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Elucidation of Molecular Basis of Neutrophil Apoptosis during *Staphylococcal Mastitis* in Crossbred Cows

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ABSTRACT

Neutrophil apoptosis is a dynamic process following their recruitment to the site of infection that varies depending upon the type of challenge. The proposed study was designed to elucidate the role of classical mediators of apoptosis in neutrophils isolated from milk samples of crossbred Karan Fries cows suffering from subclinical (SCM) and clinical mastitis (CM). Milk samples were collected from 12 KF cows suffering from clinical mastitis caused by *Staphylococcal aureus*. Clinical mastitis was confirmed on the basis of CMT scoring, bacteriological evaluation, gross and morphological changes in milk and by counting milk somatic cells (SCC). Milk Poly Morpho-Nuclear Cells (PMNs) were isolated and apoptosis was studied. Neutrophil apoptosis was evaluated by studying the exteriorization of phosphatidyl serine, mitochondrial transmembrane potential, Caspase 3, 7, 8 and 9 by fluorescent microscopy. Results showed that apoptosis in neutrophils were mediated through exteriorization of membrane phosphatidyl serine; increased mitochondrial transmemebrane potential and activation of caspases 3, 7, 8 and 9 like other somatic cells. From the study, it was evident that neutrophils undergo induced apoptosis during *Staphylococcal* mastitis. The findings of the study provide an insight into the molecular basis of neutrophil apoptosis and form a basis to enhance the host immunity by the process of apoptosis modulation to combat the infections caused by the pathogen. The study provided a base for future studies by which neutrophil apoptosis can be modulated so as to enhance the phagocytic clearance of the microbes from the site of infection.

Keywords: Neutrophil, Mastitis, Apoptosis, Caspase, Mitochondrial transmembrane potential, Phosphatidyl serine.

Neutrophils are first line of cellular defense which forms integral part of innate nonspecific immune system (Aitken *et al.,* 2011). These are the first cells to be recruited into the mammary lumen following entry of the pathogens (Paape *et al.,* 2003). Neutrophil recruitment occurs by the process of tethering and extravasation along the vascular endothelium (Sadik *et al.,* 2011) which results in dynamic crosstalk among the neutrophils, mammary epithelial cells, macrophages and pathogens (Paape *et al.,* 2002) and responsible for mediating the host defense. Thus the neutrophil dynamics is a significant factor that decides the resolution of the inflammation (Rainard and Riollet, 2006).

Neutrophil defense depends on two factors: First, timely recruitment of the neutrophils to the site of pathogen entry and second, timely removal of the neutrophils from the site of inflammation so as to prevent the onset of the secondary inflammation (Segal, 2005). The timely removal of neutrophils is mediated by macrophages and subsequently they undergo apoptosis. Pathogens also modulate the neutrophil apoptosis so as to prevent their removal from the site of infection and a long standing stay at the site of entry to elicit the infection (Boutet *et al.,* 2004; Kennedy and DeLeo, 2009; Sadik *et al.,* 2011). *Staphylococcal aureus* is considered as the most common causative factor of mastitis in India. During the *Staphylococcal aureus* caused clinical mastitis apoptosis of neutrophil takes place which is poorly understood. The free radicals generated by the neutrophils during *Staphylococcal* infection may be one of the factors responsible for induction of neutrophil

apoptosis (Yamamoto *et al.,* 2002). The study on molecular events of neutrophil apoptosis during the *Staphylococcal* mastitis appears to be scanty. Thus the present study was framed to elucidate the molecular processes and classical mediators involved in apoptosis of neutrophils isolated from milk of crossbred cows suffering from clinical mastitis.

MATERIALS AND METHODS

Animals

The present study was conducted in crossbred Karan Fries (KF) cows reared under semi intensive system of management. A total of 146 KF cows were screened during the entire study out of which 12 cows have been selected as per the requirement of the study. All cows were screened for mastitis by California Mastitis Test (CMT), gross/morphological changes in milk, bacteriological culture and its evaluation and microscopic milk somatic cell count (SCC) (Harmon, 2001; Swain *et al.,* 2014). The determination of CMT score was based on the specifications of the commercial CMT solution and accordingly the scoring was done (Masti Check Reagent, GEA Westfalia Surge, India). Cows having CMT score of triple positive based on the intensity of gel formation and SCC count of 6.14 to 7.86 (10^5/ml) were classified as clinical mastitis group (CM). This group of cow's milk was showing the presence of flakes and clots with very high milk SCC.

Sampling of milk

Milk samples were collected from infected cows as per the protocols approved by the animal ethics committee. Teats were disinfected with 70% of ethyl alcohol prior to collection of milk. Fifty ml of milk was collected in sterile plastic centrifuge tubes. The tubes were collected in ice box and immediately transferred to the laboratory for further processing and evaluation.

Bacteriological examination of milk samples

For bacteriological studies 0.02 ml aliquot of milk sample was spread on 5% sheep blood agar. The plates were incubated aerobically at 37°C and examined after 24 and 48 hrs. In case of *Staphylococcus aureus*, isolates from one colony per inoculums were considered positive. Growth of two different types of colonies per type was considered as a mixed culture. Growth of three or more bacterial types was considered as a contaminated culture and was rejected. The plates having monoculture colonies were further confirmed by morphological assessment, Gram's staining and by performing standard biochemical tests. Further the bacteria were confirmed by inoculating the culture to mannitol salt agar.

Isolation of milk neutrophils

Isolation of Polymorpho Nuclear Cells (PMNs) from milk was performed as per method described earlier (Mehzard *et al.,* 2004; Swain *et al.,* 2014). Briefly, milk was filtered separately through a nylon filter (40 µm pore size) and diluted to 50% with cold Dulbecco's PBS (volume/volume). Isolation of PMN was performed using 3 centrifugations steps. Ten ml of milk was poured into a centrifuge tube and centrifuged (600 xg, 15 min., 4°C). The fat was removed with supernatant and the remaining cell pellet was washed twice in cold Dulbecco's PBS (300 xg, 10 min., 4°C and 200 xg, 15 min., 4°C). The final pellet was resuspended in Dulbecco's PBS containing 0.5 mg/ml gelatin. In the third step, two Histopaques were used for the isolation of milk PMNs. In a 15 ml falcon, 3 ml of Histopaque 1119 (Sigma, Germany) was taken over which 3 ml of Histopaque 1077 (Sigma, Germany) was layered slowly. A ring was formed at the junction of both the Histopaques. Over the Histopaque 1077, 3 ml of cell suspension was layered slowly and carefully from one side of the tube. The tube was gently placed in the centrifuge and centrifugation was carried out at 2000 xg at 4°C for 30 minutes. The cell pellet formed at the interface between the Histopaque 1119 and 1077 was taken as milk PMNs. The collected cells were washed twice with PBS and resuspended in RPMI medium for further analysis. The purity of milk PMNs was found to be more than 85% after staining with Field's stain. The viability was determined using Trypan Blue (Sigma Chemical Co) by bright field microscopy after counting the cells using a Hemocytometer and further the viability was tested by performing Propidium Iodide exclusion test by fluorescent microscope. The cells were suspended in RPMI medium and counted.

Evaluation of apoptosis in milk PMNs

Apoptosis was evaluated by employing the following protocols.

Translocation of Phosphatidyl Serine: One of the hallmarks of early apoptosis of cells is characterized by the externalization of membrane Phosphatidyl serine. This was evaluated by using the Annexin V FITC apoptosis detection kit as per the protocol described in the kit specifications.

Evaluation of transmembrane mitochondrial potential of milk neutrophils: The mitochondrial transmembrane potential was measured by employing the MitoPT^{TM} JC-I kit (Immuno-Chemistry Technologies, USA, Cat no- 924). The basic principle of the assay is JC-I reagent dye easily penetrates the cells, healthy and intact mitochondria. Once inside a healthy non-apoptotic cell, the lipophilic MitoPT-JC-I reagent, bearing a delocalized positive charge, enters the negatively charged mitochondria where it aggregates and fluoresces red. The fluorescent cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyaniniodide, commonly known as JC-I stains the mitochondria and detects the change in the transmembrane potential of the mitochondria. Cells bearing the healthy/polarized mitochondria appeared orange red while cells bearing the depolarized mitochondria appear green.

Evaluation of Caspase 3 and 7 activity in milk neutrophils: Caspase 3 and 7 activities in neutrophils were estimated by Magic Red Caspase 3/7 detection kit (ImmunoChemistry Technologies, USA, Catalog no- 936) as per kit instructions. The kit measures intracellular Caspase activities and the degree of intracellular apoptosis. The kit employs the "Magic Red" (MR) reagent which is a non cytotoxic substrate that fluoresces upon cleavage by active Caspase 3/7 enzymes.

Evaluation of Caspase 8 and 9 activities in milk neutrophils: Caspase 8 activity in the milk neutrophils was estimated by FAM-FLICA™ *in vitro* Caspase Detection Kit (ImmunoChemistry Technologies, USA, Catalog no-910) as per kit instructions. FLICA™ is a powerful method to assess cell death by detecting apoptosis *in vitro*. FLICA probes are non-cytotoxic Fluorescent Labeled Inhibitors of Caspases that covalently bind with active caspase enzymes. FLICA measures the intracellular process of apoptosis.

Evaluation of fluorescence of cells

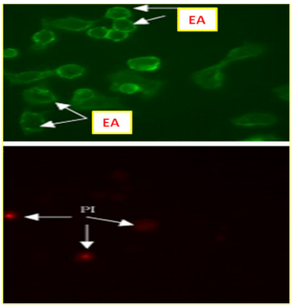
All the parameters were visualized under IX51 inverted Olympus microscope equipped with a blue filter/FITC filter. The background fluorescence was minimized by the use of auto exposure mode of microscope. Annexin V FITC cells were visualized under 100X along with the cells for mitochondrial transmembrane potential. Caspase 3, and 7 activities were measured by using 40X objective. The fluorescence exhibited was evaluated by using IX51 Inverted Olympus Microscope.

RESULTS AND DISCUSSION

Molecular basis of apoptosis has been studied by employing number of protocols to validate the process of induced apoptosis in milk PMNs isolated from milk samples of cows infected with *Staphylococcus aureus*.

Apoptosis is a controlled programmed cell death which is energy dependent and occurs naturally under normal physiological conditions where cell is an active participant in its own demise (also known as cellular suicide). This phenomenon is characterized by distinct biochemical and morphological changes in cell that includes membrane blebbing, shrinkage, cytoskeletal disassembly, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Kroemer *et al.,* 2008). Phosphatidyl serine in the intact and non apoptotic cells remain towards the cytoplasm, whereas, during early stages of cell apoptosis, the phosphatidyl serine gets translocated towards the outer site of the plasma membrane. Annexin V acts as the potential binder to phosphatidyl serine and this can be easily evaluated by flow cytometry or by fluorescence microscopy (Van Oostveldt *et al.,* 2002).

Annexin V FITC fluorescent test is considered as one of the most common techniques to study the cellular apoptosis. This assay perceives the early stages of apoptosis by detecting the externalization of membrane phosphatidyl serine. The results of present study showed apple green fluorescence under the microscope at the surface of plasma membrane exhibits flipping out of membrane phosphatidyl serine of PMNs (Figure 1). Apoptosis of milk PMNs via the involvement of phosphatidyl serine reported earlier (Sladek *et al.,* 2005a; Sladek *et al.,* 2005b) substantiates the results of this study**.**



EA- ANEEXIN V FITC POSITIVE- EARLY APOPTOTIC CELLS
PI- PROPIDIUM IODIDE POSITIVE CELLS- NECROTIC CELLS

Figure 1. Annexin V FITC staining for detection of early apoptotic changes in the neutrophil cell membrane (100x).

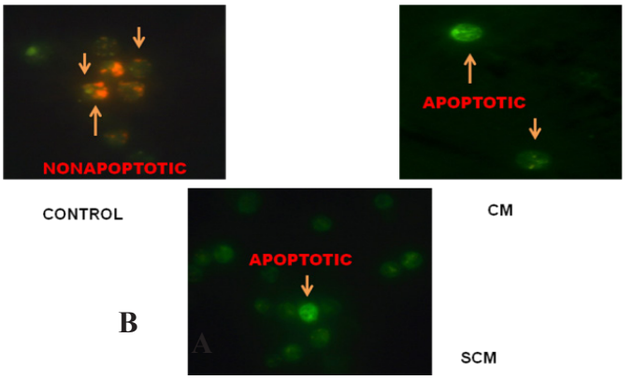


Figure 2. JC-I staining of milk neutrophils (40x). A- Control, B- Sub clinical mastitis, C- Clinical mastitis. Arrows are showing the cells with altered mitochondrial potential. Apoptotic cells appeared orange red in the cytoplasm, whereas, nonapoptotic cells appeared green.

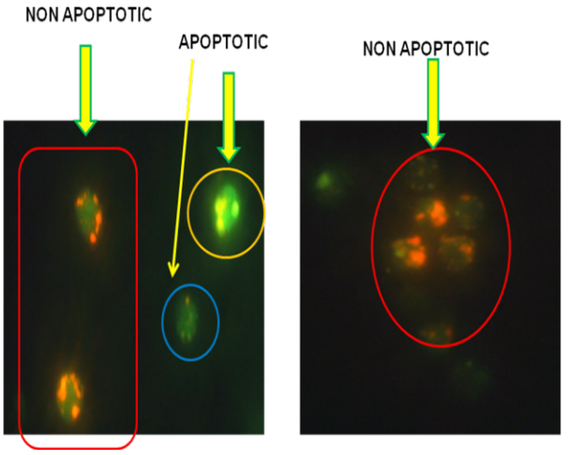


Figure 3. JC-I staining of milk neutrophils (100x). In higher magnification, apoptotic cells appeared orange red in the cytoplasm, whereas, nonapoptotic cells appeared green.

The increase in mitochondrial transmembrane potential acts as a marker of apoptosis which can be easily detected by using the lipophilic JC-I dye. With an intact and reduced mitochondrial transmembrane potential, the JC-I accumulates inside the cells and fluoresce orange red, whereas, in apoptotic cells, the JC-I accumulates in the cytoplasm and other parts of the cell giving green fluorescence. The results of JC-I staining showed appearance of green fluorescence indicated increase in mitochondrial transmembrane potential in isolated milk PMNs (Figure 2 and Figure 3). This signifies that the neutrophils undergo the apoptosis through the alteration of the mitochondria transmembrane potential leading to induction of apoptosis and its downstream signalling cascades.

The mitochondrial enzymes are the regulators of apoptosis. The oxidative events in mitochondria maintains a depolarisation potential of -80 to -120 mV in the inner membrane of mitochondria. This negative potential prevents the opening of mitochondrial permeability transition pore (PTP). Rise in the transmembrane potential causes opening of the PTP and release of the inter membrane proteins including the cytochrome c. This enzyme is involved in the induction of apoptosis through the formation of apoptosome.

Caspases are members of cysteinyl dependent aspartate directed proteases family which plays essential roles in regulating apoptosis at cellular level, and finally causes cellular demise through a cascade of molecular events. These are considered as intracellular proteases which are involved in disassembly of the cells into apoptotic bodies during apoptosis. Caspase activation is also appears to be associated with the increased mitochondrial transmembrane potential (Luthi and Martin, 2007). Caspases, major mediators of apoptosis, are synthesized as pro enzymes and get activated by various internal and external stimuli (Li and Yuan, 2008). Inside the cells, caspases are present in inactive form and they are activated by the process of proteolytic cleavage. The activation of the procaspases is mediated by the involvement of mitochondrial cytochrome enzymes as well as different death signals coming from inside and outside of the cells.

Caspase-3 is a member of the apoptosis executioner group that also includes caspases-6 and caspase-7. The inactive zymogen form of caspase-3 is efficiently processed by any of the initiator caspases (caspase-8, caspase-9, and caspase-10). All executioner caspases will target the various cytoskeletal structural proteins as well as PARP and ICAD leading to the morphological and biochemical changes that drive the apoptotic process**.** Caspases are categorized in two groups: the initiators (caspases 8, 9, and 10) and the effectors caspases (caspases 1, 2, 3, 4, 6, 7, 12, and 13). The initiator caspases 8 and 10 are also referred to as the extrinsic apoptosis pathway that originates upon activation of cell surface death receptors. Caspases-8 and caspase-10 are monomers that bind to death receptor proteins through their death effectors domain (DED) structure (Kurokawa and Kornbluth, 2009). Activity of Caspase 3, 7, 8 and 9 in the process of apoptosis and their involvement mostly mediates the constitutive apoptosis.

In present study, the PMNs isolated from milk of mastitic cows showed orange fluorescence with Magic Red reagent revealing activities of caspase-3 and 7. The activity of these caspases indicated that the milk PMNs undergo induced apoptosis and the process is programmed via the activation of initiator and executioner caspases (Fig 4).

The activity of caspase-9 depends on release of mitochondrial cytochrome c due to rise in mitochondrial transmembrane potential. Similarly the results of FLICA measures showed green fluorescence (Fig 5 and 6) in PMNs isolated from milk of mastitic cows indicating

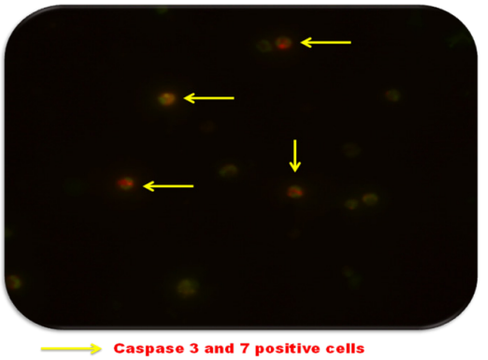


Figure 4. Caspase 3 and 7 activities in milk neutrophils (40x). Caspase 3 and 7 positive cells exhibited red fluorescence, whereas, cells negative exhibited no fluorescence or green fluorescence. Arrow marks show the cells positive for caspase 3 and 7.

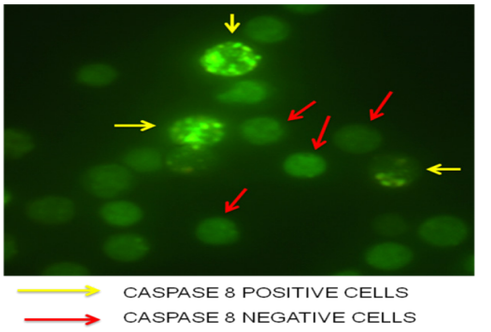


Figure 5. Caspase 8 activity in milk neutrophils (100x). Yellow arrows showing Caspase 8 positive cells, whereas, red arrows indicated Caspase 8 negative cells. Cells with high green fluorescence were Caspase 8 positive and with no bright green fluorescence were caspase 8 negative.

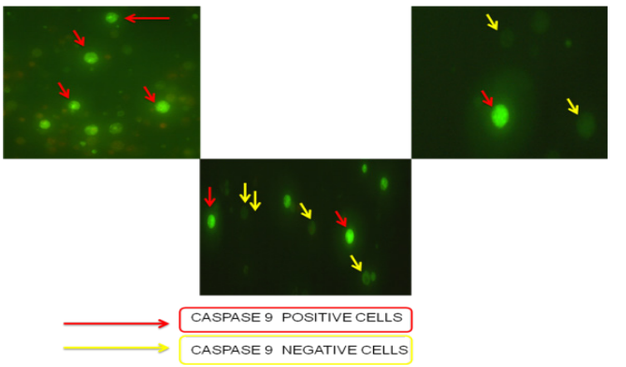


Figure 6. Caspase 9 activity in neutrophils (Fig A and B: Cells showing Caspase 9 activity (20X); Fig C: Cells showing Caspase 9 activity (40X). Cells with high green fluorescence were Caspase 9 positive and with no bright green fluorescence were caspase 9 negative.

higher caspase-8 and caspase-9 activity. The rise in caspase-8 may be due to death signals coming from outside that are from the pathogen to the host cells or may be due to leakage of mitochondrial enzymes causing the procaspase activation whereas increase in caspase-9 may be due to increase in mitochondria transmembrane potential which instead validates the caspase activation.

Pathogens modulate milk PMN apoptosis and this modulation depends on the involvement of the toxin as well as the differential activation of the apoptotic pathway (Kebir and Filep, 2013). Though in present study apoptosis was observed in the neutrophils but the apoptosis was induced or constitutive is not studied. It may be assumed

that the process of apoptosis is induced by the organism. Further studies are required to validate this statement so as to reach to a conclusion regarding the involvement of pathogen and its associated molecules in programming the process of cell death.

CONCLUSION

Staphylococus aureus elicits a delayed immune response of the host immune system as reported earlier in many studies. It is also known that, the pathogen employs a number of protective mechanisms by which it bypass the host immune system. The role of PMN apoptosis in the survival of the pathogen has been emerged as a mode of immune tolerance of the pathogen in the host body. In this study, apoptosis like changes reported in milk PMNs suggests that the recruited PMNs undergo the similar mode of classical apoptotic processes like other somatic cells. The findings of the study provide an insight into the molecular basis of neutrophil apoptosis and form a basis to enhance the host immunity by the process of apoptosis modulation to combat the infections caused by the pathogen. Further, more studies are required to understand the key molecules involved in initiation of neutrophil apoptosis and their signaling cascades regulating the neutrophil functions during clinical mastitis in bovines.

Conflict of Interest Statement

The authors declare that they have no conflict of interest with any other people or organizations in any financial or personal relationship.

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