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CHARACTERIZATION OF AN INTRAVENOUS LIPOPOLYSACCHARIDE INFLAMMATION MODEL IN BROILER CHICKENS

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Characterisation of an intravenous lipopolysaccharide inflammation model in broiler chickens

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Short title: Characterization of an avian inflammation model

Figs 6 to 9 to be on single page for printed version as per pdf sent to production

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Abstract

Intravenous administration of lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) O127:B8 at a dose of 1,500,000 units/kg BW evoked a hypothermic response followed by a fever phase in five-week-old broiler chickens. The hypothermic phase coincided with a severe decrease in blood pressure. We assume that this decrease in blood pressure is, at least partly, responsible for the hypothermic phase of the body temperature curve. LPS administration also caused a decrease in circulating white blood cells. The heterophils were predominantly sequestered in the lungs. In LPS-treated chickens, far more apoptotic leukocytes were present in the circulation, compared to control chickens. The molecular players responsible for the LPS-induced inflammatory response could be TL1A, IL-1β and IL-6, since a slight increase in their mRNA levels in white blood cells was already seen 1 hour after LPS administration. In accordance with these observations, the levels of secreted IL-6 were maximal 3 hours after LPS administration. These parameters characterize this LPS-induced inflammation model in broiler chickens.

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Introduction

Broilers come into contact with large amounts of lipopolysaccharide (LPS) by inhalation of dust and in cases of airosacculitis, after colonization of the airsacs with *Escherichia coli* (*E. coli*) (Lorenzoni and Wideman, 2008; Dwars *et al.*, 2009). LPS is a major component of the Gram-negative bacterial cell wall, and is capable of eliciting a plethora of effects including, changes in body temperature, blood pressure and circulating leukocytes. Exposure of leukocytes and other cell types to a pathogen is followed by the appearance of pyrogenic cytokines or endogenous pyrogens in the circulation among which tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 are considered the most important. These cytokines signal to the brain, where the level of prostaglandin E_2 is elevated, which is recognized as key mediator to induce fever (Coceani and Akarsu, 1998; Netea *et al.*, 2000). In chickens, TNF-α has not been identified but TNF-like ligand 1A (TL1A) produces effects similar to TNF-α such as production of IL-1β and IL-6. TL1A also promotes inflammation by raising α_1 acid glycoprotein, ceruloplasmin and nitric oxide (NO) (Takimoto *et al.*, 2008). After intravenous (iv) LPS administration in chickens, the IL-6 plasma concentration peaks 3 hours after treatment and precedes the febrile phase (De Boever *et al.,* 2008a). In the spleen, mRNA levels of IL-1β were significantly elevated 1 hour after LPS administration (Leshchinsky and Klasing, 2003).

High doses of LPS tend to evoke hypothermia instead of fever and both behaviours are reported to be adaptive (Romanovsky *et al*., 1996, Almeida *et al*., 2006b; Akarsu and Mamuk, 2007). Hypothermia can potentially lower tissue hypoxia and could play a defensive role after LPS administration (Romanovsky *et al*., 1997). Another hallmark of LPS administration is cardiovascular collapse resulting in

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hypotension through upregulation of inducible NO-synthase (iNOS) and subsequent NO production (Jordan and Hinshaw, 1963; Wakabayashi *et al.*, 1991; Leturcq *et al.*, 1996; Miyamoto *et al.*, 1996; Romanovsky *et al.*, 1997; Thiemermann, 1997; Mayr *et al.*, 2008). Furthermore, LPS induces the adhesion of circulating leukocytes to the endothelial surface in postcapillary venules in rabbits, rats and mice. The accumulation of these activated leukocytes is described primarily in the lung (Haslett *et al.*, 1987; Cardozo *et al.* 1991; Walther *et al.*, 2000; Yipp *et al.*, 2002).

The current study was performed to further elucidate which players and cell types are involved in the inflammatory effects seen after LPS administration in broiler chickens. In this acute inflammation model, body temperature and blood pressure were measured as primary clinical parameters. Pro-inflammatory cytokines were measured on three levels: assessment of mRNA expression of TL1A, IL-1β and IL-6 by PCR, intracellular protein expression using a flow cytometric method for IL-1β and IL-6, and secreted protein in plasma using an IL-6 bio-assay. The characterization of this intravenous LPS inflammation model will enable us to study the influence of non-steroidal anti-inflammatory drug (NSAID) administration on the evaluated parameters in future experiments.

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Materials and Methods

Animals Twenty five one-day-old, unvaccinated, Ross chicks of both sexes were obtained from a commercial hatchery. Temperature and relative humidity accorded with the requirements of young chickens and temperature was adjusted to prevent huddling (Anonymous, 2004). The concrete floor was covered with wood shavings.

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Normal hygienic measures were taken to avoid contact with pathogens as much as possible. The lighting schedule consisted of 12 hours dark and 12 hours light. Commercial pullet feed and tap water were available *ad libitum*. All experiments and procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine (EC 2008/006 and 008).

Experiment 1: Body temperature and cytokine expression For the first experiment, chickens were divided in two groups of 6 LPS-treated and 6 control chickens. LPS-treated chickens were injected intravenously in the wing vein with LPS of *E. coli* O127:B8 (Sigma-Aldrich, Bornem, Belgium) dissolved in *aqua ad injectabilia* at a dose of 1,500,000 units/kg BW at the age of 5 weeks. In this batch of LPS, 1,500,000 units/kg BW corresponded to a dose of 2.5 mg/kg BW. For all experiments, LPS from the same batch was used. Control chickens were injected with the same amount of *aqua ad injectabilia.*

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Body temperature was measured by inserting a thermocouple probe (Physitemp Instruments Inc., Clifton, NJ, USA) 5 cm into the cloaca, before and at 1, 2, 3.5, 5, 6.5, 8, 12 and 24 h after LPS administration. The area under the body temperature versus time curve from 0 to 5 h and 0 to 24 h post administration (p.a.) ($AUC_{0\rightarrow 5\,h}$ and $AUC_{0\rightarrow 24\,h}$) was calculated to evaluate differences between treatment groups (α=0.05).

One ml of blood was withdrawn in citrate-containing tubes before and 1, 2 and 3 h after LPS administration for PCR analysis. Blood was transferred into two Eppendorf cups (Novolab, Geraardsbergen, Belgium) containing 1 ml of RNA/DNA stabilization reagent for blood/bone marrow (Roche Applied Sciences, Vilvoorde, Belgium) and frozen at -20°C until RNA extraction.

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Half a ml of blood was withdrawn in heparin containing tubes (Venoject®, Terumo Corp., Tokyo, Japan) from the leg vein before and 1, 2, 3, 4, 5, 6, 8 and 12 h after LPS administration for flow cytometric analysis. From 3 LPS-treated chickens and 3 control chickens, a drop of these blood samples was also used to prepare a blood smear.

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For determination of IL-6 in plasma using a bio-assay, 0.4 ml of blood was withdrawn in heparin containing tubes before and 1, 2, 3, 4, 5, 6, 8 and 12 h after LPS administration.

PCR analysis Blood samples were analysed for chicken TL1A, IL-1β, IL-6 and two housekeeping genes, i.e. ubiquitin (UB) and glucose-6-phosphate dehydrogenase (G6PDH) and prepared as described previously (De Boever *et al.*, 2008b). Briefly, total RNA was extracted from 1 ml citrate treated blood using a High Pure RNA Isolation Kit (Roche Applied Science, Vilvoorde, Belgium) according to the manufacturer's instructions. The reverse transcription was performed using the Reverse Transcriptase Core kit (Eurogentec, Seraing, Belgium), following the manufacturer's instructions. A fixed amount of RNA (600 ng) from each sample was converted into cDNA, using random nonamers as priming method in the PTC-100 DNA engine (Bio-Rad Laboratories, Nazareth Eke, Belgium). The primers were designed with the LightCycler Probe Design Software 2.0 (Roche Applied Sciences) using the GenBank sequences for TL1A, IL-1β and IL-6 and G6PDH. The specific primer sequences for TL1A, IL-1β and IL-6 are listed in Table 1. The primer for UB was based on the sequences used by Li *et al.* (2007).

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Real-time RT-PCR was performed using a LightCycler 2.0 System (Roche Applied Science). All reactions were run in duplicate and were quantified by including a standard curve (5 points in duplicate) in the real-time RT-PCR analysis. The concentration of IL-1β, IL-6 and TL1A was divided by the normalisation factor (NF), calculated as the square root of the multiplication of the concentration of both housekeeping genes.

Deleted: Table 1: Primer sequences used for real-time RT-PCR analysis¶ Gene ... [1]

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Flow cytometric analysis Intracellular IL-1β and IL-6 were analysed in chicken leukocytes using a flow cytometric method (De Boever *et al.*, submitted).

Reagents and antibodies

Polyclonal antibodies, raised in rabbits and directed against chicken IL-1β and IL-6, were used as primary antibodies (AHP941Z and AHP942Z, AbD Serotec, Cergy Saint-Christophe, France). Since no antibodies against TL1A are commercially available, this analysis could not be included.

Allophycocyanin (APC)-conjugated $F(ab')_2$ goat antibodies to rabbit immunoglobulin were used as secondary antibodies (Santa Cruz biotechnology Inc., Heidelberg, Germany).

RPMI 1640, goat serum and bovine serum albumin were purchased at Invitrogen (Merelbeke, Belgium). BD FACS Lysing solution, BD FACS Permeabilizing solution 2 and BD CellFIX were purchased from Beckton Dickinson Biosciences (Erembodegem, Belgium).

Staining procedure and flow cytometric analysis. Chicken leukocytes were obtained after lysis of the erythrocytes with FACS Lysing solution; 4.5 ml of FACS Lysing

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solution (1/10 diluted with distilled water) was added to 0.5 ml of heparin treated blood in a falcon tube and kept for 10 min at room temperature. After washing, the pellet was resuspended in 500 µl of RPMI and 50 µl was pipetted into a flow cytometer tube. Leukocytes were separated from the remaining red blood cell nuclei, using phycoerythrin (PE) - conjugated Cell Lab mouse anti-chicken CD45 antibodies (Beckman Coulter, 733074), which were added in a concentration of 6 µg/ml. Samples were incubated for 30 min at 4°C. Permeabilization was performed using 500 µl FACS Permeabilizing solution 2 (1/10 diluted with distilled water) for 10 min, again at room temperature. Primary antibodies were added in a concentration of 50 µg/ml and samples were incubated for 30 min at room temperature. Next, goat serum at a concentration of 2% in RPMI was added for 30 min and samples were incubated at room temperature. Finally, the corresponding APC-labelled secondary antibody was added at a concentration of 6 µg/ml.

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The cells were resuspended in 450 µl CellFIX (1/10 diluted with distilled water) and pipetted into Trucount Tubes (BD Biosciences). Samples were analysed using a FACSCanto flow cytometer (BD Biosciences). The different cell populations were demarcated in an FL-2/Side Scatter (SSC) dot plot, based on a positive staining for CD45 and their granularity. The PE fluorescence dot plots of the gated cells were then plotted and all data were corrected for autofluorescence. Cytokine-specific fluorescence was defined as events to the right of the autofluorescence marker and the mean fluorescence intensity (MFI) and proportion of cells staining positive for the cytokine in question was recorded.

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IL-6 measurement by bio-assay Concentrations of secreted IL-6 were measured in plasma using IL-6 dependent murine 7TD1 hybridoma cells (Van Snick *et al.*, 1986;

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De Boever *et al*., 2008a). Briefly, cells were cultured in 96 well microtitre plates for 72 h in medium with different dilutions of the plasma samples. The number of living cells was measured using a colorimetric hexosaminidase enzyme reaction(Landegren, 1984). The concentration of IL-6 in plasma samples was calculated from a standard curve obtained using a dilution of recombinant chicken IL-6.

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Microscopic analysis of blood smears Blood smears were prepared with heparinised blood and coloured with Hemacolor® (Merck, Darmstadt, Germany) according to the manufacturer's guidelines. Slides were evaluated using a light microscope (Zeiss, Zaventem, Belgium) equipped with a x100 objective. On each slide, 100 leukocytes were evaluated.

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To demonstrate the presence of apoptotic cells in circulation after LPS administration, a blood sample of a LPS-treated chicken was incubated with Annexin-V-FLUOS labelling reagent (Annexin-V-FLUOS Staining Kit, Roche Applied Science) after lysis of the red blood cells as described above. Leukocyte nuclei were stained using DAPI (1/100 diluted) (Roche Applied Science). After this double staining, cells were mounted on microscope slides using glycerin–PBS solution (0.9/0.1, v/v) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium) and analyzed using a Leica DM RBE fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

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Experiment 2: Blood pressure Blood pressure was measured before and every hour until 12 h after LPS administration using a non-invasive Doppler technique in 6 LPS-treated and 2 control chickens at the age of 5 weeks (Christensen *et al.*, 1987). A Doppler crystal probe coated with ultrasound gel was placed over the plantar arcuate

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artery on the ventral side of the foot web (Ultrasonic Doppler flow detector, model 811-B, Parks Medical Electronics Inc., Oregon, USA). The crystal was connected to a medium gain amplifier. The bird was positioned on its side and an inflatable cuff, connected to a sphygmomanometer, was placed around the upper part of the hind limb. Chickens were gently handled, and adapted to this procedure to avoid stress and treatment-related effects on the measurement as much as possible. The pulse in the foot web was located as an audio signal. The cuff was inflated until the sound was extinguished. Return of the pulse sound during deflation of the cuff indicated systolic blood pressure. The area under the blood pressure versus time curve from 0 to 5 h and from 0 to 24 h p.a. ($AUC_{0\rightarrow 5\ h}$ and $AUC_{0\rightarrow 24\ h}$) was calculated to evaluate differences between treatment groups.

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Experiment 3: Histology Sequestration of heterophils in tissues of LPS-treated chickens was demonstrated by histological examination of the lung after Giemsa staining. Twenty four one-day-old, unvaccinated, Ross chicks of both sexes were obtained from a commercial hatchery and raised as described in section 2.1. At the age of 5 weeks, chickens were divided in three groups. At 2, 3 and 4 h after iv LPS administration at a dose of 1,500,000 units/kg BW, 3 chickens were euthanized. At 2, 3 and 4 h after pyrogen free water administration, 3 chickens were euthanized. At the same time points, 2 control chickens were euthanized. Lungs were collected from LPS-treated, pyrogen free water treated and control birds and stored in 10% formaldehyde for 24 h.

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After embedding in paraffin, tissues were cut in sections of 3 µm and stained with a Giemsa staining according to standard procedures. Slides were evaluated

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microscopically, by counting the amount of heterophils in 10 microscopic fields using a 100x objective (Zeiss).

Statistical analysis The Student's t-test ($\alpha = 0.05$) was used to demonstrate possible differences in the $AUC_{0\rightarrow 5\ h}$ and $AUC_{0\rightarrow 24\ h}$ of the body temperature and blood pressure between LPS-treated and control chickens. The results of the PCR, flow cytometric and histological analyses were also statistically evaluated, using the same test.

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Statistical comparison of the IL-6 concentration between the different treatment groups was done 3 h after the administration of LPS or saline, the time point at which the secreted IL-6 concentration reached its highest value. A Mann-Whitney test was performed to demonstrate possible differences in cytokine concentration.

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Results

Experiment 1: ***Body temperature.*** The course of the body temperature was characterized by a hypothermic phase followed by a fever phase in LPS-treated chickens, as shown in Figure 1. There was a significant difference in $AUC_{0\rightarrow5\ h}$ between LPS-treated chickens and control chickens expressed as mean ± SD (LPS: 204.03 ± 1.52 °C x h; control: 207.50 ± 0.96 °C x h with $P < 0.05$). For the $AUC_{0\rightarrow24\ h}$, no significant difference could be found between LPS- treated and control chickens, since the hypothermic phase cancels out the hyperthermic phase.

PCR analysi. The mRNA levels of TL1A, IL-1β and IL-6 are depicted in Figure 2. They were slightly increased in leukocytes and thrombocytes of LPS-treated chickens. However, no statistical significance could be demonstrated for any of the three cytokine levels.

Flow cytometry. A decrease in circulating CD45 positive white blood cells was observed as shown in Figure 3 (A). Specifically, in LPS-treated chickens there was a significant decrease in circulating heterophils as demonstrated in Figure 3 (B). In control chickens, an increase in circulating heterophils could be found from 4 h after aqua ad injectabilia administration, this increase was attributed to stress due to handling of the animals.

In Figure 4, significant differences in MFI 3 h after treatment between LPS-treated chickens and control chickens are shown for intracellular IL-1β and IL-6 in the heterophils.

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Bio-assay. LPS-treated chickens had significantly higher levels of IL-6 in plasma at 3 h post-treatment compared to control chickens (P<0.05) as shown in Figure 5.

Light and fluorescence microscopy. Microscopic examination of blood smears revealed a decrease in circulating heterophils in LPS-treated chickens. Moreover, 4 h after LPS administration, pregranulocytes appeared in circulation, characterized by the presence of blue granules in their cytoplasm and a less lobulated nucleus. In LPS-treated chickens, dark coloured cells could be observed from 2 h p.a. These cells were identified as apoptotic cells using annexin V staining as illustrated in Figure 6. In control chickens, an increase in circulating heterophils was seen. No marked difference in circulating monocytes and lymphocytes could be observed between LPS-treated and control chickens.

Experiment 2: Blood pressure. The administration of LPS resulted in a profound and sustained hypotension as depicted in Figure 7. Both the $AUC_{0\rightarrow 5\ h}$ (LPS: 352.92 ± 39.38 mmHg x h; control: 552.5 ± 38.89 mmHg x h) and $AUC_{0\rightarrow 24\ h}$ (LPS: 1986.25 mmHg x h ± 212.47; control: 2607.5 mmHg x h ± 272.24) were significantly different between LPS-treated and control chickens (P<0.05).

Experiment 3: Histology. LPS administration significantly increased the number of heterophils in the lung (P < 0.05), 2 h after administration as shown in Figure 8. Figure 9 illustrates the difference in presence of heterophils in lung tissue after Giemsa staining of an LPS-treated chicken (A) in comparison to a control chicken (B).

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Discussion

Recognition of microbial components by Toll-like receptors triggers a cascade of cellular signals which culminates in the activation of NF-κB leading to the expression of genes encoding pro-inflammatory cytokines like TNF-α, IL-1β and IL-6, inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), and phospholipase A_2 and clearance of the infectious agent (Witkamp and Monshouwer, 2000; Verstrepen *et al.*, 2008).

IL-1β, IL-6 and TNF-α are considered the most important pyrogenic cytokines or endogenous pyrogens in the circulation (Dinarello, 2000, Witkamp and Monshouwer, 2000). In mammals, TNF-α is considered one of the first mediators released during inflammation, which in turn promotes the production of IL-1β and IL-6 (Witkamp and Monshouwer, 2000). To date, TNF-α has not been identified in the chicken. However, chicken TL1A belongs to the TNF ligand superfamily, a family of cytokines regulating cell growth/survival/apoptosis, and shows a similar mode of action as TNF-α (Gaur and Aggarwal, 2003; Takimoto *et al.*, 2008).

In the current experiment, mRNA levels of IL-1β, IL-6 and TL1A in white blood cells were slightly elevated 1 h after iv injection of the Toll-like receptor 4 ligand, LPS. It should be mentioned that mRNA levels do not necessarily equate to protein levels and cytokines can be produced by other cell types than leukocytes (Kogut *et al.*, 2005). In the current study, the IL-1β and IL-6 intracellular protein levels in white blood cells were found to be significantly elevated in heterophils, the avian counterpart of mammalian neutrophils, 3 h after LPS administration. These results from *in vivo* administration of LPS confirm the *in vitro* results from heterophils, macrophages and thrombocytes (Dil and Qureshi, 2002; Kogut *et al.*,

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2005; Ferdous et al., 2008). The concentration of secreted bioactive IL-6, which produced by white blood cells as well as by other cell types such as endothelial cells, was also significantly elevated 3 h after LPS administration. This could be expected since there is generally a good correlation between intracellular detection of cytokines and the secreted amounts of cytokines (Schuerwegh *et al.*, 2003).

As a result of the production of these cytokines, a change in body temperature occurs. In the first experiment of this study, a biphasic response in body temperature, i.e. a fever phase preceded by a hypothermic phase was seen. The rise in body temperature of the control chickens could be attributed to several factors including circadian rhythm and handling of the chickens (Dawson and Whittow, 2000; Cabanac and Aizawa, 2000). It is proposed that hypothermic and pyrogenic mechanisms are independently activated during an LPS-induced acute phase response and that both behaviours are adaptive (Romanovsky *et al.*, 1996, Almeida *et al.*, 2006b; Akarsu and Mamuk, 2007). Hypothermia can potentially lower tissue hypoxia and thus plays a defensive role in septic shock and is likely aimed at preserving the host's vital systems (Romanovsky *et al.*, 1997; Almeida *et al.*, 2006a). The similarities in time course of the hypothermia and the reduction in oxygen consumption strongly suggest that hypothermia is a reflection of reduced thermogenesis (Derijk *et al.*, 1994), but the time course of the LPS-induced hypothermia coincides with the time course of arterial hypotension. Intravenous LPS administration triggers widespread inducible nitric oxide synthase (iNOS) expression, resulting in copious production of nitric oxide (NO) (Bowen *et al.*, 2007; Saia *et al.*, 2008). NO seems to be involved in LPS-induced hypothermia, since it may participate in vasodilatation, increasing heat loss and intensifying the magnitude of hypothermia (Vayssettes-Courchay *et al.*, 2003). As time passes after the administration of a high dose of LPS, the level of

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endotoxemia decreases, the arterial pressure returns to its normal values and along with the alleviation of tissue hypoperfusion, cold-seeking behaviour changes into warmth-seeking behaviour and fever emerges (Romanovsky *et al.*, 1996; Almeida *et al.*, 2006a). We agree with the concept proposed by Almeida *et al.* (2006a) that hypotension evokes hypoperfusion and less oxygen consumption, whereby there is less oxygen for heat production, resulting in hypothermia. In this way, hypothermia can be seen as a result of the hypotension, another hallmark of LPS administration in various species such as chickens, rats, dogs, rabbits, primates and humans (Jordan and Hinshaw, 1964;Wakabayashi *et al.*, 1991; Leturcq *et al.*, 1996; Miyamoto *et al.*, 1996; Romanovsky *et al.*, 1997; Mayr *et al.*, 2008). NO is also believed to be responsible for the hypotension after endotoxin administration (Doursout *et al.*, 2008), so either way, NO seems to be the causative agent of both hypotension and hypothermia. However, in this experiment a sustained hypotension was seen over a longer period than the hypothermic phase. Probably in the initial phase of the inflammatory response, hypotension has a profound effect on the body temperature while later on the mechanisms evoking fever overrule the effect of hypotension.

In dogs and pigs, hypothermia by cooling of the animals induced neutropenia (Fedor *et al.*, 1958; Biggar *et al.*, 1983; Biggar *et al.*, 1984). Also in our experiments, hypothermia was accompanied by a decrease in circulating granulocytes. More in general, there was a decrease of all CD45 positive cells. In sheep and dwarf goats, LPS administration was shown to induce a decrease in peripheral white blood cell counts, which was caused by margination and accumulation of leukocytes in the lungs (Meyrick and Brigham, 1983; Van Miert *et al.*, 1997). Normal lungs contain a large pool of marginated neutrophils since they are less deformable than erythrocytes, which have similar dimensions but pass the lung capillary segments faster (Hogg and

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Doerschuk, 1995; Kitagawa *et al.*, 1997; Klut *et al.*, 1998; Kuebler *et al.*, 2000; Walther *et al.*, 2000; Suwa *et al.* 2001; Jones *et al.*, 2002). Another key feature in a localized response to LPS or inflammation is sequestration of leukocytes from the circulation to the endothelium, resulting in emigration into the surrounding, inflamed tissue. In the case of sepsis or endotoxemia, the leukocytes become trapped in the lungs and are not able to recirculate, as seen in rabbits, rats and mice (Haslett *et al.*, 1987; Cardozo *et al.*, 1991; Yipp *et al.*, 2002). Endotoxin decreases neutrophil deformability by stiffening of the cytoskeleton by formation of microfilaments and increases the density of neutrophils in pulmonary capillaries (Hogg and Doerschuk, 1995; Kitagawa *et al.*, 1997; Klut *et al.*, 1998; Kuebler *et al.*, 2000; Walther *et al.*, 2000; Suwa *et al.* 2001). Interestingly, in rabbits, injection of recombinant human IL-6 induces a decrease in deformability of neutrophils (Suwa *et al.*, 2001). Also L-selectin mediated leukocyte/endothelial interaction, endothelium derived Toll-like receptor 4 and the adhesion molecules ICAM1 and ELAM1, that bind neutrophils on endothelial cells, are reported to participate in the process of sequestration (Osborn 1990; Hogg and Doerschuk, 1995; Kuebler *et al.*, 2000; Andonegui *et al.*, 2003).

The decrease in circulating white blood cells, seen in our experiments, is at least partly caused by the sequestration of these cells as shown by the abundant presence of especially granulocytes in the lungs 2 h after LPS administration. A second reason could be apoptosis of these cells, which is reported *in vivo* in pigs and chickens and *in vitro* for fish lymphocytes (Norimatsu *et al.*, 1995; Shini *et al.*, 2008a; Xiang *et al.*, 2008). In the current study, an annexin-V staining of isolated white blood cells of LPS-treated chickens showed more apoptotic cells compared to the samples from control chickens. In mice, it was suggested that these apoptotic cells have

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beneficial effects against LPS-induced endotoxic shock and even have therapeutic potential by reducing the release of pro-inflammatory cytokines (Ren *et al.*, 2008).

From 4 h after LPS administration, granulocytosis was seen in LPS-treated chickens. Ushiyama *et al.* (2005) reported granulocytosis in quails 3 h after LPS administration and Shini *et al.* (2008b) demonstrated an increased heterophil:lymphocyte ratio at 1, 3 and 24 h after LPS treatment, which is earlier than in our experiment. LPS accelerates the release of neutrophils from bone marrow pools and stimulates an increase in immature heterophils or band cells, in the bone marrow and peripheral blood as confirmed in chicken blood in the present experiment (Biggar *et al.*, 1983; Klut *et al.*, 1996; Klut *et al.*, 1998; Shini *et al.*, 2008). In rabbits it was demonstrated that administration of recombinant human IL-6 accelerates the release of PMN from the bone marrow between 9 and 24 h after treatment (Suwa *et al.*, 2001).

It should be remarked that in the control group, an increase in circulating heterophils was observed 4 h after the administration of pyrogen free water. This can be attributed to stress due to the frequent handling of the chickens. An increase in the blood heterophil:lymphocyte ratio is used as a general stress indicator and is related to corticosterone production in chickens, which can rise due to simple experimental procedures (e.g. confinement, isolation and multiple blood sampling) in birds such as pigeons and geese (Machin *et al.*, 2001; Takahashi *et al.*, 2002).

In conclusion, the parameters characterizing this iv *E. coli* LPS-induced inflammation model in chickens are profound hypotension accompanying the hypothermic phase of the body temperature curve, slight elevation of mRNA levels of TL1A, IL-1β and IL-6 at 1 h after LPS administration, peak of expression of intracellular IL-1β and IL-6 in hetreophils by 3 h after LPS administration, and also

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peak levels of secreted IL-6 by 3 h post-LPS treatment. Furthermore, LPS induced a decrease in circulating CD45 positive cells, partly caused by sequestration of the heterophils in the lungs on the one hand and, apoptosis of the circulating leukocytes on the other hand. In future experiments, the influence of non-steroidal anti-inflammatory drugs (NSAID) administration on these parameters will be evaluated.

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Acknowledgements

The authors like to thank W. Burms for the quantification of IL-6 in plasma by bio-assay, S. Stuyvaert for the help with the PCR analysis and K. Demeyere for performing the flow cytometric analysis. C. Puttevils and D. Ameye are acknowledged for the help with the Giemsa staining of the tissues. The help of E. Neirinckx, D. De Clercq en K. Jonckheere with the animal experiments was greatly appreciated.

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Table 1: *Primer sequences used for real-time RT-PCR analysis*

Gene	Accession no.	Primer sequence	$MgCl_2$	Primer annealing	Primer elongatio
TL1A	**AB194710**	Forward: CCTGAGTTATTCCAGCAACGCA Reverse: ATCCACCAGCTTGATGTCACTAAC	2 mM	60°C	12 s
IL-1β	**Y15006**	Forward: AACATCGCCACCTACAAG Reverse: TACTCGGTACATACGAGATGGAAA	4 mM	60°C	7 s
IL-6	**AJ309540**	Forward: TGGAGAGCAGCCCATGTAA Reverse: CATGGACTGGAGCACAAGTA	3 mM	60°C	3 s

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IL-6	**AJ309540**	Forward: TGGAGAGCAGCCCATGTAA Reverse: CATGGACTGGAGCACAAGTA	3 mM	60°C	3 s

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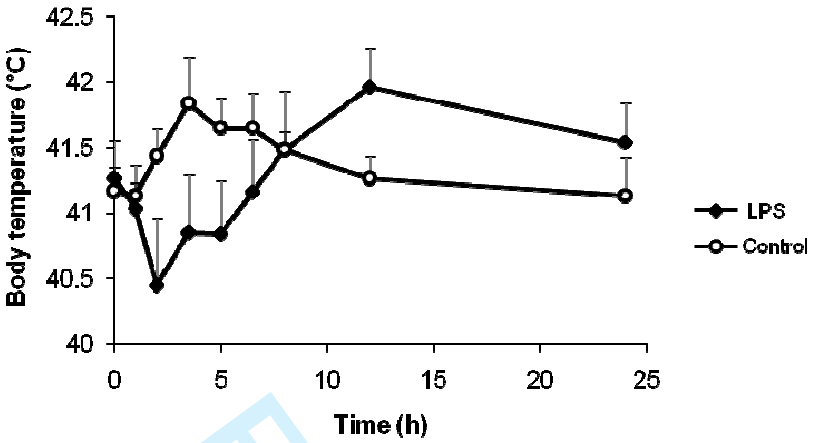

Figure 1: Body temperature versus time curve of LPS-treated chickens (n=6) and control chickens (n=6) expressed as mean (+SD). The LPS-treated chickens showed a hypothermic phase followed by a fever phase. Control animals showed a slight increase in body temperature.

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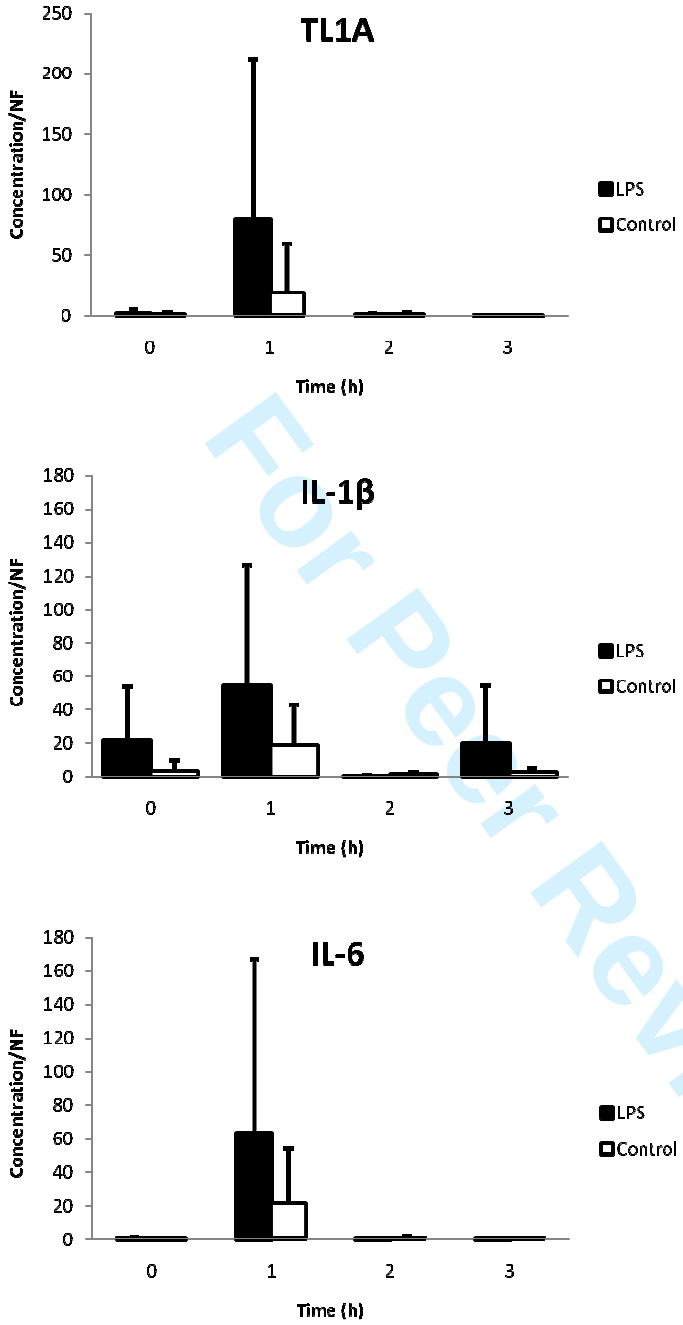

Figure 2: Concentration/NF of TL1A, IL-1β and IL-6 versus time graphs of LPS-treated (n=6) and control chickens (n=6) expressed as mean (+SD). A slight, although not significant, increase in mRNA levels in the leukocytes was seen 1 h after LPS administration.

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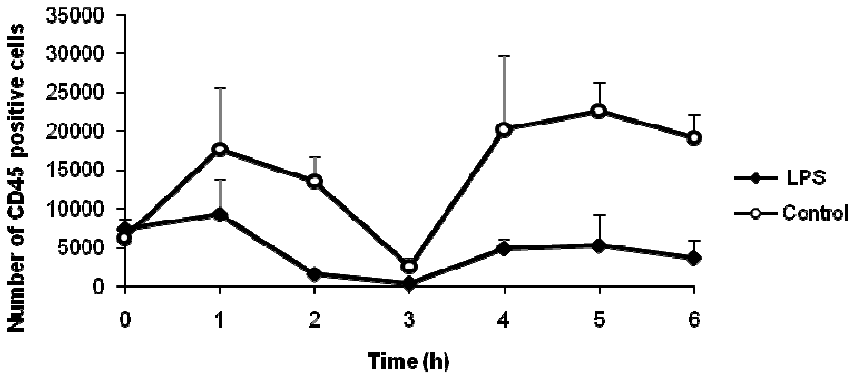
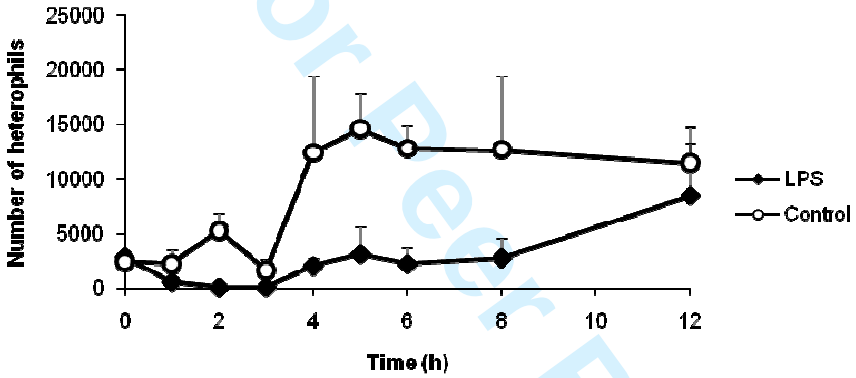


(A)



(B)

Figure 3: (A) Number of CD45 positive white blood cells versus time and (B) number of circulating heterophils in LPS-treated (n=6) and control chickens (n=6) expressed as mean (+SD). A significant decrease in circulating leukocytes was seen in LPS-treated chickens due to sequestration and apoptosis.

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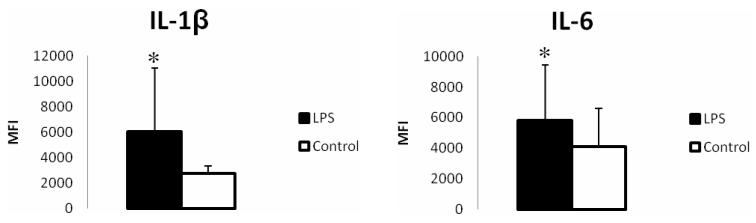

Figure 4: Intracellular expression (MFI) of IL-1β and IL-6 3 h after treatment in the heterophils of LPS-treated chickens (n=6) and control chickens (n=6) expressed as mean (+SD) * Means differ significantly from those of the control group (P < 0.05). A significant increase in intracellular IL-1β and IL-6 expression in the heterophils was seen 3 h after LPS administration.

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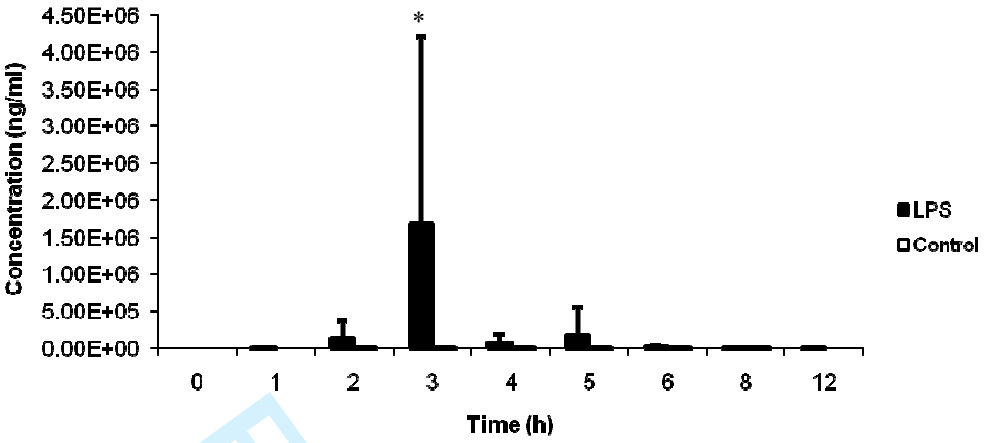

Figure 5: Concentration of secreted IL-6 versus time graph of LPS-treated chickens (n=6) and control chickens (n=6) expressed as mean (+SD) * Mean differs significantly from that of the control group ($P < 0.05$). A significant increase in plasma IL-6 concentration was seen 3 h after LPS administration.

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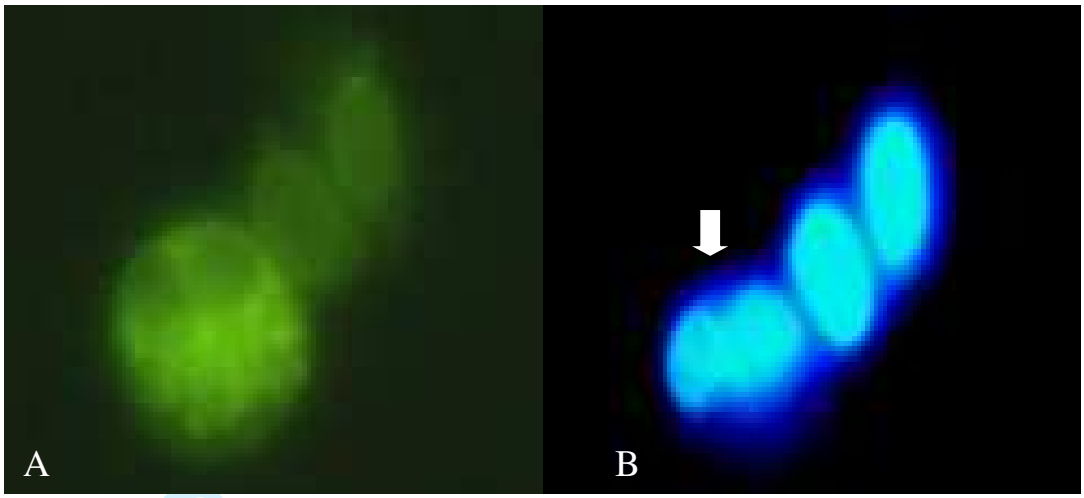

Figure 6: Fluorescence microscopic image of a fluorescein isothiocyanate (FITC) - annexin V stained apoptotic heterophil (A), as identified by the shape of the nucleus (indicated with an arrow and on the right, two red blood cell nuclei can be remarked) (B). Intravenous LPS administration to broiler chickens induced heterophil apoptosis .

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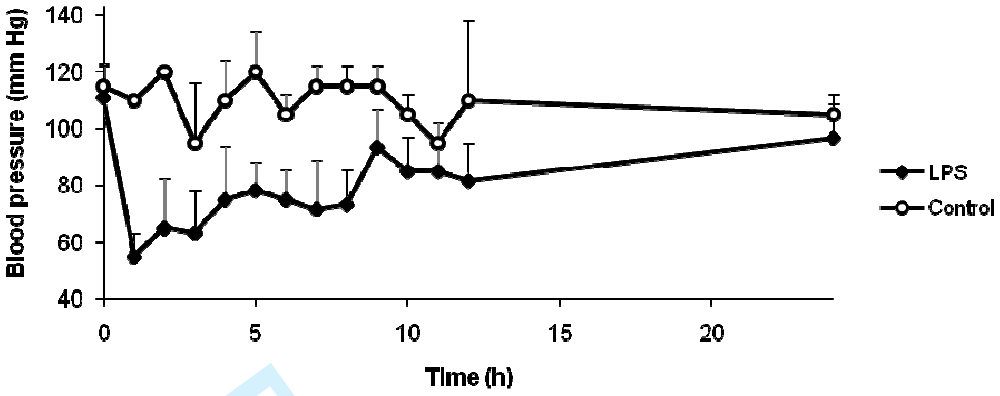

Figure 7: Blood pressure versus time curve of LPS-treated chickens (n=6) and control chickens (n=2) expressed as mean (+SD). LPS administration to broiler chickens induced a profound and sustained hypotension.

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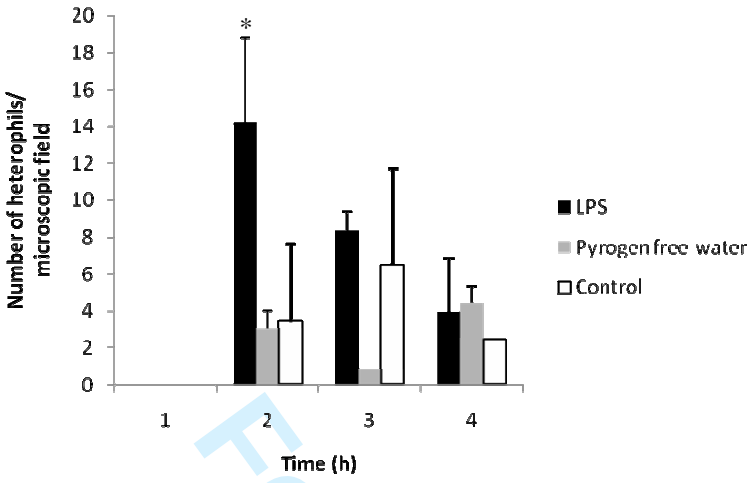

Figure 8: Number of heterophils / microscopic lung field (100x) versus time curve for LPS-treated chickens (n=3), pyrogen free water treated chickens (n=3) and control chickens (n=2) expressed as mean (+SD) * Mean differs significantly from those of the control group and the pyrogen free water treated group (P < 0.05). Two h after intravenous LPS administration to broiler chickens induced a significant sequestration of heterophils in the lungs was remarked.

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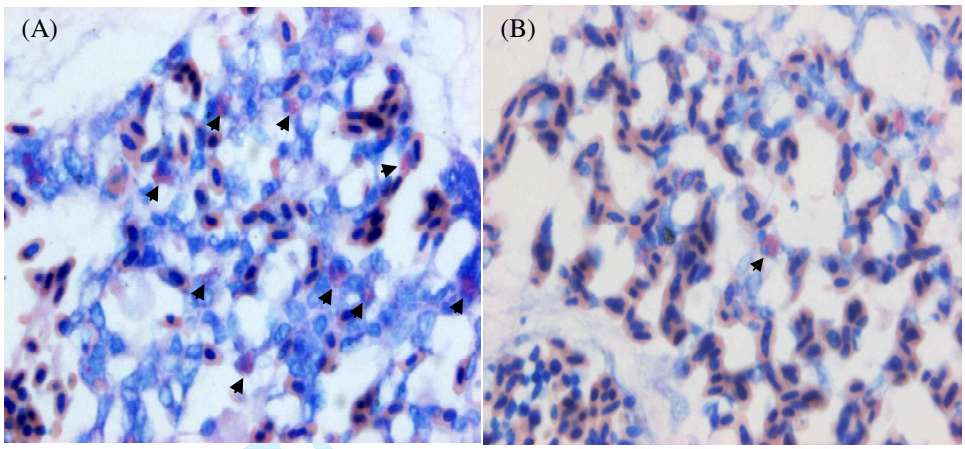

Figure 9: Giemsa staining of the lung of an LPS-treated chicken (A) and a control chicken(B). The black arrows indicate heterophils. Significantly more heterophils can be remarked in LPS-treated chickens than in control chickens.

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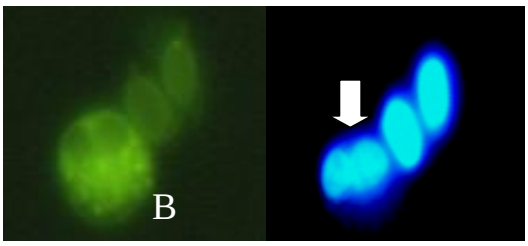

Figure 6. *Fluorescence microscopic image of a fluorescein isothiocyanate (FITC) - annexin V stained apoptotic heterophil (A), as identified by the shape of the nucleus (B).*

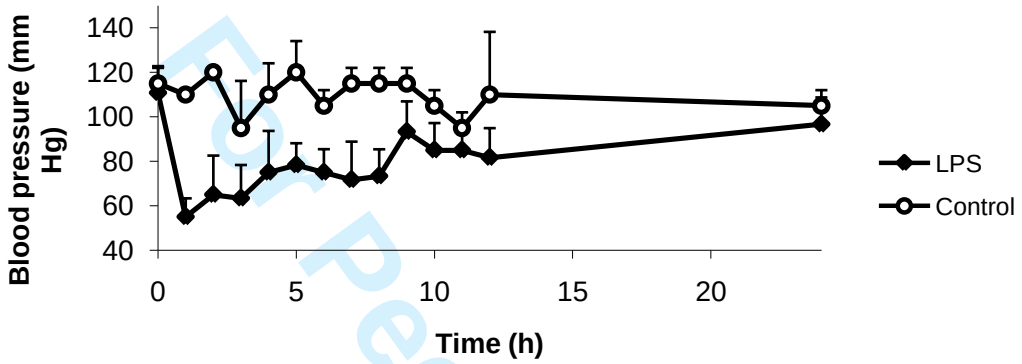

Figure 7. *Blood pressure versus time curve of LPS-treated chickens (n=6) and control chickens (n=2) expressed as mean (+SD).*

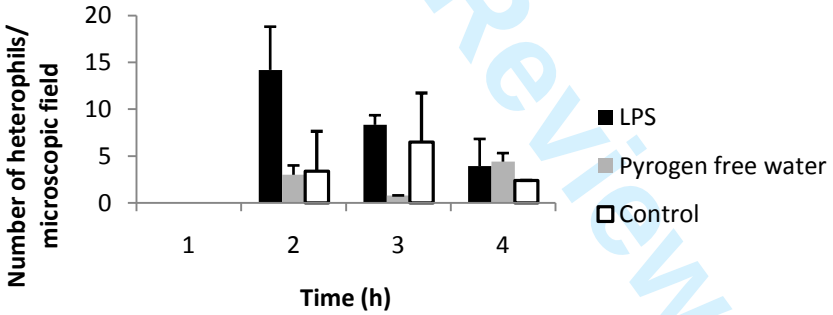

Figure 8. *Number of heterophils / microscopic lung field (100x) versus time curve for LPS-treated chickens (n=3), pyrogen free water treated chickens (n=3) and control chickens (n=2) expressed as mean (+SD) * Mean differs significantly from those of the control group and the pyrogen free water treated group (P < 0.05).*

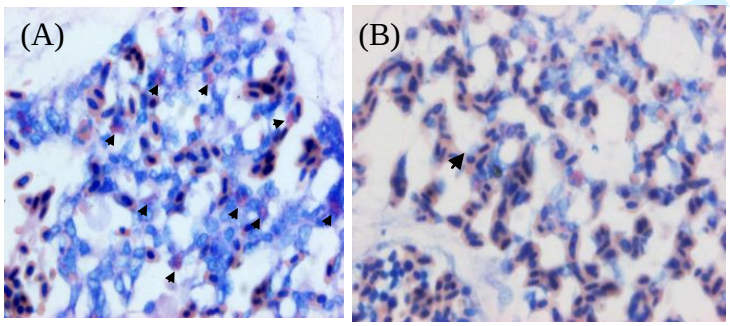

Figure 9. *Giemsa staining of the lung of an LPS-treated chicken (A) and a control chicken (B). The black arrows indicate heterophils.*

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