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# Apoptosis of Blood Polymorphonuclear Leukocytes in Cows with Acute Mastitis

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**Abstract:** The objective of the present study was to investigate the apoptotic process in the polymorphonuclear neutrophils leukocytes (PMN) in cows with acute mastitis. Sixteen cows with *Staph. aureus* and *E. coli* acute mastitis were examined and results were compared with six healthy controls. Animals were referred because of anorexia, swelling and gangrene of the mammary gland, abnormal milk secretion and recumbency. Apoptosis of PMN was assessed using the Comet assay. Compared to control cows, PMN in diseased animals showed an accelerated rate of DNA damage. Undamaged DNA remained within the core; however broken DNA migrated from the core toward the anode, forming a tail of a comet. In undamaged cells, the DNA was tightly compressed and maintained the circular disposition of the normal nucleus. This study showed an accelerated level of apoptosis of blood PMN in cows with acute *Staph. aureus* and *E. coli* mastitis using the Comet assay.

**Key words:** Apoptosis · Cattle · Comet Assay · Mastitis · Neutrophils

## INTRODUCTION

Acute bovine mastitis involves an initial phase, which includes an inflammatory reaction and a resolution phase. Initiation of the inflammatory reaction is caused by the production and release of chemoattractants by macrophages and epithelial cells for the rapid recruitment of neutrophils in order to eliminate invading bacteria. Polymorphonuclear neutrophil leukocytes (PMN) form the first line of immunological defence of the bovine mammary gland against Gram-positive and Gram-negative pathogens [1]. Neutrophils rapidly accumulate on the site of infection and there is a concomitant potential to cause severe tissue destruction, necrotic lysis and release cytotoxic granule contents into mammary gland tissues [2]. Therefore, it follows that timely and vigilant execution of a controlled programmed cell death in neutrophils is important in order to prevent damage to healthy tissues and is necessary for resolution of infection [3]. Previous elimination of bacterial pathogens extravasated inflammatory cells (neutrophils) and their contents from tissue are necessary for resolution of acute infection. Therefore, mammary gland neutrophils undergo apoptosis and they are phagocytosed by macrophages [4, 5].

The life span of PMN migrating in tissues is only 1 to 2 days; thereafter, they undergo either apoptosis, or necrosis as an alternative form of cell death. Apoptosis of PMN is a non-pathologic mode of cell death characterized by unique morphological and biochemical features. There are a number of studies reported that deregulation of apoptosis is an important feature of bacteria [6-8]. Apoptosis in such cases is marked by cellular shrinking, condensation and margination of the chromatin, roughing of the plasma membrane and translocation of phosphatidylserine on the cell membrane [9]. Apoptosis of PMN is accompanied by the loss of a number of fundamental functions: a reduced capability to respond to stimulation, a reduced intensity of phagocytosis (ranging to a total inability to phagocytise) and a lowered degree of degranulation and respiratory burst [10]. Therefore,

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these fundamental functions are critical for the maintenance of host defenses and in reaction to an insult by infectious agents. Under these circumstances, the immune system of the mammary gland may be weakened in its fight against infection and a transition into chronicity may occur.

Single-cell gel electrophoresis (comet assay) is a sensitive method for measuring DNA strand breaks [11, 12]. The clear advantage of comet assay over other techniques that measure DNA strand breaks is its ability to measure heterogeneity within complex populations. In the comet assay a damaged cell takes on the appearance of a comet, with head and tail regions. A variety of geometric and densitometric parameters are provided by the image analysis software, which allows an estimation of the amount of DNA in the head and tail regions and the extent of migration into the tail region. Because the tail length and density reflect the number of single-strand breaks in the DNA, the percentage of DNA in the tail provides a quantitative measure of the damaged DNA. A variety of modified comet assays using several parameters have been developed to evaluate the extent of DNA strand breaks. The alkaline version of comet assay [13] primarily detects single-strand breaks of DNA. The effect of mastitis on PMN apoptosis has only been studied in models of acute reversible bovine mammary gland injury caused by phosphate buffered saline and in experimentally induced mastitis. Therefore, the present study was aimed to evaluate apoptosis of PMN in cows during naturally occurring *Staph. aureus* and *E. Coli* acute mastitis using the comet assay.

## MATERIALS AND METHODS

**Animals, History and Physical Examination:** Sixteen Holstein cows (6 to 8 years old), weighed 500-680 kg were examined in the present study. Animals were referred because of anorexia, swelling and gangrene of the mammary gland, abnormal milk secretion and recumbency. According to the owners, duration of illness ranged from 6 to 24 hr after parturition. Animals were treated with parenteral and intrammary antibacterial therapy, corticosteroids and systemic fluid therapy. All animals underwent a thorough physical examination, which included general behavior and condition, auscultation of the heart, lungs, rumen and intestine, measurement of heart rate, respiratory rate and rectal temperature, swinging auscultation, percussion auscultation of both sides of the abdomen and rectal examination. After milk culture *Staph. aureus* was identified in 10 and *E. coli* in 6 cows. Sex healthy cows were enrolled in this study as controls. Due to the very poor prognosis and based on the owners' request, animals were euthanized and thoroughly examined postmortem.

**Isolation and Preparation of Polymorphonuclear Neutrophils Leukocytes:** Polymorphonuclear neutrophils leukocytes were isolated from the heparinized blood by Ficoll-Conray solution (specific gravity 1.078), followed by hypotonic red blood cell lysis, as previously described [14]. Neutrophils were represented in Hanks' balanced salt solution (HBSS, containing $ca^{2+}$ and $Mg^{2+}$; Nissui pharmaceutical company, Japan) to a concentration of $5\times10^6$ cells/ml. The resulting cell population comprised < 95% neutrophils, as determined by Wright-Giemsa staining and < 99% of the cells were viable when assessed by tryptan blue dye exclusion.

**Evaluation of Apoptosis:** Under alkaline conditions, the comet assay of isolated PMN in both diseased controls was performed as recently reported [11, 12]. Briefly, the isolated PMN were embedded in 1% low-melting-point agarose (Life Technologies Co., Ltd., Japan) and deposited on top of a 1% agarose base layer (Nakarai Techs Co., Ltd., Osaka, Japan) on fully frosted slides (Matsunami Glass Indust. Ltd., Tokyo, Japan). After solidification of the top layer of agarose, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10mM Tris-HCl, 1% Na-sarcosinate, 10% dimethyl sulphoxide and 1% Triton X-100, pH 10.0) for one hour at 4◎ in a dark room. After this, the cell membrane and cytosol were lysed and isolated nuclei remained in the agarose. The slides were incubated in an electrophoretic buffer (0.3 M NaOH, 1 mM EDTA) for 30 min. Electrophoresis was carried out at 25 V and approximately 400 mA for 25 min at room temperature. The slides were neutralized in 0.4 M Tris-HCl solution (pH 7.5) for 20 min, stained with propidium iodide (PI) and then photographed under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Images were captured with a Sony CCD camera and saved using Image Pro Plus software. ImageJ, open source and available free of charge for multiple operating systems at http://rsb.info.nih.gov/ij/, was used to quantify the different parameters of the images. Generally, 100 images were analysed per slide. The migration lengths of nuclei and total lengths including the nucleus and tail were determined and then tail length was determined for each cell. DNA strand

breaks measured by this assay are expressed as the "tail moment" which is the product of the fraction of DNA that has exited the nucleus multiplied by the distance migrated and expressed as the extent of DNA damage.

**Statistical Analysis:** Data are presented as mean ± SD. All statistical analyses were performed using computer software (SPSS version 18.0, Chicago, Illinois, USA). The significance of differences between the means were compared between diseased and control cows using Student's *t*- test. The level of significance was set at $P < 0.05$.

## RESULTS

In all examined cows, inflammation of the mammary gland occurred within the first 24hr of calving. There were a remarkable degree of swelling of the gland and the milk was bloody in 10 cows. There were hemorrhagic patches on the sclera in 6 cows. In all cows, a severe systemic reaction with elevation of the temperature to 40-42°C, accelerated heart rate to 100-120 bpm was found. Anorexia, profound depression, absence of ruminal movements and muscular weakness, often to the point of recumbency within 6-8 hr after the onset of signs were also observed in all cows.

The affected quarter was grossly swollen, hard and sore to touch and caused severe lameness on the affected side. In seven cases, a bluish discoloration of the affected gland developed as early as hours from the onset of the disease. Gangrene involved the floor of the udder in 7 cows and the whole or part of the teat in 5 animals, sometimes was restricted to patches on the sides and floor of the udder in 3 cases. Within 24hr, the gangrenous areas became black and ooze serum freely with the formation of blisters. In three cases, the gland secretions were reduced to small amount of bloodstained serous fluid without odor, clots or flakes. In 6 cows, the mammary secretions were thin, yellow serous fluid containing small flakes and the gland was moderately swollen.

Necropsy revealed a grossly swollen gland that contained bloodstained milk. There was extreme vascular engorgement, hemorrhages and necrosis of the parenchyma. Other postmortem findings included fatty infiltration of the liver in 8 cows, abomasal ulceration in 2 cows and hemorrhagic infarction of the kidney in 1 cow.

Figure 1 shows tail moment in PMN isolated from diseased cows and measured by the comet assay, compared to control neutrophils (0.52±0.27 vs 0.32±0.21)

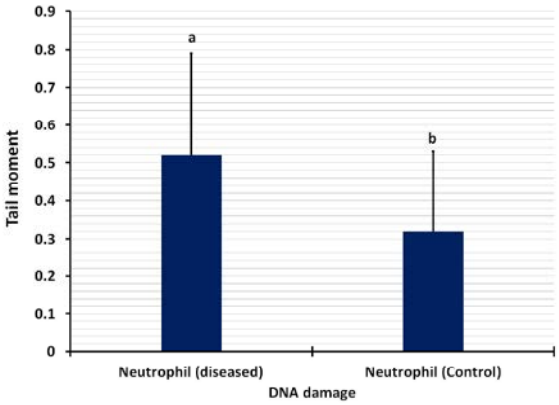


Fig. 1: DNA damage in neutrophils of cows with acute mastitis ($n$=16) measured by the comet assay compared to healthy cows ($n$=6).

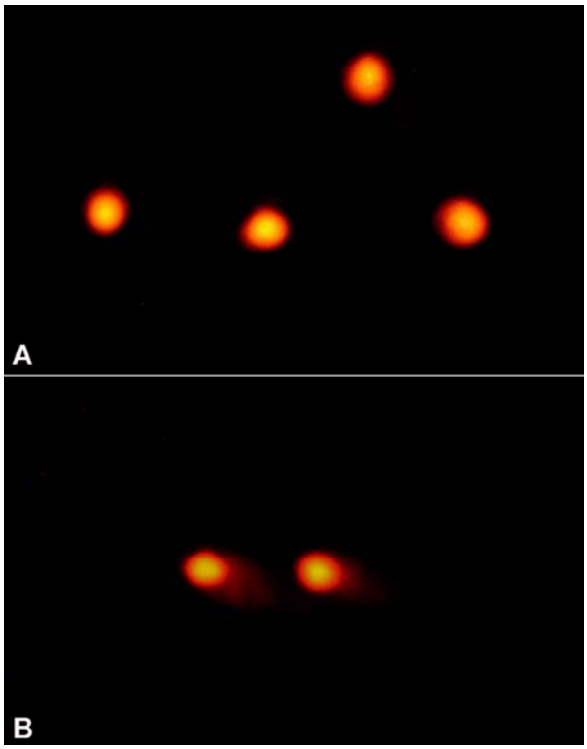


Fig. 2: Comet images of neutrophils showing undamaged cells, the DNA is tightly compressed and maintains the circular disposition of the normal nucleus (A). The damaged DNA migrates from the core toward the anode, forming the tail of a comet (B).

($P < 0.01$). Undamaged DNA remained within the core; however the broken DNA migrated from the core toward the anode, forming a tail of a comet. In undamaged cells, the DNA was tightly compressed and maintained the circular disposition of the normal nucleus. The increase in

DNA damage was mostly evidenced by an increase of comet moment. When cells processed for the comet assay were examined by fluorescence microscopy, fluorescent structures corresponded to the PI–stained nuclear DNA of the PMN cells (Figure 2).

## DISCUSSION

Apoptosis is an active process involving gene transcription and protein synthesis, which trigger a sequence of events resulting in typical morphological and biochemical changes including nuclear chromatin condensation, cell shrinking, blebbing of the cytoplasmic membrane, break-up of DNA into 180- to 200-base fragments and the final cell fragmentation into apoptotic bodies [15]. Apoptosis of PMN differs from necrosis. Necrosis of PMN is induced if the cells are affected by apoptosis-blocking signals. It results in a release of the histotoxic content of granules and subsequent damage to the surrounding tissue [16]. Necrotic PMN are not quickly removed by macrophages. The role of apoptosis in the fate of PMN in vivo has been described in human [17] and in laboratory animals [18]. During PMN apoptosis, the organelles and the cytoplasmic membrane remain intact and the histotoxic content of granules is not released into the extracellular compartment. Moreover, apoptotic PMN are promptly removed by the macrophages before they can support the development of cellular damage [19].

There is compelling evidence that PMN apoptosis plays a key role in resolution of the inflammatory response in both humans [19] and cows [20]. The end result of apoptosis is fragmentation of PMN into small membrane bound bodies that are quickly cleared by phagocytotic cells [21]. The main functions of PMN are to engulf pathogens and destroy them. At the same time, PMN can potentially harm the mammary gland. Neutrophils may promote tissue injury and disturb mammary function, via reactive oxygen metabolite generation and granular enzyme release [22]. There is increasing evidence that pathogens use various mechanisms to impinge upon cell death pathways. These virulence factors induce cell death by a variety of mechanisms, which include 1) pore-forming toxins, which interact with the host cell membrane and permit the leakage of cellular components; 2) toxins that express their enzymatic activity in the host cytosol; 3) effector proteins delivered directly into host cells by a highly specialized type-III secretory system; 4) superantigens that target immune cells and 5) other modulators of host cell death [23].

*Escherichia coli* and *Staph. aureus* is one of the most important pathogens causing mastitis in dairy cows [24], resulting in inflammation that ranges from chronic, subacute to peracute. Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E. coli* mastitis, as well as during severe experimental *E. coli* mastitis [25]. During experimentally induced *Staph. aureus and E. coli* mastitis, migration of PMN through the secretory epithelium was correlated with extensive morphological damage [26]. Neutrophils isolated from mammary glands of nulliparous heifers given an injection of *E. coli* endotoxin were incubated with mammary tissues from noninfected quarters. Microscopic examination indicated that epithelial cell damage resulted from treatment with intact, lysed, or phagocytosing PMN. The greatest morphological damage resulted from treatment with phagocytosing PMN. Activated blood PMN were cytotoxic for mammary epithelial cells [27], possibly via the release of extracellular reactive oxygen species, such as hydroxyl radicals [28]. Oxidative stress can damage all types of biomolecules (e.g., DNA, proteins, lipids and carbohydrates) and therefore induce tissue injury [29].

Apoptosis of blood leukocytes has been used previously using the flow cytometric technique [6-8, 26]. In this study, apoptosis PMN isolated from cows with acute mastitis was investigated and assessed by comet assay. The results obtained show a statistically significant difference of DNA damage between PMN isolated from cows with acute mastitis and that isolated from healthy cows.

In humans, Whyte *et al.* [10] have shown that apoptotic PMN have decreased cell functions. Cell spreading, chemotaxis, phagocytosis, production of myeloperoxidase and the release of superoxide radicals following zymosan stimulation are decreased in a PMN population with an increased percentage of apoptotic PMN [10]. In cows, the reduced viability of PMN close to parturition [20] may explain the high apoptotic process detected in the present study, the high incidence of infectious diseases and the high prevalence of intramammary infections in periparturient dairy cows. In conclusion, our findings suggest that increased percentages of apoptotic blood PMN just after parturition in cows may be a possible risk factor for severe mastitis shortly after birth. The mechanism of PMN apoptosis in cow with acute mastitis was not established in the current study; therefore further studies are warranted to elucidate such mechanism.

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