German Journal of Veterinary Research

eISSN:2703-1322

Short Communication

In vitro effects of non-steroidal anti-inflammatory drugs (Meloxicam and Flunixin Meglumine) and phytochemical (Harpagoside) on the respiratory burst of porcine polymorphonuclear neutrophils (PMNs)

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Article History:
Received: 26-Feb-2021
Accepted: 19-Apr-2021
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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in veterinary medicine. Additionally, interest regarding the anti-inflammatory properties of phytochemicals has emerged in recent years. In the present study, we aim to investigate the *in vitro* effects of meloxicam, flunixin meglumine, and harpagoside on the respiratory burst of porcine polymorphonuclear neutrophils (PMNs). We observed that harpagoside was able to suppress the respiratory burst, similarly to flunixin meglumine. Conversely, meloxicam enhanced the PMNs response. However, these effects were only detected at concentrations higher than those achievable in plasma and tissues. The present study intends to offer insights into the role of these molecules on phagocytosis mechanisms in animals to complement what is already known regarding human PMNs.

Keywords: Swine, Polymorphonuclear neutrophils, Non-steroidal anti-inflammatory drugs, Harpagoside, Respiratory burst

Citation: Marruchella, G., Mosca, F., Hattab, J., Trachtman, A. R., and Tiscar, P. G. 2021. *In vitro* effects of non-steroidal anti-inflammatory drugs (Meloxicam and Flunixin Meglumine) and phytochemical (Harpagoside) on the respiratory burst of porcine polymorphonuclear neutrophils (PMNs). Ger. J. Vet. Res. 1 (2): 8-12 https://doi.org/10.51585/gjvr.2021.2.0008

Introduction

Polymorphonuclear neutrophils (PMNs) play a leading role in innate immunity, and the generation of Reactive Oxygen Species (ROS) represents a powerful antimicrobial tool that promotes pathogen killing by direct oxidative damage (Hampton et al., 1998). Non-steroidal anti-inflammatory drugs (NSAIDs) represent a heterogeneous group of therapeutic agents. Their main mechanism consists of the inhibition of cyclooxygenase enzymes (COX), but additional anti-inflammatory mechanisms have been described in PMNs (Díaz-González and Sánchez-Madrid, 2015).

In recent decades the immunoregulatory properties of phytochemicals have been investigated with great interest in human medicine. Iridoid glycosides are a group of chemical compounds known to have anti-inflammatory properties, particularly abundant in extracts of the tuberiferous xerophyte "devil's claw" [Harpagophytum procumbens (Hp)]. Although Hp extracts consist of a heterogeneous mixture of iridoid glycosides (i.e., harpagoside, procumbide, harpagide, and 8-para-coumaroyl-harpagide), their anti-inflammatory activity is mainly related to the harpagoside content (Menghini et al., 2019). In the present study, we aim to investigate the in vitro effects of meloxicam, flunixin meglumine and harpagoside on the respiratory burst of porcine circulating PMNs, considering that little data is available in veterinary literature about the influence of these molecules on phagocytosis mechanisms.

Materials and Methods

Ethical approval

All samples were collected from animals routinely slaughtered in an abattoir approved by the European Community (Centro Carni Val Tordino, CE IT 2425 M, Mosciano Sant'Angelo, Teramo, Italy). Slaughtering procedures were performed strictly respecting the European legislation about the protection of animals at the time of killing (European Community Council Regulation No 1099/2009).

Animals and PMNs isolation

A total of 35 pigs were included in the present study. Pigs were homogeneous in terms of breed (Landrace x Large White), age (9-10 months), and body weight (155-165 kgs). Animals were characterized by normal clinical presentation and absence of pathological findings at postmortem examination. Pigs were regularly slaughtered at a commercial abattoir, and blood was collected just after jugulation directly from the draining carotid artery into EDTA-containing tubes. Sam-

ples were refrigerated at 4°C and referred to the laboratory within 15 min.

PMNs were isolated according to previous methods (Bonilla et al., 2020) with slight modifications. Briefly, blood samples were diluted with Phosphate Buffered Saline (138 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.2, 1:1 v/v), layered onto Histopaque®-1077 (Merck, Germany), and centrifuged at 400 xg for 30 min. The pellet was resuspended and incubated in lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, 0.12 mM EDTA, pH 7.4) to completely remove the erythrocytes. After additional centrifugation at 400 ×g for 10 min, a pellet mainly containing PMNs was obtained, washed twice, and finally resuspended in Phosphate-Buffered Saline (PBS). The purity of PMNs from each sample was microscopically assessed by a modified Giemsa stain (Hemacolor® rapid stain kit, Merck, Germany) by direct counting cells in at least 10 different microscopic fields. In addition, an automated cell analyzer (Vi-Cell, Beckman Coulter, USA) was used to measure the concentration of the PMNs from each sample and the cell viability using the trypan blue dye exclusion method. Finally, the PMNs concentration was adjusted to $5x10^6$/ml.To evaluate the effect of the single molecules on the cell viability and count, PMNs of each sample were incubated for 2 hrs with 400 μg/ml of meloxicam, flunixin meglumine, and harpagoside (Merck, Germany)(i.e., the highest concentrations used in the present study). Then, the viability and concentration of the PMNs were measured by a cell analyzer (Vi-Cell®, Beckman Coulter, USA).

Effects of single molecules on the respiratory burst of PMNs

The effect of the single molecules on the respiratory burst of PMNs was evaluated employing a chemiluminescence-based assay (Di Teodoro et al., 2018). PMNs suspensions from each pig were aliquoted, placed in 96-well plates ($1x10^6$ cells/well) and incubated with harpagoside, flunixin meglumine and meloxicam at different concentrations (1, 10, 50, 100, 200, 400 µg/ml in PBS) for 2 hours at 37°C. Next, the PMNs were washed twice and resuspended with PBS supplemented with 1 mM CaCl2, 0.2 mM MgCl2, and 5 mM glucose. After pre-incubation for 10 min with 1 mM luminol (Merck, Germany), the PMNs respiratory burst was stimulated with Saccharomyces cerevisiae (Baker's yeasts) at the final concentration of $5x10^7$ yeasts cells/well for 2 hrs.

The chemiluminescence was monitored by a multimode plate reader (Sinergy H1, Bio-Tek, USA) for 2 hours, with 1 min time intervals between consecutive readings of the same well. The intensity of the chemiluminescence was calculated by integrating the area under curve (AUC). Data from all animals were aggregated and graphically reported as the mean AUC ± standard deviation (SD) for each concentration of the molecules. For each sample, positive control was represented by yeasts-stimulated PMNs, which were not previously incubated with the molecules. On the other hand, the negative control was represented by non yeasts-stimulated PMNs, both previously incubated and not with the molecules. Blank controls were represented by wells containing only luminol and by wells containing luminol plus the single molecules. All the reagents were purchased from Sigma-Aldrich, except for the PBS and lysis buffer, which were homemade.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test (XLSTAT software). The level for accepted statistical significance was $p<0.01$.

Results

The purification protocol yielded PMNs suspensions with a mean purity and viability ≥90% for all blood samples under investigation. The exposure of PMNs to the highest concentration of the single molecules (400 μg/ml) did not induce significant changes in their viability or count (data not shown).

As graphically shown in Figure 1, pre-incubation with higher concentrations of harpagoside (between 100 to 400 μg/ml) significantly decreased the chemiluminescence response of PMNs challenged with yeasts in comparison to positive control ($p<0.01$).

Similarly, flunixin meglumine was able to inhibit the respiratory burst ($p<0.01$) at concentrations ranging from 50 to 400 μg/ml when compared with the positive controls (Figure 2). On the contrary, meloxicam induced a dose-dependent increase of the chemiluminescence response, significant ($p<0.01$) at 100, 200, and 400 μg/ml (Figure 3).

Discussion

Little data is currently available regarding the anti-inflammatory activity of phytoextracts on PMNs of domestic animals (Kori et al., 2009). The present study indicated that in vitro harpagoside was able to suppress the respiratory burst of porcine PMNs, as was previously described by other authors in a macrophage cell line (Huang et al., 2006) and murine peritoneal macrophages (Gyurkovska et al., 2011). The molecular basis of harpagoside's effect on the respiratory burst is still largely unknown, but it is unlikely to be related to any scavenger property towards ROS (Grant et al., 2009). Instead, it more likely could be mediated by the inhibitory effects on NADPH-oxidase (Mahomoodally et al., 2012) and myeloperoxidase (Tsumbu et al., 2012).

In agreement with previous studies (Qi et al., 2006; Gyurkovska et al., 2011), we observed a significant effect of harpagoside at concentrations of ≥100 μg/ml, which are much higher when compared with the plasma levels (25-55 ng/ml) detected in experimental animal studies (Axmann et al., 2019). However, as prolonged duration therapy is recommended for humans, the cumulative dosing effects must be considered *in-vivo* (Grant et al., 2009). In addition, other iridoid glycosides in the Hp extracts could contribute to the anti-inflammatory activity of the harpagoside through additive mechanisms (Abdelouahab and Heard, 2008). Flunixin meglumine is a non-selective inhibitor of COX enzymes; however additional anti-inflammatory

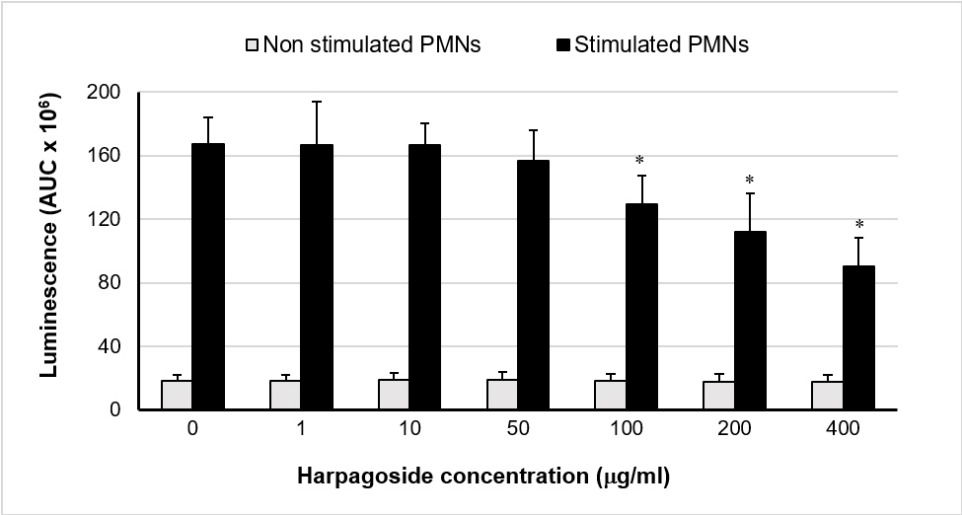

Figure 1: The luminescence response of yeasts-stimulated PMNs after pre-incubation with harpagoside at different concentrations for 2 hrs. The negative control corresponded to PMNs w/o stimulation. The positive control was represented by PMNs stimulated but not pre-incubated with harpagoside (*p<0.01 vs. positive control). Data represent the mean aggregate values of the PMNs luminescence from 15 animals ±SD

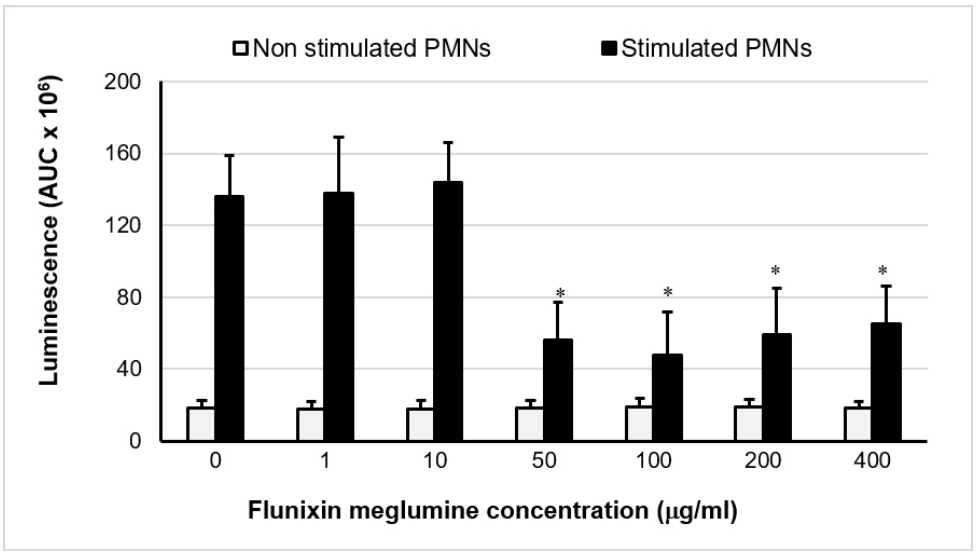

Figure 2: The luminescence response of yeasts-stimulated PMNs after pre-incubation with flunixin meglumine at different concentrations for 2 hrs. The negative control corresponded to PMNs w/o stimulation. The positive control was represented by PMNs stimulated but not pre-incubated with flunixin meglumine (*p<0.01 vs. positive control). Data represent the mean aggregate values of the PMNs luminescence from 10 animals ± SD

mechanisms have also been presumed. In this regard, Flunixin meglumine decreases the serum levels of cytokines (Bednarek et al., 2003) and acute-phase serum proteiens during inflammation (Peters et al., 2012).

It also inhibits the activation of the nitric oxide synthase gene (Bryant et al., 2003) and more generally, seems to possess antioxidant activity (Konyalioglu et al., 2007).

Meloxicam is considered to be a COX-2 preferential NSAID in several species. As with the activities observed for FM, additional anti-inflammatory actions have been described (Bednarek et al., 2003; Van Engen et al., 2014). In the present study, Flunixin meglumine exerted a negative influence on the luminescence activity of PMNs, whereas meloxicam strongly enhanced the PMNs response. The opposite effects of different types of NSAIDs on the respiratory burst were previously described in human PMNs (Parij et al., 1998). The ability of some NSAIDs to inhibit NADPH-oxidase was considered a primary mechanism for the inhibition of the respiratory burst in PMNs (Umeki, 1990). Besides, scavenging effects on free radicals have been described for various NSAIDs (Fernandes et al., 2004). On the contrary, the ability of some NSAID to enhance the luminescence response of PMNs has been thought to be an indirect consequence of COX inhibition, thus causing the accumulation of arachidonic acid, which shifts versus LOX pathways with the generation of leukotrienes (Leone et al., 2007).

In the present study, the effects of NSAIDs were

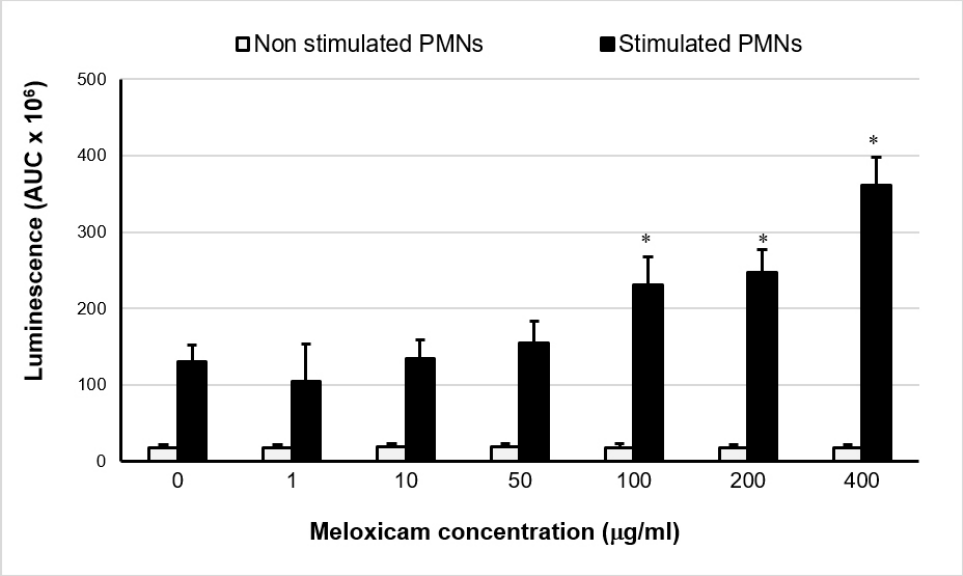

Figure 3: The luminescence response of yeasts-stimulated PMNs after pre-incubation with meloxicam at different concentrations for 2 hrs. The negative control corresponded to PMNs without stimulation. The positive control was represented by PMNs stimulated but not pre-incubated with meloxicam (*p<0.01 vs. positive control). Data represent the mean aggregate values of the PMNs luminescence from 10 animals ± SD

observed at concentrations of ≥50 μg/ml and ≥100 μg/ml for flunixin and meloxicam, respectively. These levels are not achieved in plasma or target organs. Indeed, peak plasma concentrations in pigs after intramuscular administration are in the order of 1.8-2.8 μg/mL for flunixin meglumine and 0.7-2.8 μg/mL for meloxicam. In contrast, tissue concentrations are in the order of ng/g (Nixon et al., 2020). However, the accumulation of NSAIDs in inflamed tissues represents a variable that should be considered (Brune, 2007).

In conclusion, the present study highlights that NSAIDs are characterized by additional effects with a different activity profile on inflammatory cells in animals, as seen in investigations in human medicine. To the best of our knowledge, this is the first study focusing on the effects of harpagoside on the respiratory burst of PMNs in domestic animals.

Article Information

Funding. This research received no funds.

Conflict of Interest.The authors declare no conflict of interest.

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