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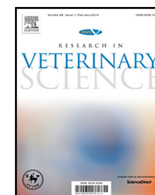

Morphine and buprenorphine do not alter leukocyte cytokine production capacity, early apoptosis, or neutrophil phagocytic function in healthy dogs

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ABSTRACT

Opioids have immunomodulatory properties in many species, but there is little information pertaining to these properties in dogs. Our objective was to compare the in vivo effects of morphine, buprenorphine, and control solution on innate immune system function and apoptosis in healthy dogs. Six adult dogs received a 24-hour infusion of morphine, buprenorphine, or control solution (saline) in a randomized, controlled, crossover block design. Leukocyte apoptosis, phagocytosis, and oxidative burst were evaluated using flow cytometry. Lipopolysaccharide, lipoteichoic acid, and peptidoglycan-stimulated leukocyte production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 were determined using canine specific multiplex assays. No significant treatment effects were detected among groups. These data suggest that healthy dogs could be less sensitive to the immunomodulatory effects of acute opioid administration compared with other species. Larger investigations in healthy and immunologically challenged dogs are recommended prior to application of these results in clinical patients.

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1. Introduction

Opioids are among the world's oldest known drugs and are the most powerful and effective analgesics used in both human and veterinary medicine (Downing, 2011; Koo, 2003; Odunayo et al., 2010; Pascoe, 2000; Stephens, 2003). Clinical indications for opioid administration include the prevention and control of acute pain, such as in the perioperative or emergency setting (Dyson, 2008; Hellyer et al., 2007; Pekcan and Koc, 2010; Quandt, 2013). Opioids are also used for long-term management of chronic pain associated with many common diseases, including osteoarthritis and neoplastic disease (Downing, 2011; Gaynor, 2008; Mathews, 2008).

In addition to their potent analgesic properties, opioids can have a variety of physiologic and systemic effects, including modulation of the immune system. These secondary effects occur following endogenous and exogenous opioid ligand binding to opioid receptors (OP1: delta opioid receptor; OP2: kappa opioid receptor; OP3: mu opioid receptor). Through both central and peripherally mediated mechanisms, or via direct interactions with immune cells, opioids are able to suppress or stimulate the innate and adaptive immune response in humans and rodents (Bidlack, 2000; Eisenstein and Hilburger, 1998; Fecho et al., 1996; Odunayo et al., 2010; Roy et al., 1998a).

The immunomodulatory properties of opioids appear to be dependent upon chemical structure (Filipczak-Bryniarska et al., 2012; Odunayo et al., 2010). For instance, some opioids are more immunosuppressive than others. In human and rodent models, morphine has been shown to increase infection rates and tissue damage by promoting pro-inflammatory mediator production, while buprenorphine is reported to have minimal effects on the immune system (Donaldson et al., 1988; Franchi et al., 2007; Hilburger et al., 1997; Ocasio et al., 2004; Sacerdote et al., 1997, 2000; Tsai et al., 2003; Tseng and Tso, 1993; Welters et al., 2000). Such evidence has raised the question of whether or not strategic selection of opioids in the clinical setting could improve outcomes in critically ill patients or those undergoing invasive procedures.

Despite extensive work in other species, there is little information pertaining to the immunomodulatory properties of opioids in dogs. Investigations in our laboratory have evaluated the in vitro effects of morphine, fentanyl, and buprenorphine on immune function following incubation in canine blood. We found increased oxidative burst intensity of neutrophils following incubation with morphine in a concentration dependent fashion. Morphine, buprenorphine, and fentanyl inhibited neutrophil apoptosis and

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promoted LPS- or lipoteichoic acid (LTA)-induced TNF-α and IL-10 production (DeClue et al., 2014). Given the inherent limitations of in vitro work, further investigations are necessary to evaluate the immunomodulatory properties of opioids in dogs.

The objective of the current study was to compare the in vivo effects of morphine, buprenorphine, and control solution on innate immune function and apoptosis in healthy adult dogs. Our null hypothesis was that there would be no significant difference in the immunomodulatory effects of morphine compared with buprenorphine. To test this hypothesis, we compared TNF-α, IL-6, and IL-10 production capacity of leukocytes following pathogen associated molecular pattern (PAMP) motif-stimulation; early apoptosis of neutrophils and lymphocytes; neutrophil phagocytic function; and oxidative burst activity following a 24-hour infusion of three treatments in a randomized, controlled, crossover block design. A continuous rate infusion was chosen for the present study to avoid changes in peak plasma drug concentrations which could potentially interfere with the chosen outcome measures (Menzenbach et al., 2003; Sharp et al., 1985). In addition, it allowed for a more controlled treatment design and consistency between drug timing and duration of treatment for each dog at all treatment points. Lastly, we wanted to utilize a clinically-relevant drug delivery system in order to emulate how opioids are commonly administered to hospitalized small animal veterinary patients (Dyson, 2008; Hansen, 2008).

2. Materials and methods

2.1. Animals

All portions of this study were approved by the Institutional Animal Care and Use Committee of the University of Missouri. Six healthy adult dogs were recruited for enrollment into this study by personal and electronic solicitation to the faculty, staff, and students of the University of Missouri College of Veterinary Medicine. The health status of each dog was confirmed prior to enrollment based on a health history, physical examination, and minimum database laboratory testing, including complete blood count, plasma biochemical profile, and urinalysis. Dogs were excluded if they were <2 years old, < 10 kg, receiving medications other than routine parasitic preventives, or immunized within 6 weeks of the study period. All clients read and signed an informed-consent form prior to enrollment of their dogs into the study.

2.2. Experimental design

All dogs received three treatments as a constant rate infusion over a 24-hour period in a randomized, controlled, crossover block design: morphine sulphate (West-ward, Eatontown, NJ) (0.5 mg/kg i.v. bolus followed by 0.1 mg/kg/h; diluted in saline to a volume of 1 ml/kg/h), buprenorphine hydrochloride (Reckitt Benckiser Healthcare (UK) Ltd., Hull, England) (0.015 mg/kg i.v. bolus followed by 1.7 mcg/kg/h; diluted in saline to a volume of 1 ml/kg/h), and saline (Abbott Labs, Chicago, IL) (negative control; 1 ml/kg i.v. bolus followed by 1 ml/kg/h). There was at least a 10-day washout between treatments to allow adequate time for drug clearance based on the reported half-life for each drug (Abbo et al., 2008; Andaluz et al., 2009; Krotscheck et al., 2008; Kukanich et al., 2005).

2.3. Blood collection and processing

Blood was collected aseptically from the jugular vein and into sodium heparin collection tubes at baseline and at completion of the 24-hour i.v. infusion. Blood was kept at 25 °C (room temperature or RT) and immediately processed for evaluation of leukocyte cytokine production capacity, apoptosis, phagocytosis, and oxidative burst.

2.4. Leukocyte cytokine production capacity

Heparinized blood was diluted 1:2 with complete Roswell Park Memorial Institute cell culture medium (RPMI, 200 U Penicillin/ml, 200 mg streptomycin/ml, and 200 mM L-glutamine; Life Technologies Corp., Grand Island, NY) and added to 12-well plates for stimulation of cytokine production. LPS (final concentration, 100 ng/ml) from *Escherichia coli* 0127:B8 (Sigma-Aldrich, St. Louis, MO), LTA (final concentration, 1000 ng/ml) from *Streptococcus faecalis* (Sigma-Aldrich), peptidoglycan (PG) from *Staphylococcus aureus* (Sigma-Aldrich) (final concentration, 1000 ng/ml), or control (PBS) were added to the wells as previously described (DeClue et al., 2014; Deitschel et al., 2010; Fowler et al., 2011). The samples were mixed thoroughly and incubated for 24 h at 37 °C in 5% CO_2. After incubation, the well contents were centrifuged (300 × *g* for 15 min) at RT and the supernatant was collected and frozen at –80 °C until batch analyses.

2.5. Assays

2.5.1. Cytokine assays

Leukocyte production capacity of TNF-α, IL-6, and IL-10 were determined in duplicate using a multiplex canine immunoassay according to manufacturer's instructions (MILLIPLEX® MAP Canine Cytokine/Chemokine Panel, EMD Millipore Corp., Billerica, MA). This assay has been previously validated for use in dogs (Karlsson et al., 2012). Briefly, an aliquot (300 ul) of each supernatant sample was thawed. The samples were centrifuged (3000 rpm for 6 min) to pellet debris and the supernatant was removed. Each aliquot was admixed with anticytokine antibody-charged polystyrene microspheres in a 96 well plate. Following overnight incubation at 4 °C with agitation, a biotinylated detection antibody was added, as well as streptavidin-phycoerythrin. Median fluorescence intensity was measured using a MAGPIX® multiplex system with xPONENT 4.1 software (Luminex, Austin, TX) and analyzed using MILLIPLEX® Analyst v3.5 Software (EMD Millipore Corp.).

2.5.2. Neutrophil and lymphocyte apoptosis

Following sample collection, heparinized blood was diluted 1:1 with RPMI cell culture medium. Samples were incubated for 5 hours at 37 °C in 5% CO_2. After incubation, the percentage of apoptotic, necrotic, and live cells was determined using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, San Jose, CA) according to manufacturer's instructions (DeClue et al., 2014; Vermes et al., 1995). Briefly, the erythrocytes were removed with ACK Lysis Buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA in deionized distilled water brought to a pH of 7.4 with 1 M NaOH). The cells were washed twice, resuspended in Binding Buffer, and dual-labeling was performed by adding FITC Annexin V and propidium iodide (PI) to each tube. The cells were gently vortexed and incubated for 15 min at RT in the dark. Binding Buffer was added to each tube and flow cytometry was performed within 30 minutes.

2.5.3. Phagocytosis of *E. coli* by neutrophils

Phagocytic function was determined using the Phagotest® commercial test kit (Orpegen Pharma, Heidelberg, Germany) according to manufacturer's instructions. This test kit has been previously validated for use in dogs (LeBlanc et al., 2010). Briefly, heparinized blood was incubated in a 37 °C water bath for 10 min with FITC-labeled, opsonized *E. coli* bacteria. Control samples were incubated and placed in an ice bath (approx. 4 °C) to inhibit phagocytosis. Phagocytosis was arrested by placing the sample on ice and adding ice-cold quenching solution in order to remove the FITC fluorescence of surface bound bacteria. After two washing steps, erythrocytes were removed by adding lysing solution and the samples were incubated for 20 min at RT, followed by additional washing. A DNA

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