

RESEARCH ARTICLE

Expression of Cytokeratin 8, 18 and 19 in the Period of Late Lactation and Involution in Cow Mammary Gland

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Abstract: Cytokeratins are intermediate filament proteins found in epithelial cells. Specific cytokeratin expression has been suggested to mark different epithelial cell line and also to associate with mammary stem/progenitor cells. However, a comparative analysis of the expression of cytokeratins in the mammary gland during the late lactation and involution periods was limited. Here, the aim of the present study is to evaluate CK8, CK18 and CK19 expressions of the mammary gland in cows during late lactation and involution periods, immunohistochemically. The lobe and lobule structure were prominent in the mammary tissue in the late lactation period, but the amount of connective tissue started to increase, and epithelial cells found on the walls of the alveoli had different appearances based on their secretion status. The walls of the ducts were covered by simple columnar epithelial cells during late lactation. CK8 and CK18 showed strong expressions in the epithelial cells of several alveoli and ducts inside the lobes and lobules in the late lactation and involution periods. In both late lactation and involution periods, there was no CK19 expression in the mammary gland tissue. In conclusion, it was demonstrated that CK8 and CK18 were expressed in the alveolar and ductal epithelial cells of the mammary gland in cows in the late lactation and involution periods, but CK19 was not expressed. Thus, our study findings revealed the role of CK8 and CK18 in mammary epithelial differentiation and maintenance of the normal mammary epithelial layer.

Keywords: Mammary gland, Cytokeratin, Cow, Immunohistochemistry

İnek Meme Bezinde Laktasyon Sonu ve İnvolyasyon Döneminde Sitokeratin 8, 18 ve 19 Ekspresyonu

Öz: Sitokeratinler, epitel hücrelerinde bulunan ara filament proteinlerindedir. Ekspresyonları organ veya dokuya özgüdür ve epitel hücrelerinin tanımlanmasına izin verir, ayrıca meme kök/progenitor hücreler arasındaki ilişkiyi ortaya koyar. Bununla birlikte, geç laktasyon ve involüsyon dönemlerinde meme bezinde sitokeratinlerin ekspresyonunun karşılaştırmalı bir analizi sınırlıydı. Bu çalışmanın amacı, geç laktasyon ve involüsyon dönemlerinde ineklerde meme bezinde CK8, CK18 ve CK19 ekspresyonunu immünohistokimyasal olarak değerlendirmektir. Geç laktasyon döneminde meme bezi dokusunda lob veya lobül yapıları belirgindi, ancak bağ dokusu miktarı artmaya başlamıştı ve alveol duvarında bulunan epitel hücreleri salgı durumuna göre farklı görünümlere sahipti. Geç laktasyon süresince kanalların duvarları tek katlı kolumnar epitel hücreleri ile örtülmüştü. CK8 ve CK18, geç laktasyon ve involüsyon dönemlerinde lob ve lobüllerin içindeki kalın epitel hücrelerinde güçlü ekspresyonlar göstermiştir. Hem geç laktasyon döneminde hem de involüsyon dönemlerinde meme bezi dokusunda CK 19 ekspresyonu yoktu. Sonuç olarak geç laktasyon ve involüsyon döneminde ineklerde meme bezinin alveolar ve duktal epitel hücrelerinde CK8 ve CK18'in eksprese edildiği, ancak CK19'un eksprese edilmediği gösterildi. Böylece, çalışma bulgularımız CK8 ve CK18'in meme epitel farklılaşması ve normal meme epitel tabakasının korunmasındaki rolünü ortaya koydu.

Anahtar sözcükler: Meme bezi, Sitokeratin, İnek, İmmünohistokimya

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INTRODUCTION

The mammary gland is a complex organ that consists of various groups of tissues and cells, which undergo certain processes like growth differentiation as the organ develops in distinct stages. These physiological stages are regulated both systemically and locally, by means of steroid hormones of ovarian origin -like estrogen and progesterone-, as well as several other molecular factors. The sustainability of milk yield in farm animals depends on the reproduction strategies implemented for the herd at large, but the aforementioned cellular mechanisms that determine the numbers and activities of secretory cells in the mammary gland determine the final milk output and its sustainability ^[1]. The mammary gland of cows is composed of a network of alveoli and ducts localized in the stroma, which show a high degree of branching ^[2]. This system consists of epithelial and myoepithelial cells that cover the ducts and alveoli ^[2,3]. The epithelial cells are surrounded by the stroma at the perimeter, where some cell types responsible for the homeostasis of the mammary gland (adipocytes, fibroblasts, and immune cells) can be found. Alveolar epithelial cells synthesize and secrete milk. Myoepithelial cells surround alveolar epithelial cells, adhere to the base membrane, and contract to transfer the milk secreted in the alveolar lumen to the ducts. Myoepithelial cells in the mammary gland also mediate the proliferation, survival, and differentiation of alveolar cells, and contribute to breast morphogenesis by modulating stromal cells ^[4]. In dairy cows, the mammary tissue undergoes comprehensive morphological and functional transformation stages that occur concerning cellular and structural changes of the lactation period ^[5]. In the early lactation period, mammary cells multiply at a rate that exceeds the rate of apoptosis and increase their total number. With the decrease in milk yield in the late lactation period, the loss of mammary cells (apoptosis) starts. This situation leads to the involution process where the mammary alveoli are effaced ^[6].

Three types of filaments constitute the cytoskeleton of all mammalian tissues. These are microfilaments, microtubules, and intermediate filaments. Depending on both the function of the tissue and the type of epithelial cells, intermediate filaments regulate the growth status and differentiation of the tissue they are in ^[1]. Cytokeratins (CKs) are intermediate filament proteins that are present in most epithelial cells. Their expression is largely organ or tissue specific. The CK expressed by an epithelial cell is mainly dependent on the type of the epithelial cell, its state in the terminal differentiation process, and its developmental stage. Accordingly, determining CK expressions allow the identification of epithelial cells ^[3,7,8]. There are two types of CKs which are usually found in the form of heterodimers among acidic type I CKs (CK9–

CK20) and basic or neutral type II CKs (CK1–CK8). Some CKs act as lineage markers in the mammary epithelium. While CK8 and CK18 are found characteristically in the luminal cells of a normal gland, CK5 and CK14 are localized in basal or myoepithelial cells. CK7 and CK19 are generally expressed in luminal cells, and sometimes basal cells ^[9]. Additionally, it has been accepted that CK6 is strongly expressed by mammary gland alveolar epithelial cells in mice, and it can be a marker for multipotent/bipotent mammary epithelial progenitor cells in the pregnancy, lactation, and involution periods ^[10-12]. CK14/CK8-positive suprabasal/luminal cells have rarely been detected in adult mouse mammary glands, and it was proposed that they are progenitor cells ^[13]. CK19 is known as a neutral CK, and its expression is accepted to be a lumen marker in human mammary glands ^[14]. Studies conducted in cows about the mammary gland have also reported that some CKs are expressed in various cell groups in the lactation or involution period ^[15].

Throughout the lifecycles of female mammals, the mammary gland goes through a set of periodical changes in pregnancy, lactation, and involution. These changes are regulated by the complex interaction of hormonal and molecular factors ^[16,17]. Cows are arguably the most significant animals for the dairy industry, and the cow mammary gland is a complex tissue with has various physiological, biochemical, and immunological functions ^[18]. The presence and amounts of molecular factors localized in the mammary gland may significantly influence the milk yield; therefore, understanding their physiological roles carries importance. This cellular and functional complexity make up for an interesting research topic regarding the contributions of different components in the functioning of the mammary gland. In this context, the aim of this study was to determine the localization of the CK8, CK18, and CK19 in the mammary gland cells of cows in the late lactation and involution periods, and to determine whether the localization and expression intensities of these locally expressed factors within the mammary glands are influenced by the lactation and involution periods.

MATERIAL AND METHODS

Experimental Animals and Samples Collection

The animal material of this study consisted of 10 Holstein cows between the ages of 2 to 8. The animals had been transported to local slaughterhouses in the province of Diyarbakır, Türkiye had normal reproductive performances, and did not show any noticeable mammary problems in their macroscopic examination. Through clinical observation, the mammary glands of the animals were examined to determine the presence or absence of diseases such as mastitis. The owners of the animals were questioned to obtain information regarding the

periods in which the mammary glands of the animals were [4,19]. The study animals were selected based on the statements of their owners and clinical inspections, and the animals were slaughtered. Samples were then collected from both the right frontal and left posterior lobes of the mammary glands of the animals. Tissue samples from different parts of mammary glands (upper, middle and lower parts) were also taken. The specimens were fixed in a 10% formaldehyde-alcohol solution after collection. Following fixation, the tissues were dehydrated, cleared and embedded in paraffin blocks. Five- μm -thick serial sections were then taken from these blocks at intervals of at least 100 μm . To help identify the periods of the mammary glands histologically, sections samples were stained with Crosman's triple stain and examined. For immunohistochemical (IHC) analyses, 3 preparations were made from each mammary gland specimen. The tissue sections were placed on slides coated with 3-aminopropyl-ethoxy silane (APES) (Sigma-Aldrich Chemicals, St. Louis, MO, USA) and were left to dry at room temperature for a day.

Determination of the Mammary Glands Periods

In the identification of the periods of the mammary glands of the animals, in addition to the clinical examinations and the statements of their owners, histological examination of the lobes and lobules in the mammary glands was used. The amount of connective tissue, formations of large vacuoles in epithelial cells (as a result of the intracellular accumulation of droplets of fat), and of secretory vesicles, the histological appearances of alveolar and ductal epithelial cells, and the presence of casein concretions (in the form of colloidal masses or a concentric structure in alveolar lumina) were used as determination criteria [4,19]. Based on the examinations, the animals were divided into two groups as late lactation (N:4) and involution (N:6) groups.

Immunohistochemical Staining

The Avidin-Biotin-Peroxidase Complex (ABC) procedure was performed for immunohistochemical staining. To inactivate endogenous peroxidase, the sections were kept in 3% hydrogen peroxide prepared with methanol for 30 min and washed with 0.01M PBS for 3 x 5 min. For antigen retrieval, the sections were boiled in citrate buffer solution (pH:6) for 20 min. Next, they were cooled and washed with PBS for 3 x 5 min. The sections were incubated with UV block for 10 min. The prepared specimens were incubated overnight at +4°C with 1/100-diluted CK8, CK18 and CK19 primary antibodies (Mouse Monoclonal Antibody, Cytokeratin 8, catalogue no: ab2530 Abcam, Mouse monoclonal Antibody Cytokeratins 18, catalogue no: ab668 Abcam, Mouse Monoclonal Antibody Cytokeratin 19, catalogue no: MA5-12663 Invitrogen). After incubation, the sections were washed with 0.01M PBS for 3 x 5 min.

The samples were then kept at room temperature for 20 min with biotinylated secondary antibodies (Histostain Plus Bulk Kit, Zymed) and washed with 0.01M PBS for 3 x 5 min. Next, the sections were incubated with streptavidin-peroxidase (HRP- Histostain Plus Bulk Kit, Zymed) for 20 min, followed by washing with PBS for 3 x 5 min. Finally, the sections were incubated with 3,3-diaminobenzidine (DAB) chromogen solution for 5-15 min according to the manufacturer's protocol, counterstained with Harris Hematoxylin for 3 min, dehydrated through an alcohol series, cleared in xylene, and mounted in Entellan (Merck, Darmstadt, Germany, Cat. No:107960) under a coverslip.

The specificity of the immunohistochemical staining process was analyzed using negative and positive controls. Tongue and skin sections of the cows were utilized as the positive control. For the negative control, non-specific immune serum was dripped instead of primary antibodies, and the remaining staining steps were repeated as in the normal staining procedures. The same protocol was applied to all sections.

The immunoreactivities of CK8, CK18, and CK19 were examined and photographed using a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope equipped with a DS-RI1 video camera (DS-U3, Nikon, Tokyo, Japan).

Semi-Quantitative Analysis

The immunohistochemical staining results were examined based on the intensity score. The cells that showed a positive reaction for the expressions of CK8, CK18, and CK19 were qualitatively analyzed. The positive cells were scored on four levels as (-) negative; (+) weak; (++) medium or (+++) strong, based on their intensity of staining. The assessments of the positively stained cells were made by two experiment-blind researchers (A.A.A. and U.T.), and average scores were calculated. In the mammary gland sections, the expression of CK8, CK18, and CK19 were examined at 40x, 100x and 400x magnifications. In each zone of the mammary gland specimen, three randomly selected areas were examined. The results were separately examined for alveolar and ductal epithelial, stromal, and myoepithelial cells. The results are presented in the results section (Table 1).

RESULTS

Histological Results

In all specimens, the mammary gland was surrounded by a capsule formed by connective tissue with compound tubule-alveolar structures, as described in previous studies. It was observed that the lobe-and-lobule structure was prominent in the mammary tissue during the late lactation period, but the amount of connective tissues was slightly elevated, and epithelial cells found on the walls

of the alveoli had different appearances based on their secretion status. Accordingly, in the alveoli, both simple columnar epithelial cells filled with secretion could be seen, along with empty, flatter cells that had released their secretion. Casein concretions in the form of colloidal masses were detected within the lumens of some of the alveoli. A thin layer of connective tissue surrounded the walls of the ducts, which in turn as covered by simple columnar epithelial cells. The ducts' lumens were still wide, the branching continued. Lumens of some ducts were filled with secretory material.

In the mammary tissue in the involution period, as a result of the intracellular accumulation of droplets of fat and secretory vesicles, large vacuoles in the simple cuboidal epithelial cells were noticeable. The lumen of the alveoli had become narrower, and the inter-alveolar connective tissue amount was elevated. The lumens of many of the mammary alveoli were narrower than their counterparts in the late lactation period samples. The ducts were lined with simple cuboidal or simple squamous epithelium, and these ducts were filled with secretory material (Fig. 1).

Immunohistochemical Results

Late lactation: It was revealed that CK8 and CK18 showed strong expressions in the epithelial cells of several alveoli and ducts within the lobes and lobules. These expressions were particularly localized in the cytoplasm. There were no CK8 or CK18 expressions in the myoepithelial cells. No CK8 or CK18 expression was detected in the cells and blood vessels of the connective tissues forming the lobes and lobules, either. Some individual cells localized in the connective tissue displayed positive reactions of CK8 and CK18 at varying intensities (Fig. 2, Fig. 3-A). There was no CK19 expression in any tissue component constituting the mammary glands of the cows in our study (Fig. 4-A).

Involution: CK8 and CK18 were found to show strong

Table 1. Semiquantitative evaluations of immunohistochemical staining intensities of CK8, CK18 and CK19

Mammary	Cells	CK8	CK18	CK19
End of Lactation	Alveolar epithelium	(+++)	(+++)	(-)
	Channel Epithelium	(++)/(+++)	(+++)	(-)
	Stromal Cells	(-)	(-)	(-)
	Myoepithelial Cells	(-)	(-)	(-)
Involution	Alveolar epithelium	(++)/(+++)	(+++)	(-)
	Channel Epithelium	(++)/(+++)	(+++)	(-)
	Stromal Cells	(-)	(-)	(-)
	Myoepithelial Cells	(-)	(-)	(-)

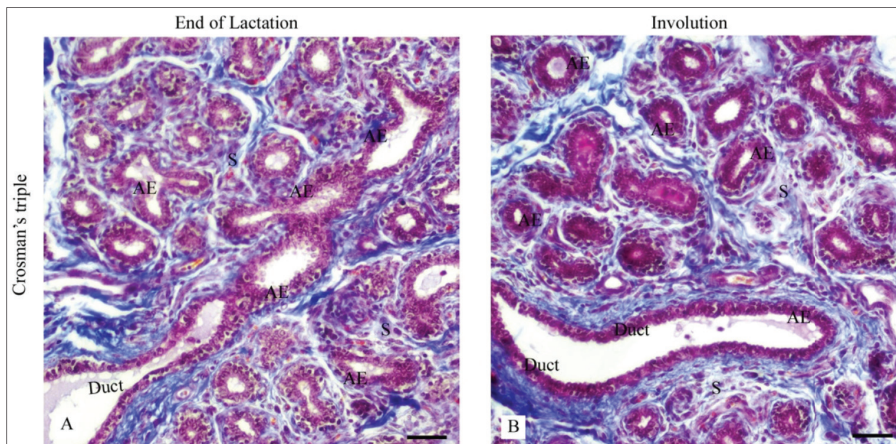


Fig 1. Crosman's triple stain. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alveolar ductus. Bar: 50 µm (A,B)

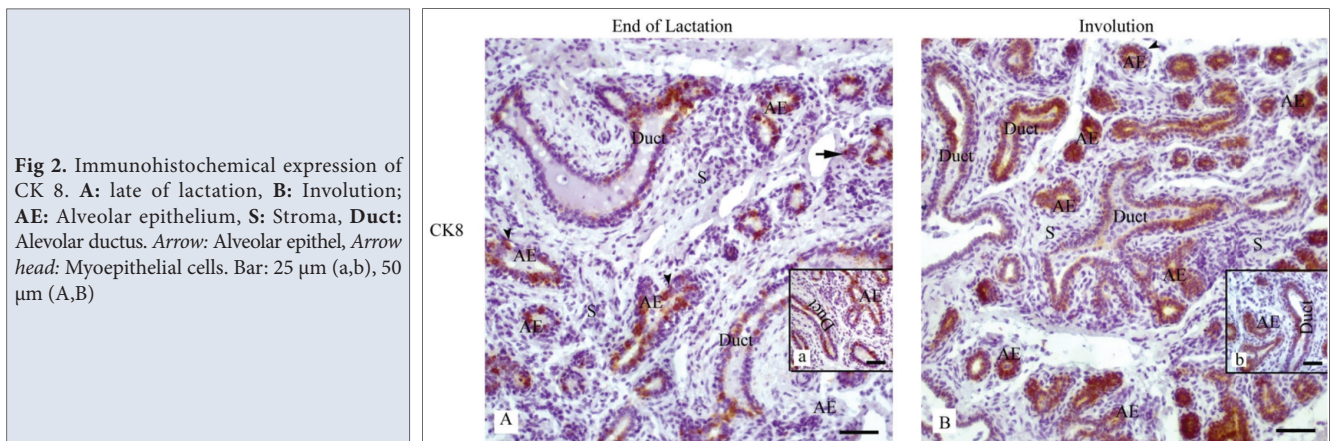


Fig 2. Immunohistochemical expression of CK 8. **A:** late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alevolar ductus. **Arrow:** Alveolar epithel, **Arrow head:** Myoepithelial cells. Bar: 25 µm (a,b), 50 µm (A,B)

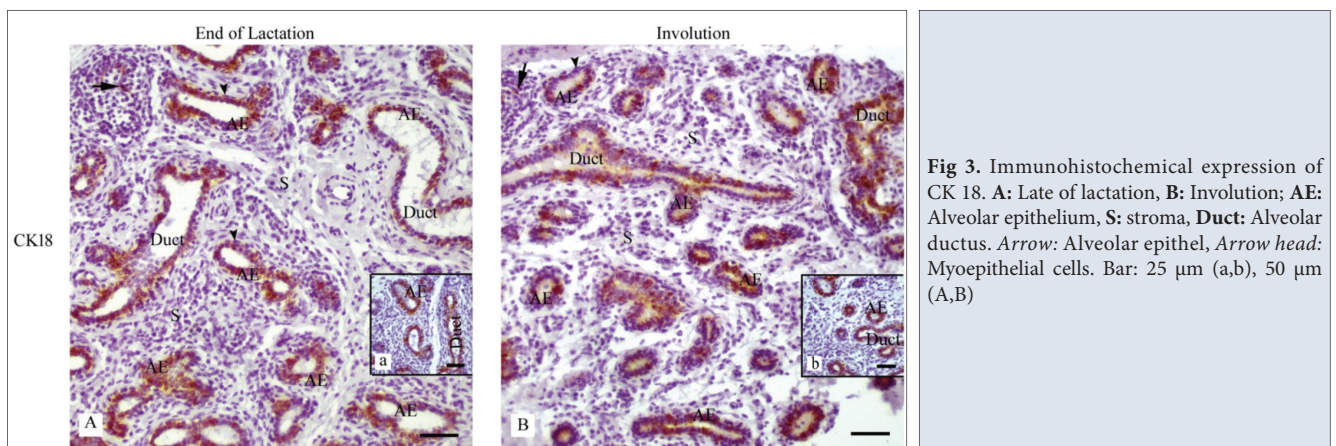


Fig 3. Immunohistochemical expression of CK 18. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** stroma, **Duct:** Alveolar ductus. **Arrow:** Alveolar epithel, **Arrow head:** Myoepithelial cells. Bar: 25 μm (a,b), 50 μm (A,B)

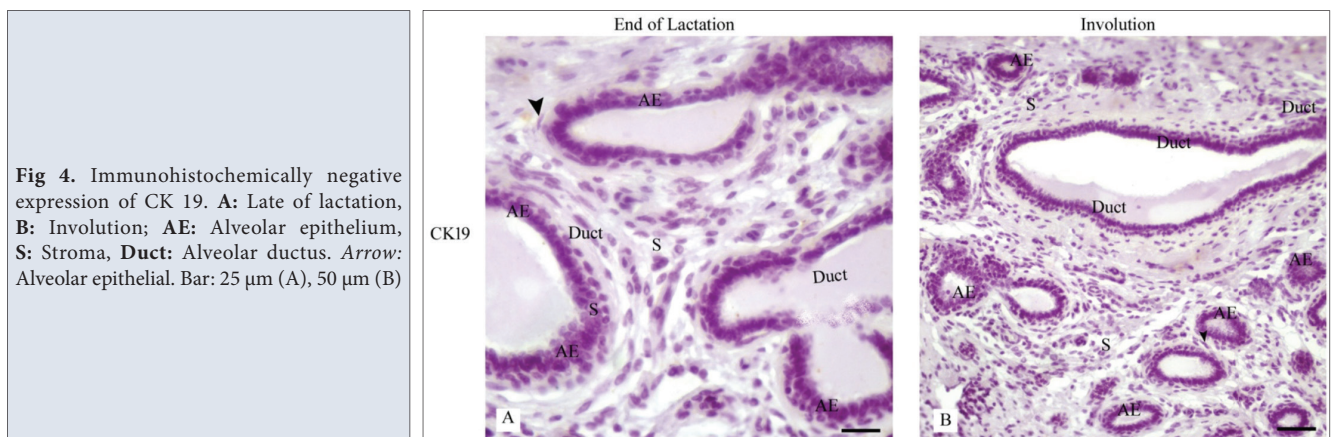


Fig 4. Immunohistochemically negative expression of CK 19. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alveolar ductus. **Arrow:** Alveolar epithel. Bar: 25 μm (A), 50 μm (B)

cytoplasmic expressions in the epithelial cells covering the alveoli and ducts. CK8 and CK18 had positive reactions in some individual cells belonging to the fibrocyte/fibroblast, macrophage (probably) in the septa of the connective tissue (Fig. 2, Fig. 3-B). There was no expression in myoepithelial cells, connective tissue cells or blood vessels. There was no CK19 expression in the mammary gland tissue in this period either (Fig. 4-B).

DISCUSSION

In the mammary gland tissue, molecular regulations influence the process of lactation and involution periods. Expressions of genes that regulate hormonal and molecular factors in the mammary gland are important for milk production in ruminants. In the development (mammogenesis) and lactation (galactopoiesis) periods of the mammary gland in dairy cows, each group of cells has a certain property related to the plasticity of the mammary gland. As in most glandular tissues, the mammary glands of adults also contain several types of cells that interact to shape the organ and make it functional. This study revealed the expressions and localizations of CKs among the intermediate filaments taking part in the cytoskeleton, especially in groups of cells that play a role in mammary gland plasticity, as well as their potential roles

in cell differentiation, in the late lactation and involution periods.

The ruminant mammary gland is organized in terminal duct-lobular units. A terminal duct lobular unit is a group, or lobule, of mammary acini with dead-ends, along with intralobular and extralobular portions of the subtending terminal ducts of the mammary gland [20]. The involution period has some dramatic changes occurring within the mammary gland at the end of lactation. Involution is initiated after sudden or gradual weaning among all species in both experimental and natural settings. Understanding the biological process of mammary gland regression is important as it supports livestock measures taken at weaning time to reduce the incidence of mastitis in all ruminants, including cows [21]. A typical lactation curve in cows is defined by a peak point in milk yield that lasts between the 30th and 90th days, followed by a decrease in milk yield, which is known as the late lactation period. In this study, it was determined that lobe formation was prominent in the late lactation and involution periods as stated by Holst et al. [19] and Hurley [22] for cows, but the amount of connective tissue was found to be elevated. The epithelial cells covering the walls of the alveoli and ducts also had different appearances based on their secretion status. Casein corpuscles were seen in the lumina of some

alveoli. The walls of the ducts were found to be covered by simple columnar epithelial cells, and the lumina were filled with secretory material. Furthermore, a large reduction in the volume of the mammary gland was observed with the onset of involution, and the residue of the collapsed alveolar structures was more prominent compared to that of the late lactation period. The alveoli and ducts also had narrower lumina [19,22].

A literature review performed as part of this study revealed that most studies about the localization of CKs have been conducted on mammary gland tumors [23,24]. Some studies have reported that alveolar and ductal epithelial cells and myoepithelial cells in the normal mammary tissue (in humans and mice) expressed CK8 and CK18 at different intensities and to a noticeable extent [16,25]. CK8 and CK18 were shown to be expressed in epithelial cells in the mammary glands of cows in the involution period [2]. Similarly, it was reported that CK18 was strongly expressed in mammary gland primary epithelial cells and cell cultures obtained from the mammary gland during the lactation period in cows. This was surmised as a potential characteristic marker for mammary epithelial cells in cows [26]. In other studies, performed on the human mammary gland in resting state, it was reported that CK8 and CK18 were expressed in alveolar and ductal epithelial cells, but no information was provided about the intensity of expression [3,27]. It was revealed that CK18 was strongly expressed in the *in vitro* cultures of cow and mouse mammary epithelial cells [21]. In agreement with the studies mentioned above, in this study, CK8 and CK18 were strongly expressed in the alveolar and ductal epithelial cells of the mammary gland in the late lactation and involution periods in cow mammary tissues. However, as opposed to reports on humans and mice, no CK8 or CK18 expression was detected in the myoepithelial cells of the cows in this study [16,25]. Additionally, in our study, no cellular localization of either of these two factors was observed in connective tissue cells and blood vessels in the late lactation period or the involution period.

The complex events that develop in the normal mammary gland throughout pregnancy, lactation and involution can be distinguished based on the expressions of CKs as markers of epithelial cell differentiation [28,29]. Mammary epithelial cells may also be recognized by their expression of intermediate filaments, although this may vary between species and between locations within the mammary gland. For example, the expression of specific CKs may be different between epithelial cells in ducts and within alveolar units [30]. In both normal and neoplastic mammary gland tissues, the biological importance of heterogeneous CK expressions has not been completely understood, and there is a need to shed light on the control mechanisms of these expressions in normal mammary growth and malignant transformation [31,32].

CK filaments take part in providing the mechanical scaffolding that is needed by cells in epithelial tissue [33]. In any case, they regulate morphogenesis and cell differentiation in tissues or organs. In particular, CKs show molecular and functional expressions in tissues depending on epithelium type and differentiation characteristics. These situations provide us with information about different cytogenetic changes in tissues [16,33]. Among intermediate filament in the CK family, CK18 is the most prevalently encountered member in tissues and organs, and it is usually found alongside CK8. Both are expressed in simple epithelial cells in the organism, and they are known as significant markers for alveolar and ductal epithelial cells [16,22]. Considering the information given above, the presence of CK8 and CK18 expressions in the late lactation and involution periods in our study suggested that these CKs may also perform similar functions in the mammary glands of cows.

CK19 is known as an important marker for alveolar epithelial cells [32]. CK19 expression is mostly seen in cells with an epithelial origin that has high plasticity such as stem cells, cells with a high capacity to differentiate, and tumoral cells [8]. Alveolar and ductal epithelial cells in human and mouse mammary glands were reported to express CK19 at varying intensities [16]. In their study on cows, Ontsouka et al. [32] demonstrated that CK7 and CK19 levels were either at very low or undetectable levels in the primary cells (epithelial cells) of the mammary gland. The localization of CK7 and CK19 expressions have been shown in cell cultures that were subcultured and made immortal after collection from the mammary gland, especially in the pregnancy, lactation or involution period of cows [32-34]. In our study, neither alveolar nor ductal epithelial cells in the cow mammary glands displayed CK19 expressions in either of the late lactation or involution periods. The absence of CK19 expressions in the mammary glands of cows in this study and clear contradictions between this study and other studies demonstrated the complex nature of cells with the mammary origin and suggested that analyses conducted based solely on the expressions of cell type markers may be risky. Moreover, as opposed to the results of this study, the information that CK19 is a marker for epithelial cells in both mammary gland epithelial cells and cell cultures obtained from the mammary gland still holds.

In conclusion, in this study, it was demonstrated that CK8 and CK18 were expressed in the alveolar and ductal epithelial cells of the mammary gland in cows in the late lactation and involution periods, but CK19 was not expressed in any of these scenarios. This gave rise to the idea that CK8 and CK18 expressions may be effective -rather than CK19- on the differentiation of epithelial cells for the cytoarchitecture in the mammary gland,

at the very least for cows. We humbly believe that by increasing our knowledge about the regulation of these molecular factors in the mammary gland, we will be able to develop methods for increasing milk yield and lactation sustainability, shorten the length of the dry period between two consecutive lactation periods, and increase milk productivity. Furthermore, data on cytokeratins in the mammary glands of cows may constitute an excellent alternative model for biochemical studies that investigate their potential roles in the physiological functions of the breast and carcinogenesis.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study available from the corresponding author (A. Arkaş Alklay) on reasonable request.

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COMPETING INTEREST

The authors declare that they no conflict of interest.

AUTHOR CONTRIBUTORS

AAA and UT planned the study, designed the experiments and helped manuscript writing; FÇ helped with data analyses and bioinformatics and wrote the manuscript; UT, FÇ and NA collected samples and conducted laboratory process; BB and ÖA analysed the statistics data. All authors read and approved the final manuscript.

ETHICAL APPROVAL

The materials used in our study were collected from the slaughterhouses of the province of Diyarbakir, and in accordance with the regulation on the working procedures and principles of animal experimentation ethics committees in the official gazette published on February 15, 'Procedures with dead animals or tissues, slaughterhouse materials, waste fetuses' are not subject to HADYEK permission.

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