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Research Article,

Therapeutic Effect of Camel Milk in Children with Autism: Its Impact on Serum Levels of Vasoactive Intestinal Peptide

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Abstract:

Background: Camel milk (CAM) regulates the inflammatory process, apoptotic pathways and oxidative stress. Thus, it is a therapeutic possibility for many autoimmune disorders, including autism. Vasoactive intestinal peptide (VIP) is an anti-inflammatory peptide that facilitates the immune regulatory functions by recruiting regulatory T cells to induce immune tolerance and prevent the occurrence of autoimmunity. This study aimed to investigate the effect of CAM consumption on both serum VIP levels and the severity of autism assessed by measuring the Childhood Autism Rating Scale (CARS).
Mehods: Sixty-five autistic children, aged between 3-12 years, were studied. Forty-seven patients received 500 mL of CAM (either raw "24 patients" or boiled "23 patients") in their regular daily diet for two weeks. Eighteen patients received 500 mL of cow milk for two weeks. For all patients, CARS and serum VIP levels were assessed before and after milk consumption.
Results: Although there was a decrease in CARS scores in patients who received raw CAM, this decrease was non-significant (P=0.070). Serum VIP levels were increased in patients who consumed CAM, either raw (P=0.076) or boiled (P=0.065), but this increase was non-significant.
Conclusions: CAM consumption for 2 weeks neither significantly decreased the severity of autism nor increased serum anti-inflammatory VIP levels. The lack of a significant response to CAM in this study may be attributable to the short duration of CAM consumption. Further studies are required to investigate the effect of CAM consumption for a longer duration on serum VIP levels and the severity of autism.

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Keywords: autism; camel milk; childhood autism rating scale; vasoactive intestinal peptide.

Introduction:

Oxidative stress and polymorphism in genes encoding antioxidant enzymes might be involved in the development of autism **(Mostafa et al., 2010; Mandic-Maravic et al., 2019; Bjørklund et al., 2020).** In addition, neurogenic inflammation, which is orchestrated by a large number of neuropeptides, may play an important role in some autoimmune neuroinflammatory diseases, including autism **(Mostafa and Al-Ayadhi, 2011; Bou Khalil, 2019; Mostafa et al., 2021).**

The camel is an animal that has been integrated into daily life of many societies in the pre-modern world and is still greatly respected in the cultures of these societies **(Zibaee et al., 2015).** Camel milk (CAM) is the closest to a human mother's milk. It is different from other milks, however, having low sugar and cholesterol, high minerals (calcium, phosphorus, sodium, potassium, iron, copper, zinc and magnesium) and a lot of fat and water-soluble vitamins, especially vitamin C.

CAM has many benefits, especially for children **(Khaskheli et al., 2005; Abdoun et al., 2007; Konuspayeva et al., 2009; Shamsia, 2009; Abbas, 2013; Zibaee et al., 2015).** Camel milk products in the world are popular due to increasing demand and are typically available in pharmacies **(El-Agamy, 2006).** CAM possesses anti-inflammatory activity **(Ahamad et al., 2017; Badawy et al., 2018)**. It ameliorates the inflammatory responses and oxidative stress via downregulation of mitogen-activated protein kinase (MAPK) signalling pathways **(Zhu et al., 2016)**. Thus, CAM has therapeutic activities in treatment of diabetes, and autoimmune disorders such as Crohn's disease, multiple sclerosis and autism. **(Agrawal et al., 2012; Al-Ayadhi and Elamin, 2013; Abdulrahman et al., 2016; Mansour et al., 2017).** In addition, CAM induces protective activity against hepatotoxicants and diverse carcinogens **(Korashy et al., 2012)**. Vasoactive intestinal peptide (VIP) is an anti-inflammatory neuropeptide produced by neurons, endothelial cells, epithelial cells and immune cells. VIP has multiple functions, such as nervous communications, digestive functions, and immune regulation. VIP facilitates immune regulatory functions by recruiting Tregs to induce immune tolerance and to prevent the occurrence of autoimmunity **(Grasso et al., 2014)**. Clinical findings indicate that serum VIP levels are lower in patients with autoimmune disorders **(Seoane et al., 2014; Jayawardena et al., 2017)**.

We hypothesized that the anti-inflammatory mechanism of CAM may be through the up-regulation of serum levels of VIP. The present study aimed to investigate the effect of CAM consumption, for two weeks, on both serum levels of VIP and the severity of autism assessed by measuring the Childhood Autism Rating Scale (CARS).

Methods:

Study population

This double-blind, randomized, and placebo-controlled clinical trial included 65 children with autism. All participants in the study were patients from the Autism Research and Treatment Center (ART Center) at King Khalid University Hospital, King Saud University in Riyadh, Saudi Arabia. A psychologist and a pediatrician examined all patients in the study. Autism spectrum disorder was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) **(American Psychiatric Association 2013).** The study did not include patients with neurological diseases (including tuberous sclerosis and cerebral palsy), metabolic disorders (such as phenylketonuria), seizures, allergic manifestations, autoimmune diseases, or concomitant infections. The Ethical Committee of King Khalid Hospital at King Saud University in Riyadh, Saudi Arabia, approved the present study. The legal tutors or the parents of all the enrolled patients signed an informed written consent.

The participants in the present study were randomly included in three groups, where Group I (n=24) received raw CAM. Group II (n=23) received boiled CAM, and Group III (n=18) received cow milk as a placebo. All study groups received the same instructions, the same milk volume in the same type of containers. The parents were instructed to regularly include 500 mL CAM in the children's daily diet for two weeks. During the research period, the children did not begin with any other therapies or interrupted their current therapies, including pharmaceuticals and supplements.

Milk sample used in the study: Raw or boiled CAM was delivered in liquid form. The children in the placebo group received cow milk, which in appearance was identical. The children received 500 ml milk/day. Half of the milk was given to the children in the morning, and the other half of the total administered dose was given in the evening. The regimen for the dosing of CAM was derived from pediatric nutrition as the recommended starting dose for children. The instructions for the dosing were placed on each liquid preparation that was given to the study subjects. The fresh CAM used in the study came from a trusted farm with camels, which regularly were controlled by veterinarians. All CAM used in the present study was controlled microbiologically to ensure that the milk was without commonly found pathogens **(Eberlein 2007).** The pathological screenings were conducted to detect *Campylobacter*, *E. coli* O157:H7, *Listeria*, *Salmonella*, *Listeria*, and *B. Brucella*. Batches that tested positive for pathogens were not used in the study. The CAM supplied to the participants in group 2 was pasteurized by heating to 65 °C for 15 s, then removed, cooled in an ice pot initially, and then

stored in the freezer at −80 °C. Milk supplied to group 1 was not heated to avoid losing beneficial nutrients and proteins **(Elagamy 2000).**

Study measurements:

Clinical evaluation of autistic patients:

The clinical evaluation of autistic patients was based on the individuals' clinical history taking from caregivers, clinical examination, and neuropsychiatric assessment.

Assessment of Childhood Autism Rating Scale:

CARS was utilized to measure the autism severity. It rates children from one to four on a scale for 15 different symptoms or dimensions (including activity level, object use, body use, imitation, verbal and non-verbal communication, emotional response, relation to other people, response to listening, nervousness or fear, reliability, and intellectual response level, adaptation to changes, visual responses, responses to touch, smell, and taste, as well as general impressions). According to this scale, scores of ≥30 strongly indicate that autism is present. The degree of autism for children who scored 30-36 is considered mild to moderate and severe for those who scored 37-60 points **(Schopler et al. 1986; Ozonoff et al. 2005).** For all patients, CARS was assessed before and after the consumption of milk for two weeks.

Biochemical assay of serum vasoactive intestinal peptide:

After an overnight fasting, a 3-ml blood sample from each patient was collected before and immediately after milk consumption for two weeks. The blood samples taken were injected immediately into containers with heparin, including 2000 KIU aprotinin (Cas 9087-70-1; Shanghai ZiYi Reagent Factory, Shanghai, China). After this, eight minutes of centrifugation of the samples was performed with 250 × g at 4°C. The resulting supernatant serum was until analyzing stored at −20°C. The serum levels of VIP were analyzed using a commercially accessible enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions. The samples' optical density (degree of absorbance) was measured at the wavelength 450 nm using an automatic microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). Following standard levels, a standard curve was drawn, and sample levels were calculated in accordance with optical density values. No significant interference or cross-reactivity was observed. To increase accuracy, all samples in the present study were double-blind in two experiments that were independently analyzed as duplicates to ensure reproducibility and determine inter-assay variations in the results ($P < 0.05$).

Statistical analysis:

Data were analyzed using the statistical software StatView (Abacus Concepts, Inc., Berkley, CA, USA). Mean and standard deviation (SD) were used to present parametric data. On the other hand, the presentation of the nonparametric data was at median levels, and the interquartile range which is between the 25th and 75th percentiles. A paired t-test was utilized to compare the parametric data, while the Wilcoxon signed-rank test was used to compare nonparametric data before and after consumption of milk for two weeks (Kolmogorov-Smirnov parametric test). A probability (P) for all tests that were less than 0.05 was weighed as significant.

Results:

The autistic patients comprised 48 males and 17 females. Their ages ranged between 3 and 12 years (mean±SD = 6.01±2.28 years). The degree of the disease severity was assessed by using CARS and according to this scale, 39 children had mild to moderate autism and the remaining 26 children had a severe degree of autism (table 1).

Mean values of CARS and serum VIP before and after consumption of raw CAM for 2 weeks in autistic patients

Before consumption of raw CAM, the mean value of CARS scores in autistic patients was 36.13±3.91. Although this level decreased to 34.79±1.59 after consumption of raw CAM for 2 weeks, this decrease was non-significant (P=0.070), table 2. Before consumption of raw CAM, the mean value of serum VIP was 89.88 ± 30.04 pg/ml. This value was increased after consumption of raw CAM for 2 weeks to 109.70± 35.64 pg/ml, but this elevation was non-significant (P=0.076), table 3.

Table 1. Basic clinical data of all autistic patients

		Patients who consumed raw CAM (n = 24)	Patients who consumed boiled CAM (n = 23)	Patients who consumed cow milk (n = 18)
Age (years)	Range	3 – 11	4-12	3.5 – 11
	Mean ± SD	5.58 ± 2.20	6.04 ± 2.4	6.09 ± 2.21
Sex	Female	6 (25%)	6 (26%)	5 (27.78%)
	Male	18 (75%)	17 (74%)	13 (72.22%)
Degree of autistic severity	Mild to moderate	14 (58.3%)	15 (65.2%)	10 (55.6%)
	Severe	10 (41.7%)	8 (34.8%)	8 (44.4%)

CAM, camel milk

Table 2. Comparison between CARS scores of autistic patients before and immediately after consumption of milk for 2 weeks.

Scores of CARS		Before milk consumption	After milk consumption	P-value
Patients who consumed raw CAM (n=24)	Range	30 – 43	33 – 39	0.070
	Mean±SD	36.13±3.91	34.79±1.59	
	Median (IQR)	36 (3)	35 (1)	
Patients who consumed boiled CAM (n=23)	Range	30 – 43	30 – 41	0.79
	Mean±SD	35.13±3.672	35.41±2.89	
	Median (IQR)	36 (5)	36 (4)	
Patients who consumed cow milk (n=18)	Range	30 – 39	30 – 39	0.64
	Mean±SD	35.83±2.99	35.11±2.76	
	Median (IQR)	36 (3)	36 (2)	

CAM, camel milk; CARS, Childhood Autism Rating Scale; IQR, interquartile range.

Table 3. Comparison between serum VIP levels of autistic patients before and immediately after consumption of milk for 2 weeks.

Serum VIP levels (pg/ml)		Before milk consumption	After milk consumption	P-value
Patients who consumed raw CAM (n=24)	Range	43–153.35	51.46–188	0.076
	Mean±SD	89.88 ± 30.04	109.70± 35.64	
	Median (IQR)	102.38 (42.06)	109 (39.05)	
Patients who consumed boiled CAM (n=23)	Range	44.43–163.51	74.93–252.80	0.065
	Mean±SD	102.53±39.86	126.96±47.83	
	Median (IQR)	102.29 (94.36)	126 (57.34)	
Patients who consumed cow milk (n=18)	Range	71.52–137.70	74.54–134.80	0.37
	Mean±SD	95.89±24.99	96.70 ± 40.16	
	Median (IQR)	88.30 (44.45)	89.38 (70.94)	

CAM, camel milk; CARS, Childhood Autism Rating Scale; IQR, interquartile range; VIP, vasoactive intestinal peptide.

Mean values of CARS and serum VIP before and after consumption of boiled CAM for 2 weeks in autistic patients

There was non-significant difference between values of CARS scores before and after (35.41±2.89) consumption of boiled CAM for 2 weeks (P=0.79), table 2.

Table 3 shows that before consumption of boiled CAM, the mean value of serum VIP was 102.53±39.86 pg/ml. This value was increased after consumption of raw CAM for 2 weeks to 126.96±47.83 pg/ml, but this elevation was non-significant (P=0.065), table 3.

Mean values of CARS and serum VIP before and after consumption of cow milk for 2 weeks in autistic patients

There was non-significant difference between values of CARS scores before (35.83±2.99) and after (35.11±2.76) consumption of cow milk for 2 weeks (P=0.64), table 2. Similarly, there was non-significant difference between serum VIP levels before (95.89±24.99 pg/ml) and after (96.70 ± 40.16 pg/ml) consumption of cow milk for 2 weeks (P=0.37), table 3.

Discussion:

Studies revealed that CAM is safer for children, improves general well-being, has therapeutic effects and may be effective in treatment of autism. More large clinical trials are needed to support these findings. The researchers hope that these reports for pediatricians will lead to increased research on camel milk and its uses for children **(Zibaee et al., 2015).** In the current study, although there was a decrease in CARS scores in patients who received 500 mL of raw

CAM for 2 weeks, this decrease was non-significant (P=0.070). CAM is traditionally used in autism treatment in some areas of the world. A study published in the 2005 observed the effects of camel milk consumption, instead of cow milk, on several cases of children and adults with autism. Researchers discovered that, when a 4-year-old female autistic child consumed camel milk for 40 days and a 15-year-old boy autistic child consumed camel milk for 30 days, and several 21-year-old autistic patients consumed camel milk for two weeks, their autistic symptoms significantly improved, including cognitive and communication skills Some parents noticed a significant improvement of autistic symptoms of their autistic children after consuming CAM such as better sleep, increased motor planning and spatial awareness, increased eye contact, better language and improved gastrointestinal function **(Shabo and Yagil, 2005).** In a case report published in 2013, a boy was diagnosed as a case of autism in the third year of his life. The mother of this boy started, from the age of nine years, to give him a glass of camel milk at night. The mother noticed rapid and sustained symptom improvements for 6 consecutive years (2007-2013). Interruption of CAM consumption on several occasions resulted in behavioral and physiological lapses **(Adams, 2013**). The etiology of many autistic cases is based on a primary autoimmune disease, affecting an intestinal enzyme responsible for the formation of amino acids from the milk protein casein. Instead, the breakdown of the caseins, primarily beta-casein and beta-lactoglobulin, is to a powerful opioid, casomorphin. The opioid leads to typical cognitive and behavioral symptoms. Eventually the casomorphin causes brain damage. Animal experimentation has shown that casomorphin causes autistic-like symptoms. It is therefore, advisable to restrict milk and milk products that can lead to the formation of casomorphin. As CAM does not contain beta-casein and beta-lactoglobulin, CAM does not lead to autism symptoms. In addition, CAM contains protective proteins, including the immunoglobulins necessary for maintaining the immune system and nutritional advantages for brain development **(Shabo and Yagil, 2005).**

Oxidative stress and polymorphism in genes encoding antioxidant enzymes might be involved in the development of autism **(Mandic-Maravic et al., 2019).** CAM and its exosomes result in induction of apoptosis (through its cytotoxic effect on MCF7 cells) and inhibition of oxidative stress, inflammation and angiogenesis (**Badawy et al., 2018)**. CAM was reported to be a rich source of proteins with antioxidative activities **(Moslehishad et al., 2013; El Hatmi et al., 2016; Homayouni-Tabrizi et al., 2016)**. Casein peptides derived from camel milk showed higher antioxidant and ACE-inhibitory activities after enzymatic digestion **(Jrad et al., 2014; Rahimi et al., 2016)**. Higher ACE-inhibitory and antioxidant activities were observed in cultured camel milk than bovine milk as a result of structural differences and the presence of higher proline content in camel milk caseins **(Moslehishad et al.. 2013)**. A more recent study identified two novel antioxidant peptides from camel milk proteins using digestive proteases **(Homayouni-Tabrizi et al., 2016)**. A study conducted on 60 patients with autism aged between 2 to 12 years in Saudi Arabia, investigated the effect of CAM consumption on oxidative stress biomarkers in autistic children, by measuring the plasma levels of antioxidant glutathione superoxide dismutase, and myeloperoxidase before and 2 weeks after CAM consumption. All measured parameters exhibited a significant increase after CAM consumption. These findings suggest that CAM could play an important role in decreasing oxidative stress by increasing the antioxidant molecules levels, as well as the improvement of autistic behaviour as demonstrated by the improved CARS scores **(AL-Ayadhi and Elamin 2013).**

Plasma levels of VIP were reported to be significantly higher in children with autism compared to the healthy subjects **(Tostes et al., 2012).** Clinical studies indicate that microduplications of VIPR2, encoding the VIP receptor VPAC2, confer significant risk for schizophrenia and autism spectrum disorder. Overactivation of the VPAC2 receptor in the postnatal mouse results in a reduction in synaptic proteins in the prefrontal cortex and selective alterations in prepulse inhibition. These findings suggest that the VIPR2-linkage to mental health disorders may be due in part to overactive VPAC2 receptor signaling during a critical time of synaptic maturation **(Ago et al., 2015).** Tregs are

immune regulatory cells. Bregs are also an important part of immune regulatory cells, and IL-10 is the immune regulatory molecule for Bregs **(Ray et al., 2015)**. Besides its anti-inflammatory effect, VIP facilitates immune regulatory functions by recruiting Tregs **(Grasso et al., 2014)** and stabilizing IL-10 mRNA in Bregs to induce immune tolerance and to prevent the occurrence of autoimmunity. Insufficient VIP levels in the microenvironment speeds IL-10 mRNA decay to cause Breg dysfunction **(Xiong al., 2019)**. Thus, the anti-inflammatory and immune regulatory effects of VIP may explain its elevation which was reported in serum of children with autism **(Tostes et al., 2012)** to counteract the neuroinflammation **(Mostafa et al., 2016; Bou Khalil, 2019)** and the formation of brain specific autoantibodies **(Singh et al., 2002; Cohly and Panja, 2005; Mostafa et al., 2008; Mostafa and Al-Ayadhi, 2015; Bjorklund et al., 2016; Kern et al., 2020; Erden et al., 2021)** which were reported in many children with autism**.**

We hypothesized that one of the anti-inflammatory mechanisms of CAM may be through up-regulation of the level of the anti-inflammatory VIP. Thus, this study was the first that investigated the effect of CAM consumption on serum VIP levels in a group of children with autism. In the present work, serum VIP levels were increased in patients who consumed CAM, either raw (P = 0.076) or boiled (P = 0.065), but this increase was non-significant. The anti-inflammatory mechanisms of VIP are broadly studied and it is known to have multiple actions including inhibition of neutrophil and macrophage migration and activation **(Abad et al., 2012)**. In addition, it is also known to play a role in modulating the expression of pathogen recognition toll-like receptors in the intestinal epithelial cells **(Gomariz et al., 2005).** The anti-inflammatory properties of VIP have been demonstrated in animal models of several immune disorders such as multiple sclerosis **(Fernandez-Martin et al., 2006)** rheumatoid arthritis **(Arranz et al., 2008)** and Crohn's disease **(Abad et al., 2005).** However, the results of the current study may indicate that the anti-inflammatory effect of CAM consumption may not be through the up-regulation of the levels of the anti-inflammatory VIP, but this is an initial report that requires other studies that investigate the effect of CAM consumption for longer duration on serum VIP levels. Limitations of this study include the short duration of CAM consumption. Other studies that investigate the ameliorative effect of CAM consumption on autistic behavior and neuroinflammation for different and longer durations are warrented. A further potential limitation of the present study is the exact mechanism of action of CAM which was not elucidated from the present study. An additional potential limitation of this study is the fact that the dose of CAM used may not have been optimal.

Conclusion:

CAM consumption for two weeks neither significantly decreased autism severity nor increased the levels of serum anti-inflammatory VIP. The lack of a significant response to CAM in this study may be attributable to the short duration of CAM consumption. Further studies are required to investigate the effect of CAM consumption, for a longer duration, on the severity of autism, levels of serum VIP and the other anti-inflammatory markers. This study may also pave the way to other studies that will measure levels of the inflammatory cytokines both before and after CAM consumption in relation to the severity of autism.

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Abbreviations

(CAM): camel milk; (CARS): Childhood Autism Rating Scale; (IQR): interquartile (VIP): vasoactive intestinal peptide.

Competing interests

The authors declare no potential conflicts of interest with respect to the authorship, and/or publication of this article.

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