Effects of inactivated *Parapoxvirus ovis* on polymorphonuclear leukocyte function and myeloperoxidase activity in horses

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ABSTRACT: Immunomodulatory products have been used for years in veterinary medicine. Inactivated *Parapoxvirus* ovis (iPPVO) is currently used in equine medicine as an immunomodulator to improve the immune system and as a prophylactic treatment to prevent or treat infectious diseases. This study was designed to determine the effects of iPPVO on polymorphonuclear leukocyte (PMNL) function (phagocytosis and intracellular killing activity) and the myeloperoxidase (MPO) activity of PMNLs in horses. Twenty-four healthy English thoroughbred horses with an average age of 11 years were included in the study. Venous blood samples (10 ml) were taken before (agent-free controls) and after the administration of iPPVO (2 ml *i.m.* injection on Days 1, 3, and 5). PMNLs (1×10^7 cells/ml) were isolated from venous blood containing EDTA (0.1 g/ml) with Ficoll-Hypaque gradient centrifugation. Cellular phagocytosis and intracellular killing activities were assayed using a modification of Alexander's method before and after treatment with iPPVO. MPO activity was also measured. The administration of iPPVO significantly increased the phagocytic, intracellular killing, and MPO activities of equine PMNLs ($P = 0.0058$, $P = 0.0050$, and $P = 0.0070$, respectively). This study demonstrates a strong correlation between MPO activity and PMNL function. The administration of iPPVO to horses has a supportive effect on their cellular immunity and an immunomodulatory effect against equine viral infections.

Keywords: equine; inactivated *Parapoxvirus ovis*; phagocytosis; intracellular killing activity; myeloperoxidase

Immunomodulatory agents and substances have been successfully used in medicine for centuries. Until recently, their use has been considered controversial and their mechanisms of action have not been fully understood (Weber et al. 2007). Their activity is based on the activation of innate cells and consequent cytokine production. Inactivated *Parapoxvirus ovis* (iPPVO) stimulates and regulates the secretion of cytokines by leukocytes (Rush and Flaminio 2000).

The immune system can be modulated in different ways (endogenously or exogenously) depending on the type of biological agent used to stimulate the cellular signalling events, and is typically termed 'immunomodulation'. The immunostimulator PPVO induces a non-specific enhancement of the immune system (Adams and Horohov 2013).

Inactivated *P. ovis* shows strong immunomodulatory activity in several species and is used in veterinary medicine as an immunostimulatory biological agent for the prevention and/or treatment of infectious diseases (Schutze et al. 2009). PPVO stimulation has been shown to induce strong antiviral activity in laboratory animal models both *in vitro* and *in vivo*. Its use as an immunomodulator has been recommended for many conditions, from canine mammary tumours, feline leukaemia, equine sarcoidosis, to furunculosis in rainbow trout. Its currently approved indication is to reduce herpesvirus respiratory infections in horses. It is well documented that some viruses induce vigorous immune reactions (Weber et al. 2003; Ziebell et al. 1997).

The immunomodulatory properties of PPVO are mediated by its activation of the innate immune response which results in the recruitment and activation of natural killer cells and neutrophils (Horohov et al. 2008). Polymorphonuclear leukocytes (PMNLs) include neutrophils, eosinophils and basophils, and PMNLs are the first cells to be activated in the host immune response to infection. The primary function of PMNLs is phagocytosis and destruction of microorganisms. The release of myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) into phagosomes containing ingested microorganisms usually has a rapid microbicidal effect (Castrucci et al. 2000).

MPO participates in innate immune defense by forming microbicidal reactive oxidants and diffusible radical species. Neutrophils contain MPO, and the enzyme is released during their degranulation or lysis. MPO catalyzes the reaction between H_2O_2 and chloride ions to form hypochlorous acid, a highly oxidising agent, to kill intracellular microorganisms (Malle et al. 2007; Prokopowicz et al. 2012).

A biological response modifier can have immunomodulatory effects that increase, reduce, or do not affect the immune system, depending on the aim of the therapy. There has been limited research into the immunomodulatory efficacy of iPPVO on PMNL function (phagocytosis and intracellular killing) either *in vivo* or *in vitro*, or into the MPO activity of PMNLs in horses. The aim of our study was to investigate whether iPPVO affects the phagocytosis, intracellular killing activity and MPO activity of PMNLs in horses.

MATERIAL AND METHODS

Immunomodulatory agent. A preparation of chemically inactivated *Parapoxvirus ovis* (iPPVO) strain D 1701 (commercially known as Zylexis®, Pfizer Animal Health), was used for non-specific immunisation.

Animals. Twenty-four healthy English thoroughbred horses from the Vocational School of the Faculty of Veterinary Medicine at Istanbul University were included in this study and the animals were treated in compliance with ethical standards. The mean age of the 24 horses was 11 (± 0.6) years. The horses had free access to water and were fed forage (hay) and concentrates (oat, barley, corn, bran) in their stables. Horses were exercised for 20 min two times per day. iPPVO was administered at a dose of 2 ml by intramuscular (*i.m.*) injection under aseptic conditions on Days 1, 3, and 5, according to the therapeutic dose recommended by the manufacturer. Venous blood samples (10 ml) were taken from the horses by jugular vein puncture into heparinised Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA; 0.1 g/ml) before (as the agent-free control) and after treatment with iPPVO, on Days 0 and 7. The PMNL function of the horses after treatment with iPPVO was determined and the values were compared with those of the agent-free controls. The MPO activity of the PMNLs before and after treatment with iPPVO was also measured and compared.

Preparation of PMNLs. PMNLs were isolated from the venous blood using the Ficoll-Hypaque gradient centrifugation method (Alexander et al. 1968; Barbior and Cohen 1981). In this study, we used a method to evaluate neutrophil function modified from that of Alexander et al. (1968) to measure PMNL functions. In this modified method, Ficoll was used rather than dextran and the PMNLs were counted under a microscope rather than with the standard pour plate technique.

Phagocytosis and intracellular killing activity. Whole blood in 0.1 g/ml EDTA was centrifuged at 1050 × *g* for 30 min. The buffy coat layer was removed and transferred to a solution of Ficoll–Hypaque and Polymorphoprep™ and centrifuged at 1510 × *g* for 30 min to isolate the PMNLs. After centrifugation, the PMNL layer was removed, washed three times in ice-cold phosphate-buffered saline (PBS) and resuspended in Hanks's buffered salt solution (HBSS) at a cell density of 1×10^7cells/ml. The viability of the PMNLs was tested with the Trypan blue exclusion method (0.5% Trypan blue in 0.9% saline solution) by counting the stained (dead) and unstained (live) cells on a haemocytometer (Alexander et al. 1968; Barbior and Cohen 1981).

A clinical strain of *Candida albicans* was used to determine the phagocytosis and intracellular killing activities of the PMNLs. The isolate was grown on Sabouraud agar plates for 24 h at 35 °C before the experiments. Under these conditions, *Candida* cells only form blastoconidia, with no germ tubes or pseudohyphae. The viability of the yeast cells was determined with methylene blue (0.01%; Sigma) staining. The yeast cells were suspended in HBSS and their concentration was adjusted to 1×10^7 CFU/ml after counting with a haemocytometer. Pooled human serum was added

to the suspension of *C. albicans* at a ratio of 4 : 1, and the mixture was incubated at 38 °C for 30 min to opsonise the yeast cells. The PMNLs collected from equine blood were incubated for 30 min at 38 °C in a shaking incubator. The opsonised yeast cells were then added to the PMNLs and the final cell densities of the mixture were 5×10^6 PMNLs/ml and 5×10^6 yeast cells/ml. The intracellular destruction of the blastoconidia was tested with methylene blue staining (0.01%). Wet mounts were prepared and the phagocytic activity of the PMNLs was determined by counting the PMNLs that ingested live and dead yeast cells. Intracellular killing activity was measured by counting the PMNLs that contained killed yeast cells on a slide under a microscope. The results are expressed as percentages (Alexander et al. 1968; Barbior and Cohen 1981). All samples were analysed in triplicate.

Preparation of PMNL homogenates. The PMNL suspension was frozen at –80 °C and thawed six times, then homogenised with a motor-driven Teflon® glass homogeniser (13 584 × *g* for 5 min at 0 °C) (Kurutas et al. 2005).

Measurement of PMNL MPO activity. The MPO activity of PMNLs isolated from blood samples taken before and after treatment with iPPVO was measured in a procedure similar to that documented by Hillegas et al. (1990). The protein content of the homogenate was measured with Lowry's method (Lowry et al. 1951). Two tubes were used in the experiment. Into the control tube was placed 10 µl of K_2HPO_4 (pH 6), and into the test tube was placed 10 µl of PMNL homogenate. Into each tube was then added 0.3 ml of 0.1M K_2HPO_4 (pH 6, 0.3 ml of 0.01M H_2O_2 and 0.5 ml of 0.02M *o*-dianisidine. The final volume was made up to 3 ml with distilled water and the samples were placed in a shaking water bath at 38 °C for 3 min. The colour reaction in each tube was then stopped with 0.5 ml of 2% sodium azide. The tubes were removed from the water bath and each tube was centrifuged at 41 400 × *g* for 10 min at 4 °C and 0.3 ml was taken from the upper phase of each tube. The samples were then read spectrophotometrically three times at 460 nm and the mean values were calculated. All measurements were made in duplicate. One unit of MPO was defined as that causing an increase in absorbance of 0.001 per min and its specific activity was given in IU/mg protein (Kettle and Winterbourn 1991; Hartert et al. 1998; Klebanoff 2005; Senoglu et al. 2009).

Statistical analysis. The data were expressed as means ± SD (standard deviations). The statistical analyses were performed using the paired *t*- test and Student's *t*-test. *P*-values ≤ 0.05 were considered statistically significant (GraphPad InStat, version 3.05).

RESULTS

Phagocytosis and intracellular killing activity

iPPVO (2 ml dose given *i.m.*) significantly increased the phagocytic and intracellular killing activities of PMNLs in healthy horses ($P = 0.0058$ and $P = 0.0050$, respectively) compared with their activities before the administration of iPPVO (Table 1).

Measurement of PMNL MPO activity

iPPVO (2 ml dose given *i.m.*) significantly increased the MPO activity of PMNLs in healthy horses ($P = 0.0070$) compared with their MPO activity before the administration of iPPVO (Table 1).

DISCUSSION

Immunostimulants have been defined as agents that stimulate the responses of effector immune cells, including macrophages, lymphocytes, and

Table 1. Comparison of polymorphonuclear leukocyte (PMNL) function (phagocytosis and intracellular killing activity) and myeloperoxidase (MPO) level of PMNLs in horses before and after administration of iPPVO

	n	Phagocytic activity (%)	Intracellular killing activity (%)	MPO level (IU/mg protein)
Before iPPVO	24	52.875 ± 7.177	6.833 ± 2.04	269.7 ± 47.9
After iPPVO	24	57.333 ± 4.135*	8.375 ± 1.689**	310.5 ± 56.3***

*$P = 0.0058$, **$P = 0.0050$, ***$P = 0.0070$ (the results were expressed as means ± SD; statistical analysis was performed using the paired *t*-test and Student's *t*-test)

neutrophils, which then activate one or more terminal immune responses, such as antigen uptake, cytotoxicity, phagocytosis, cytokine release, and the antibody response (Rush and Flaminio 2000). Some of the drugs used in the treatment of clinical infections can overcome viral resistance and some well-known antiviral drugs exert their immunomodulatory effects by interacting with neutrophils. However, although some drugs suppress PMNL functions, others do not affect them at all (Boogaerts et al. 1986).

iPPVO stimulates and regulates the secretion of cytokines by leukocytes. Its mode of action is based on a non-antigen-specific interaction with the innate and/or adaptive immune responses (Paillot 2013). Novel immunomodulatory agents that are based on active or inactivated poxviruses might have therapeutic potential in various diseases in which the immune system is unbalanced (Weber et al. 2007).

The stimulation of endogenous, non-antigen-related defense mechanisms by poxvirus- and parapoxvirus-based immunomodulators offers new possibilities for the control of infectious diseases in domestic animals. Several *in vitro* and *in vivo* studies, as well as controlled clinical trials and studies under field conditions, have shown the efficacy of these agents in target animals, such as cattle, pigs, dogs, cats, and horses. Prophylactic and metaphylactic treatments with immunomodulators can significantly reduce the incidence of infection in stressful situations (e.g., crowding, transport) or in immunodeficient animals, such as newborn foals. Beneficial effects are even observed when an immunomodulator is used as an additional treatment for infectious diseases, such as equine herpesvirus infections (Glitz 2003).

As an immunomodulator, PPVO has been shown to limit the severity of infectious disease outbreaks among horses and to enhance their cell-mediated immunity (Adams and Horohov 2013). The efficacy of PPVO as an antiviral immunotherapy for the treatment of specific infections has also been demonstrated. Weber et al. (2003) showed that Balb/c mice treated intraperitoneally with PPVO were protected from a lethal intranasal challenge with *Herpes simplex* virus. Similarly, PPVO treatment reduced the severity of herpes disease in guinea pigs and the degree of *Hepatitis B virus* replication in a transgenic mouse model. Therefore, PPVO might also effectively limit the severity of infectious disease outbreaks among horses under crowded conditions (Ziebell et al. 1997).

A study that investigated the effects of iPPVO on horses suffering from *Equine herpesvirus* 1 infection demonstrated an actual reduction in their clinical signs, represented by nasal discharge, rather than a reduction in viral shedding (Said et al. 2011). In their study, Horohov et al. (2008) found that the treatment of horses with a three-dose regimen of PPVO elevated the expression of the interferon (*IFN*) gene, which was detected 24 h after the first dose and declined thereafter. Their results indicated that PPVO stimulates IFN production *in vitro*, and the consequent increased cytokine expression could account for its immunomodulatory activity.

iPPVO, used as a model immunomodulatory agent in our study, significantly increased both the phagocytosis and intracellular killing functions of PMNLs in horses relative to the levels in the agent-free control PMNLs. These results are consistent with the results of Schutze et al. (2009), who investigated the immunostimulatory capacity of iPPVO on the innate immune system *in vitro* by evaluating the induction of the oxidative burst and the modulation of phagocytosis by canine blood leukocytes (PMNLs and monocytes) in dogs. After stimulation with iPPVO, the phagocytosis of fluorescein-isothiocyanate-labeled *Listeria monocytogenes* increased in canine blood, demonstrating that iPPVO stimulates PMNL functions in canine blood leukocytes. Since then, iPPVO stimulation has been shown to enhance the phagocytic activity of canine neutrophils and monocytes in a dose-dependent manner (Schutze et al. 2010).

There is only little known regarding the immunomodulatory efficacy of iPPVO on PMNL functions *in vivo* or *in vitro*. However, *in vitro* experiments have shown that iPPVO stimulates both phagocytic and T-cell-dependent immune mechanisms directed against bacteria in canine blood leukocytes (Schutze et al. 2009). In one study of horses, two commercial immunostimulants, iPPVO and inactivated *Propionibacterium acnes*, significantly enhanced the functions of phagocytic cells after *ex vivo* exposure to virulent *Rhodococcus equi* (Ryan et al. 2010). These data are in agreement with the results of our study.

The MPO activity of PMNLs in horses and their phagocytic and intracellular killing activities increased significantly after treatment with immunomodulatory iPPVO ($P = 0.0070$, $P = 0.0058$, and $P = 0.0050$, respectively). This increase in the PMNL functions of horses after administration of iPPVO represents one of the key results of our study.

iPPVO could alter oxidative stress parameters leading to lipid peroxidation by increasing malondialdehyde concentrations and reducing blood glutathione concentrations. Considering the possible relationships between these indicators and the immune response, further investigation into changes in these indicators is warranted (Kart et al. 2010). MPO-catalysed oxidative events are now considered as a major source of oxidative stress, and therefore oxidative reactions are directly related to inflammation (Malle et al. 2007).

PMNLs play an important role in the oxygen-dependent microbicidal system and contain MPO, which is released during their degranulation or lysis (Ponthier et al. 2012; Prokopowicz et al. 2012). No previous study has shown an interaction between PMNL functions and MPO activity in horses. In our study, the MPO activity of PMNLs in horses increased significantly after treatment with the immunomodulator iPPVO.

The results of our *in vitro* study show that iPPVO significantly increased the functions of PMNLs (phagocytic and intracellular killing activity) and their MPO activity in horses. The former result was not unexpected, but there has been limited data available on the effect of iPPVO on MPO activity. Therefore, MPO may play a significant role in the clinical activity of iPPVO against equine viral infections.

In this study, a significant correlation between MPO levels and PMNL functions was demonstrated. We conclude that iPPVO has a supportive effect on the cellular immunity of horses and an immunomodulatory effect on equine viral infections. It is likely that PMNLs contribute to the tissue damage observed in inflammatory and vascular diseases, and therefore, immunomodulatory agents that modulate PMNL activity could be of future therapeutic benefit.

In conclusion, iPPVO significantly increased both PMNL functions and MPO activity in horse PMNLs, suggesting the existence of a relationship between PMNL function and MPO activity. Treatment with iPPVO supported the cellular immunity of healthy English thoroughbred horses, increasing the MPO activity and microbicidal functions of their PMNLs. The approach of inducing oxidative enzymes with inactivated viruses might have advantages over existing immune therapies. Therefore, iPPVO could become the preferred immunomodulatory agent for the treatment of viral infections and should be investigated further so that its potential utility as an antiviral therapy can be more fully explored.

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