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Contribution of macrophages to plasmin activity in ewe bulk milk

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ABSTRACT: A total of 225 bulk sheep milk samples were collected throughout lactation to assess the contribution of macrophages to the regulation of the plasmin/plasminogen system. Samples were analyzed for composition, milk renneting parameters, and for activities of plasmin (PL), plasminogen (PG) and plasminogen activators (PA). Isolation of macrophages from milk was performed using a magnetic positive separation; separated cells were lysed and activity of urokinase-PA was determined. PL activity in milk decreased during lactation ($P < 0.001$). The reduction in plasmin activity recorded in the mid and late lactation milk matched with the increase in PG/PL ratio ($P < 0.001$). The activity of PA increased throughout lactation ($P < 0.001$), the highest value being recorded in the late lactation milk. The amount of isolated and concentrated macrophages was higher in early and mid lactation milk than in late lactation milk ($P < 0.01$). Stage of lactation did not influence the activity of u-PA detected in isolated macrophages. The activity of u-PA associated with macrophages was lower than total PA activity detected in milk. Our results lend support to the hypothesis that in ewe bulk milk from healthy flocks macrophages only slightly contributed to the activation of plasmin/plasminogen system.

Key words: Macrophages, Ewe bulk milk, Plasmin, Plasminogen activator.

INTRODUCTION – Plasmin is the main proteolytic enzyme in milk and is part of a complex system consisting of plasmin (PL), its inactive form plasminogen (PG), plasminogen activators (PA), which converts plasminogen to plasmin, PA inhibitor (PAI), which inhibits PA activity, and plasmin inhibitor, which inhibits plasmin activity (Nielsen, 2003). Tissue-type plasminogen activators (t-PA) appear to be associated with casein fraction, urokinase type activators (u-PA) are associated with somatic cells, which can convert PG to PL. In sheep, blood macrophages and neutrophils express a specific urokinase-plasminogen activator (u-PA) (Politis *et al*., 2002); also in individual ewe milk with high somatic cell count plasmin activity was found dependent on PG activators connected with milk macrophages (Albenzio *et al*., 2004). Therefore, this study was undertaken to investigate the contribution of milk macrophages to casein hydrolysis and the conversion of plasminogen into plasmin in ewe bulk milk through lactation.

MATERIAL AND METHODS – The experiment was conducted from March to July 2005 in five intensively managed flocks of Comisana ewes located in Southern Italy. Ewes involved in the trial lambed in the winter of 2005, were in parity 2 to 4, were healthy at the beginning of the experiment and monitored by veterinarians throughout the study period. The animals grazed in the morning and were supplemented with hay and concentrate in the trough during night-time. In each flock, five sampling cycles were performed during early (less than 70 d in lactation, n. of samples=75), mid (from 110 to 130 d in lactation, n. of samples=75) and late lactation (more than 160 in lactation, n. of samples=75). The ewes were machine milked twice a day; milk samples from both daily milkings were collected in 200 ml sterile plastic containers, carried to our laboratory by means of transport tankers at 4°C and immediately analyzed. For each sampling cycle, samples were collected in triplicate. The milk samples were analyzed for fat, total protein and lactose (Milko Scan 133B; Foss Electric, Denmark). Total nitrogen, non-casein nitrogen (NCN), and non-protein nitrogen

(NPN) were also determined by standard procedures using the Kjeldahl method. Casein nitrogen was calculated as the difference between total nitrogen and NCN; whey nitrogen was calculated as the difference between NCN and NPN. All nitrogen data were expressed as protein equivalent using a conversion factor of 6.38. Somatic cell count (SCC) was determined using a Fossomatic 90 (Foss Electric, Denmark). Renneting characteristics (clotting time, rate of clot formation, and clot firmness after 30 min) were measured by a Foss Electric Formagraph. PL and PG activities in milk were determined according to the method of Baldi *et al*. (1996), modified by Albenzio *et al*. (2004). Activity of total u-PA was performed in a solution containing 100 m*M* Tris Buffer (pH 8.0), 50 Mg/mL plasminogen (P5661; Sigma Chemical Co.), 0.6 m*M* val-leu-lys-p-nitroanilide (Sigma) and 10ML of milk. The reaction solution was incubated at 37°C for up to 3 h and absorbance at 405 nm was measured at 30 min intervals using a microtiter plate reader. One unit of PL, PG, and total u-PA activity was defined as the amount of the enzyme that produces a change of 0.1 in absorbance at 405 nm in 60 min. In order to isolate macrophages from milk leukocytes, milk samples were centrifuged at 2000 x g for 30 minutes at 4°C, the fatty fraction and supernatant removed and cells from the bottom layer suspended in 500 µL of PBS (pH 7.4) containing 0.02% sodium azide (NaN_3). Following two centrifugation cycles (400 x g for 15 minutes at 4°C) a cell concentration of at least 1 x 10^7 cells/mL was obtained. Isolation of macrophages from milk cells was performed by a magnetic positive separation (EasySep, StemCell Tecnologies, Canada), using monoclonal antibody mouse-IgG (MCA919, Serotec, United Kingdom) directed against macrophage surface antigens. The assay was optimized in our laboratory for concentrations of monoclonal antibody and preparation of the milk samples. After separation of macrophages, cells were lysed and activity of u-PA was determined, as previously reported. Data were processed by analysis of variance, using the GLM procedure of SAS (1999). The variation due to stage of lactation was tested. Bulk milk SCC, and isolated macrophages counts were transformed into logarithmic form to normalize their frequency distributions before performing statistical analysis. When significant effects were found (at $P < 0.05$, unless otherwise noted), the Student t-test was used to locate significant differences between means.

RESULTS AND CONCLUSIONS – Stage of lactation influenced the lactose content, which was lower in late lactation than in early and mid lactation ($P < 0.001$, Table 1), and the whey protein content, which decreased in mid and late lactation ($P < 0.01$). Considering the good milk quality, as suggested by the SCC, and whey protein concentrations recorded in the late lactation milk, the decrease in the lactose content during late lactation could be due to the reduction in milk yield with advancing lactation (Albenzio *et al*., 2004). Somatic cell count (SCC) was affected by stage of lactation ($P < 0.01$), the highest value of SCC being detected in the mid lactation milk. In our study, SCC never exceeded 600,000 cells/ml and this can be considered an indicator of good udder health and good milk ability to cheese-making (Sevi *et al*., 1999; Duranti and Casoli, 1991). Stage of lactation also affected milk coagulating behavior, with the shortest clotting time ($P < 0.001$) being recorded in mid lactation milk (16.94 *vs* 22.05 and 28.86 min in early and late lactation, respectively). Rate of clot formation was higher ($P < 0.01$) in late (2.57 min) than in early (1.76 min) and mid lactation (1.47 min), while curd firmness was lower in early (39.53 mm) and late (30.05 mm) than in mid lactation (52.09 mm, $P < 0.01$). Bulk milk composition did not undergo marked changes during lactation, probably because of both the persisting good health of ewe udders through lactation and the bulk milk ability to reduce fluctuations of individual milks. Indeed, no differences were observed for pH values (6.67) and for fat (6.01%), protein (4.99%), and casein content (3.73%) in milk during lactation. The amount of isolated and concentrated macrophages was found higher ($P < 0.01$) in early and mid than in late lactation milk. In a previous study, Albenzio *et al*., (2004) found a relationship between PG conversion and the concentration of macrophages in individual ewe milk with high SCC. These authors ascribed such a relation to the activity of u-PA associated with the macrophage membranes. In the present study, no differences were found for u-PA activity detected in isolated macrophages, while stage of lactation influenced ($P < 0.001$) the total PA activity detected in milk. In addition, u-PA activity from macrophages was lower than total PA activity in milk throughout lactation. Indeed, the activity of u-PA associated with milk macrophages remained substantially constant, while total PA activity underwent a marked increase with the advancement of lactation. This suggests that, in ewe bulk milk from healthy flocks, u-PA activity from macrophages has a limited influence in the conversion of PG into PL. In the present study, PL and PG activities were affected by stage of lactation ($P < 0.001$): in fact higher values of PL activity were recorded in early than in mid and in mid than in late lactation milk. In contrast, the highest PG activity values were detected in mid lactation compared with early and late lactation. The reduction in plasmin activity registered in the mid and late lactation milk led to an increase in the PG/PL ratio. PL activity is the result of the balance between the actions of activators and inhibitors. The reduction of plasminogen conversion into plasmin is not easy to explain; a tentative explanation is the increase in milk of plasminogen activator inhibitors, such as retinoic acid, glucocorticoids, β-lactoglobulin, α-lactoalbumin, α1-antitrypsin and α2-macroglobulin, as suggested by Le Roux *et al*. (2003). As an alternative, the decrease in PG activity during the last stage of lactation could be attributed to the reduced transfer of PG from blood to milk. In fact, the functional state of the mammary gland and the hormone actions are the main regulators of molecule transfer to milk. Finally, along with Weng *et al*. (2006), it was not excluded-that the regulation of PG

activation in the mammary gland is under the control of local factors. In conclusions, in ewe bulk milk from healthy flocks the contribution of macrophages to the regulation of plasmin-plasminogen system does not depend on macrophage concentration and stage of lactation. In addition the activity of u-PA associated with macrophage cells only slightly contributed to total PA activity in milk.

Table 1. Least square means ± SEM of some chemical parameters and enzymatic activities detected in ewe bulk milk and in isolated macrophages during lactation.

Item	Stage of Lactation			SEM
	Early	Mid	Late	
Lactose, %	4.51 ^{A}	4.37 ^{A}	4.04 ^{B}	0.05
Whey Protein, %	1.08 ^{A}	0.88 ^{B}	0.88 ^{B}	0.03
SCC, Log10cells/mL	5.25 ^{B}	5.72 ^{A}	5.29 ^{B}	0.09
Plasmin, U/mL	42.05 ^{A}	31.29 ^{B}	28.19 ^{C}	0.66
Plasminogen, U/mL	4.77 ^{C}	38.91 ^{A}	31.51 ^{B}	1.07
Total Plasminogen-Activator (PA), U/mL	37.43 ^{C}	76.53 ^{B}	260.20 ^{A}	8.66
Isolated Macrophage Count, Log10cells/mL	3.89 ^{A}	3.98 ^{A}	3.42 ^{B}	0.08
Total PA from macrophages, U/mL	7.07	6.75	6.09	1.41

^{A,B,C}*=At least P < 0.05.*

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