine the cytotoxicity, cells were exposed to serial concentrations (100, 200, 400, 800, 1600 and 3200 mg/ml) of donkey milk for 24 hours. Untreated cells served as a control group. The XTT and WST-1 assays were performed using XTT (Biological Industries) and WST-1 (Roche, Switzerland) reagents as described elsewhere (Kumbicak *et al*. 2014). A clonogenic assay which determines the ability of a single cell to grow into a colony, was performed according to Wise *et al*. (2010). Briefly, fifty thousand cells were seeded in T25 flasks and allowed to grow for 48 hours. Following treatment with donkey milk for 24 h, the cells were re-seeded at colony forming density (500 cells per well) into four pieces of a 60 × 15 mm Petri dishes. Colonies (consisted of minimum 50 cells) were allowed to grow for 10 days, fixed with 100% methanol, stained with crystal violet, and counted.

Genotoxicity assessments. Cytokinesis-block micronucleus test was performed as described elsewhere (Cavas *et al*. 2014). Briefly, the cells were seeded in T25 tissue culture treated flasks at a density of 3×10^4 cells/flask and allowed to grow for 48 hours. After treatment with donkey milk, cells were further cultured with cytochalasin-B for 24 h before harvesting. Following hypotonic treatment and fixation, the cells were stained by 5% Giemsa. The numbers of binucleated (BNC) cells with micronuclei (MN-BNC) were calculated in 2000 cells per treatment group. The nuclear division index (NDI) values were also evaluated using the following Equation (1):

$$NDI = (1 \times M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)/1000 \quad (1)$$

where: M1 through M4 represents the number of cells with one to four nuclei.

Alkaline comet assay was performed according to Singh *et al*. (1988) with slight modifications as previously described (Cavas 2014). Briefly, following treatment period, cells were harvested and embedded in 0.8% low melting agarose on slides precoated with normal melting point agarose. Following lysis, electrophoresis, neutralization and dehydration, the cells were stained with ethidium bromide. Slides were evaluated under Nikon epifluorescence microscope equipped with a digital camera (Kameram 21) using an image processing software (Arganit Mikrosistem Comet Assay).

Statistical analyses. All analyses were performed using the SPSS software (USA) version 16. ANOVA with LSD post hoc test was used to evaluate micronucleus test data. Mann-Whitney *U*-test was for evaluation of the comet assay data. The threshold for statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Milk is the primary source of nutrition for every mammalian newborn. Apart from its nutritional value, milk exhibits a wide range of biological activities mainly due to its peptide and protein content (Polidori & Vincenzetti 2012). In the present study, donkey milk was tested for its potential cytogenotoxicity on human lung cells.

The results of the clonogenic assay, WST-1 and XTT tests are shown in Figure 1A, B and C respectively. As shown in the figures, treatment with 100, 200, 400, 800, 1600 and 3200 µg/ml donkey milk signifi-

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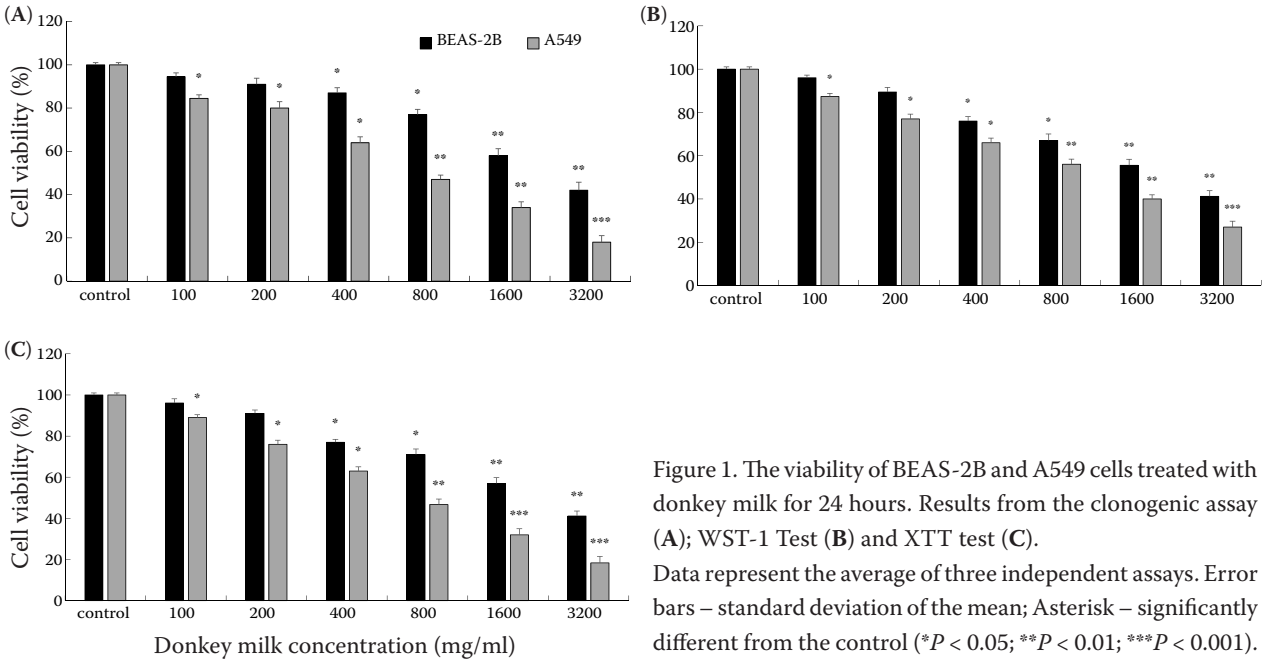

Figure 1. The viability of BEAS-2B and A549 cells treated with donkey milk for 24 hours. Results from the clonogenic assay (**A**); WST-1 Test (**B**) and XTT test (**C**).

Data represent the average of three independent assays. Error bars – standard deviation of the mean; Asterisk – significantly different from the control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

cantly reduced ($P < 0.001$) cell viability of A549 cells in clonogenic assay ($R^2 = 0.84$), WST-1 test ($R^2 = 0.86$) and XTT test ($R^2 = 0.90$). Similarly, the viability of BEAS-2B cells significantly ($P < 0.01$) decreased by 400, 800, 1600 and 3200 µg/ml concentrations of donkey milk. The two lowest donkey milk concentrations (100 and 200 µg/ml) did not affect the viability of BEAS-2B cells ($P > 0.05$).

Anticancer, antiproliferative, cytotoxic and protective effects of milk and milk related products from different mammalian species have been demonstrated. For instance, Quita Salva and Kurdi Lina (2010) reported that pretreatment with camel milk significantly reduced cisplatin-induced micronucleus frequencies in mice erythrocytes. Camel milk has been further shown to inhibit the proliferation of HepG2 human hepatoma, MCF-7 human breast cancer (Korashy *et al*. 2012). Bovine milk has also been shown to contain major and minor components, which possess anticancer properties (Gill & Cross 2000). Mare milk has also been shown to induce cytotoxicity on leukemia cells by Rahmat *et al*. (2006). However, studies on the anti-cancer effects of Donkey milk are extremely scarce.

Table 1. The IC_{50} values of donkey milk obtained by the three different cytotoxicity tests

Cell Line	Clonogenic assay	WST-1 test	XTT test
		(µg/ml)	
BEAS-2B	2.476	2.294	2.381
A549	850	1.109	830

In our study, we observed that donkey milk possesses antiproliferative effect and induce cell death in lung cells. These effects were more pronounced in A459 cancer cell line than in BEAS-2B cells. The IC_{50} values of donkey milk on healthy BEAS-2B and cancer A549 cells obtained by the three different cytotoxicity tests are demonstrated in Table 1. Similar anti-proliferative and anti-tumor effects were obtained by Mao *et al*. (2009) on A549 human lung cancer cells following exposure to different fractions of donkey milk. The authors further reported that, protein fraction with molecular weight higher than 10 kDa had the strongest anti-proliferative activity on A549-cells. Some of the main whey proteins in donkey milk, with molecular weight higher than 10 kDa are α-lactalbumin, β-lactoglobulin, lactoferrin, serum albumin and lysozyme (Fantuz *et al*. 2001; Cunsolo *et al*. 2007) which might be responsible from the observed cytotoxicity.

In fact, antiproliferative properties of α-lactalbumin has been demonstrated in several *in vitro* studies. Ganjam *et al*. (1997) demonstrated the antiproliferative effect of a-lactalbumin on the human colon (CaCo-2) and rat intestine (IEC-6) cells. Strenhagen and Allen (2001) obtained similar results on colon cancer cell lines CaCo-2 and HT-29 after 4 days of treatment period with α-lactalbumin. Lactoferrin is a glycoprotein with a molecular weight of about

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80 kDa belonging to the transferrin family. Camel milk lactoferrin was shown to inhibit the proliferation of HCT-116 colon cancer cells (Habib *et al*. 2013) and induce apoptosis in human B-lymphoma cells (Furlong *et al*. 2010). Similarly, bovine lactoferrin was shown to inhibit the growth of MCF-7, T-47D, MDA-MB-231 and Hs578T breast cancer cells (Zhang *et al*. 2015) and shown to induce apoptosis in MCF-7 cells (Zhang *et al*. 2015). Serum albumin is another component of milk whey proteins. Laursen *et al*. (1990) reported that bovine serum albumin inhibits the growth of MCF-7 human breast cancer cell line. Finally, Mao *et al*. (2009) reported that lysozyme which constitutes 26.83% of total whey proteins could be one the main component of donkey milk responsible from its anti-tumor effects.

Based on the obtained results on healthy BEAS-2B cells, three non-cytotoxic (50, 100 and 200 µg/ml) and one low cytotoxic (400 µg/ml) concentrations of donkey milk were selected for genotoxicity experiments. Our results further demonstrated that donkey milk treatment significantly induced the formation of DNA strand breaks and micronuclei in A549 cells at concentrations which are non–toxic to BEAS-2B cells.

The micronucleus (MN) frequencies and nuclear division index (NDI) values in BEAS-2B cells are summarized in Table 2. As shown in the table, positive control significantly induced MN frequencies in BEAS-2B cells ($P < 0.001$). However, no significant changes were observed in cells treated with donkey milk ($P > 0.05$). Similarly, positive control treatment significantly reduced the NDI values in BEAS-2B cells ($P < 0.01$), whereas no significant changes were observed in donkey milk treated cells ($P > 0.05$). Table 3 summarizes the MN and NDI data in A549 cells. As can be seen in the table, positive control treatment significantly induced MN formation in A549 cells. Similarly, NDI values significantly reduced following donkey milk treatment ($P < 0.01$). Donkey milk treatment significantly induced the formation of micronuclei in A549 cells ($P < 0.05$) with the exception of the lowest concentration (50 µg/ml). However, no significant differences were observed in NDI values ($P > 0.05$).

Results of comet assay performed on BEAS-2B and A549 cells are given in Table 4 and 5, respectively. As shown in the tables, positive control treatment significantly induced DNA damage in both cell line as revealed by significant increases in tail length and olive tail moment values ($P < 0.01$). Donkey milk treatment did not induce any DNA damage in BEAS-2B cells (Table 4). On the other hand, significant increased DNA damages were observed in A549 cells ($P < 0.01$). Both tail length and olive tail moment value significantly increased following donkey milk treatment, at all tested concentrations.

Although we did not analyze the mechanism underlying the observed genetic damage in A549 cells,

Table 2. Effects of donkey milk on the frequencies of MNBN and NDI values in BEAS-2B cells

Groups	MNBN (‰)	NDI
Control	8.5 ± 0.7	1.95 ± 0.1
Solvent C	9 ± 2.83	1.96 ± 0.00
Positive C	50.5 ± 2.12***	1.41 ± 0.14***
50 µg/ml	8.5 ± 0.71	1.94 ± 0.01
100 µg/ml	12.5 ± 0.71	1.95 ± 01
200 µg/ml	9.5 ± 0.70	1.95 ± 00
400 µg/ml	8.5 ± 0.70	1.92 ± 0.0

MNBN – micronucleated binucleated cells; NDI – micronucleated binucleated cells; values ± sd; ***$P < 0.001$

Table 3. Effects of donkey milk on the frequencies of MNBN and NDI values in A549 cells

Groups	MNBN (‰)	NDI
Control	11.5 ± 2.12	1.94 ± 0.14
Solvent C	17 ± 1.41	1.91 ± 0.15
Positive C	74 ± 2.83***	1.31 ± 0.14***
50 µg/ml	19.5 ± 0.70	1.85 ± 0.1
100 µg/ml	26 ± 2.83*	1.83 ± 0.13*
200 µg/ml	30.5 ± 0.71*	1.81 ± 0.1*
400 µg/ml	39.5 ± 3.54**	1.76 ± 0.1**

MNBN – micronucleated binucleated cells; NDI – micronucleated binucleated cells; values ± sd; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 4. Comet assay results in BEAS-2B cells exposed to donkey milk

Groups	Tail length	Olive tail moment
Control	9.22 ± 0.19	2.03 ± 0.07
Solvent C	9.16 ± 0.30	2.23 ± 0.16
Positive C	33.06 ± 3.7**	12.49 ± 1.72***
50 (µg/ml)	9.24 ± 0.21	2.27 ± 0.12
100 (µg/ml)	9.28 ± 0.25	2.24 ± 0.12
200 (µg/ml)	9.24 ± 0.22	2.32 ± 0.11
400 (µg/ml)	9.29 ± 0.20	2.74 ± 0.13

Values ± sd; **$P < 0.01$; ***$P < 0.001$

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Table 5. Comet assay results in A549 cells exposed to donkey milk

Groups	Tail length	Olive tail moment
Control	10.90 ± 0.34	3.06 ± 0.21
Solvent C	10.57 ± 0.23	2.71 ± 0.13
Positive C	36.24 ± 3.79***	12.75 ± 1.70***
50 (µg/ml)	16.82 ± 1.18*	5.23 ± 0.55*
100 (µg/ml)	21.82 ± 1.77*	6.94 ± 0.79**
200 (µg/ml)	25.82 ± 2.35**	7.62 ± 1.08**
400 (µg/ml)	30.46 ± 2.92***	9.21 ± 1.21**

Values ± sd; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

generation of intracellular ROS by some components of milk, which might be responsible from the observed genotoxicity, has previously been demonstrated by several authors. For instance, Yoo *et al.* (1997) reported that bovine milk lactoferrin-derived peptide induces apoptosis via triggering intracellular ROS activation in THP-1 human monocytic tumor cells. Ma *et al.* (2013) in CaCo-2 cells following *in vitro* exposure to lactoferrin obtained similar results. Stable self-assembly of bovine αLactalbumin was also shown to induce apoptosis via the formation of intracellular ROS in A549, MCF-7 and HeLa cells (Mahanta & Paul 2015).

It is known that anticancer activities of milk components are mostly related to the physical and chemical characteristics. In a study performed by Malihe Shariatikia *et al.* (2017), it was demonstrated that mare, donkey, camel and cow milk and their caseins have potent anticancer activity against MCF7 cell whereas sheep and goat milk did not. The authors further performed *in silico* analyses for caseins and reported that horse and donkey milk had the highest positive charges as well as the maximum percentage of the α-helix structure. On the other hand, functionally different milk proteins could also exert similar cytotoxic activities. For instance, human and bovine milk α-lactalbumins become selectively lethal to tumor cells when made complexes with oleic acid so-called HAMLET and BAMLET, respectively (Svensson *et al.* 1999; Fang *et al.* 2014). Similarly, equine lysozyme, based on its homologous structure to α-lactalbumin, can also form complex with oleic acid called ELOA and could induce cytotoxicity and apoptosis accompanied by DNA fragmentation in cancer cells (Winhelm *et al.* 2009; Nielsen *et al.* 2010; Clementi *et al.* 2013).

To our best knowledge, this study provides the first evidence that donkey milk has dose-depending genotoxic effects in human non-small lung carcinoma cell line A549, whereas it was not genotoxic on non-transformed counterpart BEAS-2B cells line. The observed differences between two cell lines could be due to several possible mechanisms. It is well known that the malignant and normal cells show marked differences both in their metabolism and morphology (Ertel *et al.* 2006). For instance, cancer cell membranes are more negatively charged than their normal counterparts the due to the presence of anionic molecules which makes them more attractive for the proteins and peptides possess cationic properties (Szachowicz-Petelska *et al.* 2010; Huang *et al.* 2015). The ability of cationic peptides to selectively target and disrupt cancer cell membranes due to their negatively charged cell surface was previously demonstrated (Mader *et al.* 2005; Araya *et al.* 2007; Pepe *et al.* 2013). Thus, the interaction between cationic milk components (i.e. casein) and anionic membrane of A549 cells could be responsible for the selective toxicity of donkey milk (Al-Ahmad *et al.* 2018). Furthermore, it is reported that α-helical anticancer peptides show selective apoptotic activity on malignant cells via disruption of cellular and mitochondrial membranes with electrostatic interactions (Huang *et al.* 2015). Therefore, the presence of high amount of α-helical casein components could be another reason for selective toxicity of donkey milk on A549 cells. However, the exact mechanism underlying the selective toxicity of donkey milk requires further detailed investigations.

CONCLUSIONS

This work has comparatively evaluated the cytotoxic and genotoxic effects of donkey milk on human lung cancer cells for the first time. Donkey milk significantly induced genotoxic damage in lung cancer cells, whereas the normal lung cells were not affected even at the highest concentrations. Most chemotherapeutic agents effectively used in cancer treatment, problems related with their selectivity remain as a major problem as they often affect normal cells in addition to tumor cells. Although the exact mechanisms of action have not been analyzed, the results obtained in this study suggest that donkey milk selectively cytotoxic and genotoxic on human lung cancer cells. Thus, further in vitro studies with different cell lines, followed by in vivo studies are needed to elucidate the potential effects of donkey milk and its components on cancer.

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