Emirates Journal of Food and Agriculture. 2019. 31(4): 256-262
doi: 10.9755/ejfa.2019.v31.i4.1935
http://www.ejfa.me/

RESEARCH ARTICLE

Camel milk whey inhibits inflammatory colorectal cancer development via down regulation of pro-inflammatory cytokines in induced AOM/DSS mouse model

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ABSTRACT

Camel milk (CM) has got an increasing interest by traditional healers and medical practitioners in areas where camels are raised for their therapeutic potential. To investigate the potential activity of CM against cancer on scientific bases, azoxymethane (AOM)/Dextran Sodium Sulfate (DSS) colitis Balb/c mouse model of CRC was used and CM whey was given orally during disease development. Colitis associated symptoms and tumor development were followed during the experiment and at the day of termination. Pro-inflammatory and anti-inflammatory cytokine gene expression were quantified using qPCR. The results showed a significant effect for CM whey on the reduction of early stage development of CRC and colon inflammation symptoms, as revealed by enhanced weight gain, reduced bloody stool and diarrhea. A concurrent reduction in gene expression of the inflammatory cytokine IL-6 was evident in colon tissue of CM whey treated mice. Moreover, both IFN-γ and IL-8 gene expression was also significantly reduced in treated mice. On the contrary, the expression of anti-inflammatory cytokines IL-10 was elevated in colon tissues of CM treated mice. In addition, iNOS, a marker for inflamed mucosa was down-regulated in treated mice. A control bovine milk whey treated group showed similar effect on IL-8, IL-6 and iNOS gene expression, whereas an elevation in IFN-γ was noticed in this group. Our results indicate the potential activity of CM whey in reducing the development of CRC in mice mainly by reducing colitis induction by chemical stimuli. Whether the active substance responsible for this activity is single or combined deserves further investigation.

Keywords: Colitis Induced Colon Cancer; Inflammation; Cytokines; Camel Milk Whey

INTRODUCTION

Colorectal Cancer (CRC) is one of the major public health problems. "Statistics" has shown that it is the second most common cancer (Ferlay et al., 2015). Important factors causing/affecting CRC development include low fiber and high fat diet, smoking, alcohol consumption, obesity, lack of physical activity and comorbid conditions such as inflammatory bowel disease (IBD) (Haiman et al., 2007). Many colon cancer treatment options are available for CRC depending on cancer stage and patient's profile. These include surgery, chemotherapy, and radiation. Being the most commonly used strategy, chemotherapy still has the significant limitations of high cost and lack of availability in certain countries (especially developing countries). In addition, chemotherapy cannot avoid normal cell targeting and is associated with a number of serious side effects (Chidambaram., 2011). As a result, a significant proportion of the population prefers alternative medicine for treatment or prevention of cancer development.

Camel milk is an important nutritional source. Historically, it has been used for maintenance of good health and treatment of diverse diseases (Kula & Tegegne, 2016). CM is different from other ruminants' milk in having low sugar and cholesterol levels and high minerals (sodium, potassium, iron, copper, zinc and magnesium, and vitamin C); known to be significant for the development and maintenance of a normally functioning immune system (Kula & Tegegne, 2016). Camel milk is rich in lactoferrin (LF), recognized for its potent antimicrobial and

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Received: 11 January 2019; **Accepted:** 28 March 2019

anti-inflammatory properties (Drago-Serrano et al., 2017; El-Hatmi et al.,2007; Konuspayeva et al., 2007). In addition, it contains a greater concentration of protective proteins, such as lysozme, immunoglobulin G, and secretory immunoglobulin A (Kula & Tegegne, 2016) compared to bovine milk (BM). Also, CM has insulin like activities with regulatory and immunomodulatory functions on β cells. One study elucidated the activity of LF isolated from CM against CRC (HCT-116) cell line (Habib et al., 2013). Researchers in this regard suggested that the anti-cancer agent may be a combinatorial activity of one or more of camel's milk fractions that may function by; having a direct cell cytotoxicity, by cutting the blood supply to tumor cells (anti-angiogenic action), or by decreasing the expression level of oncogenes or arrest growing of colon cancer cells by apoptosis (Habib et al., 2013 and Bader et al., 2017).

A great deal of literature describes that describe cytokines role and mechanism of action in CRC development, for example, IL-6 was over-expressed and found at high levels in serum and correlated with tumor size (Mager et al., 2016). Interleukin 8 (IL-8) is another cytokine that has been detected in CRC originating cell lines, it encourages tumor growth, metastasis, and angiogenesis (Mager et al., 2016). IL-10 plays a vital role in CRC progression by inhibiting expression of inflammatory cytokines in chronic inflammation models (Tanikawa et al., 2012; Murai et al., 2009). Berg et al*.,* (1996) reported that IFN-γ has many deleterious effects, such as altering gut physiology and decreasing epithelial barrier function. Generally, IFN-γ is able to increase cytokines production by macrophages thus enhancing inflammatory response.

Until recently, it is been traditionally claimed that drinking CM may help fighting against serious diseases and has cured numerous cases of colon cancer. These claims have never been exposed to a proper scientific investigation. To our knowledge, no study has explored the effect of CM on the development CRC in vivo. This study, therefore, has been designed to test the hypothesis that CM may prevent the development of colitis based CRC by reducing the expression of proinflammatory cytokines in the vicinity of tumor initiation site.

MATERIALS AND METHODS

Milk samples collection and whey milk preparation

Camel and cow milk samples were collected early morning from local farm in Irbid city, Jordan. Fresh camel and cow milk were pasteurized at 65ºC for 30 min using water bath (GFL, Germany). After pasteurization, milk was left at room temperature (RT) to cool down. Milk fat was separated using Rose-Gottlieb method described by Ronald et al., (1987). Two hundred milliliters of pasteurized CM were centrifuged at 10,000xg for 1hr at 4°C (Hermel Z326 refrigerated Hi-speed centrifuge, Germany). The upper cream layer was separated manually. After de-fatting of milk, casein was removed by precipitation. The skim milk was warmed up to reach 37ºC, and then 100mg/L microbial cheese rennet valiren (Valiren Rennet Ingredients, USA) was added gradually to the milk while stirring. Casein was left to precipitate for 30-60min. Finally, the casein was filtered using multiple layers of medical gauze. The remaining casein was removed by further centrifugation for 10min. The supernatant containing the whey proteins was kept at 4ºC until used.

Animal experiments

Male BALB/C mice aged 8 weeks and weighing 20– 30 g were provided by the animal facility, Yarmouk University, Irbid, Jordan. The animal groups were housed individually in plastic cages and allowed access to normal diet and water ad libitum, with a light/dark cycle of 12:12 h. Measures were taken to avoid all unnecessary distress to the animals. Housing, anesthesia, and postoperative care concurred with the guidelines established by an Institutional Animal Ethics Committee Approval (No. 16/3/3/310 Jordan University of Science and Technology).

Treatment protocol and data collection

The Azoxymethane (AOM) Dextran sodium sulfate (DSS) model (Parang et al.,2016) was based on a single intraperitoneal injection of (10 mg/kg body weight) AOM (ChemCruze, USA) and three cycles of (2.5%) inflammatory agent DSS (TdB Consultancy, Sweden) in drinking water, during a period of ten weeks. Forty mice were randomly divided into four groups (n=10 mice per group); group 1 was given drinking water as control (C), group 2 received AOM/DSS (AD) as positive control, group 3 received AOM/DSS and alternating camel milk whey (AD/CM), and group 4 received AOM/DSS and alternating bovine milk whey (AD/BM). Camel and bovine milk whey were given to mice for a whole week after every cycle of DSS.

Animals were individually weighed on weekly bases and signs of bloody stool and diarrhea were recorded when noticed. The presence of fecal occult blood (FOB) was investigated using a card assay from Helena Laboratories (USA) according to manufacturer instruction. At the day of experiment termination, colon was recovered from each mouse and inspected for presence of abnormalities including masses and deformation. Moreover, 3-5 cm of the distal part of colon was opened, washed with saline and inflammatory spots were recorded. This part was used to collect tissue for RNA extraction where tissue pieces were kept at -80º C until the day of RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from colon tissue using TRIzol reagent (Life Technologies, Carlsbad, Ca, USA) according to the manufacturer instructions. The concentration of RNA was measured using NanoDrop (Thermo fisher scientific Multiskan GO, Finland). The isolated RNA was reverse transcribed to cDNA using a Reverse Transcription kit (Applied biosystem, Lithuania) according to procedure supplied by the manufacturer.

Real time PCR

Gene expression of cytokines was quantified using Quantifast SYBR green qPCR kit according to the manufacturer instructions (Qiagen, USA). The qPCR reaction was started by adding 10µl 2X Quantifast mix, 1µl (500ng/µl) cDNA, 0.8µl forward, 0.8µl reverse primers and 7.4µl nuclease free water to PCR tubes with a final volume of 20µl. All cytokine expression levels were normalized to β-actin gene. The primer sequences for different genes under study were as follows: β-actin FW: TAAAACGCAGCTCAGTAACAGTCCG, RV: CTCTGGCTCCTAGCACCATGAAGA (Stephens et al., 2011), INF-γ FW: TTCTTCAGCAACAGCAAGGC, RV: TCAGCAGCGACTCCTTTTTCC, TGF-β FW: CCTGCAAGACCATCGACATG, RV: TGTTGTACAAAGCGAGCACC, IL-10 FW: ATAACTGCACCCACTTCCCA, RV: GGGCATCACTTCTACCAGGT, IL-6 FW: CCTCTGGTCTTCTGGAGTACC, RV: ACTCCTTCTGTGACTCCAGC (Liu et al., 2016), iNOs FW: ACATGCAGAATGAGTACCGG, RV: TCAACATCTCCTGGTGGAAC (Okayama et al., 2013), IL-8 FW: CACCTCAAGAACATCCAGAGCT, RV: CAAGCAGAACTGAACTACCATCG (Li et al., 2012). The PCR cycling conditions were 95°C initial denaturation for 30 sec followed by 40 cycles of 95°C denaturation for 10 sec, annealing at 60°C for 20 sec and template extension for 20 sec at 72°C.

The relative expressions of cytokine genes were calculated using comparative Ct ($2^{-\Delta\Delta Ct}$) analysis methods and assayed by Roter-Gene Q-QIAGEN (Germany), as in the equations below.

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \angle lCt_{(\text{treated sample})} - \Delta Ct_{(\text{control sample})}$$

$$\Delta Ct = \text{AVG. Ct}_{(\text{gene of interest})} - \text{AVG. Ct}_{(\text{housekeeping gene})}$$

Statistical analysis

Data were statistically analyzed using the Statistical Package for the Social Sciences (IBM SPSS 22) software. Experimental groups were compared with negative and positive control groups using Students t-test. Results were considered significant at $p \leq 0.05$.

RESULTS

Clinical assessment of colitis and tumor progression

The average weight of mice in the control group (C) which was given water has increased gradually every week (Fig. 1). In contrast, mice in the (AD) group showed weight fluctuations until week 8 and stayed steady afterward. In the groups treated with CM whey or BM whey, the average weight has increased during all weeks with minor weight loss noticed in weeks where DSS was given (Fig.1). At the end of experiment, the percentage of weight gain indicated that AD group had significantly the lowest weight gain (p-value ≤ 0.05) (Table1). The weight gain in AD/CM and AD/BM groups after week 8 was significantly higher than the AD group but still lower than the control group (C). Mice treated with CM whey showed higher weight gain in comparison to those treated with BM whey (Fig.1 and Table 1).

The occult blood test gave positive results in all mice in the AD, group whereas only 3 out of 8 (37.5%) were positive in AD/CM and AD/BM (Table 2). Diarrhea was observed in all mice of AD group, but only in 2 out of 8 (25%) in AD/CM and 3 out of 8 (37.5) in AD/BM group were diarrheic with P values of 0.011 and 0.044, respectively, when compared to AD group (Table 2 and 3).

Analysis of gross sections of colons on the day of experiment termination showed the development of masses in 6 out of 10 mice in the AD group (P value =0.011 compared to the C group). However, masses were observed in only in 1 mouse (12.5%) in the AD/CM (P= value 0.042) and 6 mice (75%) in the AD/BM group (P= value 0.069). No masses were observed in the water treated control group (Table 2).

Colon tissue cytokines gene expression

Camel and bovine milk whey treated mice revealed significant reduction in the fold expression levels for IL-6

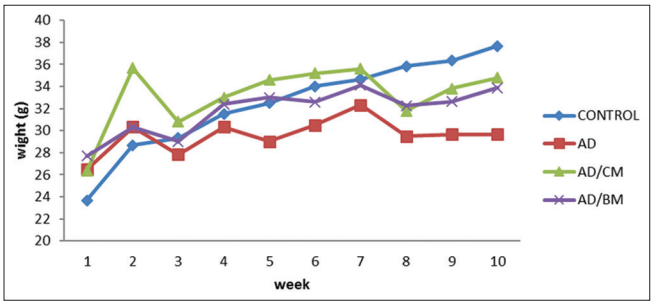

Fig 1. The average weight of each mouse group during the time of experiment. The control group (C), the group treated with AOM and DSS (AD). AD/CM and AD/BM are the groups that were given CM whey and BM whey, respectively.

and IL-8 genes in colon tissues compared to AD group (Fig. 2). Moreover, IFN-γ gene expression was reduced significantly by CMW but not by BMW treatment. In contrast, the anti-inflammatory cytokine IL-10 gene expression was upregulated in AD/CM, whereas, TGF-β expression was downregulated (Fig. 2).

DISCUSSION

The present study showed for the first time a significant effect for CM whey and BM whey on the reduction of early stage development of CRC and colon inflammation symptoms, as revealed by enhanced weight gain, and reduced bloody stool and diarrhea in AOM/DSS CRC mouse model. Moreover, the reduction in inflammation exerted by CM whey was associated with a reduction in inflammatory cytokines gene expression (IFN-γ, IL-6 and IL-8) and iNOS in colon tissue of treated animals. In line with our results, a previous study (Arab et al., 2014) which showed an anti-inflammatory effect of CM colitis mouse model (TNBS induced colitis) as indicated by reducing colon injury. The researchers returned the anti-inflammatory activity of CM to the reduction of oxidative stress and boosting of colonic glutathione and colonic anti-oxidant capacity.

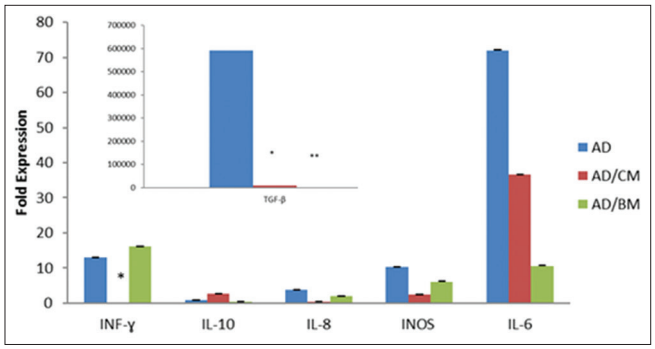

Fig 2. The fold expression level of INF-γ, IL-10, IL-8, iNOS, IL-6 and TGF-β genes in colon tissue collected from mice treated either with AOM/DSS (AD) group or alternatingly with camel milk whey (AD/CM) or bovine milk whey (AD/BM). Values are given as mean ± SD. ANOVA test was used to compare AD/CM and AD/BM groups with AD. (*) indicates significance at *p*-value ≤ 0.05.

Due to the heterogeneity of cells infiltrating CRC tumor and the cytokine network involved in the enhancement of cancer development, any cancer therapeutic or prevention approach should focus on breaching the cytokine network that promotes cancer progression. Our study revealed a significant reduction in pro-inflammatory cytokine gene expression (IL-6, INF-γ and IL-8) in colon tissues of CM whey and to a lesser extent in BM whey treated mice. Earlier studies showed that IL-6 is over-expressed in CRC tissue and its level in serum was proportionally associated with tumor size (Waldner & Neurath, 2014; Waldner et al., 2012). This agrees with the high levels of IL-6 revealed in the AD group (Fig. 2). Thus, the inhibition of IL-6 production could be an approach for treatment of CRC. The marked reduction in IL-6 gene expression associated with CM and BM whey treatment is in line with studies revealing an inhibitory effect of CM on inflammation development (Arab et al., 2014).

The most prominent effect for CM whey treatment was the complete abolishing of INF-γ, Extensive work proved

Table 1. Average weight of mice and weight gain at the beginning of the experiment, at the end of week 5 and at the end of experiment. (*) indicates significance at *p* value ≤ 0.05 in comparison to control group. (**) indicates significance at *p* value ≤ 0.05 in comparison to AD group using ANOVA test

Group	Week 0	Week 5	Week 10
C (mean weight (g) ±SD	23.7±2.3	34±2.2	37.7±1.6
% weight gain	0%	43.7%	59.2%
AD (mean weight (g) ±SD	26.5±5.6	30.5±3.2*	29.7±2.1*
% weight gain	0%	15.1%	11.9%
AD/CM (mean weight (g) ±SD	26.3±5.5	35.2±1.3**	34.8.8±1.6*/**
% weight gain	0%	33.7%	32.2%
AD/BM (mean weight (g) ±SD	28.2±4.3	32.6±2.2**	33.9±1.8 */**
% weight gain	0%	17%	22%

Table 2. The incidence of diarrhea, fecal occult blood (FOB) and colorectal masses in mice treated with CM whey or BM whey alternatingly with colitis inducing agent DSS in comparison to non-treated groups. (*) indicates significance at *p* value ≤ 0.05 in comparison to control group and (**) indicates significance at *p* value ≤ 0.05 in comparison to AD group using ANOVA test

Mouse Group	Diarrhea positive mice Number (%)	FOBT positive mice Number (%)	Masses observed in colon Number (%)
C	0/6 (0%)	0/6 (0%)	0/6 (0%)
AD	10/10 (100%)*	10/10 (100%)*	7/10 (70%)*
AD/CM	2/8 (25%)**	3/8 (37.5%)**	1/8 (12.5%)**
AD/BM	3/8 (37.5%)**	3/8 (37.5%)**	6/8 (75.0%)

C, control group; AD, AOM/DSS treated group; AD/CM, camel milk whey treated AD group; AD/BM, bovine milk

the direct anti-proliferative activity of IFN-γ on cancer cell lines. This has been proven by studies that showed higher CRC development in mice deficient in IFN-γ or IFN-γ receptor (Wang et al., 2015) and in IFN-γ gene knockout (KO) mice (Osawa et al., 2006). Although, there is a scientific consensus on the antitumor activity of IFN-γ, our study showed down-regulation of IFN-γ gene expression in colonic tissue associated with lower cancer load. We think that the reason relies on the stage at which we analyzed our samples. As indicated in this study, AOM/DSS induced CRC started with prominent colonic mucosa, erosions and ulcers that began from week 2, and result in the development of neoplasms that appear at week 10 of treatment which is consistent with data reported by other studies (Wang et al., 2015). Mouse analysis and sample collection at week 12 reflects late colitis stage and early neoplasms. According to Ito et al., (2006) DSS induced colitis was associated with a robust production of IFN-γ that was associated with weight loss and high mortality rate. Thus, CM mediated inhibition of inflammation may be explained by the reduced IFN-γ levels that result in the inhibition of immune cell recruitment to the inflammatory area and further tissue damage. As a consequence, lower mucosal inflammation may have impacted the neoplasm development in CM treated mice. In a similar manner, it has been shown that CM treatment resulted in a decrease in the level of IFN-γ in chronic hepatitis B patient (Saltanat et al., 2009)

It has been reported that IL-8 has a multifunctional role in CRC progression, and is involved in enhancing survival of cancer cells, promoting tumor cell proliferation and regulating adhesion and invasion (Rubie et al., 2007). Our result revealed that CM markedly inhibited expression of IL-8 in colon tissue, and these effects may represent the anti-inflammatory mechanism of CM. A study on immunomodulatory activity of milk proteins revealed the induction of IL-8 by Caco2 intestinal cell line by both α-lactalbumin and β-lactoglobulin from non-fat BM whey (Ustunol & Wong, 2010). This has been observed only when Caco2 cells were simultaneously stimulated by IL-1β. Whether the absence of β-LG in CM whey was responsible for the reduction of IL-8 expression in CM treated group in our model has to be addressed. However, BM whey treated group also resulted in minor reduction of IL-8 expression. One possible explanation for the contradictory results is the possible synergistic effect of all milk components in reduction of IL-8 gene expression seen in our experiment that has been reversed when purified proteins were used by Ustunol & Wong (2010).

The iNOS isoform has been shown to be the predominant enzyme that promotes tumor progression through nitrogen oxide (NO) production. Several studies indicated that the endothelial isoenzyme (eNOS) can also modulate different tumor processes including resistance, angiogenesis, invasion and metastasis (Peñarando et al., 2018; Thomsen et al., 1997). However, there is a huge controversy about the role of iNOS in colon cancer (Ambs et al., 1998; Moochhala et al., 1996; Peñarando et al., 2018) Treatment of colitis induced animals by CM whey reduced the expression level of iNOS gene (Fig.2). This indicates the anti-inflammatory activity of CM whey as iNOS expression was found to correlate with the severity of DSS-induced colitis (Seril et al., 2003). The supportive role for CM as antioxidant was confirmed in TNBS-induced colitis animal model (Arab et al., 2014). Further evidence for down-regulation of iNOS and further anti-inflammatory activity was reported in an adjuvant-induced arthritis and air pouch edema models in rats, which mimic human RA (Arab et al., 2017).

On the other side of the scene, the anti-inflammatory cytokines are considered as very critical players in the development of colitis and CRC. For this reason, we investigated gene expression levels of IL-10 and TGF-β in CM and BM whey treated colitis model. This study showed up-regulation of IL-10 gene by CM whey, but not by BM whey in colon tissue. This could be one reason for the significant reduction of mass numbers and colitis symptoms in CM whey group compared to BM whey treated group. In agreement with this, is that IL-10 KO mice developed spontaneous colitis with aberrant Th1 cytokine production in chronic colitis mouse model and exhibited higher tumor growth and metastasis in colon cancer inoculated mouse model (Tanikawa et al., 2012).

Additionally, the expression of TGF-β was down-regulated by milk whey treatment. Terzic et al (2010) mentioned that TGF-β, a cytokine with dual role in cancer development and mutation in TGF-β pathway within epithelial cells, facilitates colonic tumor development and growth. This may explain reduction in tumor development in CM and BM whey treated mice concurrently with the inhibition to TGF-β gene expression. However, contradictory results showed that TGF-β signaling in T lymphocytes infiltrating the tumor lead to growth of dysplastic epithelial cells in experimental CRC (Becker et al., 2004). Inconsistent results regarding the role of TGF-β in CRC models may be explained by the stage of CRC at which cytokine has been looked at or the signaling pathway it targets in specific immune cells.

CONCLUSION

Camel Milk whey reduced the early stage development of CRC and colon inflammation symptoms in AOM/DSS colorectal cancer mouse model that may have been

mediated by down-regulation of pro-inflammatory cytokines and up-regulation of IL-10. Also, CM whey has significant effect on the reduction of iNOS gene expression. Thus, our data represent a valid rationale for the use of CM as a complementary approach, with nutritional value, safety and no side effects, during management of CRC. In fact, further studies are warranted to elucidate the exact mechanisms of CM actions AOM/DSS colorectal cancer mouse model including the study of CM whey components individually and investigating their effects on CRC development.

ACKNOWLEDGMENT

This study was financially supported by the Deanship of Scientific Research, Yarmouk University/Jordan, grant Number 21-2016.

Authors' contributions

Al Omari M & Al Qaoud K conceived and planned the experiments, contributed to the interpretation of the results, wrote the manuscript. Al Ghariebeh R & Abu Alhaija A carried out the experiments and contributed to sample preparation. Al Zoubi H worked out the pathology data. All authors provided critical feedback and helped shape the research.

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