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Studies on effect of gadolinium chloride in *Pasteurella multocida* induced lung injury in buffalo calves

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ABSTRACT

Haemorrhagic septicemia (HS) is a fatal disease of cattle and buffaloes caused by *Pasteurella multocida* (*P. multocida*) characterized by an early stage of high fever, respiratory involvement followed by septicemia and recumbency resulting in death. Lung inflammation caused by *P. multocida* results in the expression of cytokines, chemokines and employment of neutrophils and pulmonary intravascular macrophages (PIMs). PIMs secrete various pro-inflammatory cytokines like Tumor-necrosis factor- α (TNF-α), interleukin-8 (IL-8) and IL-6, contributing to acute lung inflammation and lung damage caused by *P. multocida*. In our study, we aimed to deplete PIMs by use of drug Gadolinium chloride to study the molecular mechanisms of cytokine expression in *P. mutocida* challenged animals. The expression of genes encoding the cytokines TNF-α, IL-1β, IL-8 and IL-10 in buffalo lung tissues was studied using Real time PCR. Buffalo calves were divided into four treatment groups, viz. Group I (control), Group II (GC treated), Group III (*P. multocida* challenged) and Group IV (GC pre-treated followed by *P. multocida challenge*). The upregulation of pro-inflammatory cytokines (TNF- α, IL-1β and IL-8) in Group III indicated their role in promoting lung injury. The down regulation of pro-inflammatory cytokine expressions in Group IV revealed inhibition of PIMs. Depletion of PIMS plays a major role in pathogenesis of HS and may be targeted as a future therapeutic strategy to reduce lung damage associated with *P. multocida* infections.

Keywords: Cytokine gene expression, Gadolinium chloride, IL-1β, IL-8, IL-10, Pulmonary intravascular macrophages (PIMs), *P. multocida*, Real Time PCR, TNF-α

Hemorrhagic septicemia (HS) is a highly fatal bacterial disease that affects cattle and water buffaloes and is an important cause of livestock mortality in tropical regions of Asia, Africa and the Middle East (Sethi *et al*. 2011). It is caused by mainly two specific serotypes of Pasteurella multocida (*P. multocida*) designated B:2 (Asian serotype) and E:2 (African serotype) (Horadagoda *et al*. 2001). *P. multocida* is a small, nonmotile and Gram-negative coccobacillus with bipolar appearance. It is commonly found in the nasopharynx and gastrointestinal tract of many wild and domesticated animals (Chen *et al*. 2002). Buffaloes are more susceptible to this disease, usually occurring in countries with less developed disease surveillance (Chung *et al*. 2015). During 1974-1986, HS was responsible for the highest mortality of infectious diseases in buffaloes and cattle in India (Shome *et al*. 2019).

The majority of lung diseases like HS are accompanied by Acute Lung Inflammation (ALI) (Bertok 1998). Neutrophils have long been regarded as the first line of defense against infection and one of the main cell types involved in initiation of inflammatory responses (Rosales *et al.* 2017). Inflammation is initiated by several cytokines including TNF-α, IL-1β and oxygen radicals that are produced by neutrophils (Wagner and Roth 1999). In comparison to the effects of macrophages like pulmonary intravascular macrophages (PIMs), microvascular recruitment of neutrophils in ALI has been examined more thoroughly (Singh *et al.* 2004). The role of PIMs in systemic inflammatory responses and maintaining lung physiology are lesser known in ALI (Schneberger *et al.* 2012). PIMs are a population of mononuclear phagocytes in different animal species (Warner and Brain 1990). They are primary cells responsible for the uptake of blood-borne tracer particles, bacteria and endotoxins in pulmonary capillaries and also influence pulmonary hemodynamics (Staub 1994). PIMs are usually firmly connected to the capillary endothelium on the thicker side of the alveolar septum and range from 20 to 80 µm in diameter (Warner and Brain 1990). PIMs secrete various pro-inflammatory cytokines like TNF-α, interleukin-8 (IL-8) and IL-6 contributing to acute lung inflammation caused by blood-borne pathogens (Staub 1994, Parbhakar *et al.* 2004).

The central role of cytokine is to control the direction, amplitude and duration of inflammatory responses (Tizzard 2000). The events that follow the cellular interactions have a crucial role in initiating and developing the inflammatory

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response that results in pulmonary damage (Strieter and Kunkel 1994). This eventually leads to increased severity of disease and mortality. Depletion of PIMs inhibits the release of these pro-inflammatory cytokines leading to decreased lung damage (Carrasco *et al.* 2002). Gadolinium chloride (GC) and clodronate liposomes can be administered intravenously to deplete PIMs (Elder *et al*. 2004). A single intravenous injection of the lanthanide GC results in the depletion of PIMs *in vivo* and induces apoptosis of alveolar macrophages *in vitro* (Schneider *et al.* 2004). In the present study, we have explored the effect of GC in the depletion of PIMs and the expression of cytokines in the molecular pathogenesis of *P. multocida* challenge infection in buffalo calves. The study will help in understanding the molecular pathogenesis of *P. multocida* infections and the extent of lung damage with the depletion of PIMs.

MATERIALS AND METHODS

Animals and experiments: The study plan was approved by the CPCSEA, India vide letter no. F. No. 25/61/2015 dated 14/9/2015. Male buffalo calves (n=8) were purchased from local farms. These animals were housed in the institutional experimental animal house, stall-fed, and allowed free access to water. The animals were divided into four groups (I-IV) containing 2 numbers of buffalo calves (4-6-month-old) in each group. Each group of calves were assigned to different treatments as described in Table 1.

Tissue collection: Necropsy was performed, and lung samples were collected aseptically within 1 hour of the sacrifice of calves. The lung tissues were collected in RNA*later* and stored in -80°C for further use.

Extraction of total RNA: The total RNA was isolated from tissue samples manually using Trizol (Ambion; Life Technologies) method. The quality and quantity were assessed using Nano drop instrument (ThermoFisher). 1000 ng of total RNA was used for c-DNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, India). The confirmation of the c-DNA was checked by polymerase chain reaction (PCR) with GAPDH and all cytokine genes primers.

Real Time PCR: The c-DNA was used to study the relative mRNA expression of TNF-α, IL-1β, IL-8 and IL-10 in Real Time PCR instrument (Applied Biosystems) using SYBR green chemistry. The cycling conditions for the amplification were initial denaturation 94°C (5min), followed by 94°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec. Fold change was calculated using ΔCT method.

RESULTS AND DISCUSSION

In the control, GC-treated, *P. multocida* challenged and GC pre-treated followed by *P. multocida* challenged groups, the mRNA expression of TNF-α, IL-1 β, IL-8, and IL-10 was examined.

mRNA expression of Tumor necrosis factor α (TNF-α): The mRNA expression of the group challenged with *P. multocida* caused major increase in the expression of TNF-α by 9.78 folds in comparison to control group (Fig. 1a). The group receiving GC showed similar TNF-α mRNA expression as compared to control group. However, TNF-α mRNA expression in the group pretreated GC by followed by *P. multocida* challenge was down-regulated by 1.02-fold than the *P. multocida* challenged group.

mRNA expression of IL-1β: *P. multocida* caused an increase in the expression of IL-1 β by 5.46 folds in comparison to the control group (Fig.1b). The animals receiving GC did not reveal much difference in fold change of IL-1β as compared to the control group. However, pretreatment of animals with GC followed by *P. multocida* challenge down-regulated the IL-1β mRNA expression to 1.22.

mRNA expression of IL-8: The group challenged with *P. multocida* showed 3.2 folds increase in the mRNA expression of IL-8 in comparison to control group (Fig. 1c). The group treated with GC alone did not show much difference in IL-8 expression (1.29-fold) in comparison to control group. However, pre-treatment of GC followed by *P. multocida* challenged group down regulated the IL-8 mRNA expression to 1.08-fold in comparison to *P. multocida* challenged group.

mRNA expression of IL-10: There was a 5.38-fold increase in the expression of IL-10 in the *P. multocida* challenged group compared to the control group. Animals treated with GC showed an increase of 3.73 folds in the expression of IL-10 compared to the control group. However, pre-treatment of gadolinium followed by *P. multocida* challenged group down regulated the IL-10 expression to 1.8-fold. The relative mRNA expression of IL-10 gene in different treatment groups are indicated in Fig. 1d.

The present study is aimed to gain an insight into the mechanisms involved in the development of lung injury in buffalo calves in response to *P. multocida* B:2 infection by analyzing the effect of organism on the expression and release of cytokines in the lungs. The presence of PIMs has been reported for the first time in normal and inflamed lungs

Table 1. Treatment schedule different groups of buffalo calves

Group	Treatment
Group I/Control	No treatment was given in this group.
Group II	Gadolinium chloride (GC) (Sigma-Aldrich) was dissolved at a concentration of 100 mg/ml in sterile normal saline and was administered @10mg/kg intravenously and animals were sacrificed after 48 hrs of GC challenge.
Group III	Challenged with a suspension containing 20 x 10^9 cfu/ml P. multocida and sacrificed after 24 hrs.
Group IV	Challenged with a suspension containing 20 x 10^9 cfu/ml P. multocida challenge after 48 hrs of GC treatment and sacrificed after 24 hrs of P. multocida challenge.

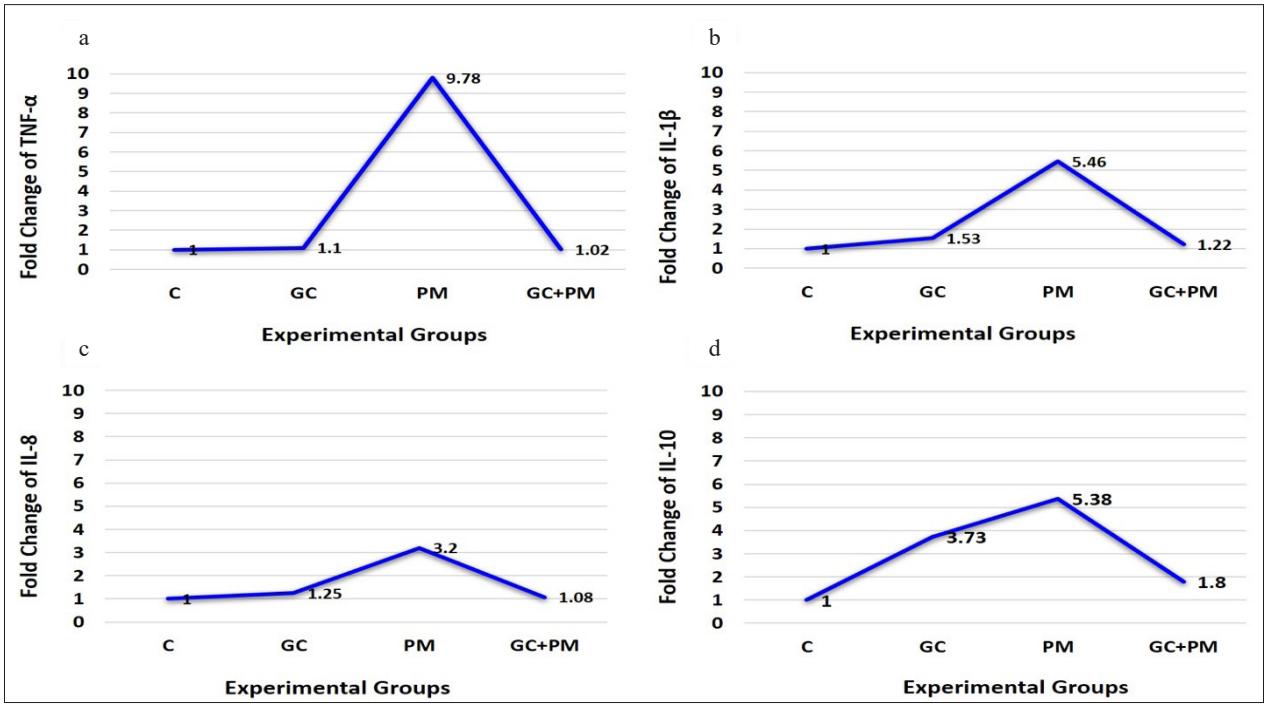

Fig. 1. Relative expression profiling of (a) TNF-α gene, (b) IL-1β gene, (c) of IL-8 and (d) IL-10 cytokine in different treatment groups.

of water buffaloes (Sethi *et al.* 2011). Our study used GC to deplete PIMs in *P. multocida* challenged buffalo calves and understand their role in modulating lung inflammation. Our study has revealed that depletion of PIMs has resulted in decreased inflammatory changes in lung tissues of buffalo calves. Similar studies were recorded in horses where depletion of PIMs by use of GC were obtained and believed to decrease their sensitivity to endotoxin-induced cardio-pulmonary shock (Parbhakar *et al.* 2004). GC pretreatment in calf model has also shown depletion of PIMs when challenged with *Mannheimia hemolytica* and reduces the microvascular buildup of inflammatory cells and related lung disease (Singh *et al.* 2004). Depletion of PIMs inhibits the release of cytokines and thus abolishes the process of lung inflammation and damage (Carrasco *et al.* 2002).

We have recorded that depletion of PIMs resulted in significant down-regulation in levels of proinflammatory cytokines TNF-α and IL-1β in Group IV (GC pre-treated followed by *P. multocida* challenge) when compared to Group III (*P. multocida* challenged) suggesting an efficient role of GC in inhibiting PIMs and abolishing the release of pro-inflammatory cytokines. Similar results were recorded in mice models where serum cytokine profiles showed significantly ($P < 0.01$) higher amount of pro-inflammatory cytokines (TNF-α, IL-1β) in *P. multocida* serotype A1 infected mice when compared to control group (Praveena *et al.* 2010). GC has been observed to inhibit the accumulation of macrophages by inducing their apoptosis and reducing the inflammatory process (Mizgerd *et al.* 1996).

Our study indicates that an increase in mRNA expressions of IL-8 in *P. multocida* challenged group may correlate with neutrophilia commonly observed in HS. Similar reports of increase in the expression of IL-8 in the inflamed lungs of buffalo were recorded when challenged with *P. multocida* compared to normal lungs (Sethi *et al.* 2011). IL-8 is the major neutrophil chemokine, which is a chemotactic factor secreted by activated leukocytes that promotes directional migration of neutrophils and T lymphocytes (Abou Mossallam *et al.* 2015). GC pre-treated buffalo calves followed by *P. multocida* challenge however decreased IL-8 expression indicating depletion of PIMs and their effect in modulating the mRNA expression of pro-inflammatory cytokines.

IL-10 is an anti-inflammatory cytokine that is a potent down-regulator of cell-mediated immune and pro-inflammatory responses (Schneider *et al.* 2004). The buffalo calves treated with GC (Group II) showed an increase in IL-10 mRNA expression (3.73-fold) as compared to control group (Group I). This indicates that PIMs are not the only source of IL-10 and suggests the possibility of IL-10 being secreted by variety of other immune cells. The levels of IL-10 were also seen to be increased (5.38-fold) in *P. multocida* challenged animals. However, IL-10 expression has been observed to be down-regulated after GC pretreatment followed by *P. multocida* challenge indicating its role in immune regulation and its effects on a broad range of pro-inflammatory factors released by PIMs.

We conclude that the increase in pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) suggests their role in promoting lung injury in HS affected buffalo calves. The decrease in the mRNA expression of proinflammatory cytokines in buffalo calves pre-treated by GC followed by *P. multocida* challenge suggests the possibility of reduced

lung injury by inhibiting PIMs further downregulating the expression of cytokines. The results of this preliminary study can help devise a treatment strategy wherein reduction in PIMs' activity may help curtail morbidity and mortality due to lung injury in HS affected animals.

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