Molecular Evidence of Anticancer Activity of Camel Milk Combined with Camel Urine

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Abstract
Camel Milk (CM) and Camel Urine (CU) is a traditional practice in the Middle East and other countries as an alternative treatment against cancer. We hypothesized that a combination of CM and CU (CM+CU), used at bioavailable concentrations, will result in cancer cell apoptosis without toxic effect on normal epithelial cell lines. Pursuant to this goal, we investigated CM+CU effect on breast (BC) and Prostate Cancer (PC) cell lines and examined its underlying mechanisms of action in-vitro. The highly metastatic BC (MDA-MB-231) and PC (PC3) cells as well as the non-tumorigenic epithelial cell line (MCF-10A) were treated with Filtered Camel Milk (FCM) and Virgin Camel Urine (VCU), both individually and in combination at bioavailable concentration levels. Although FCM and VCU were effective individually, the FCM+VCU combination significantly suppressed BC and PC cell proliferation (>80%), and surprisingly induced 100% cell death. In contrast, no deleterious effects were observed in MCF-10A control cells. More interestingly, the FCM+VCU combination upregulated Bax and downregulated expression of Bcl-2 and hypo-phosphorylated Rb expression in both BC and PC cell lines. In conclusion, this is the first study providing evidence of in-vitro chemo-preventive synergistic effect of FCM+VCU combination against BC and PC cells, supporting its anticancer property.

Keywords: Camel milk; Camel urine; Breast cancer; Prostate cancer; Cell proliferation; Apoptosis

Introduction
Breast Cancer (BC) is to the most common cancer in women worldwide reaching a rate of 25% of cancer cases [1]. While, BC is the most frequent cause of cancer-related mortality (~14%) in developing countries, it is the second cause of death in developed countries (~15%) [1]. A number of known risk factors include both environmental and genetic players associated with the onset of BC [2]. BC is a highly heterogeneous disease and gene expression profiling classified BC into four molecular subtypes: Luminal (A and B), HER2, basal-like and normal-like using hierarchical cluster analysis [3]. From the four subtypes, Triple Negative Breast Cancer (TNBC) tumour subtype is highly aggressive as it lacks or has a relatively lower expression of the three receptors, Estrogen (ER), Progesterone (PR) and HER-2/ neu. TNBC is considered as an advanced stage, a higher histologic grade, and significantly higher metastatic rates [4-7].

On the other hand, Prostate Cancer (PC) is the most common cancer in men, being the third-leading cause of cancer death in men [1]. The main risk factors associated with the onset of PC include age, race as well as family history [8]. Similar to BC, PC is morphologically heterogeneous [9]. Although, with advancement in diagnosis, they remain asymptomatic until locally advanced stages or the establishment of metastatic tumor [9].

While, systemic treatment for BC includes cytotoxic, hormonal, and immunotherapeutic agents, for TNBC, cytotoxic chemotherapy is the treatment modality of choice [6,10-12]. However surgery, radiotherapy and chemotherapy remain the curative treatment strategies for PC [13]. Although, these treatment modalities appear effective during the initial stages of therapy in around 90% of primary, and approximately half of metastatic cancer cases [14], resistance to therapy occurs leading to treatment failure and mortality in more than half of the patients with advanced/metastatic disease [14]. Hence, it is essential to develop an alternative to conventional therapy, using natural phytochemicals present in foods such as vegetables, fruits, spices and plant roots which pose to be less harmful and more efficient in preventing or eradicating tumors [15-17]. Recent studies have shown anti-oxidant, anti-inflammatory, anti-proliferative and pro-apoptotic effects of various phytochemicals [15,16,18,19], suggesting their ability to block the growth of several types of tumors of the blood, skin, brain, colon, ovaries, breast, prostate and the pancreas [20,21].

Research in Complementary Alternative Medicine (CAM) has been vital for finding natural bioactive compounds as a complementary or alternative cancer treatment modality. For several
decades, combination of Camel Milk (CM) and Camel Urine (CU) have been practiced as a traditional treatment in the Arab countries for various ailments, including cancer [22,23]. Camel (Camelus dromedarius) products, including camel milk or urine have been used for the treatment of several diseases, including chronic hepatitis [24], hepatitis C [25,26], peptic ulcers [27] as well as cancer [28]. Moreover, while, camel milk possesses anti-cancer, anti-platelet, anti-thrombotic, anti-bacterial as well as anti-viral properties [29–31], camel urine was shown to have anti-platelet, anti-bacterial and anti-fungal properties [32–34]. Importantly, virgin camel urine showed the highest degree of inhibition at the activity level, followed by lactating and pregnant camel urines [35].

To our knowledge, no study has tested the traditional combination of Camel Milk (CM) and Urine (CU) for its effectiveness as an anticancer effect. The findings described above prompted us to test the hypothesis that CM+CU has a synergistic anti-proliferative effect on breast and prostate tumours.

Materials and Methods

Cell culture

The metastatic BC (MDA-MB-231, ATCC® HTB-26®) and PC cell lines (PC3, ATCC CRL-1435®) derived from female and a grade IV adenocarcinoma male, respectively, and the non-tumorigenic epithelial cell line, MCF10-A (ATCC® CRL-10317®) derived from a female were purchased from American Type Culture Collection (ATCC). The MCF10-A cell line was used as a control normal cell line. Cells were grown and expanded in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Life Technologies), 1% penicillin/streptomycin antibiotics (Invitrogen, Life Technologies), 1% penicillin/streptomycin antibiotics (Invitrogen, Life Technologies) at 37°C and 5% CO2 levels.

Collection and sterility of camel milk and camel urine samples

Camel Milk (CM) samples were kindly provided by Mr. Mohammed Alnaaimi from Hafeet, Al Buraimi Governorate, the Sultanate of Oman. Collection of samples was performed with a careful care to avoid any contamination from the animal itself (skin or hair) or from the outside environment during the collection procedure. In camel milk, α-lactalbumin is the vital component of whey protein; previous study has indicated presence of total solids (12%), protein (3%), fat (4%), lactose, (4%), ash (1%), 4.4% lactose and acidity (0.1%) with a pH of 6.5 [36,37]. Further, gas liquid chromatography analysis of camel milk fat revealed presence of palmitic (27%), oleic (25%), myristic (11%), and palmitoleic (11%) acids [36].

Camel urine samples were kindly provided by Mr. Ibrahim Al-Busaiday (College of Agricultural and Marine Sciences, Sultan Qaboos University, Oman) from Dakhila region, the Sultanate of Oman. Mid-stream urine from Virgin Camels (VCU) was collected in sterile culture tubes. Upon receiving these samples, they were frozen at -80°C. Camel urine analysis by GC–MS identified metabolic products including benzene propanoic acid derivatives, fatty acid derivatives, amino acid derivatives, sugars, prostaglandins and canavanine [38].

According to preliminary studies in our laboratory on CM sterilization, CM was filtered through 0.22µm filter producing a clear Filtered Camel Milk (FCM). Prior to use in cell culture treatment, the sterility of both, Filtered CM (FCM) and VCU were tested by culturing each sample on Blood agar and CLED media at 37°C for 24 hours in our Microbiology laboratory within the Department of Microbiology at Sultan Qaboos University Hospital, Sultanate of Oman. After 24 hours of incubation, these cultures were examined to check for the presence of bacterial growth. Both VCU and FCM samples were sterile.

Cell proliferation assay in the presence of Filtered Camel Milk (FCU) and Virgin Camel Urine (VCU)

MDA-MB-231, PC3 and MCF-10A cell lines were plated on clear bottom black 96 well plates (2000 cells/well) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (0.1ml/well).

FCM and VCU were dissolved in DMEM media. For treatment of cells, FCM dilutions were prepared in range of concentrations (100µl/ml, 200µl/ml, 300µl/ml, 400µl/ml, 500µl/ml, 600µl/ml and 700µl/ml) and were treated individually for 48 hours. On the other hand, VCM dilutions were prepared in range of concentrations (50µl/ml, 100µl/ml, 150µl/ml, 200µl/ml, 250µl/ml, 300µl/ml and 350µl/ml). Control (untreated cells) received 100µL of media. A combined treatment of FCM (Filtered Camel Milk) and VCU (Virgin Camel Urine) was prepared at various concentrations for both the cell lines and treated for a period of 48 hours. MDA-MB-231 cells were treated with a combination of FCM/VCU at concentrations of (400µl/ml:250µl/ml) and (450µl/ml:200µl/ml), while PC3 cells were treated at concentrations of (600µl/ml:300µl/ml) and (700µl/ml:200µl/ml) for 48 hours. MCF-10A cells were treated with a combination of FCM:VCU at concentrations of 400µl/ml:250µl/ml, 450µl/ml:200µl/ml, 600µl/ml:300µl/ml and 700µl/ml:200µl/ml for 48 hours.

The suppression of cell proliferation was determined after 48 hours using the Alamar Blue cell proliferation assay (Alamar Bioscences, Sacramento, CA), according to the manufacturer’s specifications, as previously described by our group [17]. Post-incubation with the Alamar Blue dye for 4 hours, shift in fluorescence was measured at a wavelength of 570nm (excitation) and 600nm (emission) in a Fluorometer (LabSystems Fluoreskan-II). Relative cell proliferation is expressed based on the fluorescence of drug-treated cells (MDA-MB-231 and PC3) in comparison with that of control cells, MCF-10A. MCF-10A were used in all experiments as negative controls.

Trypan blue cell viability assay

To determine cell viability assay, the Trypan blue assay was performed. Post-treatment for 48 hours, cells were detached by trypsinization and the number of viable cells was counted using a Trypan blue stain reagent. The viability of the control (untreated cells) was regarded as 100%.

Western blot analysis

To understand the underlying molecular pathways including apoptosis and cell-cycle regulation, we analyzed the expression levels of proteins by Western blot analysis as previously described by our group [17]. Both, MDA-MB-231 and PC3 cells were treated with the same concentrations of the above-described FCM and VCU for 48 hours. Total cell lysates were collected using RIPA buffer (Santa Cruz, CA) and equal amounts (30µg) of total cell extracts were resolved on 10% polyacrylamide gels and electroblotted onto
Nitrocellulose membranes. The nitrocellulose membranes were probed with a number of primary antibodies as follows: anti-mouse Bax (1:200 dilution, Santa Cruz Biotechnology, CA), anti-mouse Bcl-2 (1:200 dilution, Santa Cruz Biotechnology, CA), and anti-rabbit Rb (1:500 dilution, Santa Cruz Biotechnology, CA). Anti-rabbit β-actin antibody (1:500 dilution, Santa Cruz Biotechnology, CA) was used to ensure equal loading of protein samples. ECL Western blotting substrate was used to analyze immunoreactivity as recommended by the manufacturer (Pierce Biotechnology) and blots were imaged using the iBright™ CL1000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

Relative quantification of protein expression was obtained by analyzing captured images using ImageJ software as described by our group [39].

Statistical analysis

Data are presented as Mean ± SEM (Standard Error of Mean) of at least triplicates or replicates from three experiments and the data were analyzed statistically using Student’s t-test using GraphPad Prism software (version 8.4.3) and differences with p <0.05 were considered significant.

Results

Effects of Filtered Camel Milk (FCM) and Virgin Camel Urine (VCU) on cell proliferation and apoptosis

In our laboratory, a preliminary study comparing the effect of CU from virgin, lactating and pregnant camels demonstrated that virgin CU showed the highest degree of inhibition of cancer cell proliferation than the CU from pregnant and lactating camels (Supplementary Figure 1).

Using Alamar-Blue cell proliferation assay, several combinations of FCM and VCU were examined for their effects on cell proliferation and cell death, both individually and in combination. Our results revealed that the half maximal Inhibitory Constant (IC₅₀) of FCM for MDA-MB-231 and PC3 cells was 400µl/ml and 600µl/ml, respectively. Moreover, the IC₅₀ of VCU for both MDA-MB-231 and PC3 cells was 200µl/ml. Based on these preliminary results, the combination of FCM and VCU was tested. Using these established...
individual concentrations for the combination treatment. In this assay, treatment of MCF-10A with the combination was used as a control and was considered as 100% in comparison to cancer cells treated with FCM and VCU. A moderate effect was observed on MDA-MB-231 and PC3 cell proliferation by individual treatments at day 1 (Figure 1 and 2).

On the other hand, our results showed that synergistic effect of FCM+VCU resulted in a significant reduction in cell proliferation in both MDA-MB-231 (Figure 3a) and PC3 cells (Figure 3b) as compared to individual treatments.

Furthermore, in accordance with our cell proliferation data, Trypan-Blue assay showed that MDA-MB-231 cells exhibited a smooth epithelial cell pattern with prominent nuclei on day 0 of the experiment (before treatment). In contrast, in MDA-MB-231 and PC3 cells, combination treatment (FCM+VCU), resulted in loss of cell-cell contact and cells detached from the surface of the tissue culture dish, suggesting that a decrease in cell proliferation might be the result of increased cell death.

**Figure 3**: Effect of combination of Filtered Camel Milk (FCM) and Virgin Camel Urine (VCU) treatment at varying concentration on cancer cells in response to after a period of 48 hours. a) Mean cell viability of breast cancer cells (MDA-MB-231) after treatment with FCM and VCU for a period of 48 hours. b) Mean number of Prostate Cancer cells (PC3) after treatment with FCM and VCU for a period of 48 hours. The data are expressed as mean ± standard error of mean and the experiments were done in triplicates (p <0.01).

**Figure 4**: Molecular mechanisms of synergistic effect of Filtered Camel Milk (FCM) and Virgin Camel Urine (VCU) in MDA-MB-231 cell line. Cells were treated in combination of FCM:VCU at 450µl/ml:200µl/ml for 48 hours, protein lysates were collected and examined by western blot analysis as described under methods. All bands were quantified and normalized against b-actin that was used as loading control. a) Cropped blots showing upregulation of pro-apoptotic marker Bax as well as cell cycle regulator Rb and down-regulation of anti-apoptotic marker Bcl-2 in treated MDA-MB-231 as compared to untreated cells. b) Quantification showed that treated MDA-MB-231 displayed upregulation of pro-apoptotic marker and cycle regulator as well as down-regulation of anti-apoptotic marker in comparison to untreated cells (p <0.05, p <0.01).

**Molecular mechanisms of action of in the FCM+VCU combination**

Based on the above data, we further explored the levels of protein expression by Western blot in response to the combination FCM+VCU in both MDA-MB-231 and PC3 cells. While, the expression levels of the pro-apoptotic protein Bax was significantly increased, a significant decrease in the expression of the anti-apoptotic proteins Bcl-2 was detected in both cell lines (Figure 4). Moreover, expression of the cell cycle regulator Rb was increased (Figure 4).

Taken together, these data support our hypothesis that the combination FCM+VCU might have inhibited cell growth via induction of RB expression, and induced apoptosis through an increase in the Bax/Bcl-2 ratio.
Discussion

Complementary Alternative Medicine (CAM) is effective in inhibiting proliferation of a variety of cancer cells [15,18,19,21]. Camel products including, Camel milk and urine have been used traditionally in CAM treatment, especially in the Islamic world [40] due to their anti-cancer activity, including breast, lung as well as nasopharyngeal cancers [28,29,41]. In the present study both FCM and VCU were tested, individually and in combination, on the proliferation of metastatic MDA-MB-231 BC cells and PC3 PC cells. We tested the hypothesis that FCM+VCU combination can inhibit both BC and PC cell proliferation. To our knowledge, the present study is the first to demonstrate a chemo-preventive effect of the combination of FCM+VCU using in-vitro model.

Initially, we have demonstrated that FCM, individually, induced apoptosis in both MDA-MB-231 and PC3 cells. A number of studies have well characterized the anticancer properties of FCM and its components. In fact, Nukumi et al. (2006) showed that the milk bioactive compound, Whey Acidic Protein (WAP), inhibited tumorigenesis and invasion of human BC cells MCF-7 and MDA-MB-453 [42]. In another study by the same group, WAP inhibited cell invasion via degradation of the basement membrane component, laminin matrigel [42]. Lactoferrin, another bioactive component in milk, has shown anti-cancer and anti-microbial activities [43-45]. In fact, lactoferrin blocked cell proliferation by inhibiting cytochrome P450 1a1 gene expression [46]. On the other hand, casein binds to α-lactalbumin to form a complex with oleic acid resulting in apoptosis of various cancer cells [47,48]. Interestingly, a recent study reported that FCM induced its cytotoxic effect on human colorectal and BC cells via autophagy [49]. More interestingly, FCM and its exosomes (membrane vesicles with a diameter of 40-100 nm, secreted by cells and found in milk) reduced breast tumor progression via induction of cell death, activation of immune responses as well as suppression of oxidative stress, inflammation, angiogenesis, and metastasis [50].

Likewise, a number of studies have already characterized the anti-cancer properties of Camel Urine (CU). In fact, CU inhibited cellular growth of various tumor cells, including hepatocellular, colon, glioma, and lung cancer cells [28,35,49,51,52]. In the present study, Virgin CU (VCU) inhibited proliferation of both MDA-MB-231 and PC3 cells by 40-60%. Comparable to our data, a recent study showed that CU inhibited cancer cell proliferation at a concentration greater than 100µl/ml [28]. CU encompasses several bioactive metabolites, including canavanine, erythritol, benzenepropanoic acid and melibiose [53], and Canavanine, a potent L-arginine antimetabolite, has a powerful anti-cancer activity in various human cancer cells [54-56]. Moreover, Al-Yousef N et al. [52] showed that CU inhibited cell proliferation and triggered apoptosis of breast carcinomas and medullo-blastomas cells at a rate higher than 80%. Romli et al. (2017) have demonstrated, both in-vitro and in-vivo, that CU significantly suppressed growth and metastasis of the mouse 4T1BC cells, by inducing cell death through DNA fragmentation and regulation of inflammation-related genes, as well as by downregulation of various angiogenesis-related proteins [28].

The results from the studies described above strongly support our hypothesis that the combination FCM+VCU significantly inhibited (up to 80%) proliferation of the highly metastatic MDA-MB-231 cells and PC3 cells, as compared to the individual treatments. Interestingly, no reduction in proliferation rate was observed in the control MCF-10A cells treated with the combination.

To further confirm the induction of apoptosis, changes in the expression of the apoptotic and anti-apoptotic proteins, Bax and Bcl-2, were investigated. Western blot analysis indicated that the treatment of the MDA-MB-231 BC cells with the combination FCM+VCU, not only played a significant role in apoptosis (up-regulation of Bax and down-regulation of Bcl-2), but also in other cell regulatory pathways (up-regulation of Rb) as compared to individual treatments. Our molecular results are supported by the findings from a previous study by Delou et al. (2016) showing that increased Rb levels can activate Bax and reduce Bcl-2 levels, resulting in apoptosis [57]. Other studies have already characterized the same molecular pathway (Rb/bax/Bcl-2)-regulated apoptosis in CM-treated BC cells [29,41,58]. Likewise, CU-treated cells triggered apoptosis via the intrinsic pathway (elevated Bax and reduced Bcl-2 levels) along with the down-regulation of cancer-promoting proteins including survivin, β-catenin, and cyclic D1, and the upregulation of cyclin-dependent kinase inhibitor p21 [52].

Conclusion

Despite the recent advances in conventional diagnostic and therapeutic technologies for BC and PC management, treatment of BC and PC remains challenging, particularly in triple-negative BC and metastatic PC. CAM appears to gain more interest due its safety and efficacy amid lack of sufficient scientific evidence. Nevertheless, our findings provide the first evidence supporting our hypothesis that the combination of FCM+VCU can suppress BC and PC cell proliferation via induction of apoptosis regulated by Rb/Bax/ Bcl-2 pathway. More interestingly, ongoing in vivo studies aim to validate the efficacy of the FCM+VCU combination is suppressing progression of both breast and prostate tumours.

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