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The effect of health status of the udder on plasminogen activator activity of milk somatic cells in ovine milk

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ABSTRACT

This study was conducted to examine the effect of health status of the udder on plasminogen activator (PA) activity of milk somatic cells in ovine milk. Milk samples were obtained from 4 breeds [Boutsiko, Chios, Karagouniko and a synthetic breed (50% Boutsiko, 25% Arta, and 25% Chios)]. A total of 192 milk samples were collected bi-weekly throughout the lactation period. Milk somatic cells were isolated and milk cell-associated PA activity per cell was determined. Milk samples were divided in three somatic cell count (SCC) groups: group 1 with high SCC (>1,000,000 cells/ml), group 2 with an intermediate level of SCC (from 300,000 to 1,000,000 cells/ml) and group 3 with a low SCC (<300,000 cells/ml). Results indicated that PA activity per cell was approximately 4-fold higher in the intermediate group, when compared to the low SCC group. An increase of the milk SCC from <300,000 to >1,000,000 cells/ml resulted in a 10-fold increase of PA activity per cell. The predominant form of PA in milk somatic cells is urokinase-PA. No relationship was found between breed, stage of lactation and milk cell-associated PA activity. Further research is needed to examine the possibility that increased PA activity per cell in the high SCC group may explain the higher plasmin levels, which are observed in ovine milk obtained from mastitic glands.

1. Introduction

The conversion of plasminogen (PG) to plasmin (PL) is regulated by plasminogen activators (PA) (urokinase-PA, u-PA and tissue-PA, t-PA) and at least two types of specific plasminogen activator inhibitors (PAI-1, PAI-2) (Politis, 1996). The two types of PA are present in milk (Heegaard et al., 1994b; White et al., 1995; Politis, 1996). In milk, PL, PA and t-PA are localized mainly within the casein micelle, while u-PA is closely associated with milk neutrophils (Heegaard et al., 1994a,b) or milk somatic cells (White et al., 1995). Silanikove et al. (2013) confirmed the differential localization of both forms of PA in bovine milk. Furthermore, they suggested that t-PA and not u-PA is the principal PA responsible for the conversion of PG to PL in row bovine milk. PL hydrolyzes α_s-casein (α_s-CN) and β-casein (β-CN) in milk and affects negatively the ability of milk to undergo processing. Increased activity of PL and PA has been associated with a deterioration of the coagulation properties of milk because of proteolysis of CN by PL (Srinivasan and Lucey, 2002). Milk in which casein has been broken down by plasmin will be of less value to cheese and yogurt manufacturers (Moatsou, 2010).

The PL-PG system in milk is affected by the health status of the udder. More specifically, PL and PA activities were greater by 74 and 139%, respectively, in infected vs. non-infected glands in dairy sheep (Leitner et al., 2004a). Furthermore, there was an increase (3–10-fold) in PA activity in bovine milk samples with high SCC (>750,000/ml) compared to those with low SCC (<250,000/ml) or intermediate SCC (250,000–750,000/ml) (Gilmore et al., 1995). Bianchi et al. (2004) reported that elevated SCC were associated with increased PL activity and decreased PA activity whereas PG activity did not vary with SCC in ovine milk. Albenzio et al. (2004) reported increased PL activity for milk samples with high SCC (>1,000,000/mL) compared to those with low SCC (<500,000/mL) throughout lactation in ovine milk. Zachos et al. (1992) showed that somatic cells obtained from mastitic quarters, on a per cell basis, had higher PA activity than cells obtained from healthy quarters in dairy cows. Somatic cell counts (SCC) of milk are commonly used as an indicator of subclinical mastitis and represent a marker of the sanitary state of the udder. In fact, during the course of mastitis the immune defense of the udder is activated. The two main types of somatic cells are macrophages and polymorphonuclear leucocytes (PMN), which are the first line of defense against bacterial infection, thus initiating the process of inflammation. Macrophages are more predominant than PMN in a non inflammatory state. However, a rapid influx of PMN into the milk is the response to an

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Table 1
Effect of health status of the udder on milk yield (ml) and milk composition (%) of ovine milk (mean ± SE).

SCC group (cells/ml) Parameters	$>10^6$	$3 \times 10^5 - 10^6$	$<3 \times 10^5$	Level of significance
Milk yield (ml)	835.4 ± 59.3^b	982.3 ± 107.5^b	1384.9 ± 86.4^a	
Fat (%)	6.08 ± 0.13^a	5.89 ± 0.26^{ab}	5.46 ± 0.19^b	*
Protein (%)	5.35 ± 0.07^a	5.12 ± 0.12^{ab}	4.83 ± 0.08^b	***
Lactose (%)	4.47 ± 0.04^b	4.66 ± 0.07^a	4.82 ± 0.04^a	

[a,b]Means in a column with different superscripts differ significantly ($P < 0.05$); SCC, Somatic cell count.
* $P < 0.05$.
*** $P < 0.001$.

Table 2
Effect of fibrin and amiloride on plasminogen activator (PA) activity of ovine milk somatic cells (mean ± SE).

Treatment	PA activity (units/10^6 cells)
Control	3.28 ± 0.19^a
Control + 20 μg fibrin/ml	3.74 ± 0.2^b
Control + 1 mM amiloride	0.96 ± 0.07^c
Level of significance	*

[a,b,c]Means in a column with different superscripts differ significantly ($P < 0.05$).
* $P < 0.001$.

Table 3
Effect of health status of the udder on plasminogen activator (PA) activity of ovine milk somatic cells (mean ± SE).

SCC group (cells/ml)	PA activity (units/10^6 cells)
$>10^6$	5.67 ± 0.17^a
$3 \times 10^5 - 10^6$	2.14 ± 0.31^b
$<3 \times 10^5$	0.58 ± 0.25^c
Level of significance	*

a,b,cMeans in a column with different superscripts differ significantly (P < 0.05); SCC, Somatic cell count.

acute infection of the mammary gland with subsequent inflammation, so the number of somatic cells in milk increases (Politis, 1996; Leitner et al., 2000). Albenzio et al. (2004) reported that macrophages minimally contributed to milk somatic cells in ovine milk and had the greatest levels at the beginning of lactation. An opposite trend was observed for PMN that increased throughout the lactation leading the authors to suggest that PMN may play a role in gradual involution. SCC can vary irregularly in ovine milk independently of infective processes and can reach very high levels during the colostral period and at the end of lactation (Dulin et al., 1983; Fruganti et al., 1985). An increase can also be the result of other factors: animal age, milk yield, herd management, season and stress.

Dairy sheep farming is a sector of major economic importance in Greece. Four indigenous breeds, Boutsiko, Chios, Karagouniko and a synthetic breed (50% Boutsiko, 25% Arta, and 25% Chios), were used in this study. The Chios breed is characterized by high milk yield and litter size, whereas the less-productive Boutsiko breed is known for its adaptability under harsh environmental conditions and its reduced susceptibility to mastitis (Hatziminaoglou et al., 1990; Simos et al., 1996; Kominakis et al., 1998; Ploumi et al., 1998). The synthetic breed has been created in an attempt to upgrade the Boutsiko breed by combining the high productivity of the Chios and Arta breeds with the robustness of the Boutsiko breed. There is no published information on differences of milk cell-associated PA activity among breeds.

This study was conducted to examine the type of PA present within milk somatic cells in ovine milk. Furthermore, the effect of health status of the udder on milk cell-associated PA activity was examined. Relationships between several factors (breed, stage of lactation) and milk cell-associated PA activity were evaluated.

2. Materials and methods

Four indigenous breeds, Boutsiko, Chios, Karagouniko and a synthetic breed (50% Boutsiko, 25% Arta, and 25% Chios), were used in this study. Animals were housed within the premises of the experimental farm of the Agricultural University of Athens. It was essential that all animals had completed their lactation period by the first week of July to avoid the extremely hot conditions that occur in Greece during July and August. All ewes were in their second or third lambing. After weaning (45 days after lambing), the ewes were milked twice daily at 05.00 and 17.00 h using a milking machine. Individual milk yield was recorded every 2 weeks (wk) and milk samples were collected during the morning and evening milking on the same dates during the entire lactation. Milk samples were divided into 2 aliquots; the first was immediately analyzed for major milk components and SCC and the second aliquot was used for somatic cells isolation.

Milk samples were analyzed for fat, protein, lactose, solids non-fat (SNF) and total solids (TS) by the infrared method using a Milkoscan 133 (Foss Electric, Hillerod, Denmark) calibrated against the Mojonnier method for fat, Kjeldahl method for protein, and the polarimetric method for lactose according to official methods (AOAC, 1980). SCC was determined with a Fossomatic cell counter (Foss Electric). Before and during the experiment, the equipment was standardized for sheep milk, according to FIL-IDF 141C (2000).

For isolation of milk somatic cells, milk samples (100 ml) were centrifuged at $2000 \times g$ for 15 min to produce skim milk, cream and a pellet containing somatic cells. The somatic cell pellet was collected, washed twice with phosphate-buffered saline (PBS, 0.15 M NaCl – 0.01 M NaH_2PO_4, pH 7.2) and was stored at −20 °C until used for the determination of PA per cell activity. Total cell numbers and cell viability were measured in a sample (20 μl, in triplicate), by addition of 5 μl 0.25% (w/v) trypan blue and counting the proportion of cells that were capable of excluding the dye (Zachos et al., 1992). Typically, 75% of recovered cells were viable. Phosphate-buffered saline containing 10^6 viable cells/ml was subjected to 3 cycles of freezing and thawing and was immediately used for PA activity determination.

A colorimetric assay was used to determine PA activity in milk somatic cells (Zachos et al., 1992). The assay system utilizes PA present within milk somatic cells to convert exogenously inactive PG to active PL. The PL produced in this manner is subsequently allowed to attack the chromogenic D-valyl-leucyl-lysine *p*-nitroanilide adjacent to lysine and liberate free chromophore *p*-nitroaniline. In this system, changes in color are directly related to PL levels and, therefore, to PA activity.

Typically, 10 μL milk somatic cells extracts were mixed with 150 μL 0.1 M Tris buffer, pH 8.0, containing 0.6 mM val-leu-lys *p*-nitroanilide (Sigma Chemical Co., St Louis, MO) and 20 μL plasminogen (Sigma). The assays were analyzed in duplicates. The reaction mixture was incubated at 37 °C and absorbance at 405 nm was measured at various times up to 3 h. The rate of *p*-nitroaniline formation was calculated from these values. A sample without PG served as a control. PA activity was expressed in units, with one unit being defined as the amount of the enzyme producing a change in absorbance (ΔA) of 0.1 in 60 min at 37 °C, when *p*-nitroaniline is measured in the defined reaction mixture.

To examine the type of PA within milk somatic cells, milk cell-associated PA activity was determined in the presence of fibrin (20 μg/ml) or amiloride (1 mM). Preliminary experiments indicated that these concentrations affected the PA activity optimally. All other details are as described above.

Data on PA activity per cell, milk yield and major milk components were analyzed with GLM procedure of SAS software (SAS Institute, 2005), with milk SCC, breed and stage of lactation as the fixed effects. Milk samples were divided in three somatic cell count (SCC) groups: group 1 with high SCC (>1,000,000 cells/ml, $n = 76$), group 2 with an intermediate level of SCC (from 300,000 to 1,000,000 cells/ml, $n = 20$) and group 3 with a low SCC (<300,000 cells/ml, $n = 96$). The Bonferroni adjustment was used for mean comparisons and the significance level was set at 0.05. Differences between the mean cell-associated PA activity in the presence or absence of fibrin and amiloride were evaluated using a Student's *t* test ($P < 0.05$).

3. Results and discussion

The effect of health status of the udder on milk yield and major milk components is presented in Table 1. Milk production was