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DEFENSE MECHANISMS OF MAMMARY GLAND IN SHEEP – A REVIEW

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ABSTRACT

The health of the mammary gland in animals is of paramount importance both to milk productivity and to the whole organism. The term "mastitis" refers to inflammation of the mammary gland. It is characterized by pathological changes in the parenchyma, duct system and interstitial connective tissue, leading to changes in the composition and qualities of milk. Due to its high prevalence, mastitis is considered the most important threat to dairy industry, because of its impact on animal health and milk production. The aims of this paper are to consider mammary gland defenses, including anatomical and histological features, the role of immunoglobulins and mammary epithelial cells, functions of leukocytes, lactoferrin, different enzymes such as lysozyme and lactoperoxidase, chemical antibacterial agents and complement system. As well as the factors influencing the defensive functions of the gland, such as nutrition, milking method, genetic factors and udder conformation.

Key words: sheep, mastitis, mammary, gland, defenses.

Introduction

Adequate immune functions are essential for protecting the organism from the onset of mastitis. Mammary gland immunity depends on the complex combination and coordination of nonspecific and specific protective elements, including the anatomical features of the gland, as well as the cellular and humoral defense mechanism (Sudhan N. and Sharma N., 2010; Katsafadou et al., 2019).

Innate and adaptive immunity play a vital role in protecting the mammary gland. The two most important functions are the recognition of pathogens and the ability to initiate a pro-inflammatory process, this gives rise to a cellular and humoral response, which aims to neutralize infectious agents (Strandberg G. Y., 2005; Rainard P. and Riollet C., 2006).

1. Anatomical features of the gland

The skin, papilla, and papillary canal of the mammary gland are the first line of defense against microorganisms. The gland is covered with delicate skin, and beneath it are the superficial and deep fascia. From the deep fascia connective tissue barriers are separated, which divide the mammary gland into left and right halves, these barriers also form the suspension of this organ. Nerve endings are embedded in the skin of the udder. The udder receptors are subdivided into: thermoreceptors, chemoreceptors, baroreceptors, etc. (Томов Т., 2002).

The healthy skin of the mammary gland completely isolates the parenchyma and the duct system from the influence of environmental factors. Normally, the skin surface of a healthy gland is covered by fatty acids, which have bacteriostatic and bactericidal effect (Sudhan N. and Sharma N., 2010; Alnakip, M.E. et al., 2014).

One of the main defense mechanisms of the mammary gland is the papillary canal. It acts both as a physical barrier and as a source of antimicrobial agents (Paulrud C. O., 2005). The papillary duct sphincter provides a physical barrier against bacteria. The smooth muscle surrounding the canal prevents the leakage of milk and is a blockage against the entry of pathogens through its complete closure (Sudhan N. and Sharma N., 2010). Most often the penetration of infectious agents into the mammary gland is through the papillary canal, because it is the only direct connection between the mammary gland and the environment (Zecconi A. et al, 2002). The dilated canal can remain an open entrance for pathogens for up to two hours after milking (McDonald J.S. et al, 1975). The protective role of the sphincter and papillary canal is also confirmed by Gougoulis, D.A. et al. (2007), as they found the presence of 4 times more microorganisms in the milk papillary canal after suckling, due to the prolonged dilatation of these structures. Damage to this canal due to improper handling or milking leads to a significant increase in the risk of bacterial invasion and colonization (Bramley A. J. and Dodd F. H., 1984; Zecconi and Hamann, 2005).

The antibacterial properties of the papillary canal are expressed in the fact that its inner surface is covered with hydrophobic lipids, which prevent the attachment and retention of microorganisms, as well as their further migration to the milk cistern (Paulrud C. O., 2005; Alnakip, M.E. et al, 2014; Rainard, P., Riollet, C., 2006). These bacteriostatic fatty acids belong to the groups of esterified and non-esterified – lauric, myristic, palmitoleic and linolenic acid (Paulrud C. O., 2005).

Another important factor in the protection of the mammary gland is the conformation of the udder and the location of the mammary papilla. For example, in small and horizontally directed papillae, as well as in deep and sagging udders, the risk of mastitis increases (Casu et al., 2010; Gelasakis et al., 2012).

2. Cellular defense factors

The somatic cells (SC) in milk are mainly those of the immune system, such as macrophages, lymphocytes and polymorphonuclear neutrophils. Exfoliated mammary epithelial cells are also classified as somatic cells (Boutinaud M., Jammes H., 2002). Mastitis is the inflammatory response of the mammary gland mainly against infectious microorganisms. It is a multifactorial disease characterized by varying intensity, duration and consequences depending on the cause (Fthenakis G.C. et al., 2017). Somatic cells play a role in the inflammatory response to intramammary infection. Their counting is one of the most commonly used methods for distinguishing between infected and uninfected mammary glands. The number of somatic cells reflects the health status of the animals and is considered to be the main parameter for determining the quality of milk. It is known that the number of those cells in milk obtained from a healthy mammary gland of sheep is greater than the number of somatic cells in cow's milk (Silanikove N. et al., 2010).

The composition of SC and their percentage in milk varies depending on some factors, such as animal species, breed, lactation stage, genetic characteristics, interval between milkings, sampling time, test methods, stress, trauma and breeding factors (Park YW et al., 2013). Distinguishing and identifying the different types of cells constituting the somatic cells is also an important part of distinguishing healthy from inflamed mammary glands. The greatest attention is paid to white blood cells, their type and number. Recently, interest has also been focused on mammary epithelial cells (MEC), which have been shown to be involved in the transfer of immunoglobulins (IgA), thanks to their poly-immunoglobulin receptors expressed on their basolateral surface. Compared to leukocytes, MECs account for a smaller proportion of somatic cells. Boutinaud and Jammes, (2002) concluded that MECs are 26% of the total number of cells in goat’s milk.

2.1. Mammary epithelial cells.

After passing through the papilla, microorganisms, in search of a suitable environment for development and multiplication, must overcome the next barrier in their path – mammary epithelial cells. During inflammation of the mammary gland on the apical surface of cells forming the mucosa, the presence of Toll-Like receptors, which are peptides that trigger the cellular immune response, is detected (Petzl,W. et al., 2008). Epithelial cells also show phagocytic activity, which is caused by the formation of a membrane that covers the pathogen (phagosome). (Günther, J., Seyfert H.M., 2018). Another important role of mammary epithelial cells for the protection of the mammary gland is expressed in the secretion of various mediators of inflammation: cytokines, chemokines, b-difensins (Lahouassa et al., 2007; Swanson K.M. et al., 2009; Brenaut P et al., 2014). The protective function of mammary epithelial cells is complemented by the production of serum amyloid and haptoglobin, involved in the inflammatory process (Katsafadou A.I. et al., 2019).

In addition, they respond to various pathogens by secreting cathelicidin, an antibacterial peptide that plays a critical role in the immune defense of the mammary gland (Zanetti M., 2004; Cubeddu T. et al., 2017). This peptide is synthesized by MEC and neutrophils in the mammary gland. The general rule of the mechanism triggering cathelicidin action, like that of other antimicrobial peptides, involves the disintegration (damaging and puncturing) of cell membranes of organisms toward which the peptide is active. Cathelicidin rapidly destroys the lipoprotein membranes of microbes enveloped in phagosomes after fusion with lysosomes in macrophages. In sheep, catalecidin -1, -2, -3 and cathelicidin-derived myeloid antimicrobial peptide were discontinued. During mammary gland inflammation, catalecidin levels rise rapidly and significantly compared to other antibacterial agents.

2.2. Leukocytes

The number and type of leukocytes are important for successful protection against invading pathogens in the mammary gland. All types of leukocytes can be found in it, which reach the gland through the bloodstream and are part of the so-called somatic cells (Berthelot X. et al., 2006). Lymphocytes, macrophages and polymorphonuclear neutrophils (PMNs) play an important role in inflammatory responses in the mammary gland (Albenzio M. et al., 2012). These cells provide the organ's cellular protection against bacterial invasions through its ability to recognize microorganisms and induce a rapid immune response (Dimitrov et al., 2018).

Studies have shown that somatic cell values in a healthy mammary gland are 2.5 x 10^5cells/mL milk (Pengov A., 2001), or even more than 6 x 10^5cells/mL milk. (Caroprese M. et al., 2007). In sheep not affected by mastitis, macrophages are present in the largest number (46-84%) (Cuccuru C. et al., 1997), as their levels are higher at the beginning and middle of lactation, and towards the end they decrease. The other cells that form the total number of somatic cells are PMNs (2-28%), lymphocytes (11-20%) and mammary epithelial cells 1-2%. Research has shown that in mammary gland inflammation and a total somatic cell count of 3 x 10^6 cells, neutrophils represent 90% of the total cell count (Paape M. J. et al., 2007)

2.3. Macrophages

The macrophages and neutrophils provide a first line of defense against many common microorganisms (Fthenakis G., 1988). They originate from blood monocytes, which differentiate when they reach the mammary gland. This type of cells has a pronounced phagocytic activity, absorbing pathogens and under the action of proteases they destroy them. Their phagocytic activity may increase in the presence of opsonizing specific antibodies, such as IgG. (Miller R. H. et al., 1988). In addition, macrophages secrete several different components related to the protection of the mammary gland: cytokines (interleukins) and chemokines, antimicrobial peptides (β-defensin and cathelicidin).

In order to be recognized, antigens must bind to antigen-presenting molecules and the resulting complex is located on the cell surface. Antigen-presenting molecules are also known as class I and class II major histocompatibility complexes (MHC). Macrophages, in association with MHC class II, process and present to lymphocyte antigens, so they play a role in the local immune response. (Bradley A. and Green M., 2005; Cheville N.F., 2009). In this way, they provide an increase in the permeability of blood vessels, which allows blood cell elements and molecules to penetrate the inflamed mammary gland. (Barbagianni M.S., 2017).

2.4. Neutrophils

Polymorphonuclear neutrophils (PMNs) are cells of a healthy mammary gland. Neutrophil migration is mediated by L-selectin and β2-integrin adhesion molecules (Barber M. R. et al., 1999; Paape M. J. et al., 2000). The phagocytic activity of PMNs is determined by the presence of receptors for IgG2 and IgM, as well as for C3b and iC3b components of complement (Barber M. R., and Yang T. J., 1998).

One of the main functions of leukocytes is their participation in phagocytosis, an interesting fact is that neutrophils can express their phagocytic activity in the absence of opsonizing agents, thanks to lectin carbohydrate receptors, which can recognize carbohydrate-rich fimbriae of *E. Coli* (Paape M., 1996). PMNs provides the destruction of phagocytosed bacteria by releasing reactive oxygen compounds such as superoxide radicals and hydrogen peroxide (Van Oostveld et al., 2002). In addition to these compounds, neutrophils also release peroxidases, lysozyme, hydrolytic enzymes, and lactoferrin (Harmon R. J., 1994).

The intracellular granules of these cells contain bactericidal peptides such as defensins, enzymes (myeloperoxidase) and proteases (elastase, cathepsin type B, D and G) (Linde A. et al., 2008). Neutrophils have the ability to synthesize cathelicidin (Addis M.F., 2013), which is responsible for the destruction of the lipoprotein membrane of microorganisms (Van Oostveld K ., 2018). Once PMNs play their part in the protection of the mammary gland, they undergo apoptosis. (Paape M. J., 2003).

2.5. Lymphocytes

The main representatives are T- and B-lymphocytes and natural killer cells (NK cells). Lymphocytes recognize pathogenic structures through their membrane receptors, thus mediating the immune response. The main function of lymphocytes is the immune memory and thus they provide a rapid immune response upon re-encounter with a pathogen (Stelwagen K., 2009). T-cells are subdivided into two subclasses $\alpha\beta$ and $\gamma\delta$ T-cells, and in milk $\alpha\beta$-T cells are found in larger quantities. Studies have shown that the number of cytotoxic T-cells in milk is greater than the number of T-helpers (Bonelli P., 2013).

During intramammary infection, T- and B-lymphocytes, as well as NK cells, provide the specific and nonspecific immune response. CD4+ or T-helpers produce immunoregulatory cytokines after antigen recognition, in association with MHC class II molecules. These cells provide the immune memory. (Sordillo L. M. and Streicher K. L., 2002). CD8+ cells have a pronounced cytotoxic and suppressive effect on antigens. In combination with MHC class I molecules, T-killers recognize and destroys pathogenic bacteria. In lactating animals, the pronounced cytotoxicity of T-lymphocytes was found, as well as the increased production of interferon-γ, while in non-lactating animals, the cytotoxicity of these cells was less pronounced, as they produced mainly interleukin-4 (Shafer-Weaver K. A. and Sordillo L. M., 1997).

The role of B-lymphocytes is to stimulate the formation of antibodies against infectious agents, this is provided by their specific receptors for pathogens. (Sordillo L. M. and Streicher K. L., 2002).

The NK cells are the third type of lymphocytes. These cells provide their cytotoxic activity in several ways, such as antibody-dependent cell-mediated cytotoxicity, granular exocytosis, release of cytolytic factors, and receptor-mediated antigen recognition. (Paape M. J., 2000). The effect of these cells has been demonstrated on both gram-positive and gram-negative bacteria (Sordillo L. M. et al., 2005). On the other hand, the hematological parameters in affected animals with subclinical mastitis (SCM) are mainly expressed by the elevation of the white blood cells (WBC) levels (Hristov et al., 2018).

3. Specific and nonspecific humoral defense factors

Humoral factors essential for host defense include specific antimicrobial immunoglobulin antibodies and multiple proteins.

3.1. Specific humoral defense factors

3.1.1. Immunoglobulins

Immunoglobulins (Ig) are the most important humoral factors found in milk and colostrum. These enter the mammary gland through the bloodstream or they can be produced directly in the gland. IgG1, IgG2 and IgM play a role in the opsonization of bacteria, and so these pathogens are recognized by neutrophils and macrophages in the mammary gland (Barrio M.B. et al., 2003). Immunoglobulins also play a role in complement fixation, preventing bacteria from attaching to endothelial cells, promoting agglutination of microorganisms, and neutralizing viruses and toxins (Marnila P ., Korhonen H., 2002). In a study (Lemos V.F et al., 2015) it became clear that the concentrations of immunoglobulin A in a healthy mammary gland are 0.027 mg/mL, and in the presence of intramammary infection the concentrations increase almost three times – 0.06 mg / mL. Concentrations of IgG in colostrum are between 6.2 and 65.4 mg / mL (Kessler E.C. et al., 2019).

3.2. Nonspecific humoral defense factors

3.2.1. Complement system

The complement is composed of more than 30 proteins, mainly produced by the liver. Mammary epithelial cells have also been suggested to be involved in the synthesis of the C3 component of complement (Rainard P., 2003). Components of complement provide their biological activity through complement receptors located on the cell surface (Sordillo L. M., 2003). Effector complement molecules circulate in precursor states in serum and interstitial tissues. These forms are rapidly activated in a proteolytic and cascading manner, following the recognition of pathogen-associated molecular patterns and/or noxious self-derived danger-associated molecular patterns (Arbore G. et al., 2017). The complement system is an important part of the protection of the mammary gland because it is involved in the initiation and control of inflammation, opsonization of bacteria and their disposal.

In a healthy mammary gland during lactation there is a low concentration of complement, and high concentrations during late lactation and in colostrum. In a healthy gland, complement is activated only by the alternative pathway, in which the C3 component attaches to the bacterial wall. The classical pathway is not functional in the mammary gland due to the absence or excessively low concentrations of the C1q component compared to that in the blood (Rainard P., 2003).

3.2.2. Lactoferrin (LF)

Lactoferrin is an iron-binding glycoprotein that is mainly produced by mammary epithelial cells as well as neutrophils in small amounts (Shimazaki K., Kawai K., 2017). It was first isolated in 1939 by M. Sorensen and S. Sorensen. Lactoferrin production is associated with alveolar development. The epithelial cells of the tubular system and the cistern produce most of the lactoferrin, its secretion from the lactating alveoli is minimal, while such production is absent from the proximal end of the papillary canal (Isobe N., 2017). The antibacterial effect of LF is that it competes with bacteria for free iron and with its attachment to pathogens (especially Gram-negatives such as *M. haemolytica*) (Pan Y. et al., 2007; Gelasakis A.I., 2017). In the affected gland, the concentrations of lactoferrein increase by 4.8 times compared to those in the unaffected gland, as in a healthy gland they average 24 mg/dL and in an affected gland an average of 117 mg/dL (Lemos V.F. et al., 2015).

3.2.3 Lysozyme

Lysozyme (N-acetylmuramyl hydrolase) is one of the components of the antibacterial protection of the mammary gland (Sordillo L. M. and Streicher K. L., 2002). Lysozyme reaches the mammary gland through the bloodstream or is synthesized in the mammary gland by leukocytes during inflammation. It has an inhibitory or lytic effect mainly against Gram-positive bacteria (Alnakip M.E., 2014). This component of mammary protection has its effect in synergy with antibodies, the complement and lactoferrin. In this way, the sensitivity of bacteria to various defense mechanisms increases. In sheep, lysozyme concentrations are higher in non-inflamed mammary glands (512 μg/ml), while in affected the concentrations are almost twice as low (243 μg/ml) (Moroni P., Cuccuru C., 2001; Souza F.N., 2012). Lysozyme also contributes to the regulation of the inflammatory response in immune homeostasis on epithelial surfaces by activating regulatory T-lymphocytes (Lee M., 2009).

3.2.4. Lactoperoxidase

Lactoperoxidase is an enzyme in milk that accounts for 0.5% of total whey proteins. Lactoperoxidase is synthesized locally in the mammary gland by thiocyanate (of hepatic origin) and hydrogen peroxide (of bacterial or endogenous origin) (Fox P.F., Kelly A.L., 2006). Its antibacterial properties are provided by the formation of activated oxygen products (hypothiocyanate), which are metabolites that increase the bactericidal activity of leukocytes. Lactoperoxidase levels increase during mastitis (Fox P. F., 2003).

4. Factors affecting mammary defenses

Various factors lead to a decrease in the defense mechanisms of the mammary gland, and hence to the development of mastitis. Knowledge and elimination of these factors leads to a reduction in the incidence of mastitis in sheep farms.

4.1. Nutrition

The malnutrition is a predisposing factor for mammary gland inflammation. For example, the risk of clinical and subclinical mastitis is increased in sheep whose ration is poor in vitamin A. In the absence of vitamin A, the integrity and function of the epithelial barrier of the mammary gland of affected animals is impaired (Koutsoumpas et al., 2013). Insufficient concentration of selenium in the diet leads to a decrease in the cellular protection of the mammary gland in sheep. Giadinis et al., (2011) and Barbagianni et al., (2015) recognize reduced nutritional energy as a risk factor for mastitis. Vasil et al., (2021) demonstrated the beneficial effect of oral selenium and vitamin E in pregnant sheep, with cases of postpartum mastitis halved compared to untreated sheep. Zinc is a component of teat keratin and skin; zinc deficiencies can adversely affect the integrity of the teat duct and thus facilitate bacterial entrance. Energy has been recognized as an important factor in promoting phagocytosis and intracellular killing of bacteria by leucocytes; in this context, it has been found that ewes with pregnancy toxemia were at increased risk of developing mastitis in the immediately post-partum period.

4.2. Method of milking:

Improper milking is one of the main causes of mastitis in sheep. This is due to low-skilled personnel who can serve as a mechanical carrier of pathogens. Incomplete or excessive milking also carries a risk of developing intramammary infections due to the favorable conditions for the development of microorganisms. In machine milking, predisposing factors for the occurrence of mastitis are various malfunctions in the milking systems, for example: disturbances in the vacuum and pulsating frequency. (Contreras et al., 2007). Improper cleaning and disinfection of milking machines, as well as their excessive use lead to the accumulation of pathogens. On the other hand, in manual milking, intramammary infections are caused by microorganisms transmitted by the hands of milkers, the most common are *Staphylococcus spp.* (Melero M., 1994), which are the main etiological agent of mastitis in small ruminants (Contreras et al.m 2007; Hristov et al., 2016). Machine milking could affect tissues, which in turn lowers defensive mechanisms and barriers (Zucconi and Hamann, 2002).

Suckling lambs help the transport of *M. haemolitica* from the lambs tonsils to the papillary canal of sheep (Fragkou et al., 2010). Feeding more then one lambs leads to a longer lactation period, which increases the risk of papillary wounds and lesions that favor the development of microorganisms and the development of infections (Waage et Vatn, 2008). Cross-feeding of lambs from different sheep leads to the transfer of pathogenic microorganisms between animals in the herd (Bergonier et al., 2003).

4.3. Genetic factors

Genetic differences in the susceptibility of ewes to mastitis have been reported; indeed, mastitis is considered a disease amenable for genetic studies. Differences in the susceptibility to mastitis have been associated with particular breeds of sheep. A study of the prevalence of SCM among sheep breeds in Greece found that in the Friesarta breed, the disease is most common (62%), followed by the Assaf cross breed with (60%), Karagouniko cross (45%) and Chios (30%). The breeds in which subclinical mastitis is the least commonly diagnosed are Assaf (10%) and Cephalonia (20%) (Vasileiou et al., 2018).

4.4. Udder conformation

The conformation of the udder and the location of the mammary papilla can also be predisposing factors for intramammary infections. For example, in small and horizontally directed papillae, as well as in deep and sagging udders, the risk of mastitis increases (Gelasakis et al., 2015). In the case of machine milking of sheep with this type of udder, incomplete milking and retention of milk is observed, which is a favorable environment for the development of pathogenic microorganisms.

Conclusion

From the presented information, it is clear that a number of defense mechanisms are involved in the protection of the mammary gland, which can act individually or in combination against different pathogens. Taking them into account, as well as knowing the factors that reduce the protective forces of the gland will lead to a reduction in losses in dairy farming due to mastitis.

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