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# The effect of biologically active phospholipids on the course of inflammation in porcine *in vivo* and *in vitro* systems

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## INTRODUCTION

The previous studies demonstrated that biologically active phospholipids (BAF) are involved in selective destruction of particular tumour cells and their effect is manifested by destruction of their membranes and proteinkinase C inhibition [1-3]. Our previous *in vivo* experiment in rabbits demonstrated regenerative effects of BAF after chemically induced stress monitored by changes in the percentages of peripheral blood leukocyte subpopulations and by proliferation activity of lymphocytes (Jeklova , per comm.).

The aim of this experiment was to assess the anti-inflammatory activity of a nutritional supplement composed of a mixture of BAF in the commercial product Ovosan (AREKO), on the course of experimental infection with the bacterium *Actinobacillus pleuropneumoniae* (App) in pigs. Furthermore, the anti-inflammatory activity of BAF was studied in *in vitro* experiment, by evaluating the severity of inflammation at a cellular level in a dose- and time-dependent manner.

## MATERIAL AND METHODS

***In vivo* study.** Weaned piglets from a herd free from clinical App infection over a long period, with very low levels of anti-App antibodies in the sera, were allocated into 2 experimental groups of 6 animals each. Piglets in Group 1 were orally administered pure sunflower oil for a period of 28 days (hereinafter reffered to as control group). Piglets in Group 2 were given a 15% solution of BAF at the same time and dose regimen. Fourteen days after the start of supplementation with BAF or pure oil, piglets were experimentally infected with the bacterium App via the intranasal route. The health status of animals was monitored; blood samples were collected for haematological and serological analyses at regular intervals (ELISA detection of App-Abs and interleukin-1β; Alpco Diagnostics). At the end-stage of disease or after completing the experiment (at 2 weeks postinfection) pigs were euthanized with an anesthetic overdose and necropsied. At necropsy, the status of lung parenchyma was assessed and pulmonary scores which document the extent of pulmonary parenchymal damage were calculated. At the same time, bronchoalveolar lavage fluids (BALF) were collected for serological analysis and cytology of cell infiltrate.

***In vitro* study**. Monocyte-derived macrophages (MDMF) were generated from peripheral blood monocytes of 6 pigs using Histopaque-1077 gradient centrifugation (Sigma-Aldrich) and indirect magnetic labelling on QuadroMACS™ cell separator (Miltenyi Biotec) using mAb against CD14 (clone MIL2). MDMF were derived after 6 day cultivation in Dulbecco's Modified Eagle's Medium supplemented with antibiotics (penicillin, streptomycin) and 10% porcine serum at 37°C in an atmosphere with 5% $CO_2$. MDMF were further incubated without (control) or with BAF (0.03%, 0.1%, 0.3%) for 24 and 48 h, and then washed. One half of the cultures was stimulated with lipopolysaccharide (1 µg/ml) for 4 h. Adhered cells from all cultures were lysed in TRI Reagent RT (Molecular Research Center, Inc.) or 1x Laemmli buffer for qRT-PCR and Western blotting, respectively.

**qRT-PCR.** Total RNA was obtained using 4-Bromoanisole and RNeasy Kit (Qiagen). mRNA was quantified in triplicate 3-µL reactions containing QuantiTect SybrGreen PCR Master Mix (Qiagen), using Nanodrop II liquid dispenser (Innovadyne Technologies, Inc.) and LightCycler 480 Real-Time System (Roche). Primers specific for HK genes TBP-1, HMBS-2, HPRT-1 and genes coding for pro-inflammatory IL-1ß, TNF-α, CXCL10 and anti-inflammatory IL-10 and arginase-1 were used for simultaneous measurements of gene expression activity. TBP-1 gene was selected as a reference gene among HK genes.

**Western blotting.** The involved signaling pathway under investigation was activation of caspase-1 leading to an activation of IL-1ß and phosphorylation of protein kinase Cε (PKCε). Cellular proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Primary polyclonal Abs specific for caspase-1 and total and phosphorylated PKCε (Santa Cruz Biotech.), and mouse monoclonal anti-β-actin Ab (Abcam) were used to probe the membranes. Proteins were visualized by using HRP-conjugated secondary Abs and ECL plus Western blotting detection reagents (GE Healthcare Life Sciences).

## Conclusion:

In *in vivo* experiment , we detected anti-inflammatory effect of BAF. This effect was also confirmed in *in vitro* experiment using monocyte-derived macrophages stimulated by LPS at the level of mRNA as detected by qRT-PCR.

Downregulation of caspase-1 activation and PKCε phosphorylation could explain cellular backgroud of this fenomena.

**References:**

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[3] Mikhaevich IS, Gerasimova GK, Kara J. Inhibition of protein kinase C by semisynthetic phospholipid plasmanyl-(N-acyl)-ethanolamine, a nontoxic antitumor preparation. Biochem Int 1991; 23(2):215-20

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## RESULTS AND DISCUSSION

The challenge exposure to App induced infection in all piglets. However, the clinical course of infection and the extent of lesions produced by App differed between groups. In piglets from Group 1 (control) at 6–10 h post-infection, increased respiration rate, dyspnoea, and incipient mild cough were observed. Increased body temperature observed in all 6 piglets was slowly decreasing, but was still elevated above normal values one week post-infection. The clinical signs in piglets from Group 2 (BAF) were similar at 6–10 h post-infection and disappeared during the second day after challenge (**Figure 1**).

**Figure 1: Body temperature of piglets during experiment**

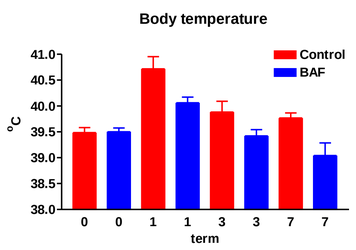
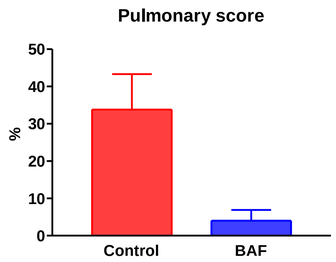



**Figure 2: Postmortem determination of pulmonary score (14 days after challenge)**

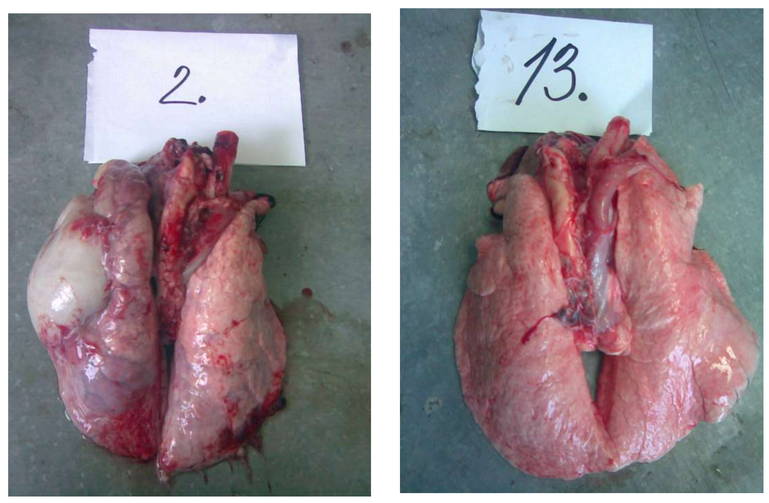



In the peripheral blood of infected animals, total leukocyte counts were increased. Differential leukocyte counts showed that the percentage of lymphocytes was decreased and the percentage of neutrophil granulocytes increased, which was indicative of bacterial infection. The white blood cell counts, including differential cell counts gradually returned to normal between day 1 and 7 post-infection, but the recovery was much slower in Group 1 than in Group 2.

In BALF samples from healthy pigs, nucleated cell count is in the range of 0.8–5 x $10^6$/ml. In differential cell count, the predominant cell types is represented by macrophages (85–98%) followed by lymphocytes (5–10%) and neutrophil granulocytes (0–5%). In postmortem BALF samples, total nucleated cell count increased in both experimental groups, being statistically significantly higher in control Group 1 than in BAF-supplemented Group 2. Analysis of differential leukocyte counts in BALF showed that the percentage of macrophages decreased whereas the percentage of neutrophil granulocytes increased which was also indicative of an ongoing bacterial infection.

Gross post-mortem examination of the lung parenchyma showed that in Group 1 (on average, 34% of tissue was affected) parenchymal lesions were chronic in nature; necrotizing fibrinous pneumonia with or without pleurisy and pericarditis was diagnosed. In Group 2, no case of pleurisy or pericarditis was found and only 4% of the lung tissue was affected on average (**Figure 2, Photo 1**). The difference between groups was statistically significant.

**Photo 1: Illustrative picture of lungs from pig No. 2 of control Group 1 (control) and from pig No. 13 of Group 2 (BAF) - 14 days after challenge**




Serological analysis was carried out to detect the levels of App-specific serum IgM and IgG antibodies and local BALF antibodies of IgA and IgG isotypes. Only in the case of IgA, increased antibody production was detected in the BAF-supplemented Group 2 in comparison with control Group 1. On the other hand, in contrast to BAF-supplemented piglets, animals from control group responded to the infection by producing the pro-inflammatory cytokine IL-1β as detected by ELISA in samples of sera .

*In vitro* incubation with BAF led to a dose- and time-dependent suppression of expression of mRNA for IL-1β, TNF-α, CXCL10 and IL-10 whereas expression of mRNA for arginase-1 remained the same (**Figure 3**). Time dependence was statistically confirmed in case of CXCL10 and IL-10.

Similarly, BAF downregulated procaspase-1 fragmentation, consequently formation of active caspase-1 and thus activation of IL-1β in LPS treatment, depending on BAF concentration and time of incubation. Similar effect of BAF was also detected in the case of phophorylation of PKCε (**Figure 4**).

**Figure 3: mRNA expression of cytokine genes**

Data are expressed as the arithmetic mean of percentage whacks of controls (without BAF) (100%) of each animal cells calculated from S/N values for respective BAF concentrations. S and N are relative expression ratios in LPS-stimulated and non-stimulated samples, respectively. Statistically significant differences ($P < 0.05$, $P < 0.01$ and $P < 0.001$) between the presented data and 100% (control) are marked by *, **and ***, respectively.

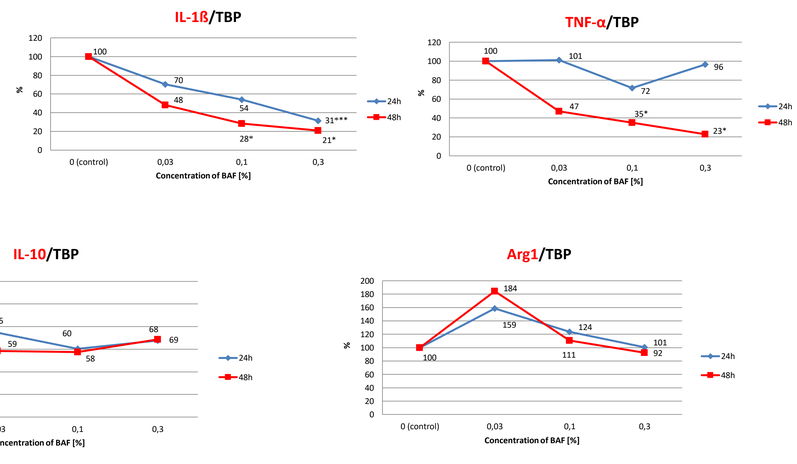



**Figure 4: Downregulation of caspase-1 activation and PKCε phosphorylation by BAF**

The expression of caspase-1 and pPKCε decreased after the highest concentration (0.3%) of BAF comparing with control (C) and cells treated with lower concentrations (0.03% and 0.1%) of BAF. A higher anti-inflammatory effect of BAF was observed after 48h of BAF incubation. β-actin served as loading control.