CD49f+ mammary epithelial cells decrease in milk from dairy cows stressed by overstocking during the dry period

CD49f^{+} mammary epithelial cells decrease in milk in overstocked-stressed dairy cows during the dry period

Mario Baratta[1*], Silvia Miretti[1], Paolo Accornero[1], Giovanna Galeati[2], Andrea Formigoni[2], Gianfranco Gabai[3], Daniele Nucera[4], Eugenio Martignani[1]

[1] Dept. Veterinary Science, University of Turin, Italy, Largo Braccini 2, 10095 Grugliasco (TO) Italy

[2] Dept. Veterinary Medical Sciences (DIMEVET), University of Bologna, 40062 Ozzano dell'Emilia (BO), Italy

[3] Dept. Comparative Biomedicine and Food Science, University of Padua, 35020 Legnaro (PD), Italy

[4] Dept. Agricultural, Forest and Food Sciences, University of Torino, 10095 Grugliasco (TO), Italy

Short title: **Epithelial cells populations in milk in dairy cows**

*Correspondence: Mario Baratta
Dept. Veterinary Science
University of Turin
Largo Braccini, 2
10095 Grugliasco (TO)
Italy
phone +39-11-670 9146
FAX +39-11-236 9146
E-mail: mario.baratta@unito.it

Summary

This study aims to describe the modification in epithelial cells populations during the first and the last month of milking in Holstein Friesian cows that have undergone different management during the dry period. We report the differential expression of $CD49f^+$ and $cytokeratin18^+$ cell subpopulations managed in overstocking during the dry period (from 21 ± 3 d to the expected calving until calving). Twenty six cows were randomly divided into 2 groups (13 animals each), balanced for the number of lactations, body condition score, and expected date of calving. Cows in the far-off phase of the dry period (from 60 to 21 d before the expected calving date) were housed together in a bedded pack. Then, animals (from 21 ± 3 d before the expected calving) until calving were housed in pens with the same size but under different crowding conditions due to the introduction of heifers (interference animals) into the pen. The control condition (CTR) had 2 animals per pen with 12.0 m^2 each, whereas the overstocked condition (OS) had 3 interference animals in the same pen with 4.8 m^2 for each animal. Cells collected from milk samples were directly analyzed for: CD45, CD49f, cytokeratin 14, cytokeratin 18 and cell viability. Milk samples were collected in two different periods of lactation: early lactation (EL = d 0–30) and late lactation (LL = 270–300).We observed a differential expression with a reduction in $CD49f^+$ ($p<0.01$) and cytokeratin 18^+ ($p<0.05$) cells in EL. These observations suggest that mammary epithelial cell immunophenotypes could be associated to different animal management in dry period and we hypothesize they may have a role as biomarkers for mammary gland function in dairy cows.

Introduction

Increased stocking density is a common practice among dairy producers. One of the main tools to evaluate the effect of such management is to determine the threshold of stressful situation that triggers a number of changes such as activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis that have been considered a well-known source of biomarkers for animal welfare (Prunier *et al.*, 2013). Very recently it has been demonstrated that overstocking during the dry period in Holstein Friesian dairy cow is associated with changes also in DHEA (Fustini *et al.*, 2017).

However, the determination of hormonal pattern to evaluate stressful situation presents some difficulties for the sampling and comparison of hormone levels in a given time interval. Also it would be interesting to include the ability to insert other physiological parameters that are to some extent related to the animal's well being. We have reported the expression of epithelial precursors and fully differentiated cells in bovine milk, highlighting possible variations in the number and features of mammary epithelial cells (**MEC**) subsets in dairy cows (Baratta *et al.*, 2015). MEC are found in milk, caused by shedding during the lactation phase, but the range of cell frequency differ from total somatic cell count (**SCC**) if only the live cell fraction is analyzed. The total amount of somatic cells in milk is affected by different factors, such as species, breeds, lactation phase, milk yield, individual animal differences, and management practices (Rupp *et al.*, 2000). A specific pattern of epithelial cell types has been found in the milk according to the stage of lactation. Cell types include an inner layer of cytokeratin 18 (**K18**)$^+$ luminal cells and an outer layer of cytokeratin 14 (**K14**)$^+$ myoepithelial cells while CD49f$^+$ cells are probably derived from a more primitive stage of cell differentiation (Martignani *et al.*, 2015). In this study we show different mammary epithelial cell types present in milk of dairy cows that have been undergone overstocking during the dry period and hypothesize that specific cell types variations may be related to stressful management.

Materials and Methods

Animals, housing and diet

Twenty six Holstein dairy cows were enrolled in this experiment. All animals were housed at the farm of the University of Bologna (Ozzano Emilia, Italy) and used according to EEC animal care guidelines. The experimental procedures had been approved by the Ethical Committee of Bologna University. Animals were randomly divided into two groups (13 animals each), balanced for

number of lactations, BCS (body condition score) and expected date of calving. Cows in the far-off phase of the dry period (from 60 to 21 days before the expected calving date) were housed together in a bedded-pack and received water and grass hay ad libitum. From 21±3 days until calving animals were housed in two bedded-pack groups where they had ad libitum access to water and were fed daily using total mixed ration. After calving cows were housed together in a bedded pack area for the first 2 weeks of lactation and then moved to a free-stall pen for the rest of lactation. The total mixed rations (TMR) were fed approximately at 7 am for lactating cows and 9 am for dry cows.

Experimental design, blood sampling and hormone assays

Animals, dried off 8 weeks before the expected calving, were housed in pens with the same size (22,5 m^2 in total with 13,5 m^2 of resting area and 9 m^2 of feeding area) but in different crowding conditions due to the introduction in the pen of heifers (interference animals) having a body weight of 450-550 Kg. Control condition (CTR) had 2 animals per pen (one animal of the study with an interference animal) with 11 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 5 m^2 for each animal. Cow were allocated to CTR or OS group based on parity, at 21 days before expected calving dates. The resting area is a deep-bedded pack with straw added twice a day. On days -30, -21, -7 before and 4, 10, 30, 60 relative to calving blood samples were collected from each cow for the determination of plasma DHEA and cortisol (C) concentrations by RIA..

Flow Cytometry Analysis: Sample Processing

Quarter foremilk samples were obtained in accordance with the Veterinary Services Standards of the Italian National Health Service, branch of the Ministry of Health. Before morning milking, teats were scrubbed with 70% ethanol and the first 2 strips of milk were discarded. Aliquots of 200 mL of milk per udder were collected aseptically.. Cells were collected and analyzed according to previously reported (Baratta *et al.*, 2015). Briefly, the determination of epithelial subpopulations in milk was carried out utilizing a 6-color flow cytometry assay. Anti-CD45 antibody (VMRD Inc., Pullman, WA) was used to gate immune cells, anti-human- CD49f–FITC antibody (anti-h-α-integrin-6-FITC, Novus Biological, Littleton, CO), monoclonal anti-CK peptide 18 antibody (clone KS-B17.2, Sigma, St. Louis, MO), and anti-CK14 antibody (Covance, Life Technology, Thermo Fisher).. Stained samples were analyzed using an Attune Acoustic Focusing Cytometer (Life

Technologies). Cells without antibody labeling served as a negative control and were regarded to be a measure for background fluorescence. Fluorescence Minus One (FMO) controls were used to identify data spread due to the multiple fluorescent signals (2000; Bayer *et al.*, 2007). Epithelial cells were identified and counted in the total living $CD45^-$ cell population.

Statistical Analysis

The two groups of cows (13 animals each) were compared on the following variables: living cells, $CD49f^+$, $K14^+$, $K18^+$, and $K14^+18^+$; values were collected at the beginning and in the last month of lactation. Considering that all variables were frequencies, non-parametric test were performed for all the analyses. In particular, Mann-Whitney U test was chosen and, firstly, variables were compared between the groups of cows at the first month of lactation. Secondly, the same analyses were repeated for measures collected in the last month of lactation, in order to explore for differences in significant results. Results were considered significant when associated at least to $p<0.05$ for all the comparisons.

Results

Hormones concentrations

In overstocking group (OS) significantly ($P<0.01$) DHEA significantly increased compared to CTR group at day -7 (2.13±0.63 vs 1.47±0.46 pmol/ml) while C did not differ between CTR and OS group (see supplementary data).

Frequency of epithelial subpopulations during the first month of lactation

Figure 1a shows total living cells (ranging from 66% to 78%) detected in the somatic cell population, identified as $CD45^-$ cells, in Holstein Friesian cows in response to stress induced by overstocking (OS) or not (CTR) during the dry period. A significant difference between the two groups was observed in $CD49f^+$ cells ($p<0.01$) with a decrease in OS group of frequency from 20 to 5 %. Interestingly, we observed a significant difference ($p<0.05$) in the level of luminal cells ($K18^+$) with a decrease in OS group. Finally, no differences were detected in myoepithelial ($K14^+$) and $CK14^+/CK18^+$ cells.

Frequency of epithelial subpopulations during the last month of lactation

Figure 1b shows total living cells ranging from 63% to 75% detected in the somatic cell population, identified as $CD45^{-}$ cells, in cows that were exposed to stress during the dry period induced by overstocking (OS). A tendency to a decrease in OS group was observed without reaching a statistical difference was (p= 0.066). Luminal cell ($K18^{+}$) were present at low frequency in both group (2-3 %) while myoepithelial cells ($K14^{+}$) still showed a greater concentration ranging from 18 to 21%. Finally, no differences were detected in myoepithelial, luminal and $CK14^{+}/CK18^{+}$ cells between the two groups.

Milk yield in response to treatment over transition period

Mean milk yield (kg/d) in response to treatment over the transition period was not different among treatments (Table 1). Among cows, treatment did not differ regarding previous lactation 305-d mature- equivalent milk yield (CTR = 10.1 ± 215.1 kg, OS = 9.5 ±187,7 kg; P = 0.35).

Discussion

It has recently been reported that DHEA secretion is affected in response to overstocking during the dry period in Holstein Friesian cows (Fustini *et al.*, 2017). We reported that DHEA concentrations were affected only during the dry period, when the stressful stimulus was applied, while no differences in DHEA secretion were observed during the first two months of lactation. Since the placenta seems the most important DHEA source in the late pregnant cow (Gabai *et al.*, 2004), it is possible that overstocking stimulates the release of DHEA from the maternal-foetal units through a still unknown mechanism. In the present work we cannot investigate on the source of this metabolite; however, we can confirm that in dairy cow DHEA plasma levels are affected during the last part of pregnancy by stressful management like overstocking that usually occurring during the dry period.

We mainly focused our attention on the frequency and differential expression of epithelial cells subpopulations in milk. We have previously reported the expression of epithelial precursors and fully differentiated cells according to the phase of lactation (Baratta *et al.*, 2015). We report now a further information that lead us to consider the hypothesis that different distributions of MEC subpopulations may provide more detailed information on the physiology of the mammary gland during lactation in dairy cows. In particular, our data suggest that stressful situations can affect the somatic cell subpopulations. We considered the first and the last month of lactation period that

212 received or not the stressful experience of overstocking monitored by the change of DHEA during
213 the last days of dry period. We observed a different pattern of expression between the two groups of
214 animal in the first month of lactation but not at the end of the physiological period indicating that
215 the stressful-experienced cows showed a lower expression in $CD49f^+$ and $K18^+$ cell populations.
216 We were interested on CD49f population evaluation since they belong to more primitive MEC
217 (mammary precursors). They appear to decrease during the decline of lactation and in this way may
218 exert a role in the reduction of the mammary secretory function, which adjusts the number of active
219 secretory cells. We hypothesize that this subpopulation may be considered the signal of a reduction
220 in mammary efficiency. The presence of CD49f positive cells, even if in a low number, may be
221 related also to the reduction in the myoepithelial compartment that indicated the modification of the
222 myoepithelial genetic program (Garbe *et al.*, 2012). This integrin has been shown to be a
223 component of a feedback circuit that regulates the myoepithelial phenotype in mammary epithelial
224 cells from humans and mice (Deugnier *et al.*, 1999; LaBarge *et al.*, 2009) suggesting that the basal
225 regulatory machinery may be disrupted in myoepithelial cells and inappropriately engaged in
226 luminal epithelial cells, maybe during the aging process. We did not observe a significant difference
227 in K14/K18 double positive cells, in term of activation of regenerative functional tissue of
228 mammary gland, in particular during the final phase of lactation. On the contrary, we have observed
229 a difference in $K18^+$ cells with a decrease in OS group during the first month of lactation. This
230 subpopulation are specifically linked to the secreting cells since they are referred as luminal cells.
231 We would expect this difference to be associated with a reduction in milk yield during the period of
232 milking, although we did not observed any decrease in milk production. The exposure to stressful
233 conditions might influence the numerical relationship between luminal cells that produce milk in
234 the mammary gland and epithelial cells that are shed in milk. One aspect that deserves to be
235 thoroughly investigated is the number of functional cells found in milk needed to detect an effect on
236 milk production.
237
238 **Conclusions**
239 In conclusion, we report the expression of epithelial precursors and fully differentiated cells during
240 the first month of lactation in dairy cows that were overstocked during the previous dry period,
241 highlighting variations in the number and features of MEC subsets in milk. Although we were not
242 able to detect a correlation with milk production, it remains interesting to observe that overstocking
243 associated with hormonal pattern during dry period shows different modulation of somatic cells

during the lactation. Further studies are necessary to determine if different distributions of MEC subpopulations may provide more detailed information on the physiology of the mammary gland during lactation in dairy cows and, potentially, have an application to evaluate mammary gland functionality as biomarkers.

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285
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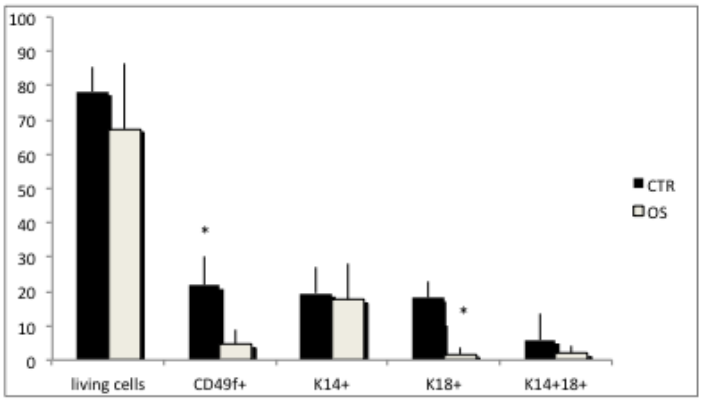
Figure Legends

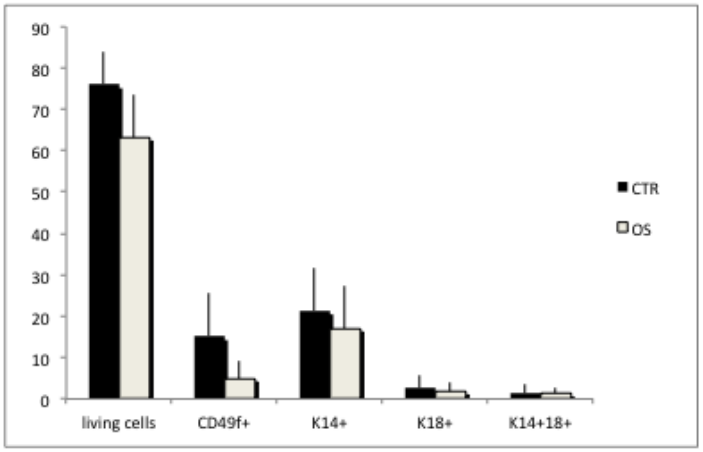
Fig.1 Frequency in percentage of cell viability and of epithelial cell subpopulations in bovine milk during the first month of lactation (**a**) and during the last month of lactation in control (**b**) in control (CTR) and overstocked condition (OS) groups. Cell subpopulations are identified according to the positive expression of CD49f, K14 and K18. * mean at least $P < 0.05$. Error bars represent SD.

Tab.1 Mean ECM yield (kg/d) in response to treatment experienced in the transition period

Fig.1



a



b

303 **Table 1**

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Week after calving	Control (CTR)	Overstock condition (OS)	SEM	P-value
1	23,5	22,3	1,4	0,65
2	34,9	31,9	1,5	0,13
3	35,8	34,4	1,6	0,23
4	37,1	35,9	1,3	0,18