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RESEARCH ARTICLE

Endotoxin exposure alters viability and apoptotic potential of buffalo neutrophils

Upkardeep Singh[1], R S Sethi[2*], CS Mukhopadhyay[3], BV Sunil Kumar[4], Dipak Deka[5] and Ramneek Verma[6]

1. Upkardeep Singh: PhD Scholar, Canadian Centre for Health and Safety in Agriculture, University of Saskatchewan, Saskatoon, Canada.
2. Professor, School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab and Adjunct Faculty, Department of Biomedical Sciences, University of Saskatchewan, Saskatoon, Canada; email: rs.sethi@usask.ca.
3. [3]Assistant Scientist, School of Animal Biotechnology, GADVASU, Ludhiana, Punjab, India 141004
4. Assistant Biochemist, School of Animal Biotechnology, GADVASU, Ludhiana, Punjab, India 141004
5. Assistant Scientist School of Animal Biotechnology, GADVASU, Ludhiana, Punjab, India 141004
6. Professor cum Director, School of Animal Biotechnology, GADVASU, Ludhiana, Punjab, India 141004

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Corresponding Author

Upkardeep Singh

Abstract

Buffalo which contributes towards the social well being of millions of South East Asian farmers commonly suffer from various infectious diseases. There is paucity of literature on the innate immune response of buffalo specially the cells like neutrophils which are the first line of defense. Buffalo neutrophils were isolated by using Hi Sep LSM-1077 along with Dextran and viability was determined by trypan blue exclusion test. Presence of myeloperoxidase suggested presence of neutrophils. The freshly isolated neutrophils showed a purity of 98.4% with viability up to 98.44 ± 0.35%. There was a significant decrease ($p<0.05$) in the number of viable neutrophils after 24 hr, 48 hr and 72 hr post-incubation in the control group. LPS challenge showed significantly ($p<0.05$) more number of viable neutrophils at 48 and 72 hr compared to non LPS group. Buffalo neutrophils undergoing apoptosis showed condensation of nucleus and presence of apoptotic bodies with intact cell membrane. LPS challenge resulted a significant decrease in the number of apoptotic neutrophils compared to control group at different time intervals. It is the first data on the viability and apoptotic potential of neutrophils of water buffalo suggesting that LPS treatment activated the neutrophils which led to significant reduction in apoptosis and increased life span of neutrophils as compared to untreated cells.

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INTRODUCTION

Buffaloes contribute to the economic well being of millions of small-scale farmers in India by providing a major component of milk and milk products. However, buffalo suffer from various bacterial diseases leading to death and economic losses to the farmers in terms of vaccination and treatment. The poor understanding of immune systems of buffalo may be a major reason for the lack of progress in developing more effective preventive and treatment strategies against infectious diseases in buffaloes. Neutrophils are the most abundant leukocytes in the blood which play an important role in the host innate immune system. Further, neutrophils are the first cells to be recruited to the site of inflammation to kill and phagocytose the invading pathogens (Appelberg, 2007). Myeloperoxidase (MPO) enzyme is present in the azurophilic granules of neutrophils and is used as cytochemical marker for the neutrophils (Bainton *et al.,* 1971).

Neutrophils take up a variety of micro-organisms by phagocytosis and kill them in intracellular vesicles using degradative enzymes and other antimicrobial substances (Baggiolini and Dewald, 1985). However, the half-life of non-activated neutrophils in circulation is very less i.e. around 6-8 hr (Keel *et al.,* 2007). Non activated neutrophils return to liver, spleen and bone marrow for phagocytosis by macrophages after undergoing the process of spontaneous apoptosis (Martin *et al.,* 2003). In addition, the neutrophil apoptosis is also critical for safe disposal of activated neutrophils from the site of inflammation without the release of harmful contents to minimize tissue (Savill and Fadok, 2000). Both extrinsic and intrinsic pathways are involved in regulating neutrophil apoptosis. Although neutrophils have very few mitochondria (Clark *et al.,* 1980), the mitochondrial or intrinsic pathways also play an important role in neutrophil apoptosis.

The apoptosis of neutrophils is delayed at the site of inflammation by a number of pathogen and host derived inflammatory mediators (Lee *et al.,* 1993) leading to accumulation and prolonged activation of neutrophils injury (Mecklenburgh *et al.,* 1999). Further, impaired apoptosis may lead to developmental and immunological disorders, neurodegeneration and cancer (Fuchs and Steller, 2011). The toll-like receptor ligands such as bacterial lipopolysaccharide (LPS) and lipotechoic acid delay spontaneous apoptosis of neutrophils and increase the life span of neutrophils (Lotz *et al.,* 2004).

However there are no reports on viability and apoptotic potential of buffalo neutrophils. Keeping in view the role of neutrophils in modulating innate immune response, the present study has been planned to study the viability and apoptosis potential of buffalo neutrophils following LPS challenge.

MATERIALS AND METHODS

The experiment protocols were approved by Institutional Animal Ethics Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Healthy adult buffalo maintained at the dairy farm of Department of Animal Genetics and Breeding, GADVASU, Ludhiana were used for this study. Blood was aseptically collected from jugular vein of buffalo in vacutainer tubes containing EDTA as anticoagulant. The blood was thoroughly mixed with anticoagulant immediately to prevent clotting and was immediately transported on ice packs to the laboratory for further processing. The blood samples were kept at 4^0C and were used within 1hr of collection. The neutrophils were isolated using HiSep lymphocyte separarting media (LSM) along with 6% dextran for sedimentation of RBC. The final neutrophil pellet was dissolved into desired volume of RPMI 1640 media (Sigma Aldrich) supplemented with 5% fetal calf serum (Gibco), antibiotic mixture 1OOX (Himedia) and counted on haemocytometer to determine the cell number. The experiment was repeated three times on separate blood collections to obtain average values after statistical analysis.

Determination of cell purity

To determine the purity of neutrophils, smear of isolated cell suspension was prepared on the slide. After air drying, slides were processed for Haematoxylin and Eosin staining (H&E). Randomly 100 cells were counted on H&E stained slides and percentage proportion of neutrophils (multilobed nucleus with intact cell membrane) was counted.

Quantifying cell number

The final neutrophil pellet was resuspended into 2ml of RPMI 1640. The cell suspension (10µl) was diluted with equal volume of trypan blue and 10µl of this mixture was loaded on haemocytometer and the neutrophils were counted in all 16 squares located at the 4 corners of haemocytometer using hand tally counter.

LPS Challenge

After calculating the initial concentration of isolated neutrophils, the cells were diluted accordingly with RPMI 1640 media to make required concentration of 2×10^6 cells/ml. After dilution, cell suspension (2×10^6cells/ml) was transferred to 12 well cell culture plate. The cells were treated with media alone (control group) or with LPS @1µg/ml (LPS group) and incubated at 37^0C in a humidified 5% CO_2 incubator.

Western blotting for myeloperoxidase (MPO)

Cell suspension (300-500µl) of freshly isolated neutrophils was transferred from the wells of culture plate into 1.5ml microcentrifuge tubes (MCT) and centrifuged at 10,000 rpm for 5min to pellet out the cells. Supernatant was discarded and pellet was air dried by inverting the MCTs for 5-10min. The air dried cell pellet was stored at -20^0C for further use. For western blot analysis, cell pellets were mixed with 2X loading dye (Laemmli buffer) in 1:1 ratio and boiled for 15 min and 20 µl sample was loaded on 12 % SDS-PAGE gel. Resolved proteins were transferred to nitrocellulose membrane and blocked with 3% BSA for 30min to block nonspecific binding. The

membrane was incubated with 1:500 concentration of rabbit anti-human antibody to myeloperoxidase (A0398; Dako) at 37°C for 2 hour, followed by horseradish peroxidise conjugated goat anti-rabbit antibody (P0448; Dako). Detection was performed with the chromogen solution (0.025g of diaminobenzidine/DAB, 10 µl of 30% H_2O_2 and 20ml PBS) and experiment was repeated three times.

Viability assay

Cell suspension was mixed with Trypan blue (0.5%) in 1:1 ratio after the selected time interval (0hr, 24hr, 48hr and 72 hr post incubation) and 10µl was loaded onto haemocytometer. Trypan blue enter inside the dead cell and imparts blue colour, however viable (live) cells excludes the dye and remain transparent. Live and dead cells were counted for control as well as LPS challenged neutrophils at the same time interval to calculate the cell viability. A total of 100 cells were counted and percentage of viable neutrophils was calculated by the following formulae.

Viability (%) = Number of viable cell/Number of Total Cell X 100

Quantification of Apoptotic cells

Slides were prepared by using cell suspension after 0hr, 24hr, 48hr, 72hr post incubation from control and LPS challenged groups. 50µl of cell suspension was placed onto uncoated slides to prepare a smear by keeping other slide at an angle of 45^0. Slides were air dried for 15-20 min. and then stained with May-Grunwald Giemsa Stain. The May-Grunwald stained slides were examined under light microscope under 40X and attention was given to normal and apoptotic cells. Each slide from selected time interval was observed and randomly a total of 100 normal, necrotic or apoptotic cells were counted by using hand tally counter and finally the percentage of apoptotic cells was calculated by following formulae:

Apoptotic cell (%) = Number of apoptotic cells/ number of total cells (normal+necrotic+apoptotic) X 100

Statistical Analysis

The data obtained from viability and apoptotic cell count from control and LPS treated groups were subjected to statistical analysis by using Fisher's Least Significant Difference test (Post Hoc Test, ANOVA) for Multiple Pair Wise comparison among LPs challenged and control groups for viable and apoptotic neutrophils. Results were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

It is the first data on the viability and apoptosis of buffalo neutrophils and changes in viability and apoptosis after challenging with lipopolysaccharide (LPS). The freshly isolated buffalo neutrophils showed a purity of 98.4% and results are consistent with the finding of Akhtar *et al.* (2010), who reported that the use of LSM and dextran for bovine neutrophils isolation produced more than 95% pure neutrophils. The isolated buffalo neutrophils were confirmed by the presence of MPO (Fig 1). Neutrophil showed presence of MPO antibody positive bands of 59kDa and 13kDa. MPO is a major component of azurophilic granules of neutrophils and isolated neutrophils are strongly positive for MPO expression at mRNA and protein level (Amanzada *et al.,* 2011). MPO is a tetrameric molecule having a pair of heavy (59 kDa) and light chains (13.5 kDa) and two iron atoms (Olsson *et al*., 1972) and is common marker for neutrophils (Haqqani *et al.,* 1999).

Viability of Neutrophils

The viability (negative by trypan blue dye exclusion test) of the freshly isolated buffalo neutrophils was found to be 98.44 ± 0.35% in the control group (Table 1, Fig 2). Earlier bovine neutrophils have been isolated with viability greater than or equal to 90% (Lichtenberger *et al.* 1999), 95% (Chambers *et al.* 1983) and >99% (Siemsen *et al.* 2014) by various methods. There was a significant decrease (p<0.05) in the number of viable buffalo neutrophils after 24 hr (92.05 ± 3.78%) and 48 hr (70.02 ± 4.66%) post-incubation compared to the freshly isolated neutrophils (Table 1, Fig 2). The viability of the neutrophils was further reduced significantly (p<0.05) to 49.91 ± 3.02% at 72 hr post-incubation. The neutrophils have only a short half life span of about 16 hr and they started dyeing after this period of time (Keel *et al.,* 1997). Non activated neutrophils do not express or express very low levels of antiapoptotic proteins and hence have relatively short half lives (Riedemann *et al.,* 2004).

LPS challenge resulted a non significant decrease in the number of viable neutrophils after 24 hr (94.49 ± 2.35%) post-incubation and significant decrease (p<0.05) after 48 hr (81.83 ± 1.41%) and 72 hr (72.62 ± 1.23%) post-incubation compared to freshly isolated neutrophils (Table 1, Fig 2). The comparison between control and LPS treated group indicated that there was significantly more number of viable neutrophils after 48 and 72 hr (81.83 ± 1.41%, 72.62 ± 1.23%) following LPS challenge compared to non LPS challenged group (70.02 ± 4.66%, 49.91 ±

3.02%), respectively. The data suggest that LPS challenge resulted in increased number of viable neutrophils specially at 48 and 78 hr post-incubation which may be due to activation of neutrophils leading to decreased cell death. Proctor (1979) reported that incubation of PMNs with LPS resulted in significant decrease in cell death, chemiluminescence, oxygen consumption and superoxide formation. LPS, bacterial products and cytokines like IL-1β, TNF-α and G-CSF enhanced the half life of PMN as compared to untreated cells (Colotta *et al.* 1992). Neutrophils activated by endotoxins and/or various microbial products prolong their life span by delaying the constitutive apoptosis (Lee *et al.,* 1993).

Apoptosis of Neutrophils

Normal buffalo neutrophils were characterized by multilobed nucleus (Figs. 3a, 3b). The light microscopic observations revealed that the buffalo neutrophils undergoing apoptosis showed condensation of nucleus and presence of apoptotic bodies along with intact plasma membrane (Figs. 3c-3f). Apoptosis is characterized by cell shrinkage, condensation of chromatin, bubbles in the cellular membrane (blebbing) and intact cell membrane (Saraste and Pulkki, 2000). During the process of apoptosis the plasma membrane of neutrophils remains intact which prevent the release of harmful contents (Savill *et al.,* 1989). Sladek and Rysanek (2001) reported that light microscopy and scanning electron microscopy are equally effective to distinguish the different stages of apoptosis i.e karyopyknosis, zeiosis and apoptotic bodies.

The freshly isolated buffalo neutrophils showed a small proportion (4.67 ± 0.63%) of apoptotic cells, however there was a significant increase ($p<0.05$) in the count of apoptotic cells after 24hr (32.36 ± 5.44%), 48 hr (49.81 ± 3.84%) and 72 hr (73.56 ± 4.04%) post-incubation compared to freshly isolated neutrophils (Table 2, Fig 4). Neutrophils undergo spontaneous apoptosis (Kirschnek *et al.* 2011) and mature neutrophils are committed to apoptotic death by virtue of their constitutive coexpression of Fas and Fas ligand (Liles *et al.* (1996). Savill *et al.* (1989) revealed that neutrophils derived from peripheral blood or acutely inflamed joints as well as aged neutrophils in culture undergo morphological and chromatin fragmentation changes indicating apoptosis.

In the present investigations, treatment of buffalo neutrophils with LPS resulted in a significant increase ($p<0.05$) in the number of apoptotic cells compared to freshly isolated cells after 24 hr (18.06 ± 2.55%), 48 hr (25.12 ± 1.71%) and 72 hr (46.14 ± 2.59%) post-incubation (Table 2, Fig 4). However, there was a significant decrease ($p<0.05$) in the number of apoptotic neutrophils in LPS treated group compared to non LPS group at 24 hr, 48 hr and 72 hr post-incubation stage suggesting that treatment of neutrophils with LPS significantly reduced the number of neutrophils showing apoptosis as compared to untreated cells at different stages. Endotoxins or microbial products such as LPS delay the apoptosis of neutrophils by activating them (Lee *et al.,* 1993; Deleo, 2004; Channabasappa *et al.* 2014)). Endotoxin/LPS reduce CD95-induced neutrophil apoptosis by cIAP-2 mediated caspase-3 degradation (Mica *et al.,* 2004), through activation of MAPK and ERK pathways (Riedemann *et al.,* 2004), NF-κB (Sabroe *et al.,* 2003; Lotz *et al.,* 2004) and subsequent transcription of anti-apoptotic factors (Miskolci *et al.,* 2007). Activation of p38 MAPK by neutral sphingomyelinase (nSMase) is mediated through binding of LPS to neutrophils, which inhibit apoptosis (Lee *et al.,* 2011). Various pathogens like *Mycobacterium tuberculosis, Anaplasma phagocytophilum* and *Chlamydia pneumoniae* have direct effect on the neutrophil apoptosis and they not only survive and replicate within the neutrophils but also delay the spontaneous apoptosis of neutrophils (Kim *et al.,* 2000). The data taken together suggest significant delay in spontaneous apoptosis due to LPS activation which increased the life span/viability of buffalo neutrophils.

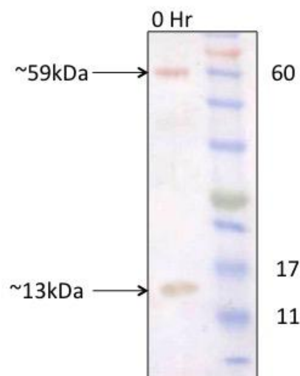

Fig 1. Western blot from freshly isolated buffalo neutrophils (0 Hr) showing bands of 59 and 13 kDa corresponding to myeloperoxidase.

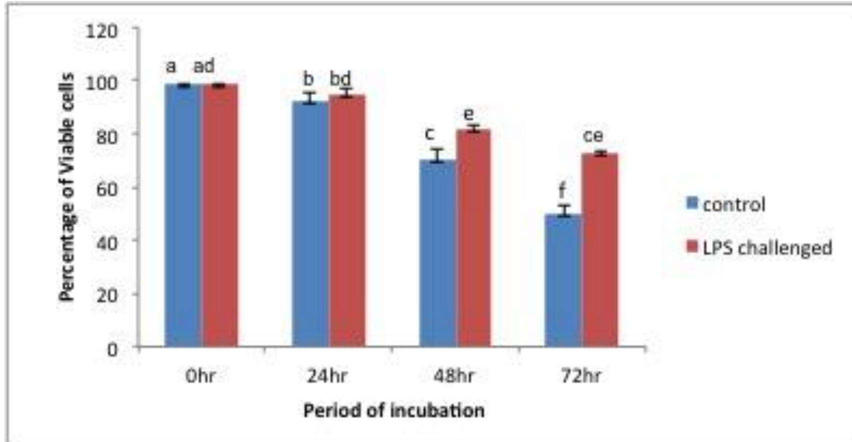

Fig 2. Percentage of viable neutrophils of control and LPS challenged groups. No common manuscript between two levels indicate the significant difference (p<0.05). The data represent the average from three separate neutrophil preparations.

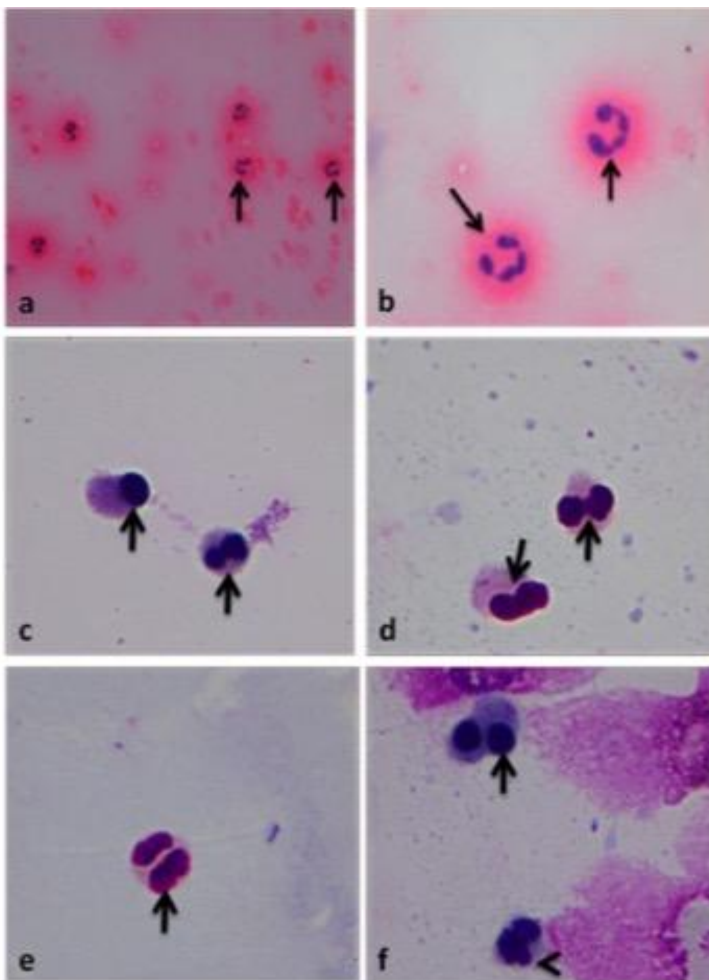

Fig. 3. Multilobed neutrophils (thin arrow) with normal histo-architecture (a,b), chromatin condensation (thick arrow) and apoptotic bodies (arrow head) in the neutrophils undergoing apoptosis (c-f). 2a: HE X 40; 2b: HE X 100; 2c-f: May Grunwald X 100

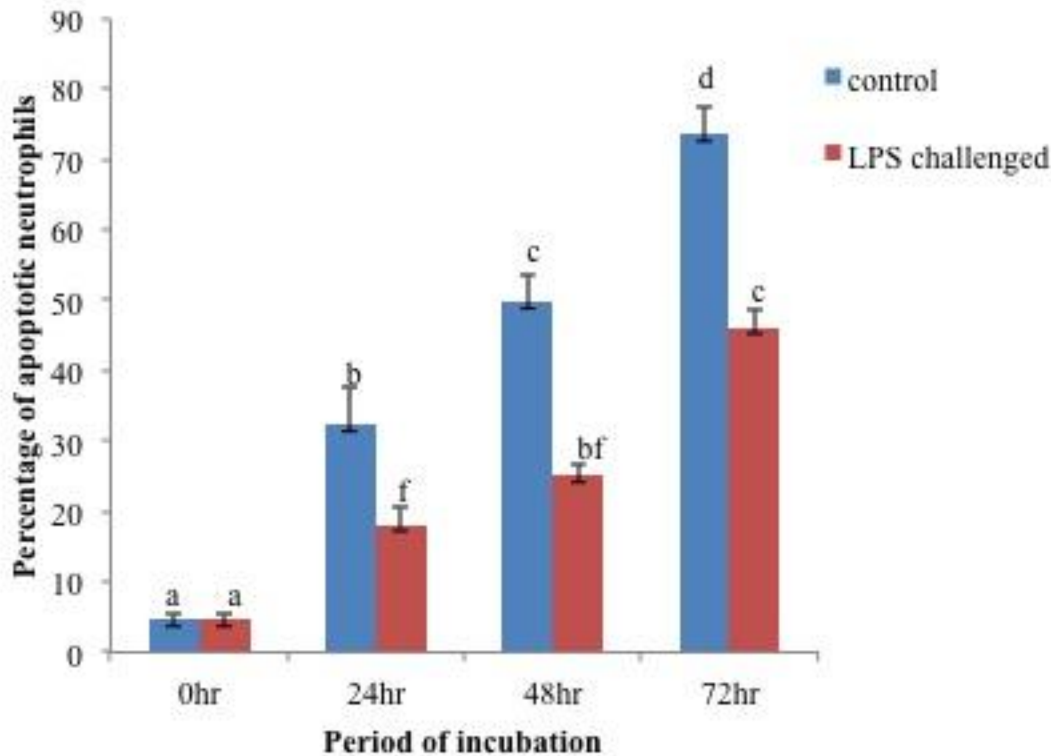

Fig 4. Percentage of apoptotic neutrophils of control and LPS challenged groups. No common manuscript between two levels indicate the significant difference ($p<0.05$). The data represent the average from three separate neutrophil preparations.

Table 1: Number of viable neutrophils expressed in percentage

	Number of viable neutrophils expressed in percentage (Mean ± S.E)	
	Control group	**LPS challenged group**
0 hr	$98.44^{a} \pm 0.35$	$98.44^{ad} \pm 0.35$
24hr	$92.05^{b} \pm 3.78$	$94.49^{bd} \pm 2.35$
48hr	$70.02^{c} \pm 4.66$	$81.83^{e} \pm 1.41$
72hr	$49.91^{f} \pm 3.02$	$72.62^{ce} \pm 1.23$

a,b,c no common manuscript between two levels indicate the significant difference ($p<0.05$). The data represent the average from three separate neutrophil preparations.

Table 2: Number of apoptotic neutrophils expressed in percentage

	Number of apoptotic neutrophils expressed in percentage (Mean ± S.E)	
	Control group	**LPS challenged group**

0 hr	$4.67^{a} \pm 0.63$	$4.67^{a} \pm 0.63$
24hr	$32.36^{b} \pm 5.44$	$18.06^{f} \pm 2.55$
48hr	$49.81^{c} \pm 3.84$	$25.12^{f} \pm 1.71$
72hr	$73.56^{d} \pm 4.04$	$46.14^{c} \pm 2.59$

a,b,c no common manuscript between two levels indicate the significant difference ($p<0.05$). The data represent the average from three separate neutrophil preparations.

CONCLUSIONS

We conclude that LPS/endotoxin exposure has been linked with activation of buffalo neutrophils leading to delay in spontaneous apoptosis and increased life span/viability which needs further investigations in terms of expression of TLRs and Caspases during such exposures.

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