Adhesion Molecules and the Cellular Population of the Normal Camel (*Camelus dromedaries*) Mammary Glands

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Abstract: The demand to camel's (*Camelus dromedaries*) milk is increasing worldwide. The new advances in the dairy industry have made the camel milk production on the commercial level is possible. The camel mammary glands immune system is not explored in detail. This study was conducted to identify the cell adhesion molecules involved in the regulation of the cells trafficking to the camel mammary glands at two different physiological stages; lactation and non-lactating periods. The expression and distribution of CD markers and the adhesion molecules, $CD4^+$, $CD8^+$, MAdCAM-1, $WC+1^+$, CD62L, CD11a/CD18 (LFA-1), VCAM-1, TCR-αβ, $CD44^+$ and $CD20^+$ in the alveolar tissues, supramammary lymph nodes, Peyer's patches and mesenteric lymph node of the healthy camels were explored. The expression of the adhesion molecules was determined *in situ* by immunohistochemical technique using immunoperoxidase staining. MAdCAM-1 was detected in almost all the tissues at the two physiological stages in which high expression was evident in the non-lactating period. $CD8^+$ T-cells were detected in both mammary alveolar tissues and the supramammary lymph nodes, with the highest expression observed in the lactating period. $WC+1^+$ expression on γδ cells were evident in mammary tissues and supramammary lymph nodes at both stages; however, the expression was higher in the non-lactating period. The detection of $CD20^+$, CD62L, VCAM-1, TCR-αβ, and $CD44^+$ expression failed despite their expression in the original species that the antibodies were raised against. The expression of $CD4^+$ and CD11a/CD18 (LFA-1) were not detected at all.

The intensive expression of the MAdCAM-1 in the camel mammary glands could indicates that camel mammary glands cells trafficking is closely linked to the intestinal immune system (mucosal) rather than peripheral as it is well known for the bovine mammary glands. The high expression of $WC+1^+$ strongly reflects the importance of these cells subset in the defense mechanism of the camel mammary glands during the late lactation.

Keywords: Camel, adhesion molecules, $WC+1^+$, $CD8^+$, MAdCAM-1, gamma delta cells, mammary glands.

1. INTRODUCTION

Dromedary camel (*Camelus dromedarius*) is one of the highly valuable domestic animals in Saudi Arabia. Camel is multipurpose animal that can be used for meat, milk, and wool production. In addition to the previous traditional commodities, modern applications in the dairy industry lead to the development of camel dairy farms that are capable of producing camel milk on the commercial level. Camel milk and meat are considered an important source of proteins for wide range of population [1]. It was estimated that world camel milk market worth 10 billion dollars [2].

Similar to cow, the camel mammary gland is consisted of four glandular quarters and it is located in the inguinal region. The left and right halves of the udder are separated from each other by fibroblastic tissue extending from the linea alba and pre pubic tendon. A groove is generally visible between the left and right halves, which is more distinct in the lactating than in the dry period. The lateral aspect of the quarters is covered by tissue from the abdominal tunica and the caudal abdominal wall. Although the anterior (front) and posterior (back) quarters are independent and totally separated, the separation between them is invisible from the outside [3]. The camel mammary gland however, has no gland cistern i.e. no milk reservoir beyond the teat cistern but consisted of compound tubule-alveolar glands, parenchyma, connective tissue stroma, ducts, and alveolar systems, similar to those of the cow [3]. Schwartz and Dioli, (1992) [4] demonstrated that the bovine teats possess only one duct cistern whereas, the teat of the camel possess 2-3 cisterns. Each teat cistern is spindle shape, tapers distally, and possess streak canal. The streak canals are short and small so that ordinary cannula used in the udder of the cow is too large for the camels [4].

Certain differences were observed between bovine and camel milk and colostrum constituents [5, 6]. Camel colostrum and milk is dominated by enzyme inhibitory subclasses IgG2 and IgG3, whereas IgA is present with lower concentration in colostrum [5, 6]. Camel milk is also rich with several inhibitory proteins, like high concentration of lysozyme, lactophorin (proteose peptone component-3), which is a member of the glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), and the peptidoglycan

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1874-3188/12

recognition protein (PGRP) that plays important role in preventing the adhesion and bacterial multiplication [6].

The nature of the immune responses in the camel mammary gland is not fully explored. Studies on the cellular populations of the camel mammary glands and the lymphocytes trafficking in health and disease are very scarce.

Lymphocyte trafficking to the mammary glands is under the influence of adhesion molecules (AM). Expression of AM such as selectins, like CD62L, and integrins, like mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) dictate the type of recruited cells and their homing destination [7]. Therefore, AM expression is vital in determining the mammary glands cellular populations in health and disease.

Studies on the bovine mammary glands revealed extensive variation in the scale and types of AM that are expressed at different of lactation period [8, 9]. For instance, $CD8^+$ T-lymphocytes prevail during the lactation period, whereas the $CD4^+$: $CD8^+$ ratio increases toward the $CD4^+$ at the end of the lactation period [10].

Lymphocytes recirculation is either of peripheral or mucosal nature [11]. Kehrli and Harp (2001) [11] have shown a difference in the lymphocyte recirculation from mucosal and peripheral tissue between the mammary glands of the ruminant and other monogastric animals. It appears that ruminant cell trafficking pathway is not part of the common mucosal immune system as it was defined for the monogastrics. In mice, MAdCAM-1 is expressed in Peyer's patches, gut lamina properia, and mesenteric lymph node, however in ruminant; MAdCAM-1 expression is missing in these tissues [12-15]. In accordance with this findings, it was therefore, clearly demonstrated that lymphocytes that home from MAdCAM-1 expressing tissues are of mucosal origin as in the monogastric animals, whereas, cells that home from tissues that lack MAdCAM-1 expression are of peripheral nature like in most of the ruminant animals [11]. The origin of the cells trafficking plays a major role in dictating route of vaccination and pathogenesis of different mastitis.

The aim of this study was to reveal types of expressed AM and the cellular population in the camel healthy mammary glands at different lactation periods. The role of the AM in normal mammary glands was seen essential in understanding the nature of the immune activities in camel mammary glands and resistance to mastitis at different physiological stages. This study also aimed to reveal the nature of the cells trafficking to camel mammary glands whether it is of peripheral or mucosal nature.

2. MATERIAL AND METHODS

2.1. Animals and Tissue Collection

Samples were collected from healthy 10 years-old multiparious female dromedary camels (*Camelus dromedarius*). Immediately after slaughter at (Alomran Slaughter House, Alahsa, Eastern Province, Saudi Arabia, the mammary glands were examined and only those free from any gross pathological changes during postmortem examination were selected.

Five lactating (mid-lactation) and other five non-lactating (dry period or late-lactation) mammary glands were excised. Immediately after slaughter, the tissue samples were collected from parenchyma, supramammary lymph node, Peyer's patches, and mesenteric lymph node. The samples were dissected out from any connective tissues before it was immersed in a fixative (4% paraformaldehyde in PBS (PH 7.4).

2.1.1. The Preparation of the Collected Samples

The fixed samples were kept for 48 hours at room temperature for the routine histological and immunohisto-chemical techniques. After fixation, the specimens were trimmed to about 0.5 cm and dehydrated in ascending grades of alcohol (70% = 3 hours, 70% = 2.5 hours, 80% = 2 hours, 80% = 1.5 hours, 90% = 1.5 hours, 95% = 1.5 hours, 100% = 1.5 hours X2) cleared in xylene (1.5 hours X2) and subsequently embedded in paraffin wax as blocks (1.5 hours X2) using automated tissue processor (TP 1020, Leica, Germany). Then sectioned at 5 µm thickness with rotary microtome (RM 2135, Leica, Germany). The sections were floated in warm water bath (41 °C). Then sections were mounted on the super frost plus glass slides for conducting both routine histological and immunohistochemical techniques.

2.2. Histology

The slides were then cleared in xylene and rehydrate in descending grades of alcohol, washed in distilled water for staining. Heamatoxylin and eosin (H&E) stain were performed for general histology to differentiate between various stages of lactation. Briefly, sections were deparaffinized and hydrated to distilled water. Sections then stained with Ehrlich Heamatoxylin for 5-10 minutes to stain the nuclei, washed in running tap water for 15-20 minutes, followed by Eosin stain for 5-10 minutes. After that, sections were dehydrated in ascending grade of alcohol, cleared with xylene and coversliped in DPX. These slides were then examined with light microscope at magnifications of 5×, 10×, 40×, and 100×. Histological images were obtained with an Olympus BX 41 microscope and Olympus DP-12 digital camera (Olympus Corp., Tokyo, Japan).

2.3. Monoclonal Antibodies

The Primary and secondary antibodies that were used for the immunohistochemical staining are listed in Table **1**. All primary and secondary antibodies are of murine origin. The secondary antibodies with 3-amino-9-ethylcarbazole (AEC) and visualized by horseradish peroxidase (HRP) (HRP-AEC) (anti-Mouse Kit, R&D systems).

2.4. Immunohistochemistry

The tissue sections for immunohistochemical techniques of 5 µm thickness were mounted on superfrost plus glass slides and were dewaxed in xylene and rehydrated in descending grades of alcohol, washed in phosphate buffered saline PBS (PH 7.4).

Then sections were processed according to the manufacturer's directions (R&D systems Inc., HRP-AEC mouse kit system, Minneapolis, Minnesota, US). Briefly, sections were bordered by Dako pen and then washed in PBS. From this point on, all the incubations except for the primary antibodies were performed at room temperature in a humidity box. The

Table 1. Primary Antibodies

Markers	Clone	Isotype	Concentration	Source
Mouse anti-bovine $CD8^+$	CC63	IgG2a	2µg/ml	AbD Serotec
Mouse anti-bovine $WC+1^+$	CC15	IgG2a	2µg/ml	AbD Serotec
Mouse anti-human MAdCAM-1	17FS	IgG1	1µg/ml	AbD Serotec
Mouse anti- human CD62L	9H6	IgG2a	2µg/ml	Abcam
Mouse anti- rat CD11a/CD18 (LFA-1)	OX42	IgG2a	1µg/ml	Abcam
Mouse anti-human VCAM-1	BBIG-VA (4B2)	IgG1	1µg/ml	R&D Systems, Inc
Mouse anti-rat TCR αβ	R73	IgG1	0.5µg/ml	BD Biosciences
Mouse anti-human $CD44^+$	2F10	IgG1	0.5µg/ml	R&D Systems
Mouse anti-bovine $CD4^+$	CC30	IgG1	10µg/ml	AbD Serotec
Mouse anti-human $CD20^+$	7D1	IgG1	1µg/ml	AbD Serotec

incubation with the primary antibodies however, was conducted over night at 4°C. First, sections were incubated with 3% hydrogen peroxide for 5 min. to quench the endogenous peroxidase activity followed by 5 min. wash in PBS. Sections were then incubated with normal blocking serum for 15 min. Then, excess serum was drained off and then the sections were incubated with avidin blocking reagent (15 min.), briefly washed, followed by incubation with biotin blocking reagent (15 min.). After brief rinsing with buffer, the slides were incubated overnight at 4 °C with optimal concentration of primary antibody diluted in reagent buffer (Table **1**). Sections were washed three times with PBS, drained carefully and were incubated with secondary biotinylated antibodies for 30 - 60 min. After washing with PBS, the sections were incubated with streptavidin-HRP conjugate (HSS-HRP) for 30 min. and finally washed. Visualization was achieved by immersing sections in freshly prepared AEC chromogen solution until desired stain intensity developed. The reaction was stopped by rinsing the sections with distilled water. Finally, slides were washed with distilled water for 5 min. mounted by aqueous mounting medium for observation. These sections were evaluated using a light microscope at magnifications of 5×, 10×, 40×, and 100×. Histological images were obtained with an Olympus BX 41 microscope and Olympus DP-12 digital camera (Olympus Corp., Tokyo, Japan).

2.5. Immunohistochemical Negative Control

The staining was performed as before except that the primary antibodies were replaced with PBS, while the rest of procedures were maintained. Controls were carried out on sections adjacent to those used in normal immunostaining protocol.

Intensity of immunoreactive staining was scored by two independent observations using the following range: Negative; (-) Weak; (+) Moderate; (++) Strong; (+++) Very strong (++++).

3. RESULTS

3.1. Haematoxylin and Eosin Staining (H & E Staining)

The H&E staining of the collected samples revealed clearly the normal structure and the cellular distribution of parenchyma in the lactating and the non-lactating mammary glands, lymph nodes and Peyer's patches (Figs. **1-3**).

The H&E staining of the non-lactating mammary glands indicated abundant connective tissue stroma. The lobules are seen to consist of intralobular ducts lined by simple cuboidal epithelium resting on a basement membrane. The epithelial lining of the lactiferous duct indicated gradual transformation from pseudostraitfied columnar in the initial part to stratified cuboidal or columnar in the lactiferous sinus. Incomplete layer of myoepithelial cells between the ductal epithelium and the basal lamina.

The H&E staining of the lactating mammary glands, however revealed that the lobules are packed with secretory alveoli, among which are seen some intralobular ducts. The intralobular and interlobular connective tissues are decreased in amount. Active alveoli are lined by low columnar epithelium and their lamina are filled with secretion, which appears as eosinophilic material containing vacuoles of dissolved fat. Resting alveoli have empty lumina and their cells are tall columnar.

3.2. The Expression of the Adhesion Molecules and the CD Markers at the Lactating and Non-Lactating Tissues Using Immunostaining

The labeling intensity of cells was scored on a subjective scale of: Negative; (-) Weak; (+) Moderate; (++) Strong; (+++) Very strong (++++).

3.3. The Adhesion Molecules and the CD Markers of the Lactating Tissues

Strong expression of $CD8^+$ was revealed in the alveolar tissue, supramammary and mesenteric lymph nodes and Peyer's patches (Fig. **1**). On the other hand, the $CD8^+$ expression in the mammary glands parenchyma was restricted to the alveoli (Fig. **1**).

The MAdCAM-1 and the work shop cluster$+1^+$ ($WC+1^+$) subtype γδ T-cells of the lactating camels indicated moderate expression in all of the tissues (Figs. **2**, **3**).

The expression of the adhesion molecules and the CD markers, $CD4^+$, TCR αβ, $CD20^+$, $CD44^+$, CD62L, vascular cell adhesion protein-1 (VCAM-1) and integrin leukocyte function antigen-1 (LFA-1) were not detected at both lactating stages. However, their expression was detected in

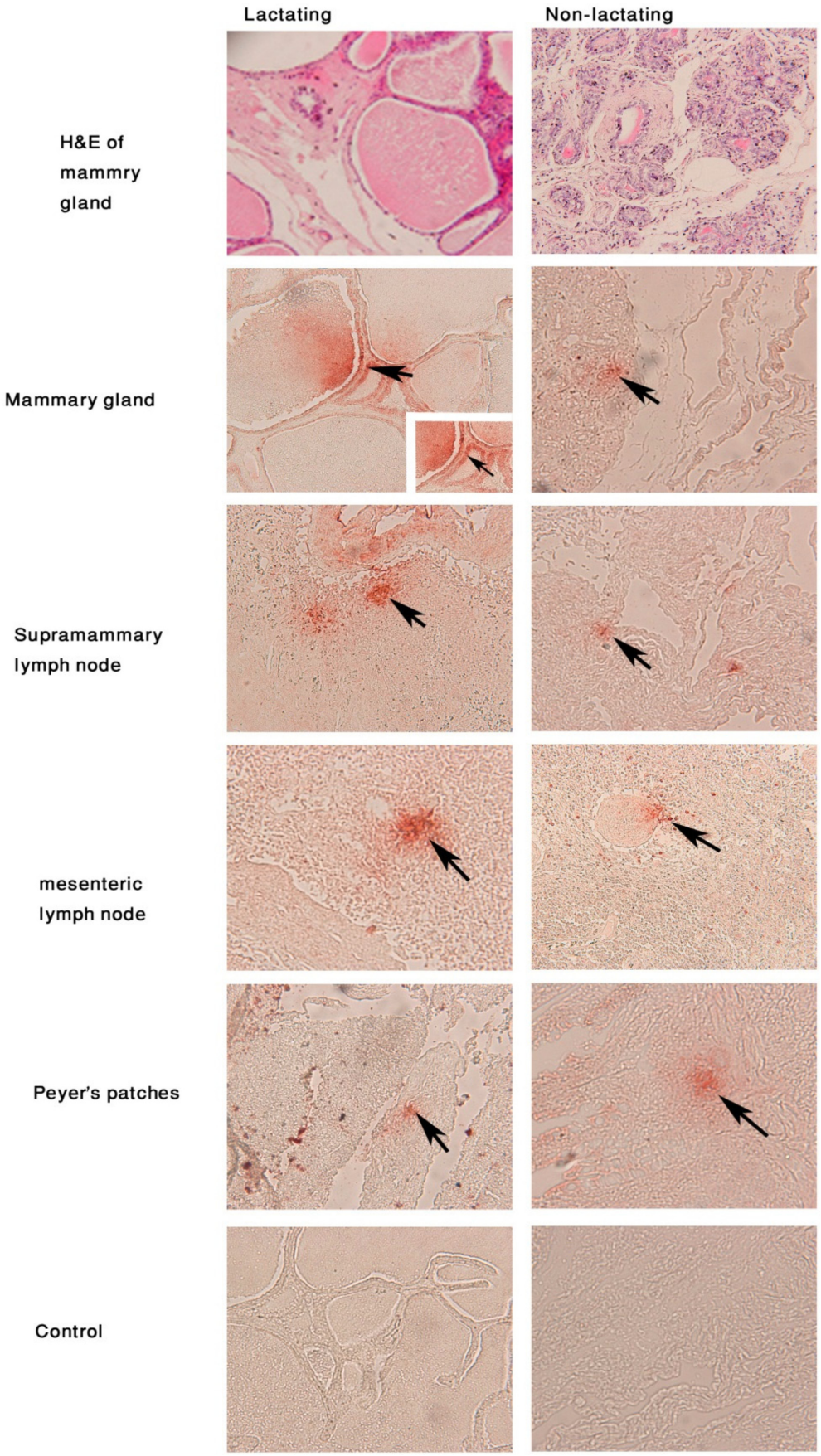

Fig. (1). The H&E staining of the mammary gland at the two stages of the lactations period is indicated in the upper panels, whereas the lower panels depict the $CD8^{+}$ expression at different tissues. The panel at the bottom is the control to the immunostained tissues.

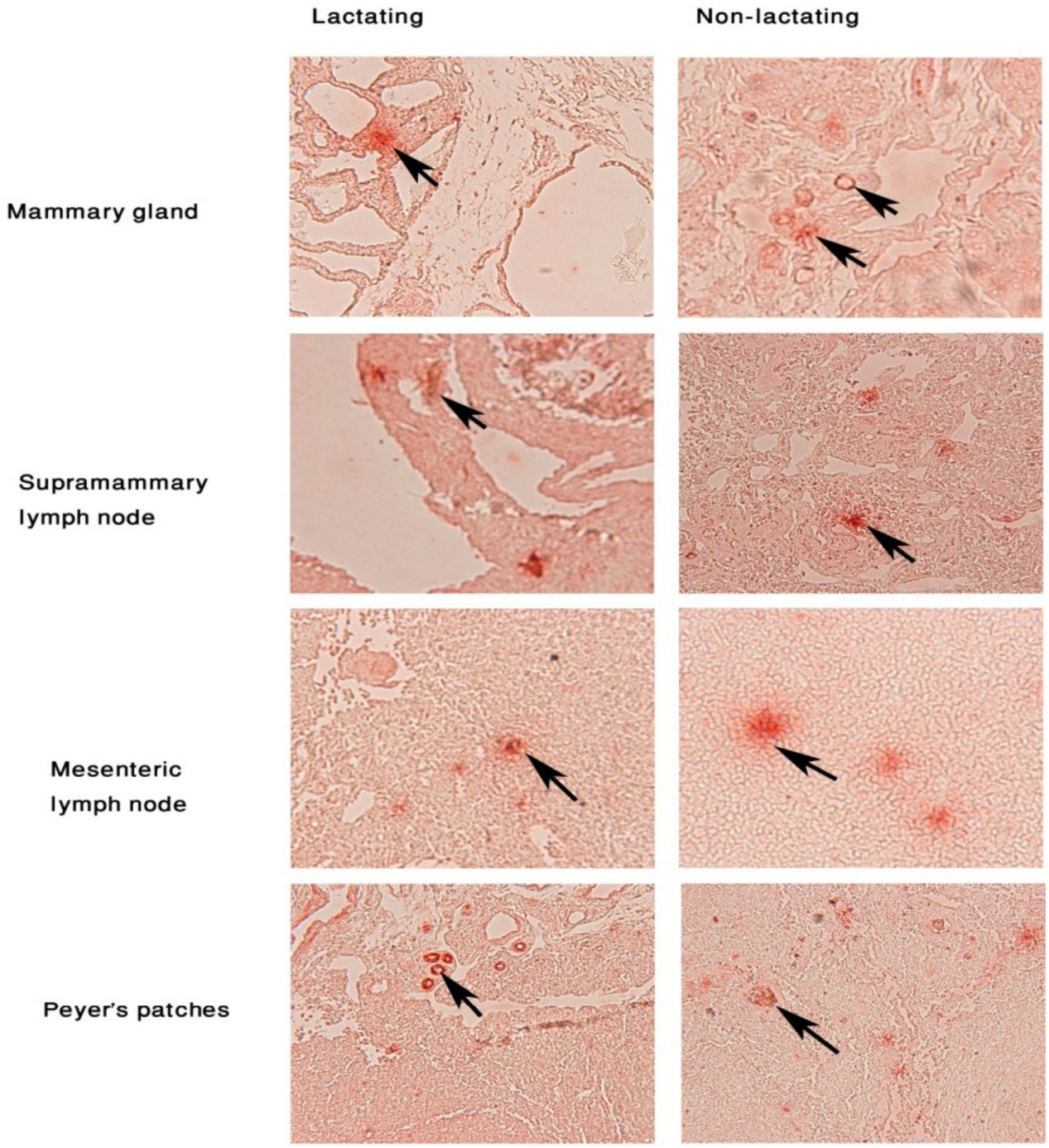

Fig. (2). The immunostaining with the anti-MAdCAM-1 antibodies indicated strong expression of this adhesion molecule in the non-lactating stage with prominent expression at the endothelium of the Peyer's patches and mesenteric lymph node.

the original species that the antibody rose against except LFA-1 and $CD4^+$ were failed to be detected.

3.4. The Adhesion Molecules and the CD Markers of the Non-Lactating Tissues

The $CD8^+$ marker was expressed moderately in the non-lactating tissues (Fig. **1**). However, the MAdCAM-1 and $WC+1^+$ γδ T cells indicated very strong expression (Figs. **2**, **3**). MAdCAM-1 was strongly expressed in the alveolar tissue, supramammary and mesenteric lymph nodes and Peyer's patches (Fig. **2**). The strong expression of MAdCAM-1 and the $WC+1^+$ γδ T cells was diffused in the vascular endothelium of mesenteric and supramammary lymph nodes. However, the expression in the vascular endothelium of Peyer's patches was restricted to the submucosal region (Figs. **2**, **3**). The strong expression of $WC+1^+$ γδ T cells in the mammary glands was localized in the connective tissue (Fig. **3**).

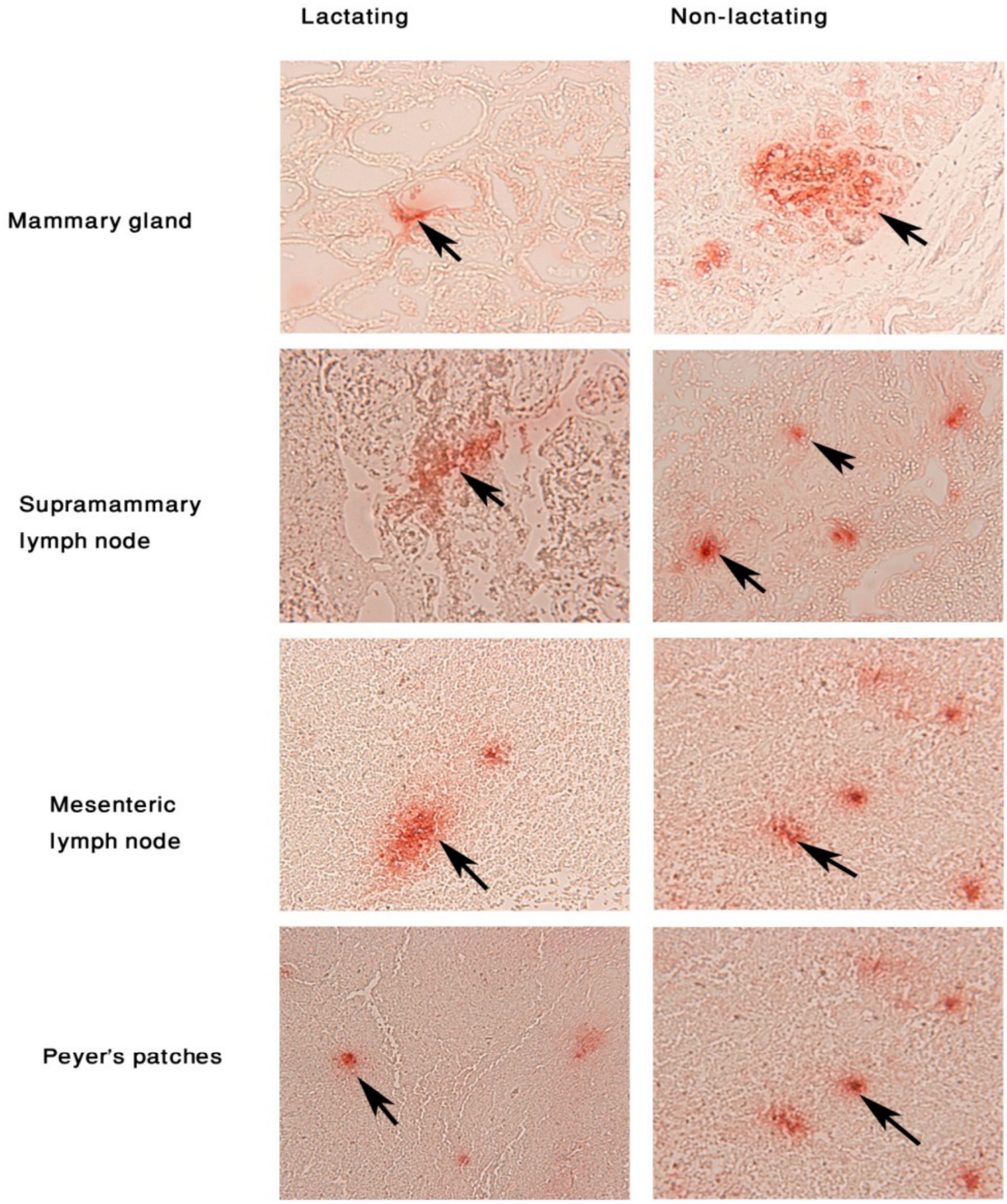

Fig. (3). The immunostaining with the anti-WC+1-antibodies indicated strong expression of this marker in the non-lactating stage with prominent expression in the alveolar tissue of the mammary gland.

4. DISCUSSION

Camel milk market was estimated 10 billion dollars annually [2]. Modern applications in the dairy industry lead to the development of camel dairy farms that are capable of producing camel milk on the commercial level. Commercializing camel milk production has created continuous pressure on the camel mammary glands to meet the increasing demand on its milk. Therefore, study of the camel mammary glands immune system becoming of high priority to understand the resistant mechanisms to mastitis.

Circulating leukocytes are the primary source of cells that transmigrate into the mammary glands of healthy and infected animals [8, 9]. The migration of leukocytes from blood to the mammary glands depends on expression of AM. Adhesion molecules such as selectins, intergrins, and members of the immunoglobulin superfamily regulate signaling between the cells of the immune system and

control the movement of leukocytes to the tissues and their interaction [7]. Hence, variations of the expressed AM indicate their role in the movement of different leukocytes into the mammary glands [10]. Overwhelming studies addressed the biological activities of the AM in the bovine mammary glands in health and disease [9, 13]. Nevertheless, there are major lack of information about the nature of immune responses and the details of the camel mammary glands immune system. One of the important facets of the immune responses in the mammary glands is the expression of AM in health and disease. Hence, this study was carried out to reveal the pattern of the AM expression in the camel mammary glands at the lactating and non-lactating periods.

Due to the lack of camel specific primary antibodies, mouse anti-bovine, human and rat were used in accordance with previous studies [9, 11, 13-15]. Overall results indicated that these xenoantibodies were of good affinity in defining the target molecules like, $CD8^+$, $WC+1^+$ and MAdCAM-1. On the other hand, the antibodies that failed to react with the camel markers were CD62L, TCRαβ, VCAM-1, $CD44^+$, $CD20^+$ but their reaction to the molecules of the original host could imply different possibilities. One of these is their lack of specificity to the camel AM or they might be weakly expressed in the non-inflamed tissues. Total failure in detecting the expression of CD11a/CD18 (LFA-1) and $CD4^+$ despite various efforts cannot be justified.

Results indicated strong and moderate expression of $CD8^+$ at all studied tissues at the lactating and non-lactating mammary glands respectively. In the bovine mammary glands, $CD8^+$ T-lymphocytes prevail during the lactation period. However, $CD4^+$: $CD8^+$ ratio tilt toward the CD4+ at the end of the lactation period [10]. Furthermore, Harp *et al*., (2004) [14] indicated over expression of the $CD8^+$ T-lymphocytes in milk in comparing to its level in the blood.

Workshop cluster$+1^+$ was strongly expressed in the tissues of the non-lactating mammary gland. Majority of young bovine γδ T-cells express WC+1+ coreceptor. The $WC+1^+$ coreceptors that are expressed on the γδ TCR are identified with respect to their $CD8^+$, $CD2^+$, and $WC+1^+$ expression and their cytokine profile [16-18]. $WC+1^+$ coreceptor is transmembrane glycoprotein of the scavenger receptor cysteine rich (SRCR), a family that is usually expressed on the γδ T cells [19, 20]. The SRCR family of the $WC+1^+$ is composed of eleven domains with interdomains homology [22]. Study with wide range of monoclonal antibodies revealed that $WC+1^+$ corecptor is made of tow nonoverlaping variants, WC+1.1 and WC+1.2, whereas WC+1.3 variant is coexpressed with the WC+1.1 [20]. $WC+1^+$ coreceptors play major role in mediating the costimulatory signal in activation of γδ T cells [21]. Further studies on the role of the $WC+1^+$ have revealed a diverse genetic variation which could reach to at least 13 $WC+1^+$ genes on the bovine chromosome 5 [22]. $WC+1^+$ γδ cells are found in blood, peripheral lymph nodes and skin. Nevertheless, there is no evidence of tissue tropism for the $WC+1^+$ expression in bovine tissue [20].

The proinflammatory activity of $WC+1^+$ γδ T cells was widely documented [18]. The copious production of interferon-γ (IFN-γ) exerts a major influence in polarizing the immune responses toward the Th1 type responses (cell mediated responses) [23]. Rogers *et al*., (2005) [23] revealed that WC+1.1 variant of γδ T cells are the source of high level of IFN-γ production with the expression of high level of interleukin-12 receptor (IL-12R).

The γδ cells coexpress different AM, like MAdCAM-1 and α4β7 integrins and selectively migrate to the lymphoid tissues that are enriched with chemokine receptors CCR7 [24]. Hence, the over expression of $WC+1^+$ in the non-lactating mammary tissues indicate the importance of this cells at this stage in which major immunological changes takes place [25]. However, the coexpression of the camel $WC+1^+$ γδ T cells of the MAdCAM-1 and α4β7 AM could refer to the mucosal nature of the cells trafficking system to mammary gland [11].

Similar to the $WC+1^+$ expression pattern, the MAdCAM-1 was also strongly expressed in the tissues of the non-lactating mammary gland. It is possible that these molecules are those that are highly expressed on the $WC+1^+$ γδ T cells. MAdCAM-1 is a ligand of the Peyer's patches adhesion molecule-1 (LPAM-1), also known as α4β7. The therapeutic humanized anti- α4β7 monoclonal antibodies, Vedolizumab, was shown to inhibit the attachment of MAdCAM-1 to the α4β7 but not to the VCAM-1 [26]. In mice, MAdCAM-1 is expressed in Peyer's patches, gut lamina properia and mesenteric lymph node [15]. Interestingly, the mucosal nature of the camel lymphocytes trafficking can be strongly advocated by the strong expression of MAdCAM-1 in the camel tissues, alveolar tissue, supramammary and mesenteric lymph nodes and Peyer's patches. However, in cattle, which their lymphocyte trafficking is of peripheral nature [11], MAdCAM-1 expression was seen missing in the above stated tissues [12].

In accordance with the above stated evidence, the over expression of MAdCAM-1 in the studied tissues of non-lactating camel mammary glands could strongly suggest that the lymphocytes trafficking to mammary glands in camel is of mucosal nature despite that it is a ruminant animal. However, absence of the secretory IgA in the camel milk could dispute this claim. Nevertheless, it was shown that the trafficking of the IgA secreting B lymphocytes to the murine mammary glands is α4β7 and MAdCAM-1 independent [27]. It was demonstrated that blocking of VCAM-1 function has influenced the accumulation of the IgA secreting lymphocytes but not blocking of the MAdCAM-1 function [27]. However, further studies appear essential to endorse this claim beyond doubts.

In conclusion, the results indicated that the xenoantibodies rose against bovine, human and rat AM are efficient enough to detect the camel markers. The over expression of the MAdCAM-1 and the $WC+1^+$ molecules at the late lactation strongly refer to essential role of these molecules in the up regulation of the immune responses at this period were mammary glands is highly susceptible to the mastitis. It was clearly demonstrated that mammary glands at late-lactation is approaching a stage of dramatic anatomical and physiological changes. These transient changes could subject the mammary glands to increasing susceptibility [25]. Hence, immunological changes at this stage could account for the enhancement in the efficiency of the mammary glands immune system.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deanship of scientific research, King Faisal University and King Abdul Aziz City of Science and Technology (KACS).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Received: March 24, 2012 Revised: May 30, 2012 Accepted: June 15, 2012