

UDDER HEALTH IMPLICATIONS OF PREMATURE BOVINE MAMMARY REGRESSION. I. CLINICAL, SUBCLINICAL AND REDUCING-SUGAR CHANGES IN MILK DURING 168 HOURS OF SUSPENDED MILKING IN MID-LACTATION

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ABSTRACT

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The investigation involved 4 mastitis-free cows, exposed to 168 h of suspended milking to induce prolonged milk stasis and premature mammary regression during mid-lactation. After 48 h the milk stasis elicited mastitis-like changes in the clinical, somatic cell count (SCC), bovine serum albumin (BSA) and *beta*-N-acetyl-D-glucosaminidase (NAG) characteristics of the udder secretions. Such changes in secretions from non-mastitic regressive mammary glands raise doubts about the present knowledge, definition, and diagnosis of so-called non-specific or aseptic mastitis. Determinations of fluctuating lacteal concentrations of lactose, galactose, mannose and glucose suggest that the secretory epithelium altered its metabolism and integrity in response to the intramammary perturbation by following a certain pattern of regressive adjustments which: (i) were apparently triggered during the initial 24 h of perturbation by disturbed Na-K-ATPase activities, followed by a cascade of changes in ion regulation, carbohydrate metabolism and increased formation of lactic acid as a metabolic end-product; (ii) advanced in a stepwise fashion during 0–24, 24–72 and 72–168 h of perturbation from recognition response to alarm reactions and manifestation of regression respectively; (iii) showed that markedly decreased carbohydrate levels preceded major increases of the SCC, BSA and NAG values; (iv) indicated that after 72 h of milk stasis leucocytic infiltrations sharply increased the SCC to more than 500 000 per ml and accelerated the manifestation of regression.

The results of this study imply that extensive premature regression of healthy, and especially, pre-irritated udders could have significant implications for the development of different types of bovine mastitis during lactation and should be further investigated.

Key words: Bovine mammary regression, reducing sugars in milk, susceptibility to udder infection, mastitis.

INTRODUCTION

Bovine udder health depends on 3 key elements, namely intramammary epithelial integrity, somatic cellular defence and bacterial challenge (Giesecke & Barnard, 1986).

A range of data on the variability of subclinical udder conditions (Giesecke & Barnard, 1986), the composition of udder secretions during lactation and regression (Lück, Giesecke, De Villiers & Mackie, 1976; Mackie, Giesecke, Lück & De Villiers, 1977; Giesecke, 1978; 1985a, b), increased rates of udder infections at the end of lactation (Natzke, 1981; Petzer & Giesecke, 1988) and other aspects suggests that bovine mammary regression affects each of the 3 key elements of udder health. Mammary regression, therefore, plays a significant role in the physiology and health of the lactating and non-lactating bovine udder. This is underlined by several metabolic changes observed by Lück *et al.* (1976) and Mackie *et al.* (1977) in milk during artificially induced premature regression (Table 1).

Little is known about the physiological mechanisms associated with normal and premature regression of the udder. Even less is known about the significance of premature regression in relation to the diagnosis, development, prevention and therapy of clinical and subclinical mastitis in dairy cattle. A more complete understanding of bovine mammary regression would be of great advantage to research on bovine mastitis. It therefore seems desirable to put into perspective the most relevant available data and augment them with new work.

The physiology of the bovine mammary gland is characterized by the 4 primary development stages

of mammaryogenesis, lactogenesis, galactopoiesis and regression.

During galactopoiesis the cow differentiates and extends lactogenic epithelium, maximizes epithelial organelles and optimizes epithelial energy production from glucose (augmented with acetate and *beta*-hydroxybutyrate) by means of the tricarboxylic acid (TCA) cycle. This facilitates the energy-efficient functioning of 2 unique mammary processes: (1) biosynthesis of *alpha*-lactalbumin, which modifies a general galactosyltransferase to lactose synthase so that lactose is formed in the Golgi apparatus at rates critical for bulk water movement into milk and for the secretion of milk as such; (2) secretion of calcium, as export-product, into the Golgi apparatus where its association with casein is critical to the formation of casein micelles (Baldwin & Yang, 1974; Bauman & Davis, 1974; Davis & Bauman, 1974; Ebner & Schanbacher, 1974; Baumrucker, 1978; Peaker, 1978; Larson, 1985).

During regression, the cow discontinues the manufacture of lacteal export-products by reducing the energy production of the TCA cycle, dismantling redundant epithelial production units and reutilizing surplus substrates.

It is thus apparent that galactopoiesis is the process which leads to lactation and facilitates the metabolic adjustments necessary to establish lactational homeostasis. Mammary regression is the opposite process, leading to involution and facilitating metabolic adjustments necessary to establish involutational homeostasis (Giesecke, 1985a, b; Giesecke, Van Staden, Barnard & Petzer, 1988). Regression occurs during all stages of lactation. Depending on conditions, it may affect focal or more extensive portions of secretory mammary epithelium and related lactational and lacteal characteristics.

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 TABLE 1 Percentage (%) changes from baseline of several metabolic key parameters monitored during 168 h of milk stasis [compiled from **Lück *et al.* (1976) and *Mackie *et al.* (1977)]

Parameters	Baseline values at 0 h	Hours of milk stasis × % change relative to baseline value			
		24	72	120	168
Lactate $\mu\text{mol per ml}^*$	0,15	73,3	153,3	546,7	1 846,7
Pyruvate $\mu\text{mol per ml}^*$	0,05	60,0	20,0	60,0	100,0
Na ⁺ $\mu\text{mol per ml}^*$	19,0	44,7	171,0	307,9	390,5
K ⁺ $\mu\text{mol per ml}^*$	43,4	-6,7	-20,5	-41,2	-59,0
ROP mV*	-218*	0,9*	5,0	9,2	13,8
O ₂ concentration*		(1,3)**	(7,3)**	(13,0)**	(20,0)**
Beta-lactoglobulin mg/ml**	5,5	-18,1	50,0	87,3*	0,0*
Alpha-lactalbumin mg/ml**	1,7	-11,8	100,0	182,4	182,4

* Estimated values ** x-fold decrease estimated

Normal bovine mammary regression usually advances more rapidly after the peaking of lactation and, particularly, after drying-off. However, it is not clear from the literature whether regression in dairy cows that are dried off abruptly at a comparatively high level of production, is as physiologically normal as that in lower yielding cows drying off gradually during the natural weaning of their calves. On this basis it can be assumed that normal mammary regression is usually characterized by rapidly declining, non-mastitic, secretory activities and diminishing concentrations of true lacteal and increasing levels of serum components in the mammary secretion.

Histological and ultrastructural changes during early drying-off have been described mainly in laboratory rodents. More recent investigations of similar regressive changes in dairy cows (Holst, Hurley & Nelson, 1987) have shown several important histopathological differences, such as: (i) earlier, more pronounced and persistent intra-epithelial formation of different stasis vacuoles; (ii) no noticeable sloughing of secretory epithelial cells and corresponding extension of myoepithelial cells in the alveolar epithelium, and (iii) intact, though less densely staining, tight junctions at all stages of regression.

Holst *et al.* (1987) have thus found that bovine regression at drying-off is generally less destructive than in rodents and is mainly aimed at: (i) reduction of cytoplasmic organelles related to milk protein synthesis and secretory functions; (ii) re-structuring of epithelial cells to more densely stained and less active involuted cells, and (iii) maintenance of an intact mammary epithelium which, though non-lactating, is nevertheless still functioning as pre-lactogenic tissue that can be readily modified to the fully lactating epithelium of the next lactation cycle.

From the point of view of natural mammary defence, the pre-lactogenic epithelium's main function, is apparently, to establish involutinal homeostasis. During regression at drying-off, lactogenic cells are remodelled to pre-lactogenic epithelium to facilitate selective, increased and active transfer of immunoglobulins, such as IgA, IgG1 and IgG2 (Sasaki, Larson & Nelson, 1977). As a result, the pre-lactogenic epithelium becomes more resistant to bacterial attachment (Brook, 1983; Nickerson & Heald, 1983a, b; Nickerson, 1986a, b) and to adverse effects associated with the attachment of pathogenic bacteria, like mastitogenic streptococci and staphylococci.

Bacterial attachment to the epithelial cells depends ultimately on characteristics of the cell membrane and, particularly, of the glycocalyx, which requires glycoproteins to facilitate the attach-

ment of oligosaccharide chains with carbohydrates acting as receptors. Differences of such receptors seem to be responsible for selective adherence of micro-organisms to certain portions of mammary epithelium (Frost, Wanasinghe & Woolcock, 1977).

The lactating mammary epithelium synthesizes a range of glycoproteins, oligosaccharides and carbohydrates. While the predominant carbohydrate in bovine milk is lactose, free monosaccharides are also present, mainly as glucose and galactose, which are closely related to lactose synthesis. More recent investigations have indicated the presence of further monosaccharides, like mannose and fucose (Kowalski & Giesecke, 1986).

Glucose, galactose, fucose and mannose are included as structural components in lacteal glycoproteins and oligosaccharides (Ebner & Schanbacher, 1974). Bovine glyco-*alpha*-lactalbumin, for example, contains residues of mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid (Ebner & Schanbacher, 1974). Glucose, galactose, fucose and mannose are also components of lacteal oligosaccharides (Ebner & Schanbacher, 1974) and epithelial membranes, such as the plasma membrane and epithelial microtubules, which are cytoskeletal organelles giving structural integrity to the Golgi apparatus and secretory processes of the cell (Patton, 1976; Cheville, 1983; Patton, Patton, Torstrup & Lange, 1984; Breau & Oliver, 1985; Fox, Timms & Schultz, 1986).

It is therefore apparent that mannose and other carbohydrates are essential components of lacteal glycoprotein and oligosaccharides associated with the cellular membranes. Fluctuations of the lacteal levels of these carbohydrates, therefore, indicate important changes in the structure and function of the mammary epithelium.

In contrast to the aspects of normal mammary regression at drying-off discussed above, regression occurring abnormally at other stages of lactation is inadequately described. However, data suggest that at all stages of lactation the gradually advancing normal regression may become more variable and more pronounced under conditions of stress, mastitis and the forced drying-off of high producing cows at the end of lactation.

Stress-related increases in mammary regression during lactation affect the yield and composition of milk, and mammary resistance to udder infections (Giesecke, 1985a, b; Giesecke & Barnard, 1986; Giesecke *et al.*, 1988). Furthermore, regression is an integral part of mastitis (Giesecke, 1978, 1985a; Nickerson & Heald, 1983a, b; Nickerson, 1986a, b; Soldillo & Nickerson, 1986; Capuco, Paape & Nick-

erson, 1986). It apparently sensitizes mammary epithelium to necrotic lesions provoked by noxious agents and it further amplifies and accelerates changes related to inflammatory leucocytic infiltrations.

More subtle regressive changes during lactation predispose udders to infection. This is obvious from data on chronic mastitis (Heidrich & Renk, 1967; Schalm, Carroll & Jain, 1971) where the pathogenesis revolves around persistent tissue irritation, repeated inflammatory flare-ups and progressive fibrosis. Under such conditions the udder secretion shows, among other things (Giesecke, 1985a, b), elevated stimulation of bacterial growth (Brown, Baetz & McDonald, 1983; Mattila, 1985a, b; Sandholm & Mattila, 1986a; Mattila, Syvajarvi, Jensen & Sandholm, 1986).

This peculiar change in lacteal secretion apparently depends on the combined effects of proteolytic and lipolytic cellular and secretional degradations, leucocytic phagocytosis of fat globules and casein particles and, especially, on scavenging of bactericidal oxygen species by catalases and peroxidases from the serous exudate (Mattila, 1985a, b; Mattila, Kaartinen & Sandholm, 1986). Inhibition of the functions of lymphocytes and immunoglobulins by H_2O_2 and $HOC1$ production (Becker, 1988) by the increased numbers of phagocytic leucocytes in mastitic milk, could further support the growth promotion of bacteria reported by Mattila (1985a, b) and Mattila *et al.* (1986).

The data suggest that irrespective of their close association with udder health, natural mammary defence, and the pathogenesis of mastitis, the regulation and related characteristics of premature mammary regression are hardly known at present. Adverse effects of this situation are for example, inadequate research on: (i) deterioration, regeneration and therapeutic restoration of secretory mammary epithelium during mastitis; (ii) differential diagnosis of subclinical non-infectious mastitis and regression; (iii) diagnosis, development and prevention of mammary regression during lactation, and other aspects.

Further research is required to advance the current understanding of the triggering and related aspects of bovine mammary regression. The aim of this study was to investigate the changes in clinical and subclinical udder health parameters and in concentrations of reducing sugars in secretions from healthy udders during induced, premature regression.

MATERIALS AND METHODS

Experimental design

The experiment was performed in 2 consecutive periods: (I) Normal lactation and normal milking from day -6 to 0 before suspension of milking; (II) cessation of milking of the right and continuation of normal milking of the left udder quarters from day 1 to 7 (Table 2).

Experimental animals

Five healthy Friesian cows were used throughout the investigation. The cows differed in age and number of lactations, were in good condition and free from tuberculosis and brucellosis. Routine testing at each milking by means of the milking cup as well as laboratory examinations conducted each month on each lactating udder, established that prior to this investigation the cows were also free

from clinical and subclinical mastitis. At the beginning of the experiment, the cows had been lactating for an average of 181 days and had a mean production of 12.8 l of milk per day. During the experiment, the animals were kept and fed with the rest of the herd. Normal milking by machine took place twice daily at 07:00 and 15:00. Prior to milking the teats were washed with running potable water and dried with disposable paper towels.

Udder health examination

Clinical examination was limited to inspection of udders at milking, routine testing of foremilk by means of the milking cup, and inspection of each sample collected aseptically for the laboratory examinations mentioned below. Subclinical conditions were monitored by means of the bacteriological, cytological, BSA and NAG determinations described below.

Sampling routine

Standard foremilk samples (10 ml each) required for the different laboratory determinations, were collected aseptically from the individual quarters, according to a predetermined schedule (Table 2).

TABLE 2 Schedule of days, samplings and hours of investigation

Day	Period	Sampling	Hour	Purpose of sample
-6	I	1	-144	Pooling of all values from all untreated right udder quarters to determine mean baseline values applicable to investigation
-4	I	2	-96	
-2	I	3	-48	
-1	I	4	-24	
0	I	5	0	
1	II	6	24	Pooling of corresponding values from right quarters to assess mean changes in right udder halves under different conditions of treatment
2	II	7	48	
3	II	8	72	
5	II	9	120	
7	II	10	168	

Laboratory examination of milk

After collection the samples were transferred without delay to the nearby laboratory for immediate processing. Initially, the original samples were subdivided to provide 2 aliquots of approximately 9 ml and 1 ml each for udder health and sugar determinations, respectively.

Bacteriological cultures from each sample were prepared by methods already described (Giesecke, Nel & Van den Heever, 1968) and interpreted, depending on similar findings in 2 and more consecutive samples, as recommended by Griffin, Morant & Dodd (1987).

Somatic cell counts (SCC) per ml of milk were assessed by means of the recommended standard method, using a Coulter¹ Model-ZM counter supported by a Coulter¹ channelyzer for correct calibration.

Levels of BSA were determined as described initially by Giesecke & Viljoen (1974). However, diameters of precipitation zones were converted to values of mg BSA per ml by means of a standard curve, corresponding to the regression equation of $x = (y-a)/b$, where $x =$ mg BSA per ml, $y =$ mm² of diameter of precipitation zone, $a = 19,2971$ and $b = 90,2941$. Under such conditions, 0.5 mg BSA per ml

¹ Coulter Electronics S.A. (Pty) Ltd., Halfway House

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TABLE 3 Clinical condition of milk monitored 144 h before (-) and 168 h during milk stasis induced in the right udder quarters of 5 dairy cows

Technique and parameters of monitoring	Hours × observations									
	-144	-96	-48	-24	0	24	48	72	120	168
<i>Milking cup inspection</i>										
Normal milky	20	20	20	18	18	20	10	19	17	
Milky with floccules				2 ⁺	2 ⁺		10 ⁺	1 ⁺⁺		
Milky with changed colour and viscosity										11
— with floccules									3 ⁺⁺	1 ⁺
Serous										8
<i>Laboratory inspection</i>										
Normal milky	20	20	20	19	20	20	12	10		
Milky with floccules							3 [*]			
Milky watery							5	9		
— with floccules								1 ⁺⁺	3 ⁺⁺	1 ⁺
— with yellow colour									10	8
Butter milk-like									7	
Serous										11

* Tiny, *small, ++more distinct floccules

of standard, showing a precipitation zone with the critical diameter of 8 mm for differentiating non-mastitic and mastitic milk (Giesecke & Viljoen, 1974), is calculated as 0,4951 mg BSA per ml. The BSA values were interpreted during Period I, depending on the triple-parameter technique of udder health diagnosis referred to by Giesecke & Barnard (1986).

NAG was evaluated according to the standard techniques and instrumental configuration recommended by Mattila (1985a) and the supplier of the commercial test kits².

Using the fluorimetric high performance liquid chromatography (HPLC) method described earlier (Kowalski & Giesecke, 1986), reducing sugars were determined against standards of 2-Desoxy-D-ribose, cellobiose, lactose, D(-)-ribose, D(+)-mannose, D(+)-fucose, D(+)-galactose, D-xylose and D(+)-glucose.

Statistical analyses

Because of doubtful udder health after suspension of milking, the laboratory results from 1 of the 5 cows were excluded from the statistical analyses. For the remaining 4 cows, basic statistics and coefficients of correlation of the values were assessed by means of BMDP Statistical Software, programme version April 1985³.

Presentation of results

The baseline values, determined during Period I (Table 2) for all right udder quarters, have been presented as pooled results.

Only the lacteal changes of SCC, BSA, NAG, lactose (LAC), mannose (MAN), galactose (GAL) and glucose (GLU), monitored during Period I and II, have been referred to below. Results of the SCC, BSA and NAG determinations used in combination with bacteriological results to identify subclinical udder conditions, have been presented together with the carbohydrate values, and are discussed using diagnostic standard terminology recommended by the International Dairy Federation (1987).

Abbreviations, enzyme terminology and interpre-

tations used, are as follows: adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP); beta-hydroxybutyrate (BHB), coenzyme A (CoA), deoxyribonucleic acid (DNA), nicotinamideadenine dinucleotide (NAD) and phosphate (NADP), tricarboxylic (TCA) and pentose phosphate (PP) cycle and Embden-Meyerhoff (EM) pathway of anaerobic glycolysis; lactose synthase (E.C.2.4.1.22), lactic dehydrogenase (E.C.1.1.1.27) and beta - N - acetyl - D - glucosaminidase (E.C.3.2.1.30), as indexed by the Enzyme Commission (EC; International Union of Biochemistry, 1979); SCC = leucocytic response to intramammary chemo-attractants; BSA = epithelial permeability response to intramammary irritants; NAG = lysosomal enzyme activity specific for hydrolysis of termi-

TABLE 4 Pooled mean (x̄) values and standard deviations (SD) of parameters monitored in 40 milk samples collected during Period I from the right udder quarters

Parameters	Statistical	designations × values
	\bar{x}	SD
SCC per ml	111 000	80 934
BSA mg per ml	0,1334	0,0733
NAG units of activity	18,3875	10,0498
LAC μmol per ml	193,9131	11,0290
MAN μmol per ml	0,0221	0,0106
GAL μmol per ml	0,2699	0,1026
GLU μmol per ml	0,1004	0,0561

TABLE 5 Correlation matrix of parameters monitored during Period I

Parameters	Parameters × correlation coefficients (r)*						
	SCC	BSA	NAG	LAC	MAN	GAL	GLU
SCC	1,00						
BSA	0,14	1,00					
NAG	0,03	0,11	1,00				
LAC	-0,12	-0,54	-0,12	1,00			
MAN	-0,17	0,16	-0,50	0,23	1,00		
GAL	-0,22	0,09	-0,32	0,32	0,91	1,00	
GLU	0,21	-0,17	-0,55	0,29	0,81	0,69	1,00

* Correlation coefficients at different limits of significance: r = 0,2573 (P<0,1), r = 0,3044 (P<0,05), r = 0,3578 (P<0,02), r = 0,3932 (P<0,01), r = 0,4896 (P<0,001)

² Seravac, Fine Chemicals Corp. (Pty) Ltd., Johannesburg

³ BMDP Statistical Software, Inc., Los Angeles, USA

TABLE 6 Mean values of parameters monitored during 168 h of milk stasis

Parameters	Hours of milk stasis × mean values					
	Baseline (Period I)	24	48	72	120	168
SCC per ml	111 000	75 875	220 125	396 875	591 500	4 475 250
BSA mg per ml	0,1334	0,181	0,211	0,441	0,816	1,981
NAG units of activity	18,3875	17,488	31,888	57,463	145,125	265,150
LAC μ mol per ml	193,9131	267,703	154,475	147,833	85,521	44,327
MAN μ mol per ml	0,0221	0,040	0,010	0,005	0,004	0,005
GAL μ mol per ml	0,2699	0,513	0,282	0,287	0,165	0,095
GLU μ mol per ml	0,1004	0,156	0,021	0,033	0,032	0,047

nal, non-reducing glucose residues of glycoprotein in response to intracellular conditions; LAC = epithelial metabolism of the disaccharide; MAN, GAL, GLU = epithelial metabolism of the monosaccharides.

RESULTS

Clinical and bacteriological results

Clinical examinations of milk from the 5 cows monitored initially, revealed several milk samples with tiny to more distinct floccules (Table 3).

Such changes (Table 3) occurred more frequently after 48 h of milk stasis, when they were augmented by changes of colour and viscosity. During milk stasis floccules were observed very irregularly. The general character of the milk tended to change from normal milky to more watery, white watery, yellow serous and eventually to distinctly serous.

During milk stasis, the frequency of bacteriologically positive samples changed from sporadic to elevated. Bacterial growth showed coliforms, micrococci, streptococci, staphylococci, *Bacillus* spp. and *Pseudomonas* spp. With the exception of one cow that was disqualified from further investigations because of the repeated presence of *Staphylococcus aureus* in milk from one of her udder quarters, the samples from the other 4 cows showed variable presence of bacteria.

Lacteal changes prior to the suspension of milking

Values of the udder health parameters SCC, BSA and NAG suggested that the 4 cows qualifying for further investigations, had healthy mammary glands (Table 4 & 5) prior to the suspension of milking.

The values (Table 4) indicated normal baseline conditions during mid-lactation. The low BSA, NAG and SCC values (Table 4) were not correlated (Table 5).

Only few parameters showed noteworthy correlations (Table 5). Values of LAC and BSA were correlated highly negatively, suggesting that under normal conditions, low lacteal levels of BSA are related significantly to the elevated normal LAC metabolism.

MAN/NAG, GAL/NAG and GLU/NAG indicated significant to highly significant negative correlations (Table 5), suggesting that in normal milk, levels of NAG (Table 4) are related mainly to the carbohydrate metabolism of the secretory mammary epithelium.

The correlations between GLU and NAG, LAC, MAN and GAL shown in Table 5, demonstrate the pivotal importance of GLU to the physiology and health of secretory mammary epithelium under conditions of normal lactational homeostasis.

TABLE 7 Percentage (%) increases and decreases (-) relative to baseline values of the parameters monitored during 168 h of milk stasis

Parameters	Hours of milk stasis × percentage (%) values of changes from baseline (=100%)				
	24	48	72	120	168
SCC per ml	-31,6	98,1	257,5	432,9	3 931,8
BSA mg per ml	35,7	58,2	230,6	511,7	1 385,0
NAG units of activity	-4,9	73,4	212,5	689,3	1 342,0
LAC μ mol per ml	38,1	-20,3	-23,8	-55,9	-77,1
MAN μ mol per ml	81,0	-54,8	-77,4	-81,9	-77,4
GAL μ mol per ml	90,1	4,5	6,3	-38,9	-64,8
GLU μ mol per ml	55,4	-79,1	-67,1	-68,1	-53,2

Lacteal changes during 168 h of milk stasis

The lacteal levels of each parameter (Table 6) changed distinctly and differently during the 168 h of milk stasis.

The percentage changes from baseline values (Table 7) suggest 2 major mean patterns of fluctuations, with the pattern of SCC, BSA and NAG (=udder health parameters) opposing that of LAC, MAN, GAL and GLU (=metabolic parameters) (Fig. 1).

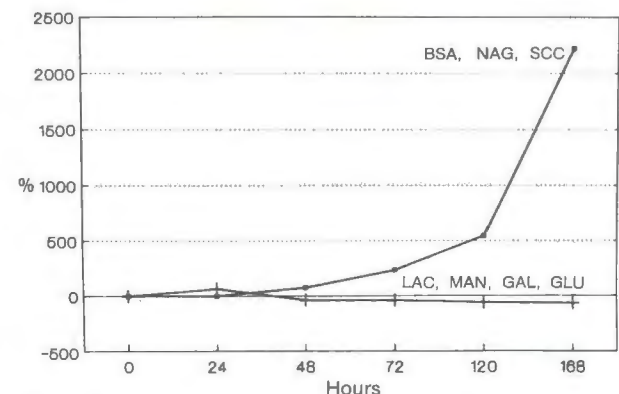


FIG. 1 Major mean patterns of comparative (%) changes of LAC, MAN, GAL and GLU (+) in contrast to a similar but opposite pattern of NAG, BSA and SCC (■)

The lacteal concentrations of BSA, NAG and SCC increased gradually during the initial 48 h, more rapidly from 48–72 h, still more distinctly from 72–120 h and, in particular, from 120–168 h of stasis (Fig. 2).

From such changes of the udder health parameters (Fig. 2) it is apparent that the milk stasis amounted

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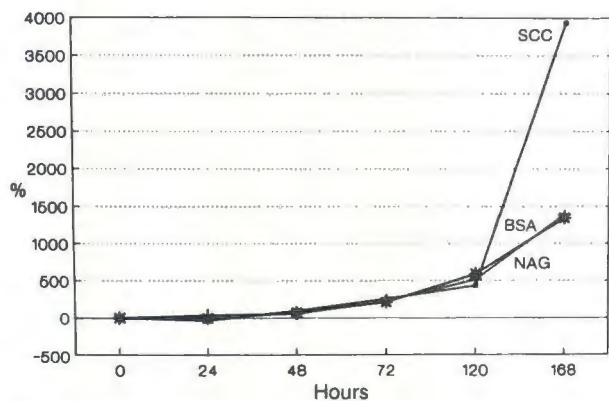


FIG. 2 Patterns of comparative (%) changes of SCC (●), BSA (*) and NAG (Δ) during 168 h of milk stasis

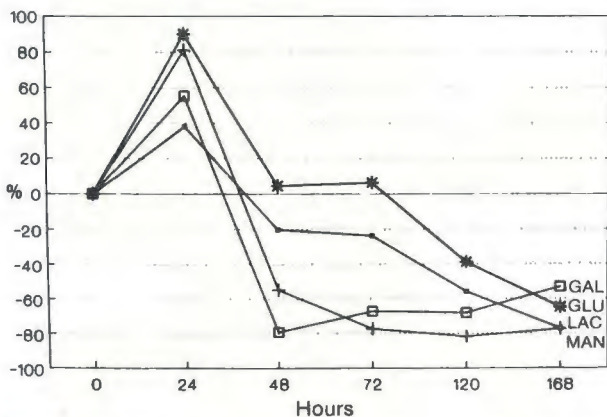


FIG. 3 Patterns of comparative (%) changes of LAC (●), MAN (+), GAL (□) and GLU (*) during 168 h of milk stasis

to a stressful perturbation which seriously affected the condition of the mammary epithelium. However, the carbohydrate mean pattern (Fig. 1) indicates that the epithelial metabolism had already reacted 24 h before the udder health parameters to the milk stasis perturbation (Fig. 3).

The changing pattern of the individual carbohydrates (Fig. 3) is noteworthy from 2 points of view, namely, consistency between the sugars and stepwise development. Concerning the consistency, this similarity indicates that the sugar fluctuations were co-ordinated by some mechanism(s). Concerning the stepwise early, intermediate and advanced adjustments of mammary carbohydrate metabolism, they imply early recognition responses, intermediate alarm reactions and manifestation of regression between 0–24 h, 24–72 h and 72–168 h of milk stasis respectively.

The fluctuations of GLU (Fig. 3) showed highly positive correlations with the values of LAC, MAN and GAL (Table 8).

Equally significant positive correlations occurred between MAN/LAC, GAL/LAC and GAL/MAN (Table 8).

Correlations between LAC and udder health parameters SCC, BSA as well as NAG (Table 8) were highly negative. The same is applicable to GAL/SCC/BSA/NAG and, to a lesser extent, to MAN/SCC/BSA/NAG values. In contrast to the highly significant negative correlations between LAC, MAN, GAL and NAG (Table 8), GLU and

TABLE 8 Correlation matrix of parameters monitored during 168 h of milk stasis

Parameters	Parameters × correlaton coefficients (r)*						
	SCC	BSA	NAG	LAC	MAN	GAL	GLU
SCC	1,00						
BSA	0,66	1,00					
NAG	0,84	0,64	1,00				
LAC	-0,62	-0,66	-0,70	1,00			
MAN	-0,29	-0,30	-0,40	0,70	1,00		
GAL	-0,47	-0,48	-0,55	0,76	0,68	1,00	
GLU	-0,22	-0,19	-0,31	0,60	0,90	0,57	1,00

* For levels of significance see Table 5

NAG showed only a significant correlaton of $r = -0,31$ ($P < 0,05$).

DISCUSSION

Clinical examinations of milk samples by means of milking cup and laboratory inspection suggest that after 48 h of milk stasis, induced suddenly during mid-lactation, the udder secretions became increasingly clinically abnormal (Table 3). From 48–168 h of milk stasis, changes like floccules, yellowish colour, watery to butter milk-like and serous appearance became more frequent and distinct. It is thus apparent that clinical changes in milk may be associated not only with mastitis but also with mammary regression.

With the exception of one quarter, apparently infected with *S. aureus*, such clinical changes occurred in milk samples with and without bacteria. The bacterial isolates were rather variable, relative to the duration of milk stasis, the micro-organisms found, and the udder quarters sampled. This inconsistent bacterial presence in milk from all but one quarter precluded the diagnosis of intramammary infections, according to standards recommended by Griffin *et al.* (1987). Nor did the udder health history and SCC, BSA and NAG values prior to (Table 4) and during (Table 6) the initial 72 h of milk stasis suggest that the clinical changes observed in milk from 48–168 h were related to conditions other than the induced milk stasis, advancing mammary regression and leucocytic infiltration. This is consistent with data from other workers, reviewed earlier (Giesecke & Van den Heever, 1974) and underlining the fact that clinical changes in milk like those reported in Table 3, may occur in the presence as well as the absence of pathogenic bacteria and are, therefore, not pathognomonic of mastitis.

Concerning the diagnosis of clinical mastitis, the results (Table 3 & 6) imply that clinical changes in milk from mastitic quarters are non-specific signs. They are primarily associated with regressive deteriorations of secretory epithelium, usually further aggravated by leucocytic infiltration, intramammary infections and related inflammatory changes.

In the light of the data above and the exclusion of the cow with an intramammary infection it seems justifiable to submit that the changes of SCC, BSA, NAG, LAC, MAN, GAL and GLU in milk discussed below were associated with induced, non-mastitic milk stasis perturbation and mammary regression in the absence of intramammary infections.

The baseline values of SCC, BSA and NAG (Table 4 & 5) suggest normal udder health with normal lacteal levels of LAC, MAN, GAL and GLU.

From correlations between the parameters it is evident that in normal milk, the SCC, BSA and NAG values were not correlated significantly. However, the normally high LAC metabolism was highly negatively correlated with low epithelial permeability to BSA. Similarly, normal concentrations of MAN, GAL and GLU were highly negatively correlated with low lysosomal NAG activities primarily of epithelial origin. Still more significant correlations indicate associations of GAL, GLU and MAN (Table 5) which were much closer than those of LAC and MAN, GAL and GLU (Table 5).

In the light of such relationships it is apparent that under normal physiological conditions, a high carbohydrate metabolism of the secretory mammary epithelium is vitally important to maintain low epithelial permeability to BSA as well as low epithelial activity of the lysosomal enzyme NAG.

Changes occurring during milk stasis (Table 6 & 7) suggest 4 particularly noteworthy developments: (i) increases of lacteal concentrations of SCC, BSA and NAG to mastitis-like levels; (ii) sharp decreases of lacteal concentrations of LAC, MAN, GAL and GLU preceded by 24 h major increases of SCC, BSA and NAG levels; (iii) consistency of the pattern of fluctuations of the reducing sugars; and (iv) stepwise progression of the fluctuations of the reducing sugars.

Concentrations of SCC, BSA and NAG showed highly positive correlations (Table 8). They escalated between 72–120 h of milk stasis to levels (Table 6 & 7) which are mastitis-like if compared with data on mastitic udder secretions (Mattila, 1985a; Sandholm & Mattila, 1986b; Pyörälä, 1986; Kaartinen, 1989). However, no research data have been published which indicate or imply otherwise that abrupt suspension of milking during mid-lactation, premature regression related to stressful conditions during lactation, or abrupt cessation of milking at normal drying-off cause non-infectious types of mastitis. Nor is such a development of non-infectious mastitis supported by earlier results from Lück *et al.* (1976) and Mackie *et al.* (1977) on various aspects of premature regression. Under these circumstances it seems reasonable to submit that the SCC, BSA and NAG increases (Table 6 & 7), though mastitis-like in magnitude, nevertheless were regressive in nature. This implies that the present understanding, concept, definition and diagnosis of so-called non-specific clinical and subclinical mastitis, described by Tolle (1971) and the International Dairy Federation (1987), are equivocal. Unless mammary regression and non-specific mastitis can be differentiated accurately depending on diagnostic parameters more specific for inflammatory changes than are SCC, BSA and NAG determinations, the data available on so-called non-specific mastitis should be considered doubtful.

The consistency and stepwise progression of the carbohydrate fluctuations (Fig. 3) suggest mechanisms of regulation which have not been reported previously and, therefore, justify further discussion below.

Changes occurring during milk stasis (Table 6 & 7) suggest early, intermediate and advanced regressive adjustments of mammary epithelium to the stasis perturbation. This is consistent with previous work (Table 1) done by Lück *et al.* (1976) and Mackie *et al.* (1977) and re-evaluated for this discussion.

The present (Table 6 & 7), and previous data (Table 1) show that the early metabolic adjustments

during the initial 24 h of milk stasis depended on recognition of the milk stasis perturbation. This recognition is apparently associated (Table 1 & 7) with increases of lactate (73 %), pyruvate (60 %), Na⁺ (45 %), BSA (36 %), LAC (38 %), MAN (81 %), GAL (90 %) and GLU (55 %) and decreases of K⁺ (–7 %), *beta*-lactoglobulin (–18 %), *alpha*-lactalbumin (–12 %), NAG (–5 %) and SCC (–32 %).

The 45 % increase of Na⁺ and 7 % decrease of K⁺ (Table 1) indicate a significant early disturbance of the epithelial sodium pump. As in other cells, this pump of the secretory mammary epithelium depends on Na⁺-activated Na-K-ATPase in the plasma membrane (Mephum, 1876; Peaker, 1978). Consequently, the data from Mackie *et al.* (1977) (Table 1) suggest inhibition of the Na-K-ATPase, increased diffusion of Na⁺ from blood into the epithelial cells and milk, as well as increased efflux of K⁺ from cells to the blood.

This change in ion-regulation would promote influx of H₂O, and intracellular vesiculation and vacuolation. Data from Holst *et al.* (1987) suggest that such symptoms of slight to severe cell injury start to develop at an early stage of milk stasis. Diminishing intracellular K⁺ enhances that degeneration, whereas the efflux of K⁺ has a vasodilatory effect (Cheville, 1983).

From the point of view of the recognition response and early changes of lacteal Na⁺ and K⁺ levels (Mackie *et al.*, 1977) applicable to this investigation (Table 1), data on Ca⁺⁺ regulation (Baumrucker, 1978; Peaker, 1978; Cheville, 1983) suggest that increases of cell Na⁺ stimulate an overall increase of free intracellular Ca⁺⁺. This would, in turn, trigger elevated ATP production, oxygen and ATP consumption, formation of prostaglandins (PG-s) (Levine, 1988) and of oxygen radicals, as well as the elevated transportation of Ca⁺⁺ into the smooth ER and Golgi apparatus.

Increases of ATP and PG-s would supplement the K⁺-induced initial vasodilatory effect, which is possibly boosted further by histamine and kinins. The elevated lacteal levels of LAC, GAL and GLU observed after 24 h of milk stasis (Table 6) are consistent with increased substrate supplies facilitated by circulatory adjustments in support of increased ATP production (Larson, 1985).

Because physiologically elevated intracellular Ca⁺⁺ levels apparently reduce permeability of the junctional complex, such changes would explain at least partially the absence of epithelial sloughing (Holst *et al.*, 1987) and the only slight increases of lacteal BSA (Table 6) when, during recognition of the milk stasis perturbation, the secretory cells showed initial alterations of their ion-regulation (Table 1), integrity of organelles (Holst *et al.*, 1987) and metabolism (Table 1 & 6).

The above-mentioned data (Table 1 & 6) imply that, after 24 h, the milk stasis precipitated a cascade of epithelial events apparently involving several steps: (1) inhibition of Na-K-ATPase, leading to influx of Na⁺ and efflux of K⁺; (2) influx of Na⁺, eliciting influx and intracellular redistribution of Ca⁺⁺; (3) Ca⁺⁺ changes, altering several metabolic processes and inducing elevated PG formation; (4) PG-s, stimulating the pumping of Na⁺ and cyclic AMP production; (5) cyclic AMP stimulation of certain enzyme activities which promote increased glycolytic conversion of glucose to pyruvate and corresponding escalations of lacteal levels of pyruvate and lactate; (6) circulatory effects of K⁺, PG-s and other

mediators (histamine, kinins), improving temporarily the availability of glucose which promotes lacteal GLU increases (Table 6) and facilitates elevated lactose (Table 6) and pyruvate (Table 1) production.

From such changes it becomes apparent that the recognition response to 24 h of milk stasis was associated with several cellular adjustments which involved increases of Na⁺, Ca⁺⁺ and water, elevated flux through the lactose, EM and PP pathways of glucose metabolism, and increased lipogenesis, which are consistent with the known physiology of the secretory epithelium (Bauman & Davis, 1974; Ebner & Schanbacher, 1974; Baumrucker, 1978; Peaker, 1978). The elevated pyruvate and lactate values (Table 1) indicate a shifting to anaerobic glycolysis, with corresponding reductions of TCA cycle activities, redistribution of citrate, accumulation of reduced metabolites (NADPH, NADH), and decrease of oxygen concentration.

Such metabolic and early structural changes (Holst *et al.*, 1987) were apparently limited to levels which were non-irritant and, hence, non-attractive to leucocytic infiltrations and non-stimulating to activities of NAG, as suggested by low SCC and NAG values (Table 6).

However, this almost harmless state of recognition response started to change rapidly during the intermediate alarm reaction elicited during 24–72 h of milk stasis (Table 6). Still more extensive changes occurred during the manifestation of mammary regression monitored after 72–168 h of the stasis perturbation (Table 6).

The alarm reaction was characterized by sudden drops of LAC, MAN and GLU values, whereas GAL decreased more gradually. Levels of NAG and SCC increased distinctly, and those of BSA more moderately (Table 6) within normal limits. Such changes showed, at differing rates, further progress after 72 h of milk stasis (Table 6). From the almost negligible availability of glucose (Table 6) and sharply increased lactate concentrations (Table 1) it is obvious that the alarm reaction coincided with further escalations of anaerobic glycolysis and related changes of electrolyte regulation, estimated ROP and oxygen concentration (Table 1). Because of decreased GLU and GLA supplies (Table 6), lactose synthesis diminished, irrespective of a 100 % increase of *alpha*-lactalbumin values (Table 1).

The most significant carbohydrate change was apparently the 79,1 % reduction of GLU in milk (Table 6 & 7). This suggests a drastically reduced glucose supply to, and concentration within, the mammary epithelium. Reduced glucose levels in blood are associated with elevated concentrations of ketone bodies in blood and milk (Schultz, 1974). Under the lipogenic conditions (Bauman & Davis, 1974; Davis & Bauman, 1974) of secretory epithelium affected by 24 h of milk stasis, even slightly reduced activities of the TCA cycle in the presence of elevated concentrations of BHB and acetate could become critical. Udder irritations are apparently related to increased lacteal levels of potentially harmful ketone bodies (Korybut-Woroniecki & Kowalski, 1987). Furthermore, the shifting to EM glycolysis, found after 24 h of milk stasis, is related to decreased oxygen concentrations (Mackie *et al.*, 1977).

This would reduce mitochondrial phosphorylation of ADP to ATP. The effects of oxygen shortage on oxidative phosphorylation are amplified by accumulating NADH, which inhibits lactic dehydrogenase,

the dehydrogenases of the TCA cycle (e.g. pyruvate dehydrogenase) and the flux of pyruvate to CoA (Cheville, 1983). Consequently, lactate and pyruvate levels start to increase after 24 h of milk stasis, as shown by Mackie *et al.* (1977).

In the light of the above findings it seems reasonable to propose that during the interval between 24 and 48 h of milk stasis, the distress situation of the mammary epithelium reached a stage where a combination of factors precipitated irreversible lesions in parts of the cellular organelles. Experience suggests that this critical stage develops at approximately 30–40 h of milk stasis. It apparently amounts to an irreversible switching of the epithelial metabolism to enhanced catabolism. Reductions of epithelial carbohydrate metabolism apparently led to sharply reduced lacteal levels of LAC, GAL, MAN and GLU (Table 6 & 7), elevated epithelial catabolism (Table 1), permeability to serum proteins and chemo-attraction of leucocytes (Table 6 & 7). The increased epithelial catabolism was associated with escalating lysosomal enzyme activities indicated by the rising NAG values and their negative correlations with LAC, GAL, MAN and GLU (Table 8). This explains the marked increase of NAG at 48 h of milk stasis (Table 6 & 7) in the presence of normal, comparatively low SCC values (Table 6).

The catabolic implications of NAG are underlined by its occurrence in mammalian tissues in association with the catabolism of hexosamine-containing substances, such as glycoproteins, mucopolysaccharides and oligosaccharides (Pugh, Leaback & Walker, 1957; Li & Li, 1970). For example, oligosaccharides formed by degradation of hyaluronic acid are further degraded stepwise by *beta*-glucuronidase and *beta*-N-acetyl-D-glucosaminidase which usually occur together with *beta*-N-acetyl-D-galactosaminidase (Pugh *et al.*, 1957; Li & Li, 1970). This supports the suggestion that the 48-h values of NAG (Table 6) are primarily related to epithelial catabolism. Furthermore, it is consistent with results from Fox *et al.* (1986) who have suggested that the secretory cells are the probable source of most lacteal NAG activity. The latter is also indicated by the fact that during milk stasis, elevations of NAG (Table 6 & 7) were correlated highly negatively with reductions of LAC and GAL (Table 8) and less negatively with decreases of MAN and GLU (Table 8). Thus increased epithelial NAG activities seem to be very closely associated with the discontinuation of lactose synthesis, as one of the initially mentioned 2 primary functions of galactopoietic epithelium. Leucocytes apparently supplement the epithelial NAG activity, depending on their concentration in the udder secretion.

The above-mentioned lesions of epithelial organelles and the catabolism indicated by the NAG values would explain the major differences between the 24-h and 48-h values (Table 6), and the sudden onset of the regressive alarm reactions. Further alterations eventually led to cytological deteriorations, presumably similar to those observed at approximately 56 h of milk stasis by Holst *et al.* (1987).

Collectively, such alterations amounted to regressive necrobiotic epithelial damage, which sufficed to stimulate increases of SCC values, permeability to BSA and activities of NAG, as indicator of lysosomal enzyme activities (Table 6). Holst *et al.* (1987) did not find noticeably increased leucocyte concentrations during early milk stasis. This is supported by

the present data (Table 6) which indicate that at 48 and 72 h of milk stasis the SCC, though increased by 98 % and 257 % respectively from baseline, was nevertheless well within normal limits.

The manifestation of regression during 72–168 h of milk stasis was characterized by changes where intermediate values, established during the alarm reactions, reached more obviously abnormal levels, particularly of the SCC, BSA, NAG, LAC and GAL values (Table 6).

This development coincides with markedly accelerated increases of lactate and Na^+ (Table 1), more moderate changes of pyruvate, K^+ , ROP (Table 1), peaking values of *alpha*-lactalbumin and immunoglobulins (Table 1), as well as advanced histological and ultrastructural symptoms of mammary regression (Holst *et al.*, 1987).

From 72–120 h and from 120–168 h the SCC increased by 49 % and 656 % respectively (Table 7), whereas the corresponding values for lactate escalated by 155 % and 201 % respectively (Table 1). Such changes suggest that, unless SCC values amount to approximately 500 000 and more per ml (Table 6), lactate levels in milk (Table 1) seem to be related mainly to changes of epithelial metabolism as such. However, higher SCC values (Table 6) apparently supplement and aggravate the epithelial lactate production (Table 1) due to leucocytic metabolism. Mackie *et al.* (1977) have shown significant negative correlations between glucose and leucocyte counts ($r=-0,87$) and positive correlations between lactate and leucocyte counts ($r=0,84$) in milk. Such correlations imply that leucocytes in milk are metabolically highly active. They could thus affect the mammary epithelium by the release of cytotoxic waste products (e.g. lactic acid, CO_2 , oxygen radicals), metabolic modulators (e.g. PG-s) as well as a range of lysosomal enzymes with hydrolytic, proteolytic and lipolytic activities. Phagocytic neutrophils are known to be associated with several cytotoxic actions; Becker (1988) has pointed out that H_2O_2 at concentrations achieved near stimulated neutrophils leads to a sequence of biochemical and morphological changes in nucleated cells, such as activation of the PP cycle, inhibition of the EM pathway with consequent loss of ATP, an increase in intracellular Ca^{++} and Na^+ , and cellular degeneration. These changes are consistent with those observed during this (Table 6 & 7) and other investigations (Lück *et al.*, 1976; Mackie *et al.*, 1977; Holst *et al.*, 1987). Whatever leucocytic factors might be involved, from the point of view of the present data, it is significant that SCC values exceeding 500 000 cells per ml and occurring at 120 and 168 h of regression (Table 6), coincided with renewed sharp reductions of LAC and GAL (Table 6 & 7).

The rate of regression and related side-effects could therefore depend significantly on the SCC level at the onset of milk stasis. This has major implications for the effect of stress on the udder health of lactating dairy cattle, the drying-off of cows, and the pathogenesis of mastitis.

CONCLUSIONS

Knowledge concerning mammary regression, as an antigalactopoietic phenomenon, is confined mainly to studies in rodents. Incomplete knowledge of bovine mammary regression under normal and abnormal lactational conditions could have serious implications for progress on mastitis in dairy cattle and related aspects, such as the understanding of so-called non-specific mastitis, attachment of pathogens

to mammary epithelium, therapeutic restoration of mastitic lesions and others.

This investigation, has shown that prolonged milk stasis leads to various changes of SCC, BSA and NAG in milk which are important from the point of view of the differential diagnosis of mammary regression and so-called non-specific clinical and subclinical mastitis. It has also become apparent that intramammary perturbation elicits profound changes of epithelial metabolism and integrity which are associated with alterations of clinical, SCC, BSA and NAG characteristics of the udder secretions that may be confused with the corresponding symptoms of so-called non-specific mastitis and which raise doubts about the present concept, understanding, definition and diagnosis of this condition.

Furthermore, fluctuating lacteal concentrations of SCC, BSA, NAG, LAC, MAN, GAL and GLU suggest that the mammary epithelium apparently adjusts metabolically to the milk stasis perturbation by means of an initial recognition response, intermediate alarm reactions and eventual manifestation of mammary regression. Such adjustments are consistent with the establishment of involuntional homeostasis, the ultimate purpose of mammary regression.

The process of regression apparently depends on epithelial ion regulation, carbohydrate metabolism and lipogenesis. It is aimed primarily at the discontinuation of 2 unique mammary functions, namely, lactose synthesis and calcium exportation. This is achieved by a progressively developing cascade of events, apparently triggered by disturbances of cellular Na^+ and K^+ regulation, extended further by intracellular Ca^+ redistribution, accelerated by shifting to EM glycolysis, amplified by increased activities of the PP cycle, and eventually aggravated to irreversible catabolic levels by changes consistent with escalating mitochondrial O_2 depletion and advancing formation of lactic acid as end-product.

Catabolic changes became apparent at 48 h of milk stasis when lacteal GLU dropped by 86,5 % and NAG increased by 82,3 %. Simultaneous increases of BSA and SCC showed that epithelial integrity had become sufficiently impaired to facilitate elevated permeability to BSA and increasing attraction of leucocytes. This explains marked re-adjustments of the parameters monitored when, at 120 h of secretional stasis, somatic cell counts exceeded the critical level of 500 000 per ml. Such re-adjustments indicate that elevated leucocytic activities, associated with abnormal cell counts in milk, aggravate the already existing epithelial responses to milk stasis.

This aggravation supports the irreversible manifestation of mammary regression. The data imply that, under regressive conditions, the main purpose of the elevated leucocytic infiltration may be to promote early manifestation of regression as a means of enhancing the natural protection of the udder from infection. This would explain the elevated susceptibility of the lactating bovine udder to intramammary infections under stressful conditions and at drying-off.

Consequently, SCC values and leucocytic activities, already increased in milk at the onset of milk stasis, could have significant implications for bovine udder health, and require further research.

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