Comparison between acutely- and naturally-induced involution of mammary gland in dairy cows

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ABSTRACT

We have hypothesized that the dissociation of the dam-offspring interrelationships (i.e., induction of mammary gland involution) is preceded by metabolic and immunological adaptive response. In the present study it was shown that genetically high-producing cows producing low amount of milk before the expected period of involution show signs of auto-defense metabolic mechanism that was associated with an orderly involution process and effective antimicrobial immune response. On the other hand, in non-adapted cows that yielded copious amount of milk before expected involution this process was associated with acute response (neutrophilia and lymphopaenia) that resembled a wood-healing response and was much less effective in terms of antimicrobial clearing capacity.

Key words: involution, mammary gland, adaptation**,** acute response

Running head: mammary gland involution in dairy cows

*Abbreviations***:** **AFI** - cows approaching forceful involution, **ANI** - cows approaching natural involution, **CF** - curd firmness, **LDH** - lactate dehydrogenase, **P** – plasmin, **PA** - plasminogen activator, **PLG** – plasminogen, **PMN** – polymorphonucler, **P-P** - proteose peptone, **RCT** - rennet clotting time, **SCC** - somatic cell count

INTRODUCTION

Most of the development of the mammary glands occurs postnatally. After weaning, which coincide typically with the end of the lactation cycle, the glands regress to its pre-lactating state (2, 21). The first stage of Inv (stage I) involves the widespread apoptosis of the alveolar epithelial cells, which reverses the striking growth of the glands during the previous cycle of proliferation (3, 21). The first phenotypic sign of induction of Inv stage I is the disruption of the tight junctions between epithelial cells, which is reflected in a marked increase in the concentration of plasma electrolytes, sodium and chlorine, and marked inflow of leukocytes into the mammary gland (40, 41). The secretion of components of the innate immune system such as immunoglobulins, lactoferrin (40), albumin (39), and many other soluble components of the innate immune system (6, 49) is also elicited at this stage.

In contrast to rodents, in which Inv is completed within less than a week (19, 21), Inv in cows is completed only 21–30 days after drying-off and never attains the histological features (extensive degradation of the secretory tissue) seen during stage II in rodents and other mammals. Reduction in milk secretion and initiation of extensive apoptosis of the epithelial cells occur considerably later than in rodents (3). However, the lack of noticeable involution stage II in bovine seems to reflect more the fact that modern cows are induced into Inv while being pregnant in the third trimester rather than real physiological differences with other mammals (13).

Involution of the mammary gland in most mammals under their natural environment begins when their offspring's stop suckling. However, most of the relevant research in bovine and mice is carried out by induction of abrupt Inv (3, 6), probably because this procedure is easier to manipulate and to be carried out under controlled conditions. Though, there is no evidence to sustain that conclusions based on abrupt Inv can be extended to the process of natural involution. This question is important because recent evidence on mice induced into Inv abruptly suggest that the Inv process resembles the course of wound healing (6, 50), which led to suggestions that mammary Inv in general increases the risk of acquiring mammary cancer (23, 36, 38).

The classical view, however, is that Inv of the ovary and mammary glands are an orderly, programmed, physiological process (26, 30); a view which coincide with basic evolutionary considerations. In mice and bovine, Inv is associated with immunemodolatory responses that are essential for clearing the sloughing apoptotic cells and preventing new infection (3, 31). Thus, while there is no doubt that Inv is intensely involve with activation of the immune system, the question whether it is an orderly tissue-remodeling process or a process that include elements of acute (wound-healing like) inflammation remained questionable in the case of natural involution.

The aims of the present experiment were: i. testing the hypothesis that Inv of the mammary glands in cows undergoing natural Inv (sudden cessation of milking in late-lactating-low yielding cows) is preceded by metabolic and immunological adaptations and ii. that the Inv induced in non-adapted cows (sudden cessation of milking in high yielding cows) is distressing and is distinctly different from that in cows undergoing natural Inv.

MATERIALS AND METHODS

Study design

All protocols were approved by the Institutional Animal Care Committee of the Agricultural Research Organization, which is the legitimate body for such authorizations in Israel.

The experiment was carried out in 10 multiparous lactating Holstein cows (at their 2nd and third lactation) at ~300 days in pregnancy (i.e., ~60 days before their expected parturition), which were scheduled to dry-off treatment (cessation of milking in order to induce Inv). At the present lactation the milk yield of all the cows was ~10,500 kg. The cow's glands were ascertained of being free of bacterial infection as previously described (16). Five of the cows that exhibit a sharp reduction in milk yield at the end of their lactation and produced less than < 14 L d^{-1} were defined as cows approaching natural involution (ANI) and five cows that produced between 25-35 L d^{-1} were defined as cows approaching forceful involution (AFI).

Three days prior to drying-off schedule, milking was stopped and milk was collected and analyzed at 0 (before last milking) 1, 2 and 3 days thereafter. Following the last sampling all cows were treated with antibiotic dry-off treatment as practiced by the veterinarian of the herd (16).

Sampling and analysis

Samples (~100 mL) of mammary glands secretion were taken from each gland (i.e., 60 glands per group) at 0700 h. Whole milk samples were analyzed within 12 h as follows:

Curd firmness (CF) and rennet clotting time (RCT) were determined by the Optigraph (Ysebaert, Frepillon, France) (24). Somatic cell count (SCC) was determined by the Fossomatic 360 (Foss Electric, Hillerod, Denmark) and somatic cells differentiation by FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA) (17, 46) using anti-bovine monoclonal antibodies (VMRD Inc., Pullman, WA, USA). Monoclonal antibodies were: anti-CD18/11a - BAT 75A (IgG-1), anti-CD4 - GC 50A1 138A (IgM), anti-CD8 - CACT 80C (IgG-1), anti-CD14- CAM 36A (IgG-1), anti-polymorphonucler (PMN)(G1)(IgM). The following secondary polyclonal antibodies (CALTAG Laboratories, Burlingame, CA, USA) were used: goat anti-mouse IgG-1 conjugated with TRI-COLOR and goat anti-mouse IgM conjugated with FITC. The amount and proportion of epithelial cells in mammary secretion was evaluated as the difference between SCC and total leukocytes ($CD18^+$ cells) in the sample (17). Apoptosis of the PMN was analyzed by means of annexin V staining by a commercial kit (BD Bioscience, Detection Kit I, provided by Bactlab Diagnostics, Caesarea, Israel) according to the manufacturer instructions. Preserved samples were analyzed for lactose, fat and total protein in a central laboratory of Israel Cattle Breeders Association (17).

A second portion of the samples were defatted and analyzed for the activity of Plasmin activator (PA), plasminogen (PLG) and plasmin (PL) (48), concentration of casein, proteose peptones (P-P) (47), nitrite, lactoferrin (45), albumin (39), lactate, malate and citrate and lactate dehydrogenase (LDH) activity by fluorimetric methods (12, 42).

Statistical analysis

The statistical analysis procedures were carried out with the JMP software (37). All measures were analyzed according to the following model:

$$\mu = \alpha i + \beta j + \alpha\beta ij + eij$$

where μ = Mean of all data, αi = effect of treatment, i (i = 1; ANI cows, or 2; AFI cows); βj = effect of sampling (j = 1, 2, 3, or 4 for days 0,1 2 and 3); $\alpha\beta ij$ = treatment × sampling interaction; and eij = residual variance between measurements (random error).

RESULTS

Milk yield and gross composition

Milk yield of ANI cows was 9.2±0.76 L d^{-1}, and 28.8±1.59 L d^{-1} in AFI cows ($P < 0.0001$). After milk cessation, only modest swelling was observed in the udders of the ANI cows. In contrast, the glands of the AFI cows, particularly at the first days, were markedly engorged, which was associated with secretion leakage and vocalization, suggesting that the cows were in pain.

At day of milking cessation, lactose concentration in the ANI cows was 6% lower compared to the AFI ones ($P < 0.05$; Table 1). After drying-off, lactose concentration dropped faster in the ANI cows than in the AFI cows. At day 3, lactose concentration in the ANI cows was 69% lower than the pre-drying value ($P < 0.001$) and 24% lower than in the AFI cows ($P < 0.05$).

Before drying-off, the concentrations of fat and protein in the milk of the ANI cows were significantly higher ($P<0.05$) than in the AFI cow's milk (Table 1). After dry-off, fat concentration in the ANI cows dropped in comparison to the pre-dry-off, and the fat/lactose concentration ratio indicated that at day 1 and 2 fat level drooped faster than lactose level whereas at day 3, the rate of reductions was about the same for lactose and fat. Fat concentration during the 3 days after drying-off in the AFI cows was significantly higher than at the beginning, and the fat/lactose ratio indicated that changes in fat secretion were minor in comparison to lactose secretion. Protein concentration increased in both groups, but more dramatically in the AFI cows, in which it almost doubled (Table 1). The protein/lactose ratio indicates that the rate of the decrease in protein secretion was lower than the decrease in lactose secretion, particularly in the ANI cows in comparison to the AFI cows.

Milk curdling, the plasmin system and formation of proteose peptone

Before the inducement of involution, RCT was longer and CF was lower in the ANI compared to the AFI cows (Figs. 1A, 1B). After induction of involution, these parameters deteriorated rapidly in the ANI cows, so that on day 3 mammary secretions did not curdle at all (Fig. 1B). In the AFI cows, significant changes in RCT and CF were observed only on day 3 after the inducement of involution, and were moderate than in the ANI cows (Figs. 1A, 1B).

Before the inducement of involution, PA activity, the conversion of PG to PL (as reflected by lower PG/PL ratio) and actual PL activity were higher in the ANI compared to the AFI cows (Table 2). Inducement of involution in the ANI cows was associated with increased conversion of PG to PL and PL activity, though PA activity was not increased and even dropped at day 3 post induction of involution. Differently, in the AFI cows, PA activity was modestly increased only on day 3 post involution (Table 2).

Before the induction of involution, P-P concentration was higher in the ANI cows than in the AFI ones (Table 2). Following induction of involution, P-P concentration increased from day 0 to day 3 in the ANI cows, in direct linear relationship to increase in PL activity ($P < 0.001$; supplementary Fig. 1), clotting time ($P < 0.001$; supplementary Fig. 2) and inverse linear relation to the parameters of milk clotting parameters ($P < 0.01$; supplementary Fig. 3). In the AFI cows, a more modest increase in P-P concentration was observed only on day 3 post-involution (Table 2). However, the interrelationships between P-P concentration and PL and between P-P and milk clotting parameters were similar in both groups (the above correlations refer to both groups).

Measures of metabolic activity

In the ANI cows, lactic and malic acid concentrations before the induction of involution were higher than in the AFI cows, whereas citric acid was lower than in the AFI cows (Table 3). After the induction of involution, the concentrations of lactic and malic acids increased with time along with reciprocal reduction in citric acid concentration. In the AFI cows, no significant change in the concentration of these acids was observed during the experiment. Consequently, the citric acid/lactic + malic acid concentration ratio that was ~ 30 before induction of involution dropped to ~ 5 on day 3 after induction of involution in the ANI cows, while it was maintained at ~40 in the AFI cows throughout the experiment (supplementary Fig. 4).

Measures of inflammation

Before the induction of involution nitrite concentration and LDH activity were higher in the ANI cows than in the AFI ones (Table 3). After induction of involution the concentration of nitrite and LDH activity increased with advance of involution in the ANI cows, whereas in the AFI cows, an increase in LDH activity was noted from days 2 after induction of involution and the increase was smaller than that of the ANI cows (Table 3).

Soluble immune components: lactoferrin and albumin

Before the induction of involution, lactoferrin and albumin concentrations were higher in the ANI cows than for the AFI ones (Table 3). After induction of involution, the concentration increased with advance of involution in the ANI cows, whereas in the AFI cows but not in the ANI cows (Table 3).

Sodium and potassium

Before Inv, the concentration of Na^+ was higher in the milk of the ANI cows than in the AFI ones (42±5 vs. 21±3), while that of K^+ was lower in the ANI cows (29±4 vs. 40±4). From day 1 and onward, dramatic changes in the concentration of monovalent ions (increase of Na^+ and decrease of K^+) occurred in the mammary secretion in both groups, so that their concentration resembled the expected concentration of their blood plasma (Na^+, 119±9 and K^+,9±4; Supplementary Fig 5, 6)

Milk leukocyte population

The SCC in milk was higher in the ANI cows than in the AFI ones before the induction of Inv. After the induction of Inv, SCC increased in both groups, but was at each stage significantly higher in the ANI cows. The numbers and the proportion of the different leukocytes are presented in Table 4 and Fig. 2. Total leukocytes ($CD18^+$ bearing cells) of SCC in milk of the ANI cows was higher than in the AFI ones before the induction of Inv. After the induction of Inv, the number of leukocytes increased in the ANI cows in a steady manner, reaching $>1.5 \times 10^3$ cells mL^{-1} and to > 85% of the SCC on day 3. The increase in leukocytes of these cows were mononuclear cells: T-lymphocytes and macrophage (Fig. 2B, 2C, 2D), in the first day and only moderately PMN (Fig. 2A), and only from day 2. Differently, in the AFI cows there was a burst of leukocyte count 24 h following the induction of INV, which increased the proportion of leucocytes to 93% of total SCC. This increase was counted for PMN only (Fig. 2A). In all cows the number of B-cells was negligible.

The proportion of live PMN (annexin negative PMN) was about the same (~23%) in the ANI and AFI cows before the induction of Inv. Following induction of Inv in the AFI cows, the percentage of live PMN remained constant in days 1 and 2 and dropped to ~15% on day 3. In contrast, in the ANI cows the percentage of live PMN increased to ~50% on day 1 and remained > 35% on days 2 and 3 (the data is not presented in graphs or tables).

DISCUSSION

Abrupt involution induces distress and non-effective antibacterial immune response

Our data suggest the abrupt Inv induced in non-adapted high yielding cows provoked a distressing response, which was associated with neutrophilia. Similar response was also observed in beef cows under abrupt weaning situation (22). Abrupt Inv in mice at the early stage (within 12 h) induced genes associated with an early acute-phase response that occurs in the mammary gland itself and resembles a wound healing process (6, 50). Consistent with the physiological evidence, behavioral research has shown that breaking the mother-offspring bond alone represents an emotional stressful experience for both (28). In addition to that, modern dairy cows are usually induced into Inv by abrupt cessation of milking while still producing considerable amounts of milk: 20, 40 and sometimes even 50 L d^{-1}. Such a practice results in the accumulation of massive amounts of milk in the udder, which leads to udder engorgement and milk leakage, and frequently causes noticeable agony to the cow, which screams loudly for several days (14).

The inflow of neutrophils to injured or infected sites forms the first line of the immunological defense (5). Thus, the question that arises is why abrupt involution induces such an urgent situation response? Typically, between 20 to 40% of the mammary glands in modern dairy herds are infected with bacteria (9). Thus, the disruption of the tight junction represents a threat for their penetration to the blood (bacteremia) where they can cause life threatening conditions.

In addition, there are evidences that disruption of the tight junction results in exchange of substances between the mammary gland lumen and systemic fluids according to their respective concentration gradients (7, 29). It has long been known that casein (the major milk protein) is a potent inflammatory mediator that induces the chemo-tactic migration of neutrophils when injected into mice tissues (25). Casein hydrolyzates (proteose peptones) are present in milk (as also shown here) and induce chemo-tactic migration of leukocytes to the mammary gland lumen (40, 41). Thus, neutrophils may serve as gate-kippers of the disrupted epithelia and prevent the penetration of pathogens and substances that might cause uncontrolled inflammation (sepsis) in the systemic fluids by phagocytosing them at the narrow opening between the disrupted cells. Evidence for release of neutrophil extracellular traps in inflamed tissue (11) indirectly supports this assumption. The fact that the PMN in the AFI cow's milk contained high proportion of apoptotic cells is also consistent with the notion that their main role is in gate keeping rather than in phagocytosing bacteria in the gland lumen

In general, our results are consistent with previous findings which show that the method of drying-off cows influences the involution process and can affect natural defense systems during the dry period (15, 32, 34, 45). According to the present study, the less effective antibacterial response in acute involution might be related to the urgent need of the organism to divert large proportion of its metabolic resources to support the large inflow of PMN to the mammary gland in response to disruption of the tight junctions, as discussed above. Secondly, it may be related to slow activation of the immune system. In previous studies, it has been shown that enzymatic cooperation between xanthine oxidase and lactoperoxidase in formation of the powerful bactericidal radical, NO_2^- plays an important role in creation of antimicrobial environment in mammary secretion of cows induced into Inv (45, 46). Consistent with the present study, it was also shown that in cows induced into Inv while producing large volume of milk the activation of this system was slower because of slower activation of the plasmin system (45).

Natural involution is preceded by metabolic adaptive responses

Previous results led us to hypothesize that natural Inv is preceded by metabolic and immune responses, which make the involution process much more effective in terms of development of antimicrobial environment in the mammary gland lumen (13, 17, 45). As discussed below, this assumption was sustained by the present results.

In the ANI cows, cessation of milking occurred without evidence of alveolar distension caused by the milk stasis. In contrast, in the AFI cows, milk cessation was associated with considerable engorgement of the mammary gland and with vocalization, which suggests that the cows were in pain (14). Lack of mammary gland distension was also found in mice undergoing natural involution (35). The results reported for mice support the idea that the gradual nature of the process had allowed autocrine control to match the rate of milk secretion progressively to the pup's falling demand for milk (35). The analogy between mice and cows suggest that the autocrine metabolic adaptation in both species are cued by a biorhythmic mechanism according to the stage of lactation and that this mechanism is maintained in some of the 'modern' cows, despite selection for milk production and persistency.

The gross composition of milk in AFI cows was within the normal range in Israeli Holstein cows and consistently, CF and RCT of the milk were in the normal range (24). On the other hand, changes in the ANI cows indicated that their milk underwent substantial changes in comparison to the AFI cows. The casein level and P-P content indicates that the casein micelle underwent considerable degradation. Furthermore, following cessation of milking (i.e., inducement of Inv), these parameters changed much faster in the ANI cows compared to the AFI cows. In previous studies, it was found that sudden and acute imposition of milk stasis resulted in more drastic reduction in lactose and milk volume than in the secretion of fat and protein, which was reflected in increased concentration of fat and protein in mammary secretion (40, 41). Thus, this type of response is consistent with the results found in the AFI cows, in which the induction of Inv occurred without pre-adaptation to milk cessation. In cows adapted to heat stress (47), or when milk is sampled from AFI cows after three weeks of cessation of milking (45), the reduction in fat and protein concentration in mammary secretion exceeded that of lactose (i.e., the ratio of fat or protein to lactose concentration became smaller than 1), as found in the ANI cows in the present experiment. Thus, the present result support the assumption that differences in gross composition of mammary secretion between the ANI and AFI cows reflect metabolic adaptations to the forthcoming involution in the formers.

Increased casein degradation and P-P content negatively affect RCT and CF which are important parameters of milk quality for cheese production (18, 24). Thus, the considerable changes in these values in the ANI cows are consistent with the evidence showing that casein underwent degradation. The main indigenous enzyme in milk that is responsible for degradation of casein is PL, which is derived from its zymogen, PLG, by PAs (43). This system was activated in ANI cows in comparison to AFI cows, which exhibit typical values to Holstein cows producing plenty of milk (16). Following induction of Inv, the activity of PL, due to conversion of PLG to PL (as reflected in the PLG/PL activity ratio), increased more firmly in the ANI cows and this was strictly related to increase in the concentration of

P-P in mammary secretion (Table 2). Our results are consistent with previous conclusions that the physiological importance of avoidance of milk clotting during milk stasis or infection may be related to prevention of uncontrolled (necrotic) inflammation (17).

The involvement of the PA-PG-PL system in regulating milk secretion (23, 43) and the induction of mammary gland Inv (8, 20, 33, 40, 41, 43) are well established. The effect of the PA-PG-PL system in regulation of mammary gland Inv in mice was related to its contribution to enhanced degradation of the extracellular matrix (8, 20). However, this explanation fell to explain its effect on milk secretion (47) and the increase in PL activity immediately after the start of active involution (i.e., cessation of milking) in the ANI cows, because in both cases there is no increase in extracellular matrix degradation; a process that starts to be notable at involution stage II (21). Involution stage II in cows starts to be notable only 3 weeks after cessation of milking (1, 3, 45). On the other hand, the present results are in line with previous reports (40, 41, 45), which suggest that the PA-PG-PL system works in mammary secretion by increasing casein degradation and liberation of active components that are involved in negative control of lactose and milk secretion. Thus, the lower lactose concentration and milk yield in ANI cows compared to the AFI ones is consistent with this explanation. A scheme that illustrates the simultaneous and proportional response for the interrelationship between plasmin activity and lactose secretion on the one hand and plasmin activity and milk quality on the other hand was recently presented (17).

Milk production is an energetic demanding process; the production of every 10 L of milk almost doubles the maintenance requirements (10). However, the mechanism responsible for spontaneous reduction in milk yield in the ANI cows, despite the fact that their food was the same as for the AFI cows as well as their genetic potential for milk yield, was not clear so far. We recently provided evidence based on reduction in citric acid secretion and its replacing by increase in lactic and malic acid secretion reflects shifting of mammary metabolism toward glycolysis on the account of mitochondrial oxidative metabolism (44). This shift under conditions of acute inflammation was regarded as important means that allows the organism to free essential metabolic resources to fight the infection. The present results suggest that the ANI cows utilized similar mechanism to reduce their milk yield towards the expected period of disconnection of the bonds with their offspring. After milk cessation, changes in these parameters suggest the trend of shifting from mithochondrial to cytosolic metabolism increased from day 0 to day 3 in the ANI cows, while no such changes were noted in the AFI cows. Thus, maintaining high rate of metabolism in the AFI cows during the first 3 days after milk cessation was associated most likely with considerable secretion, which contributed to the dramatic engorgements of their udders. Previously, we observed that the start of relaxation of udder engorgements in AFI cows started only at the 4th day after milking cessation (14). The much lower yield of ATP in glycolytic process than in oxidative process should entailed drastic drop in oxygen demand, and consequently in blood flow to the mammary gland and overall metabolism of the glands, which can explain drastic reduction in mammary gland secretion and lack of evidence for udder engorgements in the ANI cows. The increase in secretion of malic acid under anaerobic conditions is rather a new finding; however, it can be explained by classical enzymatic reaction of intermediate metabolic reactions in the cell sup (44). Leaking of mammary secretion because of inner udder pressure is a major cause for acquiring bacterial infection at the early period that follows milk cessation (34). Thus, the metabolic adaptation linked to reduced energy metabolism of the mammary glands enables to reduce mammary secretion and thus to prevent glandular engorgements and leakage.

Natural involution is preceded by immunological adaptive responses

It is suggested that the pre-Inv adaptations in the ANI cows enabled a more effective tissue remodeling cascade in response to the induction of Inv, without the negative effects of neutrophilia. Evidence that macrophages and epithelial cells involve in clearing apoptotic cells in postpartum Inv were presented (2, 26, 27), but little is known about the contribution of $CD4^+$ and $CD8^+$ lymphocytes. Further research, such as further-sub-typing the T-cells, the cytokines secreted and their role is needed for deeper elucidation of the role of the acquired immune system in the Inv process. However, the present results suggest that natural Inv is an interesting model to study the interaction between innate and acquired immunity; a subject of great interest in past years (4). Because T-cells are characterized by long-term memory, it is tempting to speculate that these cells and milk antibodies serve to recognize a wide spectrum of pathogenic microbes, their toxins and milk proteins that are repeatedly involved in posing an immune stress to the systemic system.

CONCLUSIONS

Previously, we have shown that that the disappearance on Inv stage II in modern dairy cows may be related to the fact that, unlike in most mammals, they are induced into Inv while being pregnant (13). In this study, we show that the extensive selection toward high milk yield affected in most cows the rate of Inv, particularly at stage I. Metabolic and immunological adaptation toward the expected disengagement between dams and their offspring is an auto-defense mechanism. The large scale of cell apoptosis that characterize Inv stage I require effective clearance of the dead cells to avoid necrosis and uncontrolled inflammation. The tendency of modern cows to secrete great amount of milk at drying-off increases considerably the risk for acquiring new intramammary infection soon after because of increase of milk leakage and slower build up of the mammary defense system. The metabolic and immunological adaptations described in cows which preserved the auto-defense mechanism prevent this problem to a large extent. On the other hand, when Inv is induced in non-adapted animals, the response is more hurtful and resembles the general response to wounding. Thus, finding techniques to precipitously dry up milk secretion at the induction of Inv, should be beneficial to modern dairy cows, as demonstrated in recent studies (14,15).The distressing responses to sudden Inv are similar to those described recently in mice, suggesting that our findings have wide biological implications.

FIGURE LEGENDS

Figure 1

Rennet clotting time (1A) and curd firmness (1B) in milk of cows approaching natural involution (ANI; ○) and of cows approaching forceful involution (AFI; □) before the induction of involution (milk cessation) and during the first 3 days thereafter (Mean±SD).

Figure 2

The proportion of polymorphonuclears (2A), CD4$^+$ T-lymphocytes (2B), CD8$^+$ T-lymphocytes (2C) and CD14$^+$ macrophages (2D) in milk of cows approaching natural involution (ANI; ○) and of cows approaching forceful involution (AFI; □) before the induction of involution (milk cessation) and during the first 3 days thereafter (Mean±SD).

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Table 1. Milk composition (lactose, fat and total protein) of cows approaching natural involution (ANI) and of cows approaching forceful involution (AFI) before the induction of involution (milk cessation) and during the first 3 days after the induction of involution (Mean±SD)

Treatment	Time*	Lactose g kg^{-1}	Fat g kg^{-1}	Fat/Lactose ratio	Protein g kg^{-1}	Protein/Lactose ratio
Natural involution (ANI)	0	47.0±0.8 ^{a}	39.4±1.2 ^{b}	0.84±0.02 ^{b}	42.3±1.6 ^{b}	0.90±0.12 ^{b}
	1	37.2±2.6 ^{b}	21.6±2.3 ^{d}	0.58±0.09 ^{c}	45.1±1.9 ^{b}	1.21±0.13 $^{a b}$
	2	32.9±2.1 ^{b}	22.7±2.8 ^{d}	0.70±0.09 ^{b}	47.2±1.9 ^{b}	1.44±0.15 ^{a}
	3	24.8±3.5 ^{c}	29.2±3.7 ^{cd}	1.18±0.10 ^{a}	51.7±2.7 ^{b}	2.08±0.21 ^{a}
Forceful involution (AFI)	0	49.9±1.1 ^{a}	34.9±0.1 ^{c}	0.70±0.07 ^{b}	36.6±1.4 ^{b}	0.73±0.11 ^{b}
	1	45.3±0.7 ^{a}	39.2±1.1 ^{b}	0.88±0.11 ^{b}	42.6±1.4 ^{b}	0.94±0.13 ^{b}
	2	37.1±1.0 ^{b}	44.1±1.4 ^{b}	1.19±0.13 ^{a}	56.1±2.5 ^{ab}	1.51±0.16 ^{a}
	3	32.7±1.6 ^{b}	49.3±0.5 ^{a}	1.51±0.12 ^{a}	65.4±3.3 ^{a}	2.01±0.19 ^{a}

* Days after milking cessation

$^{a-b}$ Results within columns with no common superscript differ significantly ($P < 0.05$)

Table 2. Activity of plasminogen activator (PA), plasminogen (PLG) and plasmin (PL) and the concentration of proteose peptons (P-P) in caws approaching natural involution (ANI) and in cows approaching forceful involution (AFI) before the induction of involution (milk cessation) and during the first 3 days after the induction of involution. (Mean±SD)

Treatment	Time*	PA U mL^{-1}	PLG U mL^{-1}	PL U mL^{-1}	PLG/PL	P-P mg mL^{-1}
Natural	0	443±14^{a}	147±9^{a}	32±6^{b}	4.60±0.51^{b}	1.01±0.11^{b}
involution	1	423±13^{a}	144±12^{a}	36±6^{b}	4.01±0.51^{b}	1.21±0.12^{a}
(ANI)	2	418±53^{a}	142±8^{a}	39±6^{b}	3.61±0.60^{b}	1.32±0.14^{a}
	3	272±9^{c}	94±9^{b}	66±7^{a}	1.42±0.48^{c}	1.55±0.13^{a}
Forceful	0	352±23^{b}	131±10^{a}	22±6^{c}	5.95±0.50^{a}	0.86±0.09^{b}
involution	1	345±27^{b}	128±9^{a}	23±5^{c}	5.56±0.55^{a}	0.85±0.08^{b}
(AFI)	2	298±28^{c}	136±10^{a}	18±3^{c}	7.55±0.65^{a}	0.87±0.09^{b}
	3	363±16^{b}	127±10^{a}	33±12^{b}	3.85±0.63^{b}	1.03±0.10^{b}

* Days after milking cessation

$^{a\text{-}b}$ Results within columns with no common superscript differ significantly ($P < 0.05$)

U = activity unit; 1 unit is the amount of PA/PLG/PL that produces a change in absorbance of 0.1 at 405 nm in 60 min.

Table 3. Concentration of lactate (LA), malate (MA), citrate nitrite, activity of lactate dehydrogenase (LDH) and concentration of lactoferrin and albumin in milk of cows approaching natural involution (ANI) and in cows approaching forceful involution (AFI) before the induction of involution (milk cessation) and during the first 3 days after the induction of involution (Mean±SD)

Treatment	Time*	LA (mM)	MA (mM)	Citrate (mM)	Nitrite (µM)	LDH (U mL^{-1})	Lactoferrin µg mL^{-1}	Albumin µg mL^{-1}
Natural	0	132±22 ^{c}	294±44 ^{b}	12.1±1 ^{a}	263±41 ^{b}	242±45 ^{c}	392±52 ^{bc}	255±39 ^{d}
involution	1	248±40 ^{b}	356±41 ^{b}	11.0±1 ^{a}	372±31 ^{ab}	444±58 ^{b}	610±49 ^{b}	425±49 ^{c}
(ANI)	2	287±52 ^{b}	306±46 ^{b}	10.1±1 ^{a}	541±99 ^{a}	544±99 ^{b}	990±53 ^{a}	795±51 ^{b}
	3	511±28 ^{a}	864±79 ^{a}	8.0±2 ^{b}	663±65 ^{a}	1699±30 ^{a}	1215±63 ^{a}	1110±62 ^{a}
Forceful	0	115±12 ^{c}	216±32 ^{d}	13.1±1 ^{a}	225±23 ^{b}	84±6 ^{d}	179±48 ^{d}	172±39 ^{d}
involution	1	105±8 ^{c}	297±21 ^{b}	14.2±1 ^{a}	245±27 ^{b}	113±7 ^{d}	185±38 ^{d}	174±40 ^{d}
(AFI)	2	147±13 ^{c}	239±15 ^{b}	14.1±1 ^{a}	233±22 ^{b}	267±6 ^{c}	187±43 ^{d}	175±41 ^{d}
	3	122±9 ^{a}	255±69 ^{b}	14.0±1 ^{a}	300±31 ^{ab}	478±38 ^{b}	182±49 ^{d}	174±51 ^{d}

* Days after milking cessation

$^{a-b}$ Results within column with no common superscript differ significantly ($P < 0.05$)

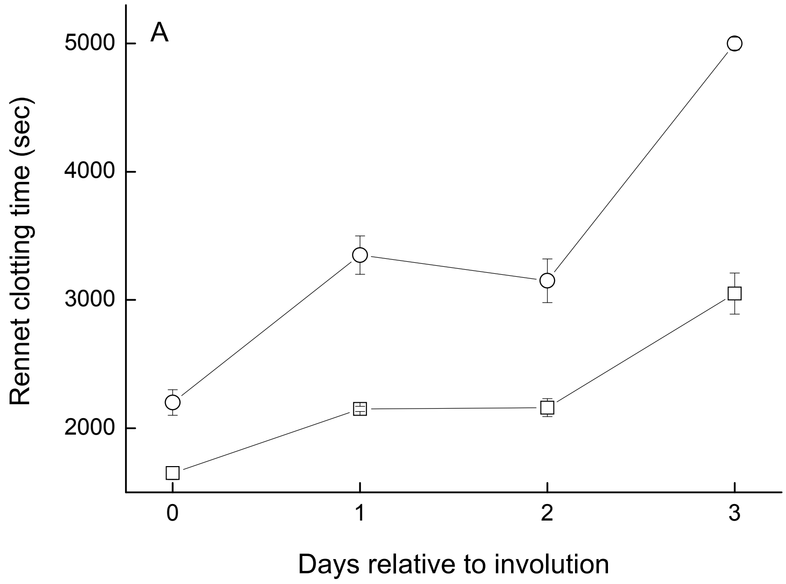
Table 4. Somatic cell count (SCC x 10^{-3}), total leukocytes (CD18^{+} x 10^{-3}), neutrophils (PMN x 10^{-3}), lymphocytes (CD4^{+} and CD8^{+} x 10^{-3}) and macrophages (CD14^{+} x 10^{-3}) in milk of cows approaching natural involution (ANI) and in cows approaching forceful involution (AFI) before the induction of involution (milk cessation) and during the first 3 days after the induction of involution (Mean±SD)

Treatment	Day*	SCC	CD18^{+}	PMN	CD4^{+}	CD8^{+}	CD14^{+}
Natural involution (ANI)	0	182 ± 77^{c}	86 ± 27^{d}	37 ± 12^{c}	9 ± 4^{c}	20 ± 7^{c}	8 ± 3^{d}
	1	650 ± 172^{b}	588 ± 164^{b}	182 ± 56^{b}	83 ± 23^{b}	140 ± 45^{ab}	82 ± 19^{b}
	2	744 ± 198^{d}	685 ± 194^{b}	399 ± 96^{b}	48 ± 17^{b}	98 ± 44^{b}	85 ± 26^{b}
	3	1873 ± 632^{a}	1626 ± 539^{a}	999 ± 342^{a}	152 ± 46^{a}	243 ± 76^{a}	327 ± 88^{a}
Forceful involution (AFI)	0	78 ± 25^{d}	53 ± 21^{d}	39 ± 19^{c}	5 ± 2^{c}	6 ± 3^{d}	6 ± 3^{d}
	1	248 ± 39^{c}	227 ± 33^{cd}	203 ± 31^{b}	3 ± 1^{c}	1 ± 1^{d}	10 ± 3^{d}
	2	479 ± 95^{bc}	414 ± 88^{bc}	382 ± 94^{b}	3 ± 1^{c}	1 ± 1^{d}	29 ± 5^{c}
	3	903 ± 179^{b}	750 ± 171^{b}	615 ± 129^{ab}	11 ± 4^{c}	2 ± 1^{d}	61 ± 16^{b}

* Days after milking cessation

$^{a-b}$ Results within column with no common superscript differ significantly ($P < 0.05$)

1A


A
Rennet clotting time (sec)
5000
4000
3000
2000
0
1
2
3
Days relative to involution

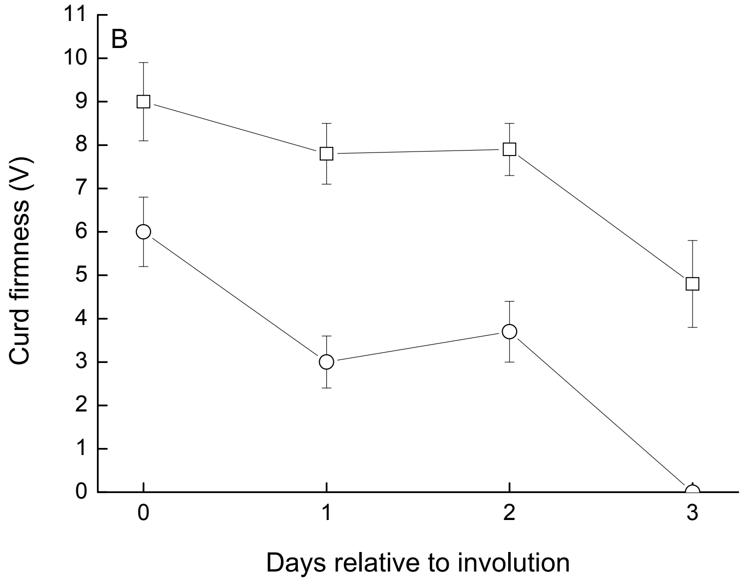

B
Curd firmness (V)
11
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
Days relative to involution

Figure 1B

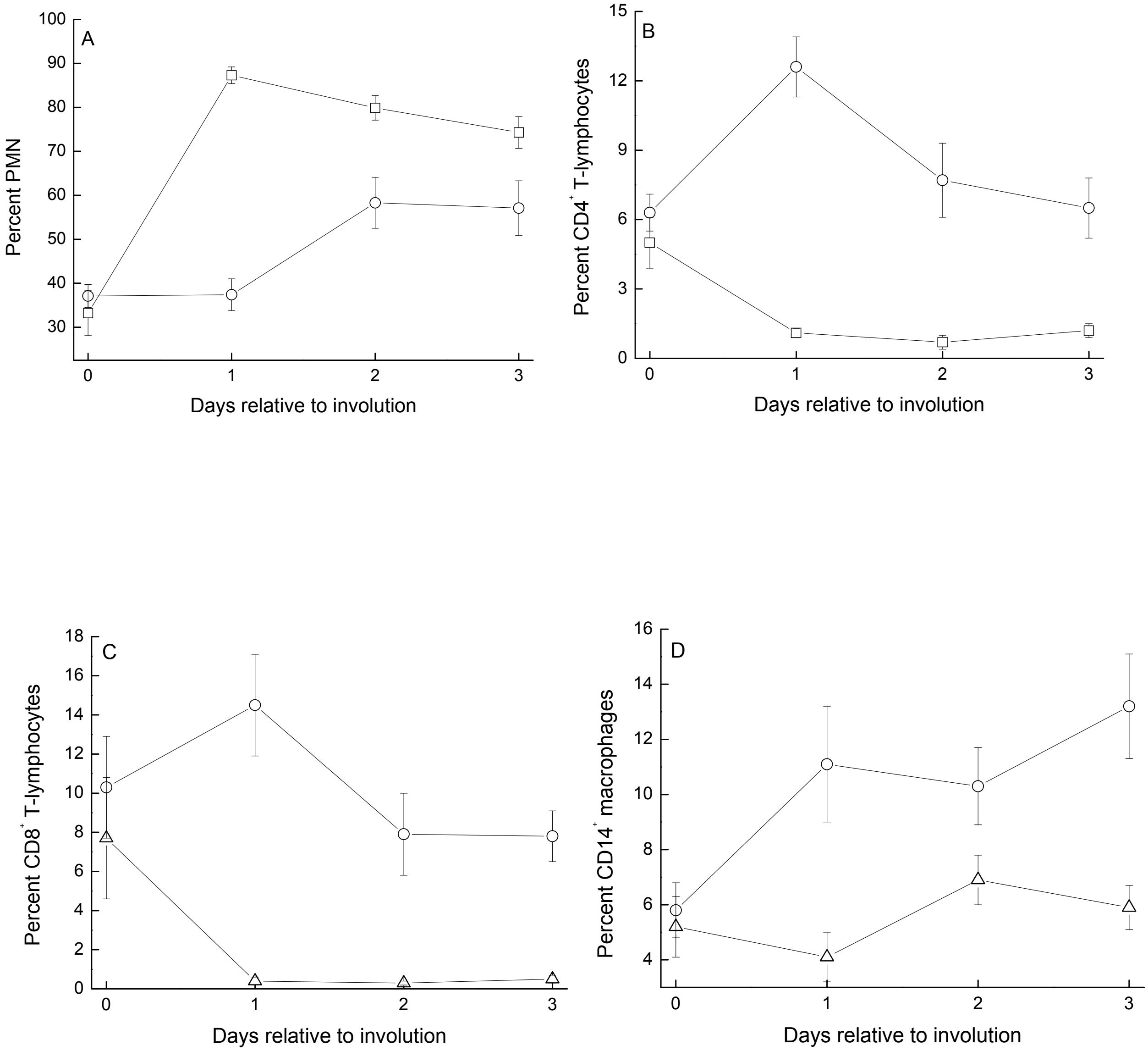


Figure 2 ABCD