Pregnancy Obstructs Involution Stage II of the Mammary Gland in Cows: General Biological Implications

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Background

Repeated research findings over the last 4 decades show that involution of mammary glands in dairy cows did not regress to the same extent as that noticed in other mammalian species.

Methodology/Principal Findings

We took advantage of a rare event in the normal modern dairy farming: A cow that was false-positively identified as being pregnant was "dried-up" (i.e., induced into involution) conventionally about 60 days before her expected parturition. This cow was culled, and samples of her mammary gland tissue were examined for gross histology. In this study we demonstrate for the first time that modern dairy cows may undergo extensive obliteration of the lobular-alveolar structure, as expected in involution stage II.

Conclusions/Significance

We conclude that lack of histological evidence for the appearance of involution stage II in the vast majority of the modern cow's population is related to the peculiar modern dairy husbandry, in which dairy cows are induced into involution still pregnant. Because retardation of involution stage II in pregnant mammals is most likely a general physiological phenomena, it might occur in other mammals, particularly in lactating humans. Thus, based on basic comparative physiology considerations, we suggest that concurrent lactation and pregnancy should be considered as an independent risk factor for breast cancer.
Introduction

Mammary glands of adult mammals undergo cycles of avolution (proliferation), lactation, and involution (regression) in coordination with the reproductive cycle of the breed [1]. Abrupt weaning or abrupt cessation of milk removal induces the rapid and acute onset of involution in the unmilked mammary gland at any stage of lactation [2-6]. In most mammals under natural conditions the weaning process is gradual and the active stage of involution starts after the mammary gland naturally produces less milk than it did at peak lactation. In nature, mammals are not often pregnant while still nursing their infant [1-3].

The modern western dairy industry has evolved in a situation which considerably departs from the natural environment of most mammals: First, virtually all cows in a given herd are pregnant during most of the lactation period, because those who fail to conceive at the start of the lactation (~80 days post partum out of 300-day lactation cycle) are culled. Secondly, cows are induced into involution (also known as "dry-off") by abrupt cessation of milking, usually about 60 days before the expected parturition [2-3]. Because of intensive selection for high milk yield and for extended milk-yield persistence during the last 4-6 decades [7], dairy cows are dried-off while still producing considerable amounts of milk: 20, 40 and sometimes even 50 liters/day. Such a practice results in the accumulation of milk in the udder, which leads to udder engorgement, milk leakage, increased risk of acquiring bacterial infection [2], it also frequently causes noticeable agony to the cow, which might scream loudly for several days [8]. The length of the dry-off period in modern dairy farming is a compromise between the farmer's wish to maximize milk production and the need for a period sufficiently long to accomplish involution and avolution of the glands and thereby to prevent a decline in milk production in the following lactation [2-3]. However, the physiological processes underlying the involution/avolution cycle in dairy cows are not fully known, and the optimal length of the dry period is under renewed consideration [2].

After the induction of the acute phase of involution, mammary regression proceeds through at least two stages of morphogenetic alterations [1], which are reflected in respective phenotypic reformation of the amount and composition of mammary secretion, stages which are well characterized in dairy ruminants [9-11]. Stage I of involution involves the widespread apoptosis of alveolar epithelial cells, which reverses the dramatic expansion of this compartment during the previous cycle of avolution [1-6]. The first phenotypic sign of induction of stage I is the disruption of the tight junctions between epithelial cells, which is reflected in marked increase in the concentration of plasma electrolytes, sodium and chlorine, and marked inflow of leukocytes into the mammary gland [9-11]. The secretion of components of the innate immune system such as immunoglobulins [9-11], lactoferrin [9-11], albumin [12], and many other soluble components of the innate immune system [13-14] is also elicited at this stage.

During the second stage of involution the lobular-alveolar structure of the gland is obliterated by proteinases, which degrade the basal membrane and extracellular matrix, resulting in regression of the gland to a morphological condition that resembles the pre-adult stage [1]. Stage II is, therefore, associated with irreversible loss of the glands capacity to produce and secrete milk [1, 5, 6]. Mammary secretion in cows at this stage become scant, watery, turbid (serum-like) and rich in leukocytes [9-11]. In comparison to stage I, stage II is characterized by increased proportions of lymphocytes and macrophages and a reduced number of polymorphonuclear cells [2].
In contrast to rodents, in which involution is completed within less than a week [1, 5, 6], involution in cows is complete only after 21–30 days after drying-off, and never attains the histological features seen during stage II in rodents and other mammals [15-18, and see Ref. 2 for a most recent review]. Reduction in milk secretion and initiation of extensive apoptosis of the epithelial cells occur considerably later than in rodents [2]. However, the most distinguishing feature between cows, on the one hand, and rodents and other mammals, on the other hand, is the maintenance of an intact lobular-alveolar structure throughout the 60-day prepartum period, after termination of milking [2, 15-18]. Consistent with the continued presence of intact alveoli, milk production in the dairy cow was partially restored by mammary secretion removal after 11 days of milk stasis [19]. For comparison, in mouse it would need only 4 days of milk stasis to reach a complete involution and irreversibility in the capacity to secrete milk [1, 5, 6].

The rate of mammary gland involution is affected by systemic hormones, local factors, pregnancy and stage of lactation [1-6]. Involution stage II can be inhibited by systemic glucocorticoids and progesterone [20, 21], and by pregnancy [22]. Thus, an interesting question raised by the above points concerns whether the involution process, particularly stage II, is fundamentally different in cows in comparison to rodents and other mammals, or whether it merely reflects the selection for high milk yield, the artificial living conditions associated with modern dairy farming, and the fact that most research in this aspect was focused on modern cows?

The present study aimed to address this question and to evaluate the general biological implications of simultaneous involution and pregnancy in cows and in humans.

Results

The histological data presented in Figs. 1 and 2 differed dramatically from those of pregnant cows induced into "dry-off" 2 days before their slaughter (Figs. 3 and 4). In fact, it would be difficult to differentiate between the histology of the former glands and that of lactating glands. This indicates that during the first 2 days of "dry-off", the mammary gland parenchyma undergoes minimal changes, which is consistent with previous reports [2]. Thus, in Figs. 3 and 4 we show that the "abnormal" features of dairy cows involution are the predominant situation when they are induced into dry-off pregnant, as shown many times in the past (15-18).

Figs. 1 and 2 show unequivocally that the mammary gland of a dairy bovine may undergo involution stage II. The main indications that the gland underwent involution stage II, were reduction in lobular size, irregular outline and collapse of the alveolar lumen and marked increases in interlobular collagen-rich fibrous stroma (red arrows in Fig. 1) and fat (blue arrow in Figure 1). Higher magnification shows attenuation of the alveolar cuboidal epithelial cells (blue arrows in Fig. 2) and diminuation of the alveolar lumen (red arrow in Fig. 2). These histological features are also reflected by marked morphological differences in the shape of the udder of a cow induced into involution pregnant versus the test cow, which was not pregnant at drying-off (supplementary Fig. 1 vs. supplementary Fig. 2).
Discussion

Interpretation of results

The present results show for the first time (to the best of our knowledge) that the mammary gland of a dairy cow may undergo extensive obliteration of the lobular-alveolar structure, as expected in involution stage II.

There are two possible explanations for the phenomenon described in this communication. First, this cow differs genetically from the vast majority of cows in the world. However, the likelihood of this being the cause is quite remote, because this cow already underwent two typical lactation cycles without anything unusual being noticed and in view of the fact that she differed from the rest of the cows in the same herd she would have had to have had a major mutation without exhibiting any phenotypic physiological defect.

Alternatively, during the 3rd trimester, pregnancy obstructs involution stage II in cows. Thus, when non-pregnant cows are induced into involution, even while yielding a lot of milk, which is the common situation in modern dairy cow husbandry, they will go through involution stages I and II as do most other mammals. This interpretation is independently supported by data from the literature.

In sheep induced into involution by weaning 5 d after parturition, the mammary glands were completely involuted after 30 d [23]. Thus, the present study has clearly shown that in a non-pregnant ruminant species, weaning was associated with complete involution. Similarly, pregnancy retarded mammary gland involution in mice. The mechanism for retarding or obstructing involution during pregnancy appears to be hormonal. Injections of glucocorticoids, progesterone, and prolactin after weaning inhibited mammary involution in mice [21], and similar results have been observed following grafting of progesterone and deoxycorticosterone implants into the mammary gland [22]. Conversely, reduction of circulating concentrations of prolactin by bromocriptine accelerated mammary involution [3]. Accelerating the rate of involution in dairy cows to 3 days, as in mice, has been achieved by treating the cows with casein hydrolyzate [10]. However, this treatment was not associated with obliteration of the lobular-alveolar structure (unpublished results). Thus, all in all, the present results support the hypothesis that in current pregnancy retards or obstructs involution stage II.

General biological implications

In the wild, pregnancy and lactation occur concurrently only when nutritional conditions are favorable. If conditions are poor, rebreeding will be delayed and lactation will continue at an energetically sustainable level, for much longer than its ‘normal’ duration. In this way the twin energetic burdens of pregnancy and lactation are separated and extremes are avoided [24]. In at least two mammalian species these natural interrelationships among pregnancy, lactation and nutritional state appeared to have been broken up. The first example as described above and by Knight [24] is the modern dairy cows, in which aggressive trait selection forces the cows to be concurrently pregnant and lactating for most of their adult life. However, it would be difficult to determine the long-term effect of such selection on the cow’s health, because the life expectancy of modern cows in a given herd is very short: two or three lactation cycles. (i.e., years). The life of humans in modern western societies is characterized by availability of abundant food, and the challenge is usually to avoid gaining weight, or to loose excess weight, rather than to cope with nutritional...
deficiency. It is also well known that breast-feeding is a "poor contraceptive". Thus, in humans concurrency of pregnancy and lactation is expected to be more common than in other mammalian species.

In women, pregnancy reduces the risk of breast cancer, but the reduction is relatively small and occurs only in women who bear their first child at an early age [25-28]. The susceptibility to breast cancer increases under conditions that reduce mammary epithelial differentiation, increase the expression of genes regulating cell proliferation, and down-regulate genes that improve DNA damage repair or induce apoptosis or differentiation [25]. Recently, evidence was presented and reviewed, which suggested that oxidative damage to the mammary gland epithelial cells is among the causes for the declining phase of lactation in mammals, even in animals that continue to be milked or suckled [29]. This highlights the physiological role of involution stage II as a means to replace oxidative damaged tissues with newly synthesized intact tissues.

All of the above-considerations suggest that retardation or obstruction of involution stage II is a risk factor for acquiring breast cancer. Thus, it is remarkable that we could not trace even a single example in the literature of a study in which concurrency of lactation and pregnancy was examined as an independent risk factor for breast cancer. To search for such a possibility is perhaps the most important suggestion that may be derived from the present communication.

Materials and Methods

We took an advantage of a rare event in the normal modern dairy farming: a cow which was falsely identified as pregnant was "dried-up" conventionally about 60 days before her expected parturition. However, after more than 60 days elapsed without the cow giving birth, it was recognized that she had not been pregnant, so that this cow’s mammary glands were induced into involution while she was not pregnant. Our attention was drawn to this cow’s short, compact udder, which more closely resembled that of a virgin heifer rather than that of a mature involuted pregnant cow (supplemented Fig. 1 vs. supplemented Fig. 2). The histology of the parenchyma of the mammary gland in this cow was examined to determine whether the lobular-alveolar structure was completely obliterated, as expected in involution stage II.

The test-case cow was a 3-year-old animal that was dried at day 279 of her 2nd lactation. Her accumulated milk yield in this lactation was 9930 liters and her milk yield at dry off was 30 liters/day. These figures are typical of many Israeli Holstein cows. The somatic cell count (i.e., total count of leukocytes and epithelial cells) were on the average 50,000 cells/ml, indicating that her udders were free of bacterial infection. The cow was sacrificed in abattoir 96 days after she was induced into dry-off.

At slaughter, the udder was removed quickly and pieces of mammary tissue parenchyma (~ 3 cm³) were dissected from each of three zones of the gland: upper, middle and lower (the latter being just above the gland cistern). Tissue samples were immediately placed in fixative and processed, and analyzed for gross histology by a professional histologist (AM, A.) as described previously [23]. Tissue samples from the mammary parenchyma of glands of three "normal" pregnant cows, induced into "dry-off" 2 days before their slaughter were taken to illustrate the "abnormal" features of involution in cows induced into dry-off pregnant. The mammary gland tissue samples from these cows were analyzed for gross histology in the same manner.
**Figure legends**

Figure 1:  
Gross histology of the mammary gland of the test cow at ×10 magnification. There is a reduction in lobular size, when compared with Fig. 4. Alveoli have irregular outline and there is a collapse of the lumen. There is markedly increased interlobular collagen-rich fibrous stroma (red arrows) and fat (blue arrow) (×10).

Figure 2:  
Gross histology of the mammary gland of the test cow at ×40 magnification. Note the attenuation of the alveolar cuboidal epithelial cells (blue arrows) and the decreased luminal size of alveoli (red arrow).

Figure 3:  
Gross histology of the mammary gland of a "normal" cow (pregnant, two days after dry-off) at ×10 magnification. Alveoli are distended. In most alveolar lumens there is evidence that proteinaceous material was secreted into the alveolar lumen (blue arrow). The interlobular stroma space is narrow (red arrow) and no interlobular fat is detectable.

Figure 4:  
Gross histology of the mammary gland of a "normal" cow (pregnant, two days after dry-off) at ×40 magnification. Note the normal cellular height, rich in cytoplasmic vacuolation of the alveolar epithelial cells in comparison with the situation in the test cow (Figs. 1 and 2).

**Supporting Information**

**Figure s1.**  
A picture of the udders of the test-case cow taken shortly before her culling.

**Figure s2**  
A typical picture of the udder of a cow after being induced into drying-off for ~3 weeks.

**Acknowledgment**  
The authors would like to thank Shamay Jacobi, the general manager of the ARO dairy research farm, for his cooperation and help in conducting this study.

**Author's contribution**  
GL and NS set in motion this study; UM carried out the pictures of the supplementary data, A-MA carried out the histology analysis; NS wrote the first version of the manuscript and GL and UM contributed to its final format.
References cited

1. **Lund LR Romer J Thomasset N Solberg H Pyke C Bissell MJ Dano K Werb Z.** (1996). Two distinct phases of apoptosis in mammary gland involution: Proteinase-independent and -dependent pathways. Develop 122: 181-193.

2. **Capuco AV, Akers RM.** (1999). Mammary involution in dairy animals. J Mam. Gland Biol. Neoplasma 4:137-144, 1999.

3. **Wilde CJ Knight CH Flint DJ.** (1999). Control of milk secretion and apoptosis during mammary involution. J. Mam. Gland Biol. Neoplasia 4: 129-136.

4. **Quarrie LH Addey CVP Wilde CJ.** (1996). Programmed cell death during mammary involution induced by weaning, litter removal, and milk stasis. J. Cell. Physiol. 168: 59-569.

5. **Li M Liu X Robinson G Bar-Peled U Wagner K-U Young WS Hennighausen L Furth PA.** (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc. Natl. Acad. Sci. U.S.A. 94: 3425-3430.

6. **Marti A Feng ZW Altermatt HJ Jaggi R.** (1997). Milk accumulation triggers apoptosis of mammary epithelial cells. Eur. J. Cell. Biol. 73: 158-165.

7. **Kadzere CT Murphy MR Silanikove N Maltz E.** (2002) Heat stress in high producing dairy cows: a review. Livestock Prod. Sci., 77: 59-91.

8. **Leitner G Jacoby S Maltz E Silanikove N.** (2007). Casein hydrolyzate intrammary treatment improves the comfort behavior of cows induced into dry-off. Livestock Sci 110: 292-297.

9. **Shamay A shapiro F Mahjeesh SJ Silanikove N.** (2002). Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. Life Sci., 70: 2707-2719.

10. **Shamay A Shapiro F Leitner G Silanikove N.** (2003). Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. J. Dairy Sci. 86:1250-1258.

11. **Silanikove N Shapiro F Shamay A Leitner G.** (2005). Role of xanthine oxidase, lactoperoxidase, and NO in the innate immune system of mammary secretion during active involution in dairy cows: manipulation with casein hydrolyzates. Free Radicals Biol. Med., 38: 1139-1151.

12. **Shamay A Homans R Fuerman Y Levine I Barash H Silanikove N Mahjeesh SJ.** (2005). Expression of albumin in nonhepatic tissues and its synthesis by the bovine mammary gland. J. Dairy Sci. 88: 569-576.

13. **Clarkson RW Wayland MT Lee J Freeman T Watson CJ.** (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Res., 6: R92-R109.

14. **Stein T Morris JS Davies CR Weber-Hall SJ Duffy MA Heath VJ Bell AK Ferrier RK Sandilands GP Gusterson BA.** (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. Breast Cancer Res., 6: R75-R91.

15. **Swanson EW Pardue FE Longmire DB.** (1967). Effect of gestation and dry period on deoxyribonucleic acid and alveolar characteristics of bovine mammary glands. J. Dairy Sci., 50: 1288.
16. **Holst BD Hurley WL Nelson DR.** (1987). Involution of the bovine mammary-gland - histological and ultrastructural-changes. J. Dairy Sci., 70: 935-944.

17. **Sordillo LM Nickerson SC.** (1988). Morphologic changes in the bovine mammary-gland during involution and lactogenesis. Am. J. Vet. Res. 49: 1112-1120.

18. **Capuco AV Akers RM Smith JJ.** (1997). Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. J. Dairy Sci., 80: 477-487.

19. **Noble MS Hurley WL.** (1999). Effects of secretion removal on bovine mammary gland function following an extended milk stasis. J. Dairy Sci., 82: 1723-1730.

20. **Ossowski L Beigel D Reich E.** (1979). Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell:16:929-940.

21. **Feng Z Marti A Jehn B Altermatt HJ Chicaiza G Jaggi R.** (1995). Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol., 131: 1095-1103 [Abstract].

22. **Capuco AV Lib M Long E Ren S Hrusk KS Schorr K Furth PA.** (2002). Pregnancy retards mammary involution: Effects on apoptosis and proliferation of the mammary epithelium after forced weaning of mice. Biol. Reprod., 66: 1471-1476.

23. **Tatarczuch L Philip C Lee CS.** (1977). Involution of sheep mammary gland. J. Anat. 190: 405-416.

24. **Knight CH.** (2004). Lactation and gestation in dairy cows: flexibility avoids nutritional extremes. Proc. Nutr. Soc., 60: 527-537.

25. **Pike MC Kraitl MD Henderson BE Casagrande JT Hoel DG.** (1983). “Hormonal” risk factors, “breast tissue age” and the age-incidence of breast cancer. Nature 303: 767-70.

26. **Ewertz M Duffy SW Adami HO Kvale G Lund E Meirik O Mellemgaard A Soini I Tulinius H.** (1990). Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. Int. J. Cancer 46: 597-603.

27. **Bain C Willett W Rosner B Speizer FE Belanger C Hennekens CH.** (1981). Early age at first birth and decreased risk of breast cancer. Am. J. Epidemiol., 114: 705–709.

28. **MacMahon B Cole P Lin TM Lowe CR Mirra AP Raynihar B Salber EJ Valaoras VG Yuasa S.** (1970). Age at first birth and breast cancer. Bull World Health Org., 43: 209-221.

29. **Hadsell D George J Torres D.** (2007). The Declining Phase of lactation: Peripheral or central, programmed or pathological? J. Mammary Gland Biol. Neoplasia 12: 59-70.
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Supplementary Figure 1

Supplementary Figure 2