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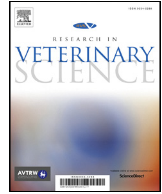

In Vitro effects of tamoxifen on equine neutrophils

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ABSTRACT

Neutrophils participate in innate immunity as the first line of host defense against microorganisms. However, exacerbated neutrophil activity can be harmful to surrounding tissues; this is important in a range of diseases, including allergic asthma and chronic obstructive pulmonary disease in humans, and equine asthma (also known as recurrent airway obstruction (RAO). Tamoxifen (TX) is a non-steroidal estrogen receptor modulator with effects on cell growth and survival. Previous preliminary studies showed that TX treatment in horses with induced acute pulmonary inflammation promoted early apoptosis of blood and BALF neutrophils, reduction of BALF neutrophils, and improvement in animals' clinical status. The aim of this study was to evaluate the *in vitro* effect of TX on functional tests in equine peripheral blood neutrophils. Chemotaxis, respiratory burst production and phagocytosis assays were performed on neutrophils isolated from peripheral blood samples from 10 healthy horses. Results showed that IL-8 stimulated cells decrease their chemotactic index when treated with TX (1 and 10 μM). Respiratory burst production was also dampened after treatment with TX. In conclusion, these results confirm that tamoxifen has a direct action on equine peripheral blood neutrophils. However, more *in vivo* and *in vitro* studies are required to fully understand the mechanisms of action of TX on neutrophils, in order to elucidate if it can be used as treatment in disorders such as allergic asthma in humans and horses.

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1. Introduction

Neutrophils play a central role in innate immunity, acting as the first line of host defense against invading organisms. They are the predominant cell type involved in the cellular phase of acute inflammation (Cassatella, 1999). They migrate to the site of infection or inflammation for containment and clearance of invading agents (Nathan, 2006). At this site, several locally-generated messenger molecules attract leukocytes from blood and direct their migration towards microbes, in a process termed chemotaxis (Baggiolini et al., 1993). This is regulated by chemokines, pro-inflammatory mediators that regulate leukocyte trafficking, among other functions (Sanz and Kubes, 2012). Once neutrophils encounter the inciting pathogen, they engulf them into a phagosome that fuses with intracellular granules to form a phagolysosome. Within the phagolysosome, pathogens are killed through exposure to enzymes, antimicrobial peptides and reactive oxygen species (ROS) (Mayer-Scholl et al., 2004). ROS are produced in what is called "respiratory burst", during which the NADPH oxidase complex assembles at the phagosomal membrane and produces O_2^-, which is readily converted to hydrogen peroxide by the enzyme superoxide dismutase (Chapman et al., 2002). It is believed that neutrophils in inflamed tissues preferentially undergo apoptosis after completing their function. This process would prevent release of cytotoxic products –such as ROS or proteases– that would contribute to tissue damage (Nathan, 2006). Neutrophil apoptosis leads to the expression of cell surface "eat me" signals such as phosphatidylserine, enabling neutrophils to be recognized and cleared by scavenger macrophages (Savill et al., 1989). When phagocytosis of apoptotic neutrophils is impaired, neutrophils undergo secondary necrosis and release their cytotoxic products, aggravating tissue injury and amplifying the inflammatory response (Filep and El Kebir, 2009). Therefore, in terms of resolution of inflammation, neutrophil apoptosis holds a central position as it brings sustained neutrophil recruitment to an end, while the phagocytic clearance of apoptotic neutrophils reprograms macrophages to an anti-inflammatory phenotype, which is characterized by the release of mediators that suppress the inflammatory response, such as transforming growth factor-β (TGF-β) and IL-10, thus contributing to the restoration of homeostasis after tissue injury or infection (Soehnlein and Lindbom, 2010).

One of the diseases in which neutrophils play an important role in the equine airways is asthma, previously termed recurrent airway obstruction (RAO) (Bullone and Lavoie, 2015, Couetil et al., 2016; Pirie et al., 2016). Equine asthma develops in mature horses following stabling and exposure to dusty hay and straw (Robinson, 2001). The disease is characterized by pulmonary neutrophilia and excessive

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mucus production, resulting in reduced dynamic lung compliance and increased pulmonary resistance and pleural pressure excursions (Derksen et al., 1985; Jackson et al., 2000). Glucocorticoids (GCs) are currently the most potent anti-inflammatory drugs for the pharmacological control of equine asthma (Ivester and Couetil, 2014). However, systemic administration of corticosteroids to horses has been associated with several systemic adverse effects, including adrenocortical suppression and dysfunction (Rush et al., 1998; Picandet et al., 2003; Dowling et al., 1993), laminitis and hepatopathy (Ryu et al., 2004), muscle wasting (Cohen and Carter, 1992), altered bone metabolism (Lepage et al., 1993), and increased susceptibility to infection (Cutler et al., 2001). Systemic corticosteroids have also been shown to affect the equine immune system by inducing transient peripheral neutrophilia and lymphopenia (Burguez et al., 1983), changes in lymphocyte subpopulations and expression of activation markers (Flaminio et al., 2009), as well as by decreasing the antibody response to vaccination (Slack et al., 2000). Moreover, there is a variable resistance to GC treatment in a number of human inflammatory diseases in which neutrophils are considered as the primary offender, such as COPD (Chronic Obstructive Pulmonary Disease), cystic fibrosis, severe neutrophilic asthma, and certain types of cancers, among others (Marchette et al., 1985; Dibbert et al., 1999; Vignola et al., 1999; Barnes, 2007). Glucocorticoids may even prolong neutrophil survival by suppression of apoptosis (Liles et al., 1995; Tintinger et al., 2013). This has prompted a search for non-steroidal drugs with the potential of modulating neutrophilic inflammation, and lesser side effects than steroids.

Tamoxifen (TX) is a synthetic non-steroidal anti-estrogen agent widely used for treating all stages of breast cancer, and has been approved for the prevention of breast cancer in high-risk women. The observed efficacy of TX has been attributed both to growth arrest and to the induction of apoptosis (Budtz, 1999; Cameron et al., 2000). However, Duffy et al. (2003) suggest that TX is worthy of further clinical trials because it might be useful in the treatment of mast cell-mediated diseases and other serious diseases, such as refractory asthma, pulmonary fibrosis, and mastocytosis. Other results shows that prophylactic treatment with TX interferes with all aspects of the allergic immune response, leading to a reduction of allergen-specific Ig levels in mice (IgE, IgG1 and IgG2a), a skewing effect in the T cell compartment with the inhibition of IL-4 and an abrogation of ear swelling responses in dermatitis (Babina et al., 2010). Our research group has previously shown that TX increased early apoptosis of equine peripheral blood and bronchoalveolar lavage fluid (BALF) granulocytic cells *in vitro* (Sarmiento et al., 2013). Furthermore, our data suggests that TX has the ability to induce apoptosis of granulocytic cells from peripheral blood and BALF obtained from horses with acute lung inflammation, with a concomitant improvement in their clinical status (Perez et al., 2016). Based on the data exposed above, we hypothesize that the clinical improvement in horses treated with TX was not only due to its effects on apoptosis, but also due to an effect on other neutrophilic functions. Therefore, the aim of this study was to evaluate the *in vitro* effect of TX on chemotaxis, respiratory burst production and phagocytosis of equine peripheral blood neutrophils.

2. Materials and methods

2.1. Horses

Ten clinically healthy adult horses ranging in age from 8 to 12 years, belonging and housed at Universidad Austral de Chile veterinary teaching hospital were enrolled in this study. There were five mares and geldings respectively, of mixed breed, weighing 420–450 kg. They were grass fed with free access to water. Physical examinations were performed before sample collection for the duration of the study by qualified veterinarians, to ensure that the animals were healthy. All procedures were approved by the Universidad Austral de Chile Bioethics Committee for the Use of Animals in Biomedical Research.

2.2. Blood sampling and neutrophil isolation

The isolation of blood leukocytes was done as previously described by our group (Perez et al., 2016). Briefly, 10 mL of blood obtained by jugular venipuncture was placed in sterile tubes containing 1 mL of 3.8% w/v trisodium citrate. Blood was placed on a discontinuous density gradient (Percoll® GE Healthcare), with 4 mL of 85% Percoll in the bottom of a 15 mL tube and 4 mL of 70% Percoll above. After centrifugation (45 min, 670 g), the upper layer contained mononuclear cells and the lower layer contained granulocytes. Both layers were aspirated for further processing. Cells were subsequently prepared for bioassays with different concentrations of TX for evaluation of chemotaxis, respiratory burst production and phagocytosis. Each assay, and each variable of each assay, was repeated ten times.

2.3. Tamoxifen

Tamoxifen was used at concentrations extrapolated from human data; chronic treatment with 20 mg of TX in women generates steady-state plasma concentrations of 2.5–5 μM (Stearns et al., 2003; Kisanga et al., 2004). Chemotactic response was evaluated with TX 0.01, 0.1, 1 or 10 μM. Respiratory burst production assays were performed with TX 0.1, 0.5, 1, 2.5, 5 or 10 μM. Phagocytosis was assayed with TX 5 μM.

2.4. Chemotactic response

For *in vitro* chemotaxis assays, 12-well Costar Transwell plates (Corning Costar) were used as previously described for equine neutrophils by Van de Walle et al. (2007). Briefly, 700 μL of Ca^{2+} assay buffer (NaCl 25 mM; KCl 5 mM; $MgCl_2$ 0,5 mM; $CaCl_2$ 1 mM; NaH_2PO_4 1 mM; HEPES 25 mM; glucose and bovine serum albumin), either containing 10 nM of IL-8 or not, was placed into the lower chambers of the wells. Wells were covered with a polycarbonate membrane with an 8-μm pore size. A 300-μL cell suspension (containing 1×10^4 cells) and treated with TX (0.01, 0.1, 1 or 10 μM) was added to the top chamber, and assay plates were incubated at 37 °C in a 5% CO_2 humidified chamber for 60 min. The positive control was a cell suspension without TX, and with IL-8 10 nM; the negative control consisted of a cell suspension with neither TX nor IL-8; the drug vehicle, dimethyl sulfoxide (DMSO), was also included as a control on its own. The concentration of DMSO was never higher than 0,1%. After incubation, cells in the lower chamber were stained, counted under a light microscope, and expressed as a chemotaxis index by dividing the amount of cells which migrated in each incubation solution, over the amount of cells which migrated in the control group without IL-8.

2.5. Respiratory burst production

Neutrophil ROS production was evaluated using the luminol-dependent chemiluminescence method adapted for equine neutrophils (Benbarek et al., 1996). Briefly, respiratory burst production by equine neutrophils was stimulated with opsonized zymosan, with or without TX (0.1, 0.5, 1, 2.5, 5 or 10 μM). The drug vehicle, dimethyl sulfoxide (DMSO), was also included as a control on its own. The concentration of DMSO was never higher than 0,1%. Finally, 6.1 mM of luminol (Sigma Chemical Co.) was added to the plates. Chemiluminescence was obtained by the interaction of O^{-}_2 or a dismutation product with luminol, which results in the emission of light that was measured by the luminometer (Perkin Elmer, Victor 2030) during approximately 1 h of incubation at a temperature of 37 °C.

2.6. Phagocytosis

After isolation, neutrophils were centrifuged and resuspended in Ca^{2+} buffer at a concentration of 1×10^6 cells/mL. Cells were maintained at 37 °C in a 5% CO_2 humidified chamber and cultured for

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