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**Original Article**

# Oxidative response of neutrophils to platelet-activating factor is altered during acute ruminal acidosis induced by oligofructose in heifers

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**Reactive oxygen species (ROS) production is one of the main mechanisms used to kill microbes during innate immune response. D-lactic acid, which is augmented during acute ruminal acidosis, reduces platelet activating factor (PAF)-induced ROS production and L-selectin shedding in bovine neutrophils *in vitro*. This study was conducted to investigate whether acute ruminal acidosis induced by acute oligofructose overload in heifers interferes with ROS production and L-selectin shedding in blood neutrophils. Blood neutrophils and plasma were obtained by jugular venipuncture, while ruminal samples were collected using rumenocentesis. Lactic acid from plasma and ruminal samples was measured by HPLC. PAF-induced ROS production and L-selectin shedding were measured *in vitro* in bovine neutrophils by a luminol chemiluminescence assay and flow cytometry, respectively. A significant increase in ruminal and plasma lactic acid was recorded in these animals. Specifically, a decrease in PAF-induced ROS production was observed 8 h after oligofructose overload, and this was sustained until 48 h post oligofructose overload. A reduction in PAF-induced L-selectin shedding was observed at 16 h and 32 h post oligofructose overload. Overall, the results indicated that neutrophil PAF responses were altered in heifers with ruminal acidosis, suggesting a potential dysfunction of the innate immune response.**

**Keywords:** cow, lactic acidosis, L-selectin, neutrophils, reactive oxygen species

## Introduction

Lactic acidosis is a key factor in the pathogenesis of acute ruminal acidosis in bovines [7,13]. The ingestion of excessive amounts of highly fermentable carbohydrates is followed by the proliferation of *Streptococcus bovis* in the rumen. This microorganism metabolizes carbohydrates to produce lactic acid, which decreases the pH of the ruminal fluid to 4.5～5.0 [19]. This environment promotes rapid growth of lactobacilli, producing two forms of lactate, D and L. The liver and heart tissues readily metabolize the L form; however, D-lactate is metabolized slowly by mammalian tissues [19]. During acute ruminal acidosis, D-lactic acid reaches the blood stream, leading to lactacidemia of approximately 5 mmol/L [8]. As a consequence of acute ruminal acidosis, several inflammatory processes are triggered, causing ruminitis, liver abscesses, laminitis and an increase in acute phase proteins [5,19].

Polymorphonuclear neutrophils (PMNs) are the first line of defense against pathogens and the primary cellular components responsible for acute inflammatory response. Neutrophils exert their antimicrobial effects *via* reactive oxygen species (ROS)-dependent and ROS-independent mechanisms. A strong respiratory burst is produced by neutrophils during phagocytosis or after stimulation with a wide variety of agents [20]. ROS production is induced following the activation of nicotinamide adenine dinucleotide phosphate (NADPH, reduced form) oxidase, which is assembled at the plasma membrane. This reaction produces superoxide anions ($O_2^-$) and hydrogen peroxide

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($H_2O_2$), generating several microbiocidal agents. Neutrophils also possess a wide variety of granules that contain enzymes, such as gelatinase-B/metalloproteinase 9 (MMP-9), which are released to destroy the extracellular matrix [2]. The oxidative and non-oxidative functions of PMNs are key components of the innate immune response that may be affected during lactic acidosis. We recently demonstrated *in vitro* that D-lactic acid interfered with ROS production, CD11b up-regulation, MMP-9 release and L-selectin shedding in bovine neutrophils induced by platelet-activating factor (PAF) [1]. Hence, we hypothesized that acute ruminal acidosis would affect the neutrophil-dependent response in cattle. The present study was conducted to examine whether acute ruminal acidosis induced experimentally by acute oligofructose overload in heifers interferes with ROS production and L-selectin shedding in blood neutrophils.

## Materials and Methods

### Animals

Twelve clinically healthy, nonpregnant black Friesian dairy heifers aged 16 to 18 months and weighing between 280 and 310 kg were provided by the Estación Experimental Agropecuaria Austral farm. The animals were free of brucellosis, leucosis and tuberculosis and were certified by the National Livestock Service of Chile. All experiments were conducted in accordance with institutional review board-approved protocols and the National Guidelines on the Use of Experimental Animals of the Comision Nacional de Ciencia y Tecnologia de Chile.

The animals were submitted to a 2-week period of acclimatization before the experiments were conducted, and were carefully handled to avoid stress throughout the experiment. The study employed a repeated measure design, in which each animal served as its own control (time 0 h) [12]. The sample size was estimated by analysis of variance (ANOVA) with a repeated mean using G*Power (ver. 3.1.3) analysis software [6]. The parameters employed were the effect size f = 0.5; $\alpha$ error = 0.05; power $(1-\alpha) = 0.8$; and number of measurements = 6.

### Housing and husbandry

The heifers were fed twice daily. The daily ration of concentrate was equally divided into two meals of 1.0 kg/d each. The heifers were grazed on naturalized pasture composed primarily of perennial grasses, mostly *Holcus lanatus* and *Agrostis capillaris*. The contribution of forage legumes was low, $<10\%$ of the dry matter.

### Oligofructose overload

Six heifers were treated with 13 g/kg of body weight (BW) of oligofructose (Beneo P95, Orafti Active Food Ingredients, Santiago, Chile) dissolved in warm tap water and administered in a volume of 2 L/100 kg of BW. The solution was administered as a ruminal drench. Prior to this, 5% of the main dose was provided twice daily for 3 days before the main overload, as previously described [25]. The experiment included a 3-day control period before the oligofructose overload and a 48 h surveillance period afterwards. We used a lower dose of oligofructose than has been previously reported [3,25] to reduce potential side effects. At the end of the study, all of the animals recovered, and none exhibited secondary pathologies after two months of observation.

The other six heifers were administered a similar volume of tap water. All animals were clinically monitored (heart and respiratory rate, rectal temperature, ruminal frequency and for signs of laminitis) by a veterinary clinician, and all procedures were performed in the ruminant unit of the Veterinary Hospital of Universidad Austral de Chile.

### Isolation of Blood Neutrophils

A total of 32 mL of blood was collected at $-72$, 0, 8, 16, 24, 32 and 48 h post oligofructose overload by jugular venipuncture, after which the PMNs were isolated as previously described [21]. Briefly, following collection into acid citrate dextrose collection tubes (Becton, Dickinson and Company, USA), the blood was gently shaken for 5 min (Nutator; Becton, Dickinson and Company) and then centrifuged at 1,000 × g at 20°C for 20 min. The plasma was then isolated and stored (see below), the buffy coat was aspirated, and the remaining red blood cells and PMN pellet were suspended in Hank's Balanced Salt Solution (HBSS). The red blood cells were removed by flash hypotonic lysis using a cold phosphate-buffered water solution (5.5 mM $NaH_2PO_4$ and 8.4 mM $HK_2PO_4$, pH 7.2). After returning the sample to isotonicity using a hypertonic phosphate buffer solution (5.5 mM $NaH_2PO_4$, 8.4 mM $HK_2PO_4$, and 0.46 M NaCl, pH 7.2), the sample was centrifuged at 600 × g at 20°C for 10 min. The remaining PMN pellet was then washed three times with HBSS, after which viability was determined by trypan blue exclusion, and was never less than 97%. The purity was at least 94%, as assessed by light microscopy and by flow cytometry according to forward scatter (size) and side scatter (granularity) parameters.

### Plasma and ruminal sampling

Plasma was collected from the samples used for neutrophil isolation. The plasma samples at 0, 8, 16, 24, 32 and 48 h post oligofructose overload were frozen at $-80$°C, and prior to analysis, 25% metaphosphoric acid (MPA) was added to the samples at a 1 : 3 ratio (MPA : plasma). The samples were then centrifuged at 2,000 × g for 10 min, after which the supernatants were filtered using a 0.22 μm RF-Jet Syringe filter (RephiLe Bioscience, China). Blood

was used for neutrophil isolation (see above).

Ten-milliliter ruminal samples were collected using rumenocentesis [18] at 0, 24 and 48 h after the oligofructose overload, and the pH of the samples was then immediately measured using a pH meter (pH 21; Hanna Instruments, USA). This procedure was not performed in animals treated with tap water due to animal welfare considerations and because no significant variations have been observed in samples taken at different times in previous studies [18]. Following measurement of the pH, 25% MPA was added to the ruminal fluid at a ratio of 1 : 3 (MPA: ruminal fluid), after which the samples were filtered using silanized glass wool (Supelco; Sigma-Aldrich, USA) and centrifuged at 2,000 × g for 20 min. The supernatant was then filtered using a 0.20-μm Whatman filter, collected into 15 mL tubes and frozen at −80°C.

### Lactic acid measurement by HPLC

HPLC analysis was performed on a LaChrom Elite HPLC (VWR International, Germany) equipped with a Hitachi L-2455 Diode Array Detector. HPLC separation was performed on a Restek Ultra Aqueos C18 column (5 μm particle size, 150 mm length × 4.6 mm internal diameter).

The mobile phase consisted of $KH_2PO_4$ (50 mM) with acetonitrile at a 99 : 1 ratio, adjusted with HCl to a pH of 2.5. The mobile phase flow was 0.4 mL/min for the first 12 min, then increased to 2 mL/min after 20 min, followed by 2.5 mL/min after 30 min until the end of the HPLC run after 40 min. Lactic acid detection was performed at 210 nm, and the temperature of the column oven was 25°C. Lactic acid recovery was estimated at 94%, and the detection limit was estimated to be 0.05 mM.

### *In vitro* ROS production in bovine neutrophils during acute ruminal acidosis

To assess the effects of ruminal acidosis on neutrophil respiratory burst, we used a luminol chemiluminescence assay as previously described [2]. Briefly, $1 \times 10^6$ neutrophils were suspended in 250 μL of HEPES buffer in a 96-well plate and pre-incubated for 15 min at 37°C. Following incubation, 80 μM luminol was added, the solution was gently mixed and the neutrophils were stimulated with 100 nM PAF or vehicle (0.01% EtOH in HEPES buffer). ROS production was measured for 2,000 sec using a Luminoskan Ascent luminometer (Thermo Scientific, USA).

### Flow cytometry analysis of L-selectin in bovine neutrophils

For the flow cytometry experiments, a sample of $1 \times 10^6$ neutrophils/mL in HEPES buffer was used. The neutrophils were stimulated with 100 nM PAF or vehicle (0.01% EtOH in HEPES buffer) for 30 min at 37°C. The cells were then washed and incubated with anti-L-selectin coupled with phycoerythrin (PE, clone DREG-56 from BD Pharmingen; BD Bioscience, USA) according to the manufacturer's instructions. This antibody showed cross-reactivity with bovine neutrophils, as has previously been described by other authors [2,24]. The analysis was performed on 10,000 cells using a FACSCanto II cytometer. First, the cells were characterized by their forward and side light scattering characteristics. We chose to present the relative mean fluorescence intensity of emission because this parameter was more discriminating for the experimental conditions than the percentage of positive cells. Non-specific background fluorescence was defined as any fluorescence associated with granulocytes upon incubation with the cross-matched isotype antibodies. The results were analyzed using FlowJo (ver. 7.6; Tree Star, USA).

### Statistical analysis

The results are illustrated in bar graphs or dot plots as the means ± SEMs of independent experiments obtained from six heifers with oligofructose overload or six heifers treated with tap water. A logarithmic transformation was previously performed because of the heteroscedasticity of data [18,29]. Afterwards, a one or two-way repeated-measures ANOVA and Dunnett's multiple comparisons were performed with a significance level of 5%. All results are depicted in bar graphs as the arithmetic mean ± SEM of the untransformed data. Statistical analyses were performed using GraphPad Prism for Mac OS X (ver. 5.0; Graphpad Software, USA).

## Results

Ruminal liquor samples for rumenocentesis were obtained from six animals. Prior to oligofructose overload, the pH of the ruminal liquor was approximately 7; however, a significant decrease in pH was observed at 24 h (4.2), and a partial recovery to approximately 5.0 was observed at 48 h after the administration of 13 g/kg of oligofructose (Fig. 1A). Before induction of acute ruminal acidosis, the mean lactic acid concentration in the rumen was 0.08 mM; however, 24 h after oligofructose overload, the mean lactic acid content in the rumen increased significantly to 57.3 mM ($p < 0.05$). This effect was not sustained, and at 48 h, the lactic acid level in the ruminal samples diminished to a mean value of 13.4 mM (Fig. 1B). At 0 h, the lactic acid in the plasma was 0.28 mM, and 8 h after oligofructose overload, a rapid and significant ($p < 0.05$) increase in lactatemia was observed (Fig. 2). The lactic acid concentration increased significantly ($p < 0.01$) to its maximum level, reaching 3.2 mM at 32 h after oligofructose overload.

In this experiment, we used luminescence detection with luminol to measure ROS production because it is a more

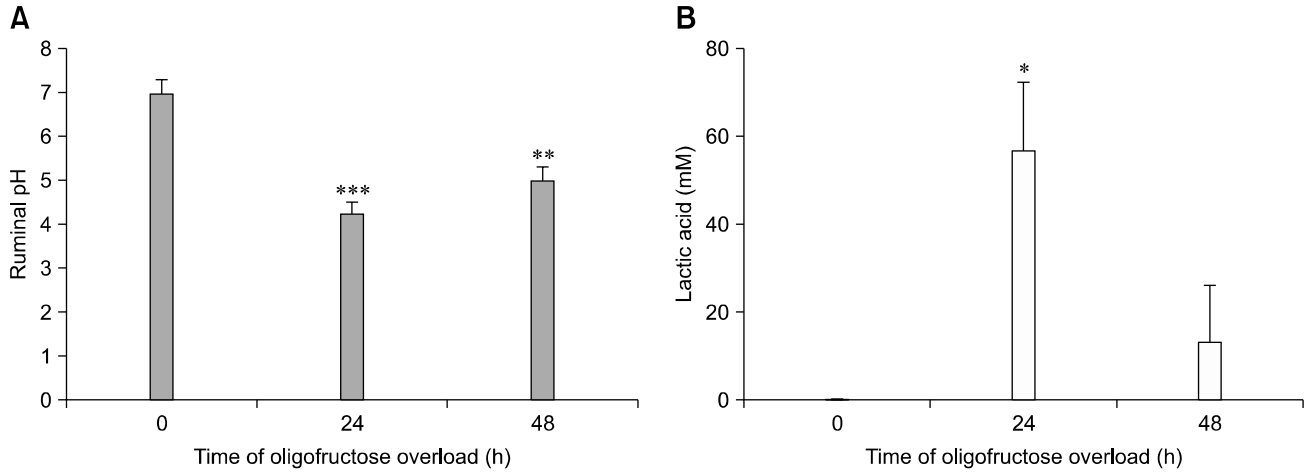


**Fig. 1.** Ruminal liquor pH and lactic acid concentrations in the rumen after oligofructose overload. (A) pH of ruminal samples obtained by rumenocentesis at 0, 24 and 48 h. (B) Lactic acid concentrations analyzed by HPLC in ruminal samples obtained at 0, 24 and 48 h. The bar graph shows the mean ± SEM of six different animals. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with 0 h.

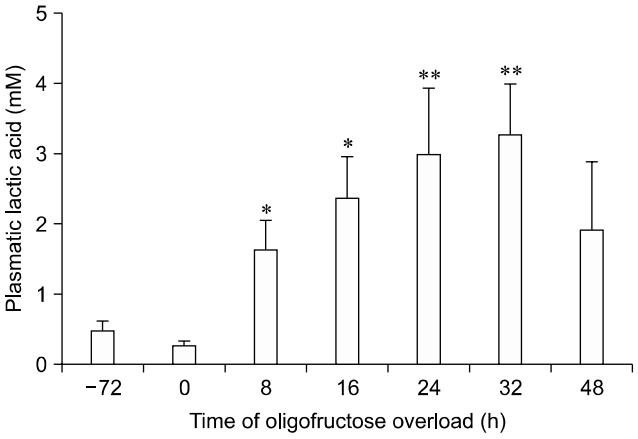


**Fig. 2.** The plasma lactic acid concentration after oligofructose overload. Plasma samples obtained at −72, 0, 8, 16, 24, 32 and 48 h after oligofructose overload were analyzed by HPLC. The bar graph shows the mean ± SEM of six different animals. $*p < 0.05$, $**p < 0.01$ compared with 0 h.

sensitive method than the rate of superoxide dismutase-inhibitable ferricytochrome c reduction [2,24]. We observed that tap water did not affect PAF-induced ROS production in the neutrophils after 24 and 48 h when compared with −72 h (Figs. 3A, C, E and G). In heifers with ruminal acidosis, a strong reduction in PAF-induced ROS production was observed at 24 and 48 h (Figs. 3B, D, F and H). In fact, at 24 h after oligofructose overload, PAF-induced ROS production was completely inhibited.

L-selectin is an adhesion molecule that is constitutively present at high levels on the neutrophil surface and interacts with inducible ligand(s) on the non-lymphoid vascular endothelium, which supports adhesion. During neutrophil activation by chemoattractants, L-selectin is shed, and this process may be affected during acute ruminal acidosis [1]. We demonstrated a PAF-dependent decrease in the expression of L-selectin in bovine neutrophils from heifers treated with tap water (Figs. 4A and B). Meanwhile, in blood neutrophils from heifers with acidosis, the PAF response was observed until 8 h post oligofructose overload, while PAF-induced L-selectin shedding was significantly inhibited at 16, 24 and 32 h, but not at 48 h after oligofructose overload (Figs. 4A and C).

## Discussion

Acute ruminal acidosis induced by oligofructose overload was previously described in a laminitis experimental model [25,26]. We demonstrated that heifers treated with an overload of oligofructose had strongly increased lactic acid levels in the ruminal fluid and plasma. It is well known that this metabolic disorder induces the onset of inflammatory processes, such as ruminitis [27], laminitis [26] and polysynovitis [4]. It has been reported that heifers subjected to oligofructose overload develop generalized sterile polysynovitis involving the exudation of fibrin and the mobilization of neutrophils [5]. Moreover, it has been proposed that the acute clinical laminitis and joint distension observed in practice may share a common pathogenesis that involves the systemic activation of leukocytes and the innate immune system [5]. Additionally, during acute acidosis induced by grain overload, an intense accumulation of neutrophils is observed in the epithelial cells and stratum lucidum [27]. These unspecific inflammatory conditions reflect the interplay between acidosis and innate immune response modulation [10,28]. In fact, the ruminal acidosis caused by oligofructose overload in dairy heifers resulted in marked

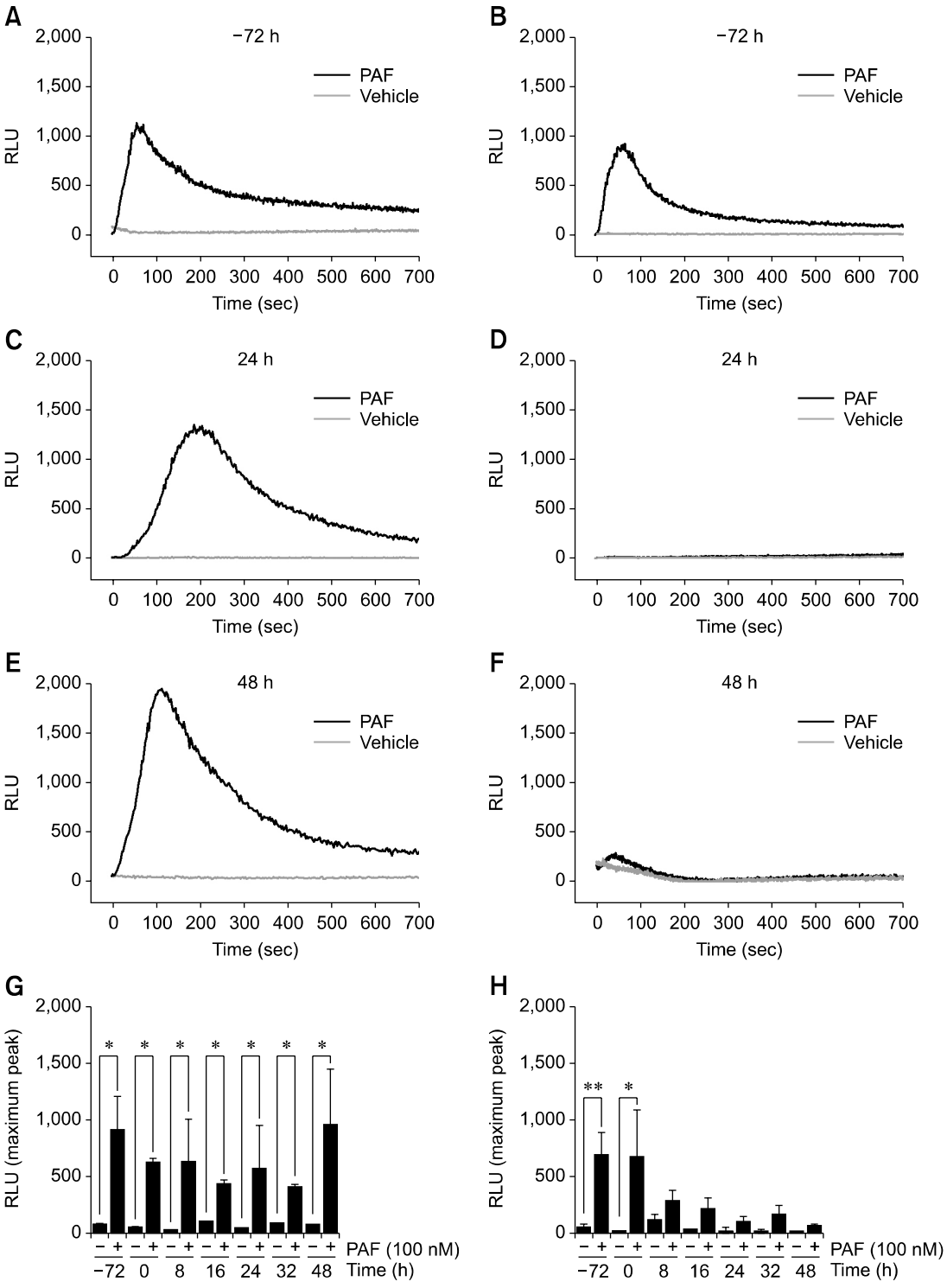


**Fig. 3.** The effects of oligofructose overload on blood neutrophil reactive oxygen species (ROS) production *in vitro*. Blood neutrophils isolated from six animals with ruminal acidosis (oligofructose overload) or six animals fed with tap water were treated with vehicle (0.01% ethanol in HEPES buffer) or platelet activating factor (PAF; 100 nM), and ROS production was measured by a luminol chemiluminescence assay. The PAF-induced ROS production in neutrophils isolated from heifers treated with tap water (A, C, and E) or oligofructose (B, D, and F) 72 h before treatment or 24 h and 48 h after oligofructose overload is shown. The bar graph of basal ROS production (without PAF induction or negative) or PAF-induced ROS production in the neutrophils (positive) isolated from heifers at −72, 0, 8, 24, 32 and 48 h after being provided with tap water (G) or after oligofructose overload (H) are shown. The bar graph shows the mean ± SEM of six different animals. *$p < 0.05$, **$p < 0.01$. RLU: relative luminescence units.

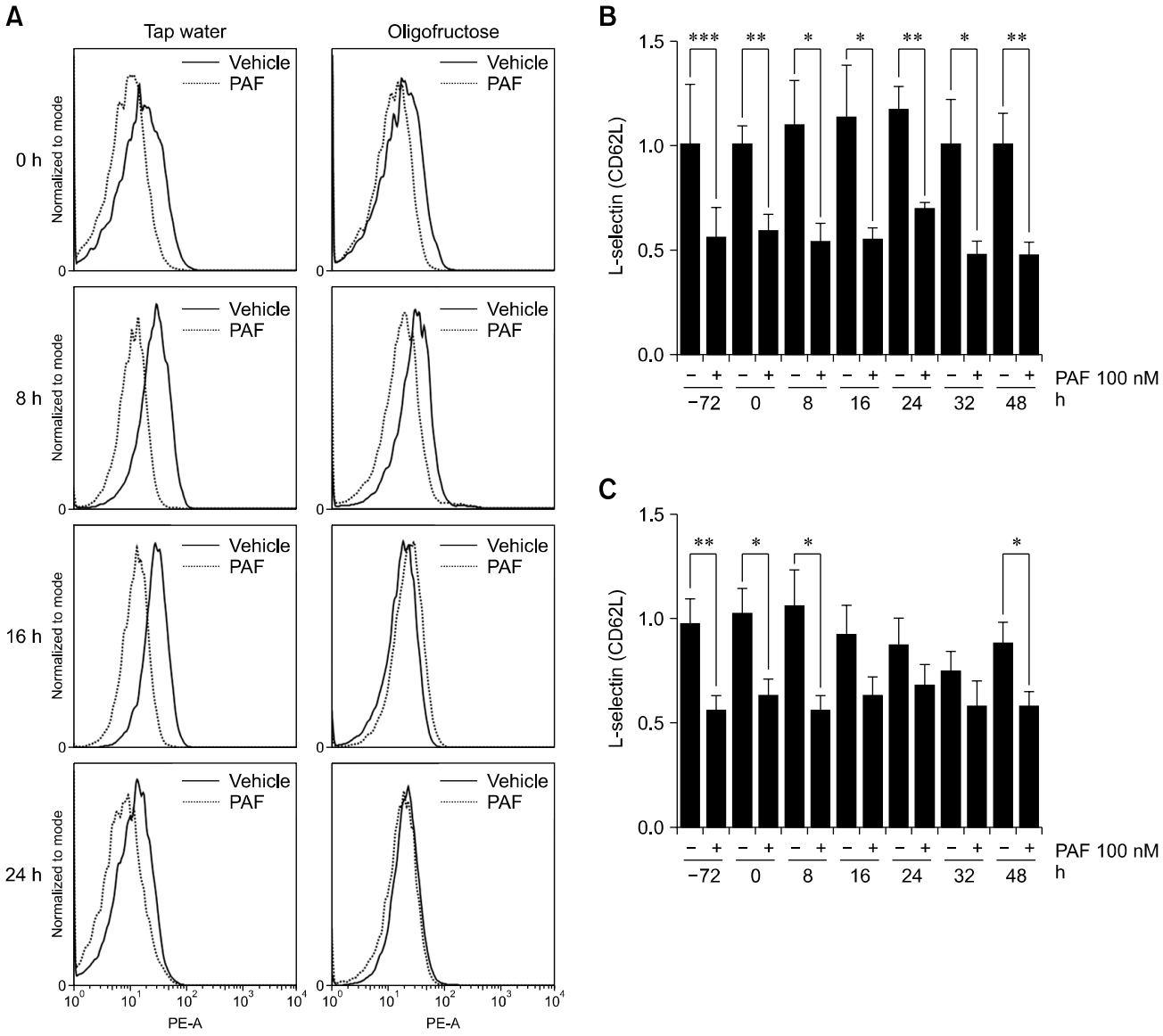


**Fig. 4.** Changes in L-selectin expression in bovine neutrophils over the course of acute ruminal acidosis. Neutrophils isolated from animals treated with tap water or oligofructose were stimulated *in vitro* with vehicle (0.01% ethanol in HEPES buffer) or PAF (100 nM) for 30 min at 37°C. Afterwards, the cells were incubated with anti-L-selectin coupled with phycoerythrin, and fluorescence was analyzed by flow cytometry. In A, representative flow cytometry histograms of PAF-induced L-selectin changes in neutrophils isolated from heifers at 0, 8, 16 and 24 h after oligofructose or tap water treatment are depicted. The bar graph represents the mean of PAF-induced L-selectin changes represented as the fold change relative to the control ± SEM from six independent experiments with tap water (B) or oligofructose (C). $*p < 0.05$, $**p < 0.01$.

systemic acidosis characterized by an increase in acute phase proteins and white blood cell counts [5]. It has been suggested that the presence of lipopolysaccharide (LPS) and other pro-inflammatory substances in the systemic circulation after translocation across damaged ruminal epithelium, as well as acidosis and the accumulation of organic acids such as lactic acid modulate the inflammatory response [1,5,19].

One of the most important mechanisms in the control of microbial killing is the production of ROS by neutrophils. Bovine neutrophils show a transient increase in ROS production induced by chemoattractants such as PAF. We demonstrated a reduction in neutrophil PAF-induced ROS production in *in vitro* and *ex vivo* experiments in heifers with ruminal acidosis induced by oligofructose overload. The lactacidemia induced by ruminal acidification affected ROS production, and in support of this finding, extracellular or intracellular acidification reduced superoxide anion production induced by fMLP in neutrophils [11,22]. This effect is associated with increases

in both cAMP and PKA activity *via* proton-sensing G-protein-coupled receptors [16]. During acute ruminal acidification, the increased lactacidemia observed in cattle is primarily attributed to D-lactic acid absorption from the rumen because the metabolism of D-lactic acid is not as efficient as that of L-lactic acid [19]. We postulated that D-lactacidemia may interfere with ROS production during ruminal acidosis. In support of this assumption, we previously demonstrated that D-lactic acid reduces PAF-induced ROS production *in vitro* with an $IC_{50}$ of 0.7 mM [1]. Therefore, during the early stages of acute ruminal acidosis, D-lactic acid may contribute to alterations in ROS production in cattle.

In our ruminal acidosis experimental procedure, bovine neutrophils showed a reduction in PAF-induced L-selectin shedding after 16 h of oligofructose overload; however, this condition was transient. D-Lactic acid at concentrations of 10 mM reduced PAF-induced L-selectin shedding [1]. It has been proposed that, in neutrophils exposed to lactic acid, a low intracellular pH induces L-selectin shedding that involves p38 MAP kinase, extracellular metalloprotease activity and $Na^+/H^+$ exchanger function [9] and may explain the minor expression of L-selectin. A decrease in L-selectin expression in cattle is observed in a bovine leukocyte adhesion deficiency, which demonstrates a reduction in neutrophil migration [17] and reduces the recruitment of neutrophils into tissues [15]. L-selectin shedding occurs *via* enzymatic cleavage "sheddases" at membrane-proximal sites and is involved in the migration of neutrophils induced by chemokines and with transendothelial processes [23]. Moreover, it is possible that the inhibition of neutrophil ROS production during acute ruminal acidosis is involved in the reduction of PAF-induced L-selectin shedding observed in our experimental procedure. In support of this, $H_2O_2$ mimicked PMA-induced L-selectin shedding in Jurkat cells and was blocked by an inhibitor of tumor necrosis factor $\alpha$ (TNF-$\alpha$) converting enzyme, a well-known sheddase [30].

In the current study, plasma concentrations of lactic acid reached values $\geq$2 mM after 8 h of ruminal acidosis. Lactic acid at 1 mM increases the protease activity and shedding in cultured trabecular meshwork cells [14], and 6 mM lactic acid decreases L-selectin expression in bovine neutrophils *in vitro* [1]. Therefore, the inhibition of PAF-induced L-selectin shedding or the decreased expression of L-selectin in neutrophils from animals with acute ruminal acidosis caused by oligofructose overload could affect the innate immune response in cattle.

Collectively, the data support the hypothesis that, in earlier stages, lactic acid contributes to the systemic acute inflammatory response during ruminal acidosis. In heifers, an increase in serum amyloid A after 6 h has been observed after an oligofructose overload with simultaneous increases in haptoglobin and fibrinogen after 36 h and 18 h, respectively [5]. Additionally, the reduction in bovine neutrophil ROS production observed during lactacidemia and the potent interference observed *in vitro* with D-lactic acid [1] may explain the higher risk of secondary infections observed in cattle with ruminal acidosis.

We conclude that ruminal acidosis induced by oligofructose increases the lactate concentration in plasma and ruminal fluid. During lactacidemia, bovine neutrophils showed a reduction in PAF-induced ROS production and L-selectin shedding *in vitro*. These findings suggest that, in addition to LPS or bacteria, lactate contributes to the onset of an acute inflammatory response during ruminal acidosis; however, higher concentrations of lactic acid in plasma also affect the innate immune response, causing greater susceptibility to bacterial infections.

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## Conflict of interest

There is no conflict of interest.

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