Acute aerocistitis induced by thioglycolate, lipopolysaccharide and inactivated *Aeromonas hydrophila* in *Piaractus mesopotamicus*: hematological effects

Aerocistite aguda induzida por tioglicolato, lipolisacarídeo e Aeromona hydrophila inativada em Piaractus mesopotamicus: efeitos hematológicos

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Abstract

The effects of swim bladder injection with thioglycolate, *Escherichia coli* lipopolysaccharide (LPS) and heat-inactivated *Aeromonas hydrophila* were assessed on hematological responses in pacu, *Piaractus mesopotamicus* (Characidae). A quantitative assessment was done on erythrocytes, thrombocytes e leucocytes at 6, 24, and 48 h pos-injection of the inflammatory agents and compared with fish injected with saline solution (control). Fish injected with inactivated *A. hydrophila* showed a reduction of erythrocytes and hemoglobin, whereas the hematocrit increased 6 h pos-injection. The results show that thioglycolate and LPS also induced a reduction on hemoglobin and an increase on the hematocrit. The thrombocytes count decreased 6 h post *A. hydrophila* injection, whereas increased 48 hours post LPS injection. The leukocytes count increased after 6 h post *A. hydrophila* injection, while the lymphocytes and PAS-positive granular leukocytes (PAS-LG) count decreased after 24 h post injection. In fish injected with thioglycolate or with LPS showed an increase in the LG-PAS counts when compared to *A. hydrophila* or control groups. The monocytes count was not affected by the different inflammatory agents.

Keywords: Inflammation. Blood. Erythrocytes. Leucocytes. Thrombocytes.

Resumo

Os efeitos da injeção de tioglicolato, lipolissacarídio de *Escherichia coli* e *Aeromonas hydrophila* inativada na bexiga natatória de pacus, *Piaractus mesopotamicus* (Characidae) foram avaliados quanto às respostas de células vermelhas, leucócitos e trombócitos do sangue. Ensaios quantitativos de eritrócitos, leucócitos e trombócitos foram realizados 6, 24 e 48 h após os estímulos e comparados com peixes que receberam solução salina 0,65% pela mesma via. Peixes inoculados com *A. hydrophila* apresentaram redução do número de eritrócitos e da taxa de hemoglobina enquanto o hematócrito aumentou 6 h após o estímulo. Os resultados mostraram que o tioglicolato e o LPS também induziram redução da hemoglobina e aumento do hematócrito. A contagem de trombócitos diminuiu 6 h após a inoculação de *A. hydrophila* inativada e aumentou 48 horas após a injeção de LPS. A contagem de leucócitos aumentou 6 h após a inoculação de *A. hydrophila* enquanto a de linfócitos a leucócitos granulares PAS positivos (PAS_LG) diminuiu 24 h depois. Peixes injetados com tioglicolato o LPS apresentaram aumento do número de LG_PAS em relação aos inoculados com *A. hydrophila* inativada ou grupo controle. A contagem de monócitos não foi afetada pelos diferentes agentes.

Palavras-chave: Inflamação. Sangue. Trombócitos. Leucócitos. Eritrócitos.

Introduction

The variables of the red series are used in the diagnosis of anaemia[1,2,3], and the leucogram are employed in the diagnosis of infections and indicators of systemic response to external stimuli[4,5,6,7,8,9,10], while the osmoregulatory disturbances[11] and other homeostatic imbalances. Currently, there are several publications that describe the hematologic characteristics of pacu, *Piaractus mesopotamicus*, fed diets supplemented with DL-α tocopheryl acetate ascorbic acid and ascorbic acid [12,13,14,15] and to the parasitism [4,10,16].

The effect of inflammatory response on the hematologic variables were described in trout, *Onchorhynchus mykiss*, inoculated with *Renibacterium salmoninarum*[17]; in goldfish, *Carassius auratus*, with *Aeromonas hydrophila*[18], with *Vibrio salmonicida* and dietary cortisol[19] or *A. salmonicida* and dietary vitamin C supplementation[20]; in hybrid tambacu (*P. mesopotamicus* x *Colossoma macropomum*) injected with carrageenin and thioglycolate[21]; in carp *Cyprinus carpio*, injected with *A. hydrophila*[5]; in Nile tilapia (*Oreochromis niloticus*) injected with carrageenin[14] and *P. mesopotamicus* inoculated with inactivated *A. hydrophila*[22].

For several reasons, the swim bladder is an excellent model-organ for studying inflammatory responses to xenobiotics exposure. First, it is a cavitary organ with terminal circulation which enables inoculation and exudates collection for evaluation of the cellular and fluid components accumulated in the inflammatory focus [23,21,22]. Second, it is less passive to be contaminated by other organs during the application[24]. Quintana; Moraes (2001)[25] observed 43% macrophage and 56% lymphocyte in the visceral cavity of unstimulated *P. mesopotamicus*, resident cells. In *P. mesopotamicus* and hybrid tambacu Martins et al.[21,14] did not observe resident cells in the swim bladder. Consequently, it was necessary to stimulate the swim bladder to obtain response.

The studies on inflammatory responses are scarce in Brazilian freshwater fish species. Reque et al.[22] related the inflammatory response and hematological parameters among Nile tilapia, *Oreochromis niloticus* evaluated six and 24 hours after inoculation with inactivated *Aeromonas hydrophila* into the swim bladder. The values of erythrocyte count, hemoglobin concentration and blood hematimetric indices did not differ statistically. The variation in circulating thrombocyte and leukocyte counts suggests that the inflammatory stimulus caused recruitment from reserve compartments to the blood.

The current study aimed to evaluate the hematological effects of the acute aerocistitis in the pacu, *P. mesopotamicus*, Holmberg 1887 induced by injection of thioglycolate, lipopolysaccharide and *A. hydrophila*.

Material and Method

After arrival, full-siblings pacu (n= 96, 115.0 ± 1.0 g weight) were randomly distributed in 12 tanks of 250-L each, supplied with a flow-through (1L/min) non-chlorinated water. Water temperature (29.5 ± 1.5ºC), pH (6.75 ± 0.25) and dissolved oxygen (5.0 ± 0.0 mg/L), alkalinity (83.0 + 1.0 mg/L) and conductivity (200.0 + 1.0 μS/cm) were measured on a daily basis.

After 1 week of acclimatization period, fish were distributed into four groups (n=24 fish/group) followed by an injection in the swim bladder of one of the three inflammatory agents: TIO – 0.5 mL of a 6% thioglycolate saline solution[23,21,14]; Bacteria – 3×10^9 colony-forming units (UFC) of heat-inactivated *A. hydrophila* (30-min bath at 40 C) dissolved in 0.5 mL of a 0.65% saline solution [23,22]; and LPS – 3.0 mg/kg of lipopolysaccharide from *Escherichia coli* dissolved in 0.5 mL of a 0.65% saline solution[23,25]. A group (control) was injected with 0.5 mL of a 0.65% sodium chloride solution.

The fish were anaesthetised by immersion in a alcoholic benzocaine solution (1.0g/L of ethanol) diluted in water (1:500 v/v)[26] and the inflammatory stimuli were injected into the anteromedial region, accessing the anterior region of swim bladder.

Blood samples were collected at 6, 24 e 48 hours post-stimuli with EDTA-treated syringes (10%) for the erythrocytes count (Modelo CC510, da Celm), haematocrit[27] and haemoglobin[28] concentration. For the differential counts of leucocytes, blood smears were stained using a May Grunwald–Giemsa–Wright (MGGW) stain, for posterior cell count (up to 200 cells) by light microscopy. Cell identification was car-

ried out according to Tavares-Dias et al.[9]. Total leucocyte and thrombocyte counts were carried out in relation to the number of erythrocytes in randomly selected fields and recalculated per unit volume (µL)[9,10] as follows:

a) Leucocytes = $\dfrac{\text{Leucocytes number x erythrocytes count}}{2000 \text{ erythrocytes}}$

b) Thrombocytes = $\dfrac{\text{Thrombocytes number x erythrocytes count}}{2000 \text{ erythrocytes}}$

Statistical analysis was done entirely by random projection. Comparison of averages was done by Tukey's test, with 5% of probability (p<0.05).

Results

Erythrocytes count and haemoglobin concentration were significantly lower at 6, 24 and 48h post-stimuli in fish injected with bacteria, compared to the other groups. Hematocrit was significantly higher at 6 hours post-stimuli in fish injected with bacteria when compared to the control group. Thereafter, hematocrit decreased at 24 hours, reaching similar values to those obtained in the other groups, and rised at 48 hours post-stimuli (Figure 1).

Fish injected with TIO and control did not show alterations in the number of erythrocytes, either at 6, 24 or 48 hours post-stimuli. After 6 hours post-stimuli haemoglobin concentration did not show significant differences in TIO group compared to the control group, whereas hematocrit where significantly higher at 6 hours and then decreased 24 hour post-stimulus, reaching similar values of control group until 48 hours (Figure 1).

Fish injected with LPS and control did not show changes in the erythrocyte count and the haemoglobin concentration. LPS group had a significant reduction in the hematocrit after 24 hours of inflammatory stimuli (Figure 1).

Fish injected with bacteria had significantly lower total thrombocytes and leukocytes counts at 6 hours pos-stimuli, when compared to the control group. Conversely, LPS induced an increase in thrombocytes count at 48 hours post-stimuli. TIO had no significant effect in the thrombocyte and leukocyte, regardless of the time post-stimuli (Figure 2).

In the differential counts of leukocytes, the lymphocytes, monocytes, PAS-positive granular leukocytes (PAS-LG) and neutrophils were identified in all treatment groups. Lymphocyte counts were significant lower in fish injected with bacteria (P<0.05), but only at 6 hours post-stimuli. PAS-LG and neutrophils counts were significantly reduced in fish injected with bacteria, when compared to fish injected with LPS (Figure 3). The differences in PAS-LG count were more pronounced at 6 hours than at 24 or 48 hours post-stimuli. Neutrophils were significantly lower in bacteria group, but only at 6 hours post-stimuli. At 48 hours post-stimuli, PAS-LG decreased sharply in all treatment groups, included the control group, in that PAS-LG was below the detection limit.

The monocyte count was numerically higher at 6 hours post-stimuli in control group (8) and decreased thereafter in all treatment groups (Figure 3).

Discussion

The results demonstrated that thioglycolate did not interfere in erythrocytes count in *P. mesopotamicus*. Nevertheless, after six hours post-stimuli there was a reduction in the haemoglobin concentration. Moreover, haematocrit count increased and then decreased after 24 hours post-stimuli to levels similar to the control group. Contrarily to that found in this assay, Martins et al.[14] observed that thioglycolate (3%) or carrageenin (500 µg) injected in the swim bladder did not alter the erythrocytes number, haemoglobin concentration and hematocrit values in the hybrid tambacu. However, the groups not injected had higher haemoglobin concentration and lower plasma cortisol levels than fish injected with

thioglycolate, carrageenin and saline solution. These results suggest that the handling and the injection itself was sufficient to cause stress without interfering in the blood count, which did not take place in the present study.

The haemoglobin concentration and the erythrocytes counts decreased between 6 to 48 hours post-stimuli in fish injected with *A. hydrophila*, whereas the haematocrit increased after six hours post-stimuli. That suggests possible lysis of erythrocytes and, as a
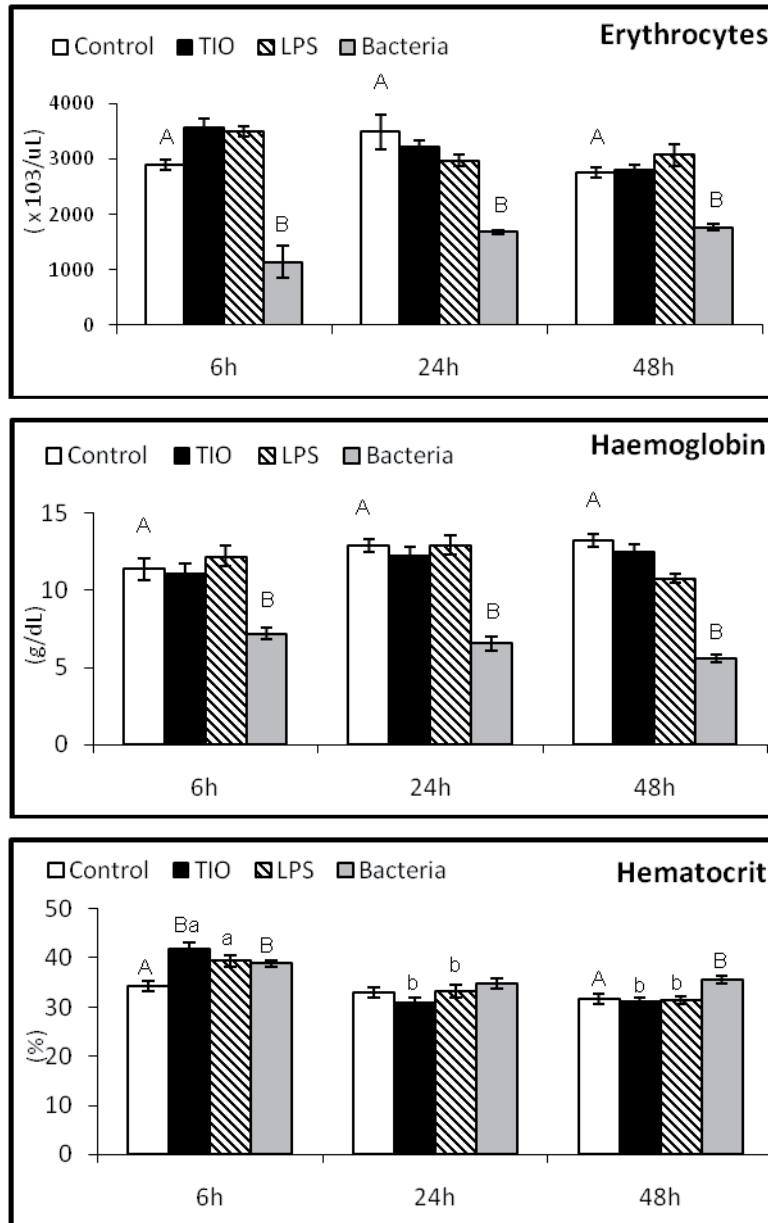

Figure 1 - Mean ± standard error of the erythrogram (erythrocytes, haemoglobin and hematocrit) of pacu (*P. mesopotamicus*) injected with thioglycolate (Tio), heat-inactivated *A. hydrophila* (Bacteria), lipopolysaccharide from *E. coli* (LPS) and sodium chloride solution without inflammatory stimulus (control). Capital letters for comparison between treatments; small letters for comparison between time post-stimuli within each treatment
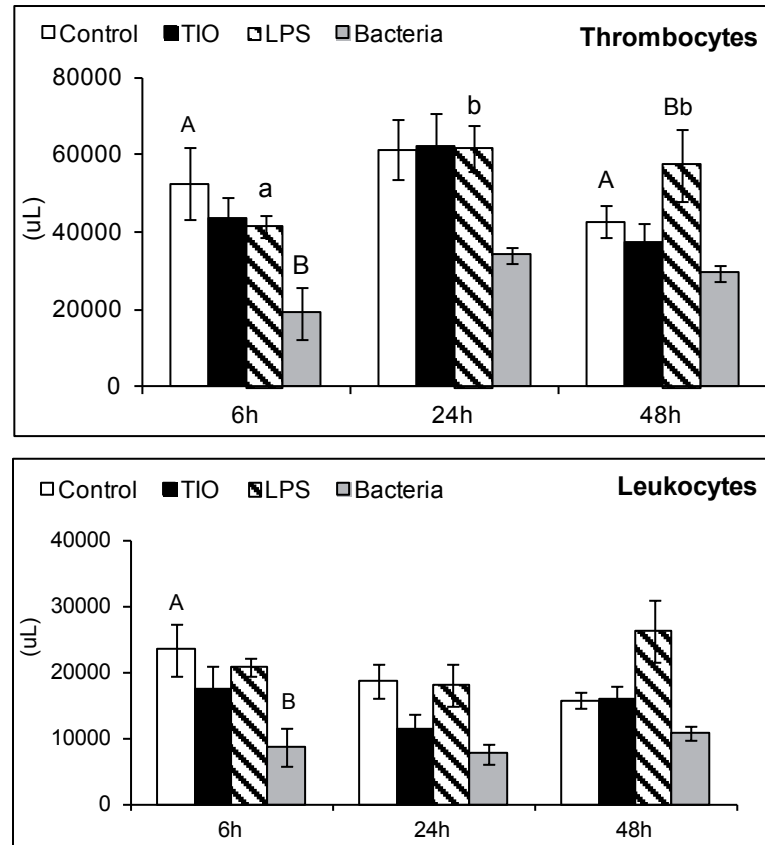
Figure 2 - Mean ± standard error of the total thrombocytes and leucocytes counts of pacu (P. mesopotamicus) injected with thioglycolate (TIO), heat-inactivated A. hydrophila (bacteria), lipopolysaccharide from Escherichia coli (LPS) and sodium chloride solution without inflammatory stimulus (control). Capital letters for comparison between treatments; small letters for comparison between time post-stimuli within each treatment

consequence, an increase of circulating erythroblasts. Although it has not been possible to determine the mechanism of destruction, it can take place for several reasons: 1) activation of the alternative pathway of the complement system caused by the LPS; 2) increase of the phagocytosis of erythrocytes in which membranes of the endotoxin is adhered; 3) direct lysis[18].

The erythrocytes destruction can be responsible for the anaemia in goldfish inoculated with *A. hydrophila* active[18]. In trout, the inoculation of *V. anguillarum* or its products caused an increase of the phagocytic activity of the erythrocytes by the splenic macrophages and, in less quantity, by renal macrophages[29]. In fish inocu-

lated with active bacteria the process is probably more severe, since bacterial multiplication may increase the endotoxine concentration. In case of inactive bacteria the concentration of LPS of the cell wall remains stable.

At the beginning of the bacterial infection there is an increase in the circulating reticulocytes and subsequently an increase in erythroblasts. The initial and continuous appearance of erythroblasts in the circulation represents the attempt of maintaining the homeostasis[17]. Such behaviour take place in moderate infections, since in this study, after six hours of *A. hydrophila* injection there was an increase in the hematocrit, influenced by the increase in circulating
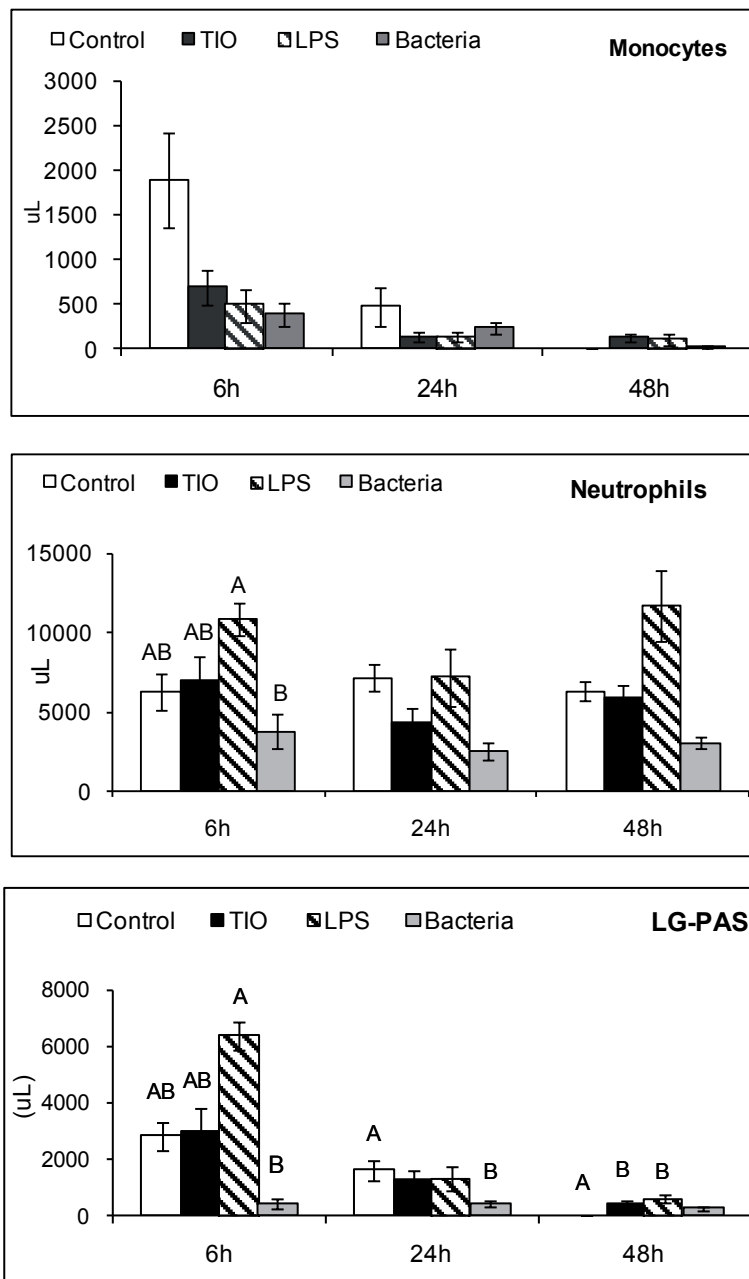
Figure 3 - Mean ± standard error of differential counts of leukocytes (lymphocytes, monocytes, LG-PAS and neutrophils) of pacu (P. mesopotamicus) injected with thioglycolate (TIO), heat-inactivated A. hydrophila (bacteria), lipopolysaccharide from Escherichia coli (LPS) and sodium chloride solution without inflammatory stimulus (control). Capital letters for comparison between treatments; small letters for comparison between time post-stimuli within each treatment

immature erythrocytes. Though the number of erythroblasts has not been determined, they appeared in great quantity in the smears. Fact not observed in the control group. The results suggest that heat-inactivated *A. hydrophila* presented relative pathogenicity, since it caused anaemia. The LPS of the cell mem-

brane of Gram-negative bacteria is thermo stable and have continuous deleterious effects, including erythrocytes lysis.

Formalin-inactivated *Renibacterium salmoninarum* injected in trout and Atlantic salmon (*Salmo salar*) did not affect the blood profile, but reduced the erythrocyte size up to 10 days after the inoculation. Such an alteration suggests that the bacterial toxin is resistant to the formalin. The inoculation of active *R. salmoninarum* in both salmonids reduced gradually the erythrocytes, haemoglobin and haematocrit counts, as an effect of the retention of the spleen cells leading to splenomegaly[17]. The injection of LPS did not alter the erythrocytes counts and the haemoglobin concentration, but triggered a reduction of the haematocrit. These results demonstrate that LPS was less stressful to the fish than the thioglycolate and *A. hydrophila*, possibly the concentration injected was not sufficient to activate the alternative pathway of the complement system[18].

In the current study, the number of thrombocytes in the LPS group increased from 6 to 48 hours post-stimuli, decreased 6 hours after the inoculation of *A. hydrophila*, reaching values similar to the controls 48 hours after the inoculation.

Trout injected with *V. anguillarum* or its products[29] and hybrid tambacu injected with thioglycolate and carrageenan[30,21] had no change in the thrombocytes counts. In trout and salmon inoculated with active or inactive *R. salmoninarum*, the thrombocytes counts varied with periods of elevation and decline[17]. As observed in the present study, thrombocytes can migrate to inflammatory site for phagocytosis of inflammatory agents[21]. In goldfish the inoculation of *A. hydrophila* did not alter the monocytes count[18]. In trout, the inoculation of *V. anguillarum* or its products provoked monocytosis, increase in the circulating immature leucocytes, vasodilatation in the kidney and spleen and mobilization of eosinophilic granulated cells[29].

The LPS is a powerful mitogen for fish lymphocytes. The cell response to LPS in fish differs from the one observed in mammals, since fish do not suffer the same toxic effect.

The inoculation of heat-inactivated *A. hydrophila* provoked reduction of the lymphocytes count six hours after inoculation. This effect can be due to the fact that the LPS used in the current study is from *Escherichia coli*, in which fish are relatively immune[1], whereas the *A. hydrophila* can cause damages to fish, such as haemorrhagic septicaemia.

In the present study, fish injected with heat-inactivated *A. hydrophila* showed a reduction in the LG-PAS and neutrophils counts when compared with fish injected with LPS. Goldfish injected with *A. hydrophila* showed lymphocytopenia, accompanied by neutrophilia[18]. Lamas et al.[29] described lymphocytopenia and neutrophilia in trout inoculated with *V. anguillarum* or its products. It seems that lymphocytopenia could be a result of 1) migration of lymphocytes to the injured tissues; 2) degeneration caused by the bacterial products/components; while the neutrophilia coincided well with the reduction of neutrophils in the head kidney. So, it is plausible to infer that the lymphocytopenia developed after injection of *A. hydrophila* has been caused by 1) migration of lymphocytes to the injured tissues; 2) anchorage of lymphocytes in the haematopoietic tissues or 3) the stress as a result of the inoculation of inactivated bacteria.

In conclusion, the challenge with thioglycolate and LPS did not interfere in erythrocytes counts. The results of this study suggest that heat-inactivated *A. hydrophila* caused anaemia, and reduction of thrombocytes and lymphocyte number in the blood.

Acknowledgments

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