SHORT COMMUNICATION

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Epidermal growth factor concentration in milk of healthy water buffaloes (*Bubalus bubalis*)

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Abstract

Epidermal growth factor (EGF) has biological roles, including embryonic organ development, breast morphogenesis, breast cell proliferation, and mammary development. This study aimed to measure EGF concentration and evaluate its relationship with somatic cell count (SCC) in healthy water buffaloes *(Bubalus bubalis)* milk. The study material was constituted of 120 milk samples obtained from 30 healthy water buffaloes between the ages of 3 - 6 years, negative for California mastitis test and SCC less than 3.00×10^5 cells mL^{-1} milk. In milk serum samples, the EGF concentration was measured using a bovine-specific enzyme-linked immunosorbent assay kit. Epidermal growth factor concentration in the buffalo milk was ranged from 4.30 to 9.80 ng mL^{-1}, with a mean of 8.30 ± 1.50 ng mL^{-1}. Positive correlation between milk SCC values and EGF concentrations was recorded in water buffaloes. Further research is required to evaluate the content of milk EGF in different species of animals because of the EGF effective role in mammary gland and intestinal mucosa.

Introduction

Epidermal growth factor (EGF) is a protein consisting of 53 amino acids. Although the EGF is mainly in epithelial cells, it has receptors in endothelium and mesodermal, fibroblast and smooth muscle cells. Epidermal growth factor has a stimulating effect on the proliferation of mammary gland cells.[1] The EGF has biological roles, including embryonic organ development, breast morphogenesis, breast cell proliferation, and mammary development. Especially, it is known that EGF possesses crucial functions in the normal development of the udder gland by controlling the growth and differentiation of the udder glands.[1,2] Furthermore, Akers has suggested that it is unclear whether EGF may be normally important in ruminants to determine effects of EGF on udder development and subsequent milk production.[3] Dehnhard *et al.,* have reported that in ruminants, studies are limited, EGF does not occur in peripheral plasma.[4] Epidermal growth factor was measured in goat milk by Dehnhard *et al*., and it was presented that its level might change during end- pregnancy and early lactation.[4] However, Sheffield has indicated that mastitis in cows induces increase in the levels of EGF and it could be essential in a variety of processes occurring during infection, such as protection against injury or tissue repair and recovery processes.[5] Therefore, EGF is a known modulator of udder function and it is thought that the knowing of normal EGF level in milk has an importance in the physiological and pathological conditions. In addition, EGF plays important roles in intestinal development of foetus, starting during gestation when the foetus swallows amniotic fluid, that includes levels of EGF and also after birth, intestinal EGF of infant is mainly obtained from breast milk.[6] Dvorak *et al*., have reported that EGF reduces the development of necrotizing enterocolitis in an experimental necrotizing enterocolitis of rats.[7] Epidermal growth factor supplementation in animals has been reported to remain biologically active in the gastrointestinal tract and also stimulate intestinal development, reduce pathogen infection and increase resistance to intestinal infections.[8] Thus, it has known that in pathophysiologic situations, EGF contributes to epithelial protection from injury and post-injury mucosal repair.

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Somatic cells (SCs) are normally present at low levels in milk, primarily consisting of cells from the udder secretory tissue (epithelial cells) and some leukocytes (white blood cells). Milk SCs such as macrophages, neutrophils, lymphocytes and epithelial cells were isolated from milk of buffaloes. These cells fight against mammary infections and repair tissue damage.[9] Gokceoglu *et al*. have indicated that high EGF level is associated with somatic cell count (SCC) in milk of cows with sub-clinical mastitis and also suggested that a milk EGF analysis might be a useful tool for the diagnosis of mastitis as well as mammary health monitoring. [10]

Water buffalo milk has an important value in human nutrition because its lipid, protein, vitamin and mineral content is higher than other milks such as human, cow, goat and camel milks.[11] Epidermal growth factor plays crucial roles in the physiological and pathological processes in the organism.[5,7,8] The somatic cell count is used as an index to predict udder health and milk quality of dairy animals.[9] To our knowledge, although there are studies about buffalo milk components, studies regarding the growth factor concentration in buffalo milk are limited. Thus, the purpose of the present study was to determine the EGF concentration and evaluate its relationship with SCC in milk of healthy water buffaloes.

Materials and Methods

Milk samples. In the study, 120 quarter milks from 30 healthy water buffaloes *(Bubalus bubalis)* between the ages of 3 - 6 years at stage of lactation in a farm in Igdir province, Turkiye were used. California mastitis test (CMT) and SCC were performed on milk samples taken from four different udder lobes of each buffalo and screening was performed for sub-clinical mastitis. Milk samples with negative CMT and SCC < 3.00×10^5 cells mL^{-1} milk constituted the study material.[12]

The California mastitis test procedure. For CMT, milk from four different mammary lobes of buffaloes was milked into each quarter of a four-cup plastic paddle and CMT reagent (Kepro, Deventer, The Netherlands) was added to each compartment in a volume equal to the retained milk. The CMT paddle was rotated in circular motions for a few sec and color changes and gel formation were observed. The test was read and recorded quickly. The degree of gel formation was scored from 0 (negative) to 3 (strong positive).[12]

The somatic cell count procedure. Somatic cell count in milk samples was performed according to the method suggested by Kilicoglu *et al*. For this purpose, 10.00 mL of milk samples taken into glass tubes were centrifuged for 10 min at 1,550 *g*, the milk cream collected at the top of the tube was poured by heating of the end of tube, and the tubes were placed in and turned upside down for 20 min.[13] The sediment collected at the bottom was collected with a loop, spread with a drop of physiological saline in circular movements on the slide, and its preparations were prepared and allowed to be dried at room temperature. The slides were then stained with 0.20% toluidine blue. Immersion oil was dropped onto the slide surface, cells in 15 - 20 fields were counted on immersion objective with a light microscope, the number of cells was divided by the number of fields and the average cell number was determined. The number of SCs in milk was calculated as summarized in Table 1.

Table 1. Evaluation of quantitative somatic cell count method.

Average counted cell number (cells per field)	Scores	Number of cells per mL of milk ($\times 10^5$)
1 - 5	+	< 3.00
6 - 20	++	> 3.00
> 20	+++	< 10.00

Preparation of milk serum. Milk samples were collected into 20.00 mL plastic vials from four separate mammary lobes of each water buffalo. Milk serums were prepared with the procedure reported by Alais.[14] One milliliter of 0.30% rennet (chymosin), (R4877; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was added to milk samples, incubated in a 37.00 ˚C water bath for 20 min and the clot was cut after standing for 80 min at room temperature in order to separate milk serum. After the milk serum was filtered with filter paper into glass tubes, it was centrifuged for 5 min at 1,550 *g*; the milk cream collected at the top of the tube was removed and the milk serums were aliquoted and kept at – 20.00 ˚C until analysis.

The epidermal growth factor analysis. In milk serum samples, the EGF concentration was measured using bovine-specific enzyme-linked immunosorbent assay kit (MBS706122; MyBioSource, San Diego, USA). To perform the analysis, the procedure was followed as suggested by the manufacturer company. The absorbance of the color formed in the microplate was evaluated at 450 nm using a microplate reader (Infinite F50; Tecan, Grödig, Austira) and milk serum EGF concentration was calculated according to its standard concentration. The results were presented as ng mL^{-1}.

Statistical analysis. Data were analyzed using the SPSS statistical package program (version 21.0; IBM Corp., Armonk, USA). The results were given as the mean ± standard deviation. Results were tested for normality using the Shapiro-Wilk normality test. The Spearman correlation coefficient was performed to determine the relationship between the milk SCC values and the EGF concentration. A minimum $p < 0.05$ was considered statistically significant for statistical evaluation.

Results

Somatic cell counts and EGF values in the milk of individual udder quarters of water buffaloes are presented

in Table 2. The EGF level of water buffalo milk was ranged from 4.30 ng mL^{-1} to 9.80 ng mL^{-1}, with a mean of 8.30 ± 1.50 ng mL^{-1}. The correlation between milk SCC values and EGF concentrations in water buffaloes is shown in Figure 1. A significant correlation was recorded between SCC value and EGF concentration in the milk of water buffaloes (r = 0.837; p < 0.01).

Table 2. Somatic cell count (SCC) and epidermal growth factor (EGF) values in the milk of individual udder quarters of water buffaloes *(Bubalus bubalis)*. Data are presented as the mean ± SD of values obtained from four different udder quarters.

Buffalo number	SCC (× 10^5 cells mL^{-1})	EGF (ng mL^{-1})
1	1.50 ± 0.60	5.90 ± 1.10
2	2.30 ± 1.00	6.40 ± 1.30
3	3.30 ± 0.50	7.70 ± 0.10
4	2.80 ± 1.30	6.80 ± 1.60
5	2.30 ± 1.00	6.90 ± 1.60
6	4.00 ± 0.00	8.00 ± 0.20
7	3.00 ± 1.20	7.70 ± 0.40
8	3.30 ± 1.00	7.90 ± 0.40
9	4.00 ± 0.00	8.10 ± 0.10
10	3.80 ± 0.50	8.30 ± 0.00
11	3.00 ± 1.40	7.60 ± 2.00
12	3.30 ± 1.50	7.50 ± 2.00
13	3.50 ± 0.60	8.60 ± 0.00
14	3.00 ± 1.20	8.80 ± 0.10
15	4.00 ± 0.00	8.90 ± 0.00
16	3.30 ± 1.50	8.10 ± 1.70
17	3.80 ± 1.30	8.70 ± 0.50
18	4.00 ± 0.00	9.10 ± 0.10
19	3.00 ± 0.00	9.10 ± 0.10
20	3.50 ± 1.00	9.20 ± 0.20
21	3.80 ± 1.00	9.30 ± 0.10
22	4.30 ± 1.00	9.40 ± 0.10
23	3.80 ± 1.30	8.60 ± 2.00
24	3.80 ± 0.50	9.50 ± 0.10
25	3.80 ± 1.00	9.40 ± 0.30
26	4.50 ± 1.00	9.70 ± 0.20
27	5.00 ± 0.00	9.80 ± 0.10
28	3.00 ± 2.30	7.20 ± 3.10
29	4.00 ± 2.00	8.40 ± 2.80
30	4.00 ± 2.00	8.40 ± 2.70

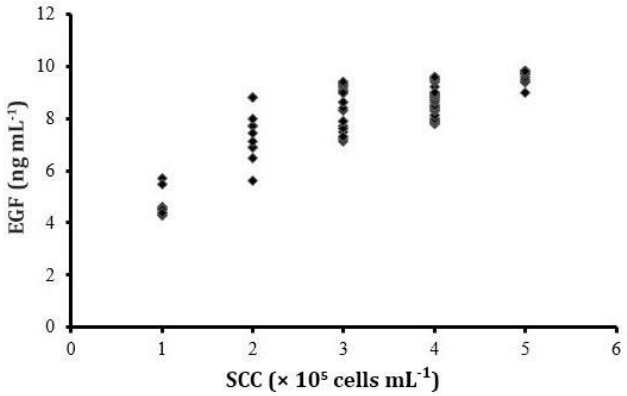

Fig. 1. Correlation between milk epidermal growth factor (EGF) concentration and somatic cell count (SCC) in the milk of water buffaloes (r = 0.837; p < 0.01).

Discussion

Milk contains not only nutritional components, but also biologically active substances including growth factors.[2] Since buffalo milk has higher fat, lactose, protein, calcium, sodium, vitamin and mineral content than other dairy animals, it has a recognized role in human nutrition.[11] Epidermal growth factor is involved in normal physiological functions and many pathological conditions.[5,7,8] The expression of EGF has been demonstrated in mammary epithelial cells. Epidermal growth factor concentration is about 1,500 ng mL^{-1} in colostrum and 150 - 250 ng mL^{-1} in porcine milk.[15] The mean values for EGF-like activity of mares have been determined as 9.70 ng mL^{-1} in post-suckle colostrum, 9.60 ng mL^{-1} in day one, 8.50 ng mL^{-1} in day two, 8.00 ng mL^{-1} in day four and 7.80 ng mL^{-1} in day eight.[16] The content of EGF has been determined as 25.00 – 38.00 ng mL^{-1} in colostral milk and 5.20 - 11.50 ng mL^{-1} in mature milk in women.[17] The concentration of EGF has been found less than 2.00 ng mL^{-1} in bovine milk and 30.00 - 40.00 ng mL^{-1} in human milk when measured by radioreceptor analysis.[18] In the present study, the EGF concentration in healthy water buffalo milk was ranged from 4.30 - 9.80 ng mL^{-1}, with a mean of 8.30 ± 1.50 ng mL^{-1}. In addition, a significant positive correlation was observed between SCC value and EGF concentration in the milk of water buffaloes.

It is well known that breast milk is essential and beneficial for intestinal mucosa of infants.[2] Higher EGF levels in maternal milk of extremely preterm group (23 - 27 weeks) compared to the values from the preterm and full-term groups during the first month of lactation compared to those of the preterm and full-term groups have been reported and milk-borne EGF has been suggested to have a potential importance for the development of extremely pre-mature infants.[8] Xiao *et al*., have reported that the EGF content in breast milk is correlated negatively with the birth weight of newborns and the gestation period of the mother.[19] Human milk from mothers of pre-mature infants has been reported to have higher EGF content compared to that from mothers of term infants (28.20 ± 10.30 nmol L^{-1} *versus* 17.30 ± 9.60 nmol L^{-1}).[19] These researchers have emphasized that the EGF content in fresh cow milk is ranged from 13.80 to 18.20 nmol L^{-1} and is similar to that in human milk; the high EGF content in pre-mature milk may represent a maternal compensatory mechanism to accelerate the growth and development of immature infants.

In human medicine, the growth factors regulating the biological response have been demonstrated by several researches indicating that they regulate the survival, differentiation, proliferation and migration of cells.[7] Although scientific reports regarding the role of growth factors in veterinary medicine using both experimental model and clinical case are more limited than human

medicine, some reports can be found.[10,20] Gurler *et al*., have reported that milk EGF concentrations increase in Anatolian water buffaloes suffering from sub-clinical mastitis.[20] Recently, positive correlation between increased milk EGF concentration and SCC has been reported in cows with sub-clinical mastitis.[10] Similarly, in this study, it was presented that EGF concentration positively correlates with SCC in the milk of healthy water buffaloes. Thus, it may be considered that this correlation does not change between milk EGF and SCC in healthy or sick animals.

In conclusion, studies are needed to determine the amount of milk EGF in different species of animals, since EGF can be applied as an effective therapeutic approach in various situations such as mastitis and intestinal damage.

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Conflict of interest

The authors have no conflict of interest to declare.

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