Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.216

RESEARCH ARTICLE

Effects of Tyloxapol in the Amelioration of Endotoxemic Effects of *Pasteurella multocida* During Hemorrhagic Septicemia in Water Buffaloes

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ARTICLE HISTORY (24-356)

Received: June 24, 2024
Revised: July 26, 2024
Accepted: July 27, 2024
Published online: August 03, 2024

Key words:
Tyloxapol
haemorrhagic septicemia
Cytokines
Lipoproteins
endotoxins

A B S T R A C T

Tyloxapol is a surfactant which increases the concentration of lipoproteins in humans and animals. These lipoproteins are the natural scavengers of the lipopolysaccharides, also known as endotoxins, secreted by outer membrane of the gram-negative bacterium and responsible for morbidity and mortality of the diseases associated with gram-negative bacterium. Therefore, the present study was designed to evaluate the therapeutic effects of tyloxapol in *Pasteurella multocida*-induced infection in water buffaloes. For this purpose, 12 healthy buffalo calves, 8-10 months old, were procured and experimentally induced with *Pasteurella multocida* infection after acclimatization period of 7 days. The buffalo calves were randomly divided into two groups ($n=6$) A & B. Group A was treated with ceftiofur HCl and flunixin meglumine @ 6 and 2 mg/kg BW, respectively. This treatment continued for 5 days. Group B was administered with tyloxapol @ 150 mg/kg BW IV QD continuously for 3 days. Efficacy of the treatment was evaluated through cardinal parameters, lipoproteins and concentrations of cytokines and endotoxins. The data obtained was analyzed statistically. Results of the study depicted that no untoward or allergic reaction was observed after intravenous administration of tyloxapol to the buffalo calves. Tyloxapol significantly elevated lipoprotein concentrations to the normal in group B and differed significantly ($P<0.05$) from its counterpart. It significantly ($P<0.05$) reduced the concentration of pro-inflammatory cytokines and endotoxins than its counterpart, thereby successfully ameliorated their deleterious effects. We concluded from findings of the study that tyloxapol is safe to administer in buffalo calves and could successfully resuscitate the calves suffering from hemorrhagic septicemia thus improving survival. It showed anti-endotoxic effects and enhanced natural scavengers of endotoxins i.e., HDL & LDL and successfully ameliorated cytokine concentration. Tyloxapol can be useful in clinical settings to contravene the effects of endotoxemia/septicemia in animals.

To Cite This Article: Shahzad MN, Zafar MA, Tahir AH and Rehman SU, 2024. Effects of tyloxapol in the amelioration of endotoxemic effects of *Pasteurella multocida* during hemorrhagic septicemia in water buffaloes. Pak Vet J, 44(3): 675-682. http://dx.doi.org/10.29261/pakvetj/2024.216

INTRODUCTION

Haemorrhagic septicemia, associated with *Pasteurella multocida* (*P. multocida*), is considered a serious, acute, highly fatal and one of the most devastating diseases of livestock especially water buffaloes (Zafar *et al*., 2012; Almoheer *et al*., 2022; Chanda *et al*., 2024) in tropical region of the world like South-East Asia, Africa and Middle East. In these countries, it is always considered of great economic importance (Chilambarasan *et al*., 2019; Shahzad *et al*., 2020; Michael *et al*., 2021). Likewise, HS causes a huge economic loss in Pakistan, accounting for about 350 million USD every year (Michael et al., 2021). According to a GTZ (*Gesellschaft für Technische Zusammenarbeit: a German company for technical cooperation*) survey, the estimated annual losses of HS are PKR. 2.17 billion accounted in Punjab. These losses rank HS as number one economically important disease. It was further reported that if 50% financial losses due to HS are reduced through adopting preventive measures like vaccination, then it can overcome the scarcity of livestock production and products obtained by them to compensate the deficit for human population (Anonymous, 1996). In Pakistan, water buffaloes are reported more prone to HS as compared to cattle (Zafar *et al*., 2012). HS has always remained a challenge to the

scientists and created nuisance to the veterinary practitioners, despite of the concreted efforts made in scientific advancements. It is now well established that clinical manifestations of this disease are due to lipopolysaccharide (LPS), also known as endotoxin, excreted due to lysis of *P. multocida* (Horadagoda *et al*., 2001; Zafar *et al*., 2012). Vaccination failure and development of resistance to antibiotics are the concerns faced by the practicing veterinarians now a days (Vercelli *et al*., 2022; Caneschi *et al*., 2023). These factors are the main hindrances in treating the animals affected with HS. In septicemia, a dysregulated immune response may initiate an aggravated pro-inflammatory process that leads to immunosuppression and continual immune disturbance (Cao *et al*., 2023; Marques *et al*., 2023).

The main unresolved and ignored aspect in sepsis treatment is the inactivation of LPS (Harper *et al*., 2011). Mammals are naturally provided with LPS scavengers in the form of "lipoproteins" which are considered a physiological defense mechanism. Lipoproteins interact with endotoxins and mask their activity and ability to induce their manifestations (Levels *et al*., 2011; Pérez-Hernández *et al*., 2021). The modulation of biological process of formation of lipoproteins in the body through any source can help to reduce the endotoxins associated fatality. To this end, detergents, like tyloxapol, are supposed to be a good source in activation of the lipoproteins and are capable to inactivate bacteria especially gram-negative bacteria. Tyloxapol is a prototype of polymeric nonionic surfactant that has been traditionally used as a dispersing agent in medicinal preparations. Recently, its potential role in the treatment of sepsis in rabbits and sheep has been reported. The mechanism by which tyloxapol exerts its effects in sepsis is multifaceted. It has been shown to modulate the immune response, reduce inflammation, and enhance the clearance of pathogens but the foremost phenomenon through which it plays a pivotal role in the resuscitation of patients from sepsis is activation and modulation of lipoproteins concentration in the mammalian body like rabbits, sheep etc. (Staub *et al*., 2001; Serikov *et al*., 2003; Chalmeh *et al*., 2016; Heidari *et al*., 2016). Keeping in view this phenomenon, it was hypothesized that tyloxapol could mask the endotoxin receptors (CD14) by increasing lipoproteins in the body, thereby reducing the severity of the disease and decreasing the mortality and morbidity associated with hemorrhagic septicemia. Therefore, this study was planned to evaluate the efficacy of intravenous administration of tyloxapol in the reduction of morbidity and mortality in experimentally induced hemorrhagic septicemia in buffalo calves and to analyze its ameliorative effects on lipoproteins, cytokine and endotoxin concentrations during hemorrhagic septicemia.

MATERIALS AND METHODS

The protocol of the present study was approved and in accordance with the guidelines of Institutional Ethics Committee (PMAS-AAUR/IEC/422), Pir Mehr Ali Shah-Arid Agriculture University, Rawalpindi, Pakistan.

The study was conducted in 8-10 months old buffalo calves (*n*=12). Calves were procured and reared at "University Research Station, PMAS-Arid Agriculture University, Rawalpindi, Pakistan. At experimental station, buffalo calves were acclimatized for a period of 7 days and offered feed and water *ad-libitum*. Animals were kept in the individual pens.

Induction of *Pasteurella multocida* infection: Nasal, blood and tissue samples were obtained from the animals showing signs of HS. All information regarding the collected samples was recorded. Swab samples were transported to the Infectious Diseases Lab (IDL) of the Department of Clinical Studies, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan in phosphate buffer saline (PBS) and blood samples were transported in vacutainers by placing on ice cubes. Swabs, blood and tissue samples were inoculated in blood agar containing 5% defibrinated sheep blood. These cultures were incubated for 24 h at 37 ºC. Primary culture colonies were further sub-cultured on blood agar (BA) using streak plate method. We repeatedly performed it until pure culture was obtained (Katoch *et al*., 2015). Identification of *P. multocida* was done on the basis of characteristics of its cultural, morphology and biochemical profiling. The *P. multocida* was then inoculated on Brain Heart Infusion (BHI) and placed in shaker incubator at 37ºC for 24h with 150 rev/min for massive growth. Bacteria were then quantified and an inoculum was prepared that contain $3x10^9$ CFU/ml of *P. multocida* (Ievy *et al*., 2013). *Pasteurella multocida* was administered intranasally. Buffalo calves were then observed for the development of infection/disease.

The criteria for the development of the disease (HS) were i.e., increased rectal temperature up to 105-107ºF, depression, swelling on the throat and upper dewlap region, dyspnea, discharge through nostrils, hypersalivation, anorexia and hesitancy to move (Zafar *et al*., 2012).

Study Design: After development of *P. multocida* infection (haemorrhagic septicemia), the buffalo calves were then randomly divided into two equal groups (n=6) as group A and group B. The treatment was started after water hosing on the head for 10-15 minutes to control the body temperature. Both groups were allocated with following treatment protocols.

Calves of group A served as control group and allocated to the treatment regimen already in vogue i.e., ceftiofur HCl, a 3rd generation cephalosporin available with brand name Excenel RTU® (Pfizer Animal Health Division, Pakistan) and flunixin meglumine, a NSAID available with the brand name of Tricure® (ICI Animal Health Division, Pakistan). These drugs were administered @ 6mg/kg BW, IM, QD and @ 2mg/kg BW, IV, bid, respectively, continued for 5 days. Group B served as experimental group and treated with Tyloxapol 20% (T8761 Lot No. MKCH0996; Sigma-Aldrich, St. Louis, MO USA) @ 150mg/kg BW, IV QD and continued for 3 days.

Preparation of Tyloxapol: A 20% (v/v) homogenous solution of tyloxapol (T8761 Lot No. MKCH0996; Sigma-Aldrich, St. Louis, MO USA) was prepared in PBS 1% with slight agitation. The solution was then filtered with the help of 0.45µm filter tube. The solution was left

overnight for suspension. At the time of administration, the solution was further diluted with isotonic saline to the desired concentration (Rasouli *et al*., 2016).

Measurements and analyses: Efficacy of the treatment regimens was analyzed through following parameters:

Cardinal Parameters: The survival of buffalo calves was observed for 5 days (t=120 h) after institution of treatment in both groups induced with hemorrhagic septicemia. Mercuric thermometer was used to measure the body temperature. Respiration rate was observed through counting ribcage movements carefully for a minute. The measurement was repeated pertinent to that if animal started sniffing or vocalizing. The pulse rate was recorded through coccygeal artery and was counted for 15 sec and then multiplied by 4. All these procedures were repeated three times at each measuring interval to obtain average value of that specific timepoint (Constable *et al*., 2016).

Lipoprotein Concentration: High-Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL) was measured through the Lipid Analyzer (Blood Lipid Analyzer®, China). The principle of this equipment for measuring HDL and LDL and other parameters is "Enzyme Chromophore Reaction". This reaction catalyzes the enzymes (alkaline phosphatase, horseradish peroxidase and β -galactosidase) and separate it from chromophore group which result in formation of distinctive colours. This enzyme chromophore reaction is used in laboratory equipment which facilitates the quantitative and qualitative identification of the enzymes and proteins (Mendoza *et al*., 2012). The test strip was inserted into the meter and 30μL of fresh venous whole blood was filled in in the triple capillary rod. Then it was moved for proper mixing of the blood. The value appeared on the screen after few seconds.

Measurement of cytokine and endotoxin concentrations: Cytokine concentration was measured with the help of ELISA kits according to the manufacturer's protocols at 450 nm (microplate reader, Stat Fax 4200). The ELISA kits used in the study were Tumor necrosis factor (TNF)-α (RAB0522; Sigma Aldrich, USA), Interleukin (IL)-1β (AB273202; Abcam, China), interleukin (IL)-6 (AB205080; Abcam, China) and interleukin (IL)-8 (A76807; Antibodies.com, Cambridge, UK).

Quantification of endotoxin concentration: The extraction of LPS, the outer membrane of *P. multocida*, was made form the cultured cells. This extraction was performed through "phenol-chloroform-petroleum ether method". The protocol of de-acylation derivatives of LPS described by Kojima *et al*., (2009) and Rezania *et al*., (2011) was adopted. The quantification of endotoxin concentration was carried on HPLC (LC-10ADVP HPLC system from Shimadzu Co. Ltd. (Kyoto, Japan) as per procedure described by Kojima *et al*., (2009) and Rezania *et al*., (2011).

Measuring Intervals: All above mentioned parameters were recorded at BL (baseline), t=0 (during HS), t=6 hours (h), t=12 h, t=24 h, t=72 h and t=120 h after administration of allocated treatment regimens.

Statistical Analyses: The data was subjected to the statistical analysis which was performed using SPSS (version 23). The level of statistical significance was set at $p < 0.05$ for all analyses. Analysis of variance (two factor completely randomized design) was used to find out the significance difference among means and interactions (group x time). Tukey test was applied at 5% level of significance for significant factors & interactions to compare their means. Pearson's correlation analysis was also performed to check the bivariate relationships.

RESULTS

Cardinal Parameters

Survival index: Group A treated with combination of ceftiofur and flunixin showed 66.6% survival (Table 1). Whereas the survival percent in group B treated with IV administration of Tyloxapol @ 150mg/kg BW was 83.3% and showed high survival percent which was significant over group A (Table 2).

Body temperature (ºF): During *P. multocida* infection, the body temperature increased significantly in all buffalo claves of both groups. After treatment, both groups A and B showed a significant decrease in the value of body temperature throughout the study and achieved baseline values at t=72 h. However, group A showed significant differences ($P<0.05$) over group B at t=12 h and t=24 h (Table-2).

Respiration rate (breaths/min): Induction of *P. multocida* infection to the buffalo calves significantly decreased the values of respiration rate in both groups. After administration of respective treatments, both groups showed improvement in recovering the respiration rate. Group A showed a continuous increase in values of respiration rate but it was non-significant throughout the study period and failed to achieve the baseline values. Group B presented better recovery trend, and showed significant differences ($P<0.05$) over group A from t=24 h till the end of study (t=120 h) and successfully attained the basal values of respiration rate (Table-2).

Pulse rate (beats/min): The pulse rate decreased significantly during *P. multocida* infection. After administration of allocated treatment helped to recover the normal values of pulse rate in both groups. Group A showed a continuous increasing trend toward recovery but in a non-significant manner and this group failed to achieve the basal values within the study period. Whereas group B showed better increasing trend and attained the basal values of pulse rate successfully within the study period and differed significantly ($P<0.05$) from its counterpart at t=120 h (Table-2).

Lipoproteins

Low Density Protein (LDL; g/dl): Induction of HS to the buffalo calves caused a significant decrease in LDL values. After administration of allocated treatments, both groups showed recovery trend but recovery pattern in

Table 1: Number of buffalo calves survived from induced hemorrhagic septicemia after treatment.

	Time after treatment (hours)						
	BL	t=0	t=6 h	t=12 h	t=24 h	t=72 h	t=120 h
Group A	6	6	6	6	5	4	4
Group B	6	6	6	6	6	5	5

Table 2: Values of cardinal parameters during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

		Time after treatment (hours)						
Cardinal Parameters	Groups	BL	t=0	t=6 h	t=12 h	t=24 h	t=72 h	t=120 h
Survival percent	Group A	100	100	100	100	83.3	66.6	66.6
	Group B	100	100	100	100	100^{NS}	83.3^{*}	83.3^{*}
Body temperature	Group A	101.9±0.3	105.3±0.2	104±0.1	103.4±0.1	$102±0.3^{*}$	101.7±0.1	101.9±0.2
	Group B	102.2±0.2	105.7±0.2	104.4±0.2	$104±0.3^{*}$	$102.9±0.3^{*}$	$102±0.3^{*}$	$102±0.1^{*}$
Respiration rate	Group A	26.67±1.1	15.33±1.1	17±0.6	18.6±1.1	20±1.2	22±1.0	24.5±0.8
	Group B	28.6±0.6	15.6±0.6	18±0.7	20.3±0.8	$24±0.5^{*}$	$28±0.8^{*}$	$28.4±0.6^{*}$
Pulse rate	Group A	76±2.0	55.3±2.6	58.6±2.4	62±2.4	65.6±2.4	71±2.5	72±1.6
	Group B	77.3±2.2	53.3±2.8	57.3±2.8	60.6±2.8	67.3±2.4	74.4±2.0	$76.8±2.6^{*}$

Note: NS for non-significant (if the *P* value >0.05); *significant (if the *P* value <0.05); **highly significant (if the *P* value <0.01).

group A was insignificant and failed to regain the normal values of LDL. Whereas group B showed a significant increase at every time point throughout the study period and attained the normal values at t=72 h. Group B showed significant differences ($P<0.05$) over group A at t=12 h and t=24 h and highly significant difference ($P<0.01$) at t=72 h and t=120 h (Fig-1).

High Density Protein (HDL; g/dl): At baseline values of HDL differed non-significantly between groups A and B which decreased significantly during induction of HS in buffalo calves. After administration of allocated treatment regimens, a mild increase was observed in group A which was non-significant and it failed to recover the baseline values of HDL. On the other hand, group B showed better recovery trend and values of HDL increased in a significant way and successfully achieved the baseline values. Group B also showed significant difference ($P<0.05$) over group A from t=12 h till the last interval of the study i.e., t=120 h (Fig-2).

Cytokines concentration

Tumor necrosis factor-α (TNF-α; pg/ml): The concentration of TNF-α increased significantly during *P. multocida* infection as compared to baseline values. Impact of treatment protocol administered to group A was non-significant in terms of amelioration of TNF-α concentration and it failed to achieve the baseline values during the study intervals. Group B showed better recovery trend than its counterpart and showed significant recovery pattern in the values of TNF-α after administration of the allocated treatment. It successfully achieved the normal values of TNF-α concentration within the study period. Group B showed significant difference ($P<0.05$) over group A at t=6 h, t=12 h and t=24 h. It continued its pattern and showed highly significant difference ($P<0.01$) over its counterpart at t=72 h and t=120 h (Fig-3).

Interleukin-1β (IL-1β; pg/ml): At baseline, the concentration of IL-1β found non-significant between groups A and B. Infection with *P. multocida* to the buffalo calves of both groups induced a significant increase in the concentration of IL-1β. When buffalo calves of groups A and B treated with allocated treatment regimens, a significant decrease started from t=12 h in group B whereas group A showed significant decrease after t=72 h. The concentration of IL-1β decreased significantly at each time point in group B and baseline values were attained at t=120 h. Whereas group A failed to achieve the basal values of IL-1β. Group B showed significant difference ($P<0.05$) over group A at t=12 h and t=24 h. At t=72 h and t=120 h, group B showed highly significant difference ($P<0.01$) over group A (Fig-4).

Interleukin-6 (IL-6; pg/ml): Induction of infection to buffalo calves with *P. multocida* significantly increased the concentration of IL-6 in both groups A and B. Administration of assigned treatment protocols ameliorated the IL-6 concentration in both groups. Group A presented a non-significant decreasing trend in the IL-6 concentration. However, the values of IL-6 were near to baseline at the end of study. Group B showed a better recovery trend which was non-significant in the start but became significant in late hours. Group B successfully achieved the baseline values at t=120 h (Fig-5) and showed significant difference ($P<0.05$) over group A at t=72 h and t=120 h.

Interleukin-8 (IL-8; pg/ml): The IL-8 concentration significantly increased during *P. multocida* infection in both groups A and B. Institution of treatment helped to decrease the IL-8 values in both groups. Group A showed non-significant decreasing trend except at t=72 h. Despite that, it failed to attain the baseline values within study intervals. Whereas group B showed better recovery and amelioration in the concentration of IL-8 than group A. Group B showed significant difference ($P<0.05$) over group A at t=24 h, t=72 h and t=120 h and successfully ameliorated IL-8 concentration to the normal (Fig-6).

Endotoxin concentration (x10^2 pg/ml): Data of this study depicted that the concentration of endotoxin was zero at baseline. Experimental induction of *P. multocida* infection significantly increased the endotoxin concentration in buffalo calves of both groups. Administration of allocated treatment to the respective groups inflexed and decreased the endotoxins concentration. Both groups showed significant decrease in the concentration of endotoxins but the recovery pattern was better in group B and it showed significant difference

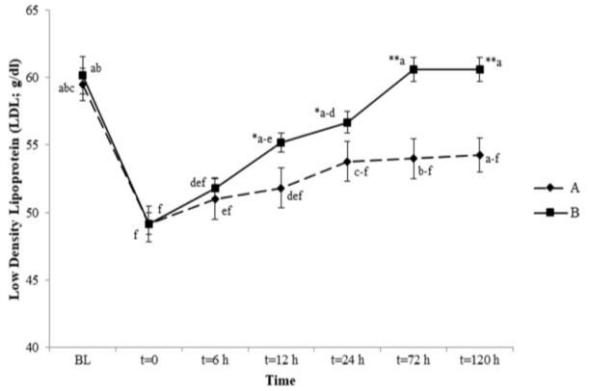

Fig. 1: Low Density Protein (LDL; g/dl) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

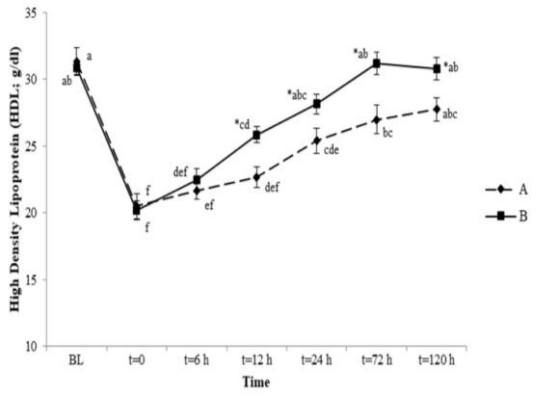

Fig- 2: High Density Protein (HDL; g/dl) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

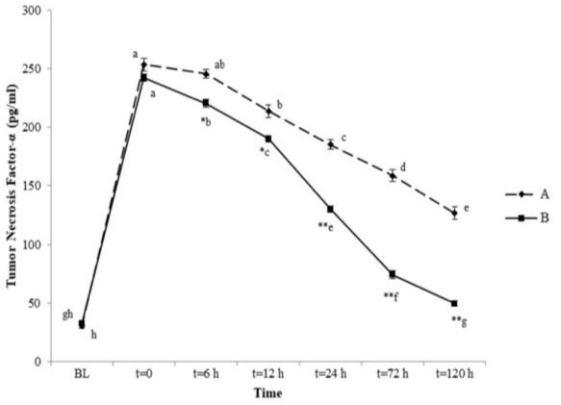

Fig. 3: Tumor Necrosis Factor-α (pg/ml) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

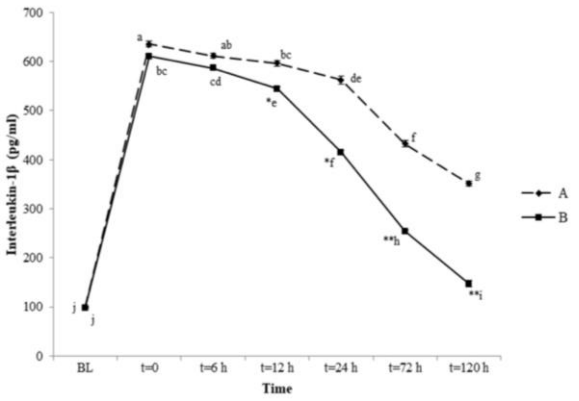

Fig. 4: Interleukin-1β (pg/ml) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

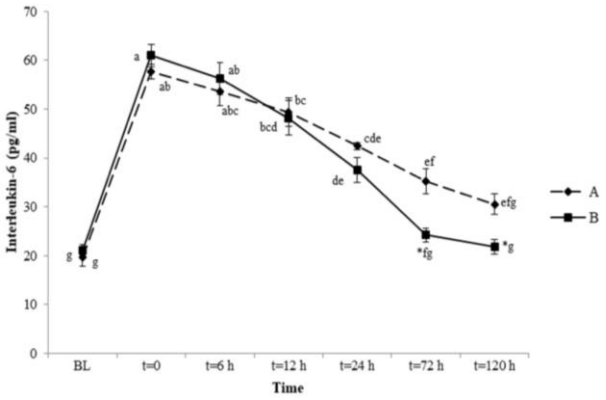

Fig. 5: Interleukin-6 (pg/ml) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol-150 (group B).

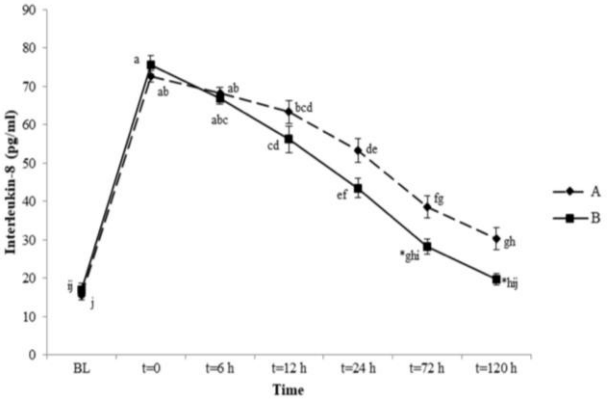

Fig. 6: Interleukin-8 (pg/ml) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

(P<0.05) over group A at t=12 h and highly significant difference (P<0.01) at t=24 h. At t=72 h and t=120 h, group B again showed significant differences (P<0.05) over group A. It attained the baseline values within study period, while group A did not recover the basal values, however, the values were near to the baseline (Fig-7).

DISCUSSION

This study was conducted to evaluate the efficacy of tyloxapol in the treatment of hemorrhagic septicemia associated with *Pasteurella multocida* in water buffaloes. To that end, our one of the major concerns was to validate the safety of intravenous administration of tyloxapol in water buffalo calves. For that purpose, the recommended protocol described by Alshammari (2016) for assessment of safety of the drug were included in the study and we found that tyloxapol is safe to administer in buffalo calves and no untoward reaction was observed. Therefore, tyloxapol gave us opportunity to evaluate its efficacy in HS as an alternative treatment regimen of this fatal disease as it is already reported in the attenuation of

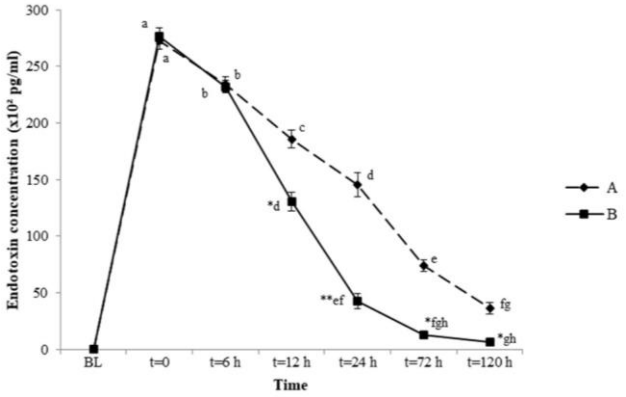

Fig. 7: Endotoxin concentration (x102 pg/ml) during induced hemorrhagic septicemia in buffalo calves in response to treatment protocols with Ceftiofur and flunixin (group A) and Tyloxapol (group B).

pathological effects of endotoxin in rabbits (Serikov *et al*., 2003) and endotoxemic sheep (Staub *et al*., 2001; Chalmeh *et al*., 2016; Heidari *et al*., 2016). Up to the authors knowledge, tyloxapol is not reported earlier in the treatment of HS and this is the first study ever conducted to evaluate its efficacy in buffalo calves suffering with HS.

The survival of buffalo calves was observed better in tyloxapol administered group (group B) of the study as compared to its counterpart (group A). The survival was significantly higher in group B which was 83.3% as compared to group A in which it was 66.6%. The improved survival rate strengthened our hypothesis that tyloxapol is capable of efficiently coping with the endotoxemic effects of *P. multocida* in hemorrhagic septicemia efficiently and will improve the survival of the buffalo calves suffering from *P. multocida* infection. The cardinal parameters were better recovered in tyloxapol treated group of the study as compared to the other group. During *P. multocida* induced endotoxemia, the release of LPS in the blood stream stimulated the arachidonic acid pathway, which led to the production of prostaglandins and leukotrienes. These arachidonic acid metabolites caused increase in body temperature (Horadagoda *et al*., 2001). Although, administration of flunixin meglumine helped in lowering the increased body temperature but it was noted that tyloxapol also decreased body temperature efficiently and there was no significant difference between flunixin treated and tyloxapol administered calves in recovering the body temperature to the normal ranges. However, the calves treated with tyloxapol showed better recovery trend towards normal in respiration and heart rates.

Cytokines are small protein molecules produced and released by a great variety of cells in the body like macrophages, B and T lymphocytes and mast cells, as well as endothelial cells and fibroblasts as an immune response to the infection (Marques *et al*., 2023; Sikora *et al*., 2023). These are important indicators of health provided by the nature to eradicate the microorganism and are designed for tissue repair. It is important to know about their mechanism of regulation and involvement in the response to infection and sepsis to maintain a balance between their role as protective inflammatory mediators and detrimental effects (Marques *et al*., 2023; Cao *et al*., 2023). Cytokines are initially considered to be immunomodulators because of their production and release from the immune cells in response to the infection and inflammation. Up till now, more than 20 different types of cytokines have been identified as sepsis biomarkers in response to the infection in both humans and animals (Burkovskiy *et al*., 2013; Doganyigit *et al*., 2022;). The first immune cells which respond to the bacterial infection are monocytes and macrophages. The foremost cytokines produced in response to the bacterial infection are tumour necrosis factor-α, interleukin-1β and interleukin-6 (Burkovskiy *et al*., 2013; Doganyigit *et al*., 2022). These cytokines facilitate the production of other inflammatory cytokines like interleukin-8, interleukin-12 and interleukin-15. These cytokines activate neutrophils and natural killer cells (Cao *et al*., 2023; Marques *et al*., 2023). Later on, T and B lymphocytes also contribute to the immune response against infection. The release of these and numerous other cytokines at high level is usually called as cytokine storm or cytokine cascade (Doganyigit *et al*., 2022; Marques *et al*., 2023). The cytokine storm leads to tissue injury and, eventually, organ dysfunction (Weigand *et al*., 2004; Cao *et al*., 2023). This study also discusses about the picture of cytokine concentration during hemorrhagic septicemia induced through *P. multocida* in buffalo calves. We studied the proinflammatory cytokines i.e., TNF-α, IL-1β, IL-6 and IL-8. Induction of *P. multocida* infection to the animals inflicted a cascade of cytokine after few hours. This cytokine cascade in response to the gram-negative bacterial infection was assumed as host immune response as reported in previous studies (Weigand *et al*., 2004; Doganyigit *et al*., 2022; Cao *et al*., 2023) and it was also observed that the level of these cytokines was high and could be lethal to the body tissues and capable to cause organ dysfunction as mentioned by Weigand *et al*. (2004) and Cao *et al*. (2023). After administration of the allocated treatment regimens, the animals administrated with tyloxapol ameliorated the concentration of cytokines which showed that it is capable to cope up with the deleterious effects of cytokines in sepsis and could be considered as one of the choices of treatment of the diseases associated with endotoxemia/septicemia.

Lipoproteins are macromolecular proteins and composed of apolipoproteins and phospholipid layer consisting a layer of lipid (Kontush *et al*., 2015; Tanaka *et al*., 2020). There are many studies which reported that lipoproteins especially high-density lipoprotein (HDL) and low-density lipoprotein (LDL) are capable to bind and neutralize the endotoxins of gram-negative bacteria and toxins of gram-positive bacteria (Staub *et al*., 2001; Serikov *et al*., 2003; Tanaka *et al*., 2020). Adequate level of these two lipoproteins i.e., HDL and LDL are believed to have a protective role during sepsis. These lipoproteins are thought to be bacterial toxin-binding proteins which inhibit the release of inflammatory cytokines. Leukocytes adhesion is considered as key step during sepsis and inflammation (Angus and van der Poll, 2013). HDL play a pivotal role in decreasing adhesion molecule expressions of the leukocytes (van Leeuwen *et al*., 2003; Tanaka *et al*., 2017; Tanaka *et al*., 2020). The reported role of lipoproteins especially LDLs and specifically HDLs demonstrate that their presence in the body is beneficial

during sepsis. In this study, values of LDLs and HDLs significantly decreased during hemorrhagic septicemia which corelates with the previous reports in which it was found that a rapid and significant decrease was observed during early phase of sepsis development (Aşar *et al*., 2021; De Geest and Mishra, 2022). After administration of tyloxapol, a significant rise in the values of HDLs and LDLs were observed. This rise in the values of lipoproteins and decrease in concentration of endotoxin well demonstrated the effects of tyloxapol in minimizing the devastation of the endotoxins. Tyloxapol blocks the lipolytic activity and inhibits lipoprotein lipase hence it helps in the breakdown of triglyceride-rich lipoproteins and prevents triglyceride uptake from plasma, respectively (Korolenko *et al*., 2010; Rasouli *et al*., 2016). These findings of our study confer the findings of previous studies that lipoproteins especially LDLs and HDLs play a protective role in sepsis (van Leeuwen *et al*., 2003; Levels *et al*., 2011; Tanaka *et al*., 2017; Asar *et al*., 2021; De Geest and Mishra, 2022).

The aim of this study was to evaluate tyloxapol as a therapeutic agent in the treatment of hemorrhagic septicemia and introduce it as a new treatment regimen for diseases associated with endotoxemia/septicemia in animals. Due to emerging issues of vaccination failure and development of antimicrobial resistance, we planned this study to look into a new but effective alternative treatment regimen so that a practitioner would be able to treat not only hemorrhagic septicemia but also a number of other fatal diseases associated with endotoxemia and/or septicemia and also be able to encounter the emerging issue of antimicrobial resistance. We successfully introduced this alternative treatment regimen in one of the gram-negative bacterial infections, however, this is just the beginning and further work is clearly warranted before its recommendation to a field practitioner.

Conclusions: The findings of the study helped to conclude that tyloxapol is safe to administer in the buffalo calves and could successfully resuscitate the calves suffering from hemorrhagic septicemia thus improving survival. It ameliorated the concentration of cytokines and endotoxins thus acted as anti-endotoxic drug and protected animals from toxic effects of the endotoxins and their manifestations. Tyloxapol efficiently enhanced natural scavengers of LPS i.e., lipoproteins especially HDL and LDL. Therefore, it should be considered as a potential alternative treatment of endotoxemia/septicemia in animals and can be useful in clinical settings pertinent to further work.

Authors contributions: MNS conducted the research work and collected and compiled the data. MAZ made a significant contribution to the idea of the study, organizing the data and interpretation of the results. AHT and SUR contributed in planning the methodology of the research and helped in drafting the manuscript.

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