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Increasing plasma methadone concentrations are associated with decreased leukocyte oxidative burst without affecting cytokine production or phagocytosis in healthy dogs

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Objective
To determine whether methadone administration affects leukocyte phagocytic function, oxidative burst, and cytokine production and if immune function is associated with plasma methadone concentrations in dogs.

Methods
This was a prospective, randomized, placebo-controlled, nonblinded crossover study. Ten client-owned healthy dogs were included in the study. Dogs were randomized to receive either methadone (0.3 mg/kg, IV) or placebo (0.9% NaCl, IV) once every 6 hours for 24 hours. Dogs were crossed over to the alternative treatment following a 7-day interval period. Blood was collected at baseline (ie, before treatment administration) and then 10 minutes, 6 hours, and 24 hours after the first treatment administration. Immune function tests and plasma methadone concentrations were measured at all time points (before treatment administration and 10 minutes, 6 hours, and 24 hours after the first treatment administration). Plasma methadone concentrations were measured with liquid chromatography quadrupole time-of-flight mass spectrometry. Whole blood cultures were performed with exposure to PBS, lipopolysaccharide, and lipoteichoic acid. Canine-specific multiplex assay was used to measure tumor necrosis factor-α, IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor concentrations in the supernatant. Granulocytic and monocytic phagocytosis and oxidative burst were evaluated via flow cytometry.

Results
There was a moderate inverse association between the percentage of granulocytes and monocytes undergoing oxidative burst and plasma methadone concentrations (r = −0.88, r^2 = 0.77, P < .001). Oxidative burst percentage increased over time regardless of treatment (F[1,67] = 7.758; P < .007). No other between treatment or time differences were identified.

Conclusions
Increasing methadone concentrations is associated with decreased leukocyte oxidative burst in healthy dogs.

Clinical Relevance
Methadone may have immunologic effects in dogs but requires additional investigation.

Keywords: inflammation, plasma methadone, leukocyte, leukocyte oxidative burst, cytokine production

Methadone is a potent synthetic opioid commonly used either alone or in combination with other analgesics for pain control in dogs. It is a μ-opioid receptor agonist, as a well as a noncompetitive *N*-methyl-D-aspartate. While it is pharmacologically similar to morphine, it has higher relative intrinsic efficacy at the μ-receptor, with fewer side effects such as vomiting or histamine release.[1,2] Methadone is generally well tolerated aside from occasional bradycardia and panting, while providing superior perioperative analgesia to other opioids like buprenorphine.[3–6] While its metabolic profile and cardiovascular effects are well documented, there is limited information regarding the immunological effects of methadone in dogs.[4,7,8] Understanding the potential immunomodulatory effects of methadone has clinical value because it is commonly used

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in dogs that have active infections or disorders with the potential for infections to develop (eg, surgery).

Some studies have indicated methadone may have weak immunosuppressive effects. An in vitro study[9] in rats found that while prolonged exposure to morphine decreased host response to *Trichinella spiralis*, this was not observed in the methadone group. However, the methadone group did exhibit elevated serum immunoglobulin G levels, and other potential indicators of immunotoxicity, such as increased weight of mesenteric lymph nodes and cell density of medullary cords. This could suggest that methadone has negative effects on humoral immunity. Conversely, another study[10] comparing in vivo macrophage function in mice treated with morphine or methadone found morphine to be strongly immunodepressive, while methadone had no significant effect on any of the parameters tested, including macrophage cytostatic activity or secretion of IL-1-α or tumor necrosis factor (TNF)-α.

To our knowledge, only 3 studies[11–13] have examined the immunomodulatory effects of opioids in dogs, although none have included methadone. DeClue et al[11] demonstrated that opioids alter immune and apoptotic pathways in dogs in vitro. In that study, incubation of blood from dogs with morphine enhanced the intensity of oxidative burst in neutrophils. In addition, morphine, buprenorphine, and fentanyl contributed to increased antigen-stimulated leukocyte production of TNF-α and IL-10 and decreased neutrophil apoptosis. Axiak-Bechtel et al[12] found that tramadol and *O*-desmethyltramadol had no effects on canine immune parameters in vitro other than decreased IL-10 production in blood incubated with *O*-desmethyltramadol. Monibi et al[13] found that a 24-hour infusion of morphine (0.5 mg/kg, IV bolus, followed by 0.1 mg/kg/h) or buprenorphine (0.015 mg/kg, IV bolus, followed by 1.7 µg/kg/h) in vivo did not affect leukocyte apoptosis, phagocytosis, oxidative burst, or antigen-stimulated concentrations of TNF-α, IL-6, and IL-10 in the supernatant.

Our study seeks to fill the gap in the literature by evaluating the immunomodulatory effects of methadone in dogs. Therefore, the primary objective was to evaluate the in vivo effects of a clinically relevant dose of methadone on several aspects of innate immune function, including granulocyte and monocyte (GM) phagocytic function, oxidative burst, and antigen-stimulated leukocyte cytokine responses. A secondary objective was to determine whether plasma methadone concentrations are associated with immune function variables. We hypothesized that methadone administration would alter at least 1 immune function variable and that these changes would be magnified over time with cumulative dose concentrations. Further, we hypothesized that plasma methadone concentrations would be associated with some of these immune function variables.

Methods

Animals

The study protocol was approved by the Midwestern University Animal Care and Use Committee (protocol No. AZ-4036). Ten healthy dogs owned by faculty, staff, or students that weighed > 10 kg were eligible for this prospective, randomized, placebo-controlled, nonblinded crossover study between June 2021 and July 2021. Health status was determined by a board-certified small animal internist (JAJ) after performing a physical examination, review of medical records, and evaluation of clinicopathological test results including CBC, biochemistry, and urinalysis (Antech Diagnostics). Dogs were not permitted to have received any supplements or medications (except monthly parasitic control) or vaccinations within 60 days of enrollment.

Experimental design and blood sample collection

Dogs were randomized to initially receive either methadone (0.3 mg/kg, IV) or placebo (0.9% NaCl) once every 6 hours over a 24-hour period in treatment arm 1 **(Figure 1)**. The volume (mL) of placebo was equal to the calculated volume of methadone for each dog. Following a 7-day interval period, dogs were crossed over to treatment arm 2 and were given the intervention they did not receive in treatment arm 1 with the same frequency and time period of dosing. Each dog had 1 IV catheter placed in each cephalic vein (ie, 2 total), the first for blood sampling and the second for the administration of the intervention. Then, 5-mL blood samples were collected into lithium heparin and EDTA anticoagulated blood tubes at baseline (before methadone or placebo administration; T0) and then 10 minutes (T1), 6 hours

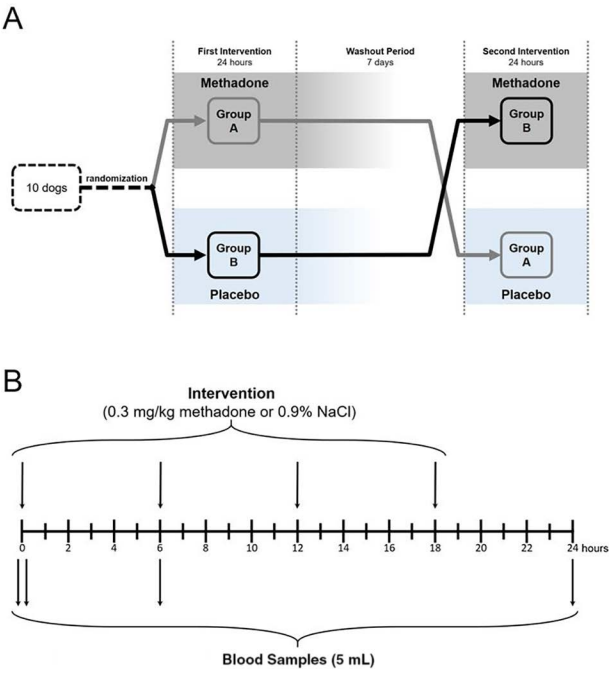

Figure 1—A prospective, randomized, placebo-controlled, nonblinded crossover study was performed with 10 healthy dogs enrolled between June 2021 and July 2021. Illustration of study design (A) and timing and frequency of intervention (B; methadone or placebo [0.9% NaCl] administration) and blood sample collection.

(T2), and 24 hours (T3) after the first intervention administration; samples coinciding with intervention time points (ie, at 6 hours) were taken immediately following administration of the intervention. Blood collected in the lithium heparin tubes was processed immediately to evaluate immune parameters; blood collected in the EDTA tubes was centrifuged, and plasma was removed and stored at −80 °C for batch analysis of methadone concentrations.

Plasma methadone concentrations

A modified methyl *tert*-butyl ether liquid-liquid extraction was used to prepare samples for the liquid chromatography-mass spectrometry analysis. Briefly, 250 µL of dog plasma was spiked with 25 µL methadone-d3 (internal standard [IS]; 0.5 µg/mL) followed by 2.5 mL cold methyl *tert*-butyl ether. The sample was vortexed for 1 minute and centrifuged (2,109 X *g*, 4 °C, 10 minutes). After the bottom layer was frozen (−80 °C, 8 to 10 minutes), the upper layer was transferred to a new vial and dried by centrifugal evaporation (40 °C for 35 minutes). Samples were reconstituted in 125 µL methanol:water (70:30).

Analyte separation was performed using a 1260 HPLC system with autosampler (Agilent Technologies). Separations of 5-µL injections of IS-spiked plasma extracts were performed using a 2.1 mm X 50 mm (2.7 µm) Poroshell 120 EC-C18 column (Agilent Technologies) at a flow rate of 0.5 mL/min using a mobile phase consisting of eluent A (water with 0.1% formic acid) and eluent B (acetonitrile with 0.1% formic acid). An isocratic elution of 67:33 (eluate A:eluate B) was started for 1 minute, which was followed first by a linear gradient to 5:95 over 2 minutes, and then re-equilibrated at 67:33 for 2 minutes.

Methadone and methadone-d3 were detected using an Accurate-Mass 6530 Quadrupole Time-of-Flight (Q-TOF) mass analyzer (Agilent Technologies). Ionization was performed with positive electrospray ionization. The transition for methadone was 310.2165 → 105.0339 (collision-induced dissociation = 40 V). The retention time for methadone was 1.01 minutes.

Methadone was quantified using a series of matrix-matched calibration controls (MMC) prepared fresh each day of analysis. Each MMC was prepared using naïve plasma and contained methadone at incremental concentrations ranging from 0.5 to 100 ng/mL and spiked with IS. Each curve was generated using a minimum of 5 MMC areas. Linear regression analysis using a 1/y weighting system to maximize accuracy, and reduce coefficient variation was performed. The coefficient of determination was > 0.99 for all calibration curves.

Leukocyte cytokine production

For cytokine analysis, whole blood samples from the lithium heparin blood tubes were diluted 1:2 in RPMI culture medium containing 200 U/mL penicillin and 200 mg/mL streptomycin (Invitrogen). In 24-well plates, diluted blood samples were stimulated with either lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 (final concentration, 100 ng/mL; Sigma-Aldrich), lipoteichoic acid from *Streptococcus faecalis* (final concentration, 1 µg/mL; Sigma-Aldrich), or PBS as a negative control.[11] Samples were incubated in the dark for 24 hours at 37 °C in 5% CO_2 and then centrifuged at 400 X *g* for 7 minutes at 21 °C. Supernatants were stored at −80 °C until subsequent batch analysis of TNF-α, IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Analysis was conducted on samples in duplicate with appropriate controls, as previously described using a canine cytokine-specific multiplex bead-based assay (Milliplex MAP; MilliporeSigma).[14] Results for the median fluorescence intensities and cytokine concentrations were analyzed using Milliplex Analyst 5.1 (MilliporeSigma). The lower limit of detection of this assay for each cytokine was 48.8 pg/mL, the intra-assay coefficient of variation was < 5%, and the interassay coefficient of variation was < 15%.

Phagocytosis of E coli

Granulocyte and monocyte phagocytic function was evaluated using the PhagoTest kit (Orpegen Pharma). One hundred microliters of heparinized whole blood was combined with 20 µL of either FITC-labeled, opsonized *E coli* strain LE392 or washing solution as a negative control. Samples were then incubated in a 37 °C water bath for 10 minutes before being transferred to ice to arrest phagocytosis; 100 µL of quenching solution was added to quench surface-bound FITC-labeled *E coli*. Cells were washed, erythrocytes were lysed, and all cells were washed again before the addition of 200 µL of DNA staining solution (containing R-phycoerythrin to bind eukaryotic diploid DNA and facilitate the exclusion of aggregated artifacts of bacteria or cells that do not have intact DNA to bind). The phagocytic function was then analyzed via flow cytometry.

Oxidative burst

Oxidative burst function was evaluated using the PhagoBurst kit (Orpegen Pharma). One hundred microliters of heparinized whole blood was combined with 20 µL of either opsonized *E coli* strain LE392 or control solution. Samples were incubated in a water bath at 37 °C for 10 minutes; after the addition of 20 µL of dihydrorhodamine-123 (a substrate for oxygen intermediates), the samples continued incubating for an additional 10 minutes until the reaction ceased. The erythrocytes were lysed, cells were washed, and 200 µL of DNA staining solution containing R-phycoerythrin was added as described above. Oxidative burst was then analyzed via flow cytometry.

Flow cytometry

Flow cytometry was performed at the Midwestern University College of Veterinary Medicine Immunology Laboratory using the Guava easyCyte HT flow cytometer (MilliporeSigma) and GuavaSoft 3.2 data analysis software (Luminex Corporation). A minimum of 20,000 events/sample were recorded. Our gating scheme has previously been reported.[15] Phagocytosis data were recorded as the percentage of GM cells

having internalized FITC-labeled *E coli*, as well as their mean fluorescent intensity (to quantify the mean number phagocytosed bacteria per cell). Oxidative burst data were recorded as the percentage of GM cells having produced reactive oxygen metabolites and the mean fluorescent intensity: the relative robustness of oxidative burst reaction produced per cell.

Statistical analysis

Statistical analyses were performed using statistical software (R Version 4.0; R Core Team, 2020). Descriptive data (age and weight) were normally distributed and presented as mean and SD. Normality was assessed using the Shapiro-Wilk test. When the measured cytokine fell below the lower limit of detection, data were recorded at the lower limit of detection for statistical purposes. Paired *t* tests were conducted to assess differences at T0 in mean immunological variables between the methadone and placebo groups given that each dog acted as their own control. To analyze the results from the first objective, linear mixed-effect models were used to determine whether immunologic variables changed over time after the administration of intervention and whether the magnitude of change varied based on intervention. Significant *P* values were further interrogated with pairwise comparisons and adjusted with the Bonferroni method to mitigate type I errors resulting from multiple comparisons. To investigate the correlation between plasma methadone concentrations and the immunologic variables, a repeated measures correlation was used, which accounted for the repeated observations of T0, T1, T2, and T3. The significance level was set at *P* value < .05.

Results

Animals

Ten dogs (5 neutered males and 5 spayed females) were included. The mean age and weight were 4.1 years (SD, 2.5) and 22.5 kg (SD, 7.6), respectively. There were 8 mixed-breed dogs and 2 purebred dogs including a German Shepherd Dog and a French Bulldog.

Effects on leukocyte cytokine production

There was no difference in leukocyte production of any cytokines at T0 (ie, before intervention) between methadone and placebo groups (*P* values > .05; **Table 1**). Results from the linear mixed-effect models indicated that only LPS-stimulated leukocyte concentrations of IL-10 had a significant interaction between time and intervention (*P* = .02; **Table 2**). However, when the *P* values for multiple comparisons for the interaction term were adjusted, there were no differences between time holding intervention constant or between intervention while holding time constant **(Supplementary Table S1)**. There were no other differences in cytokine concentrations based on intervention or time (*P* values > .05).

Effects on phagocytosis and oxidative burst

There were no differences identified in the percentage of GM with phagocytosed *E coli* or the mean number of *E coli* phagocytized per cell at T0 between methadone and placebo groups (*P* values > .05; Table 1). Similarly, the percentage of GM that had performed oxidative burst and the intensity of respiratory burst reaction were no different between methadone and placebo groups at T0 (*P* values > .05).

Table 1—A prospective, randomized, placebo-controlled, nonblinded crossover study was performed with 10 healthy dogs enrolled between June 2021 and July 2021.

	Placebo (n = 10)	Methadone (n = 10)	*P* value
Phagocytosis (%)	59.3 (18.2)	64.2 (20.1)	.63
Phagocytosis [mean fluorescent intensity (MFI)]	6,171.6 (2,047.4)	5,696.9 (1,637.6)	.64
Oxidative burst (%)	66.1 (19.4)	72.4 (18.3)	.52
Oxidative burst (MFI)	1,494.6 (335.4)	1,412.8 (334.8)	.53
GM-CSF (pg/mL)			
PBS	48.8 (0.0)	67.3 (58.4)	.34
LPS	675.7 (652.9)	678.1 (636.7)	.99
LTA	53.2 (10.5)	81.7 (83.4)	.32
IL-6 (pg/mL)			
PBS	48.8 (0.0)	63.3 (39.9)	.28
LPS	475.6 (383.8)	406.0 (246.4)	.64
LTA	115.6 (81.7)	127.5 (134.5)	.83
TNF-α (pg/mL)			
PBS	48.8 (0.0)	55.7 (17.6)	.25
LPS	1,580.2 (1,084.4)	1,421.1 (784.2)	.73
LTA	184.6 (156.2)	130.3 (97.5)	.46
IL-10 (pg/mL)			
PBS	199.5 (13.3)	213.8 (58.6)	.48
LPS	1,154.2 (444.6)	1,008.6 (304.2)	.41
LTA	293.5 (245.1)	215.5 (59.9)	.37

Data are presented as means and SD. Comparison of immunologic variables at baseline (ie, before administration of intervention) between dogs that subsequently received methadone or placebo (0.9% NaCl). Immunologic variables included phagocytosis of opsonized *Escherichia coli* (percent of cells performing phagocytosis and the mean number of bacteria phagocytosed per cell [MFI]), opsonized *E coli*–induced oxidative burst (percent cells performing reaction and intensity of reaction per cell [MFI]), and supernatant concentrations of cytokines after leukocyte exposure to lipopolysaccharide (LPS), lipoteichoic acid (LTA), or PBS.

GM-CSF = Granulocyte-macrophage colony-stimulating factor. TNF-α = Tumor necrosis factor.

Table 2—Linear mixed-effects models evaluating effects of intervention (methadone vs placebo [0.9% NaCl]) and time on various immunological variables.

	Methadone vs placebo effect	Time effect	Time X intervention effect
Phagocytosis (%)	$F(1,67) = 1.39$ $P = .24$	$F(1,67) = 0.28$ $P = .60$	$F(1,67) = 0.025$ $P = .88$
Phagocytosis (MFI)	$F(1,67) = 4.283$ **$P = .04$**	$F(1,67) = 5.890$ **$P = .02$**	$F(1,67) = 3.872$ $P = .053$
Oxidative burst (%)	$F(1,67) = 0.238$ $P = .63$	$F(1,67) = 7.758$ **$P = .007$**	$F(1,67) = 0.642$ $P = .43$
Oxidative burst (MFI)	$F(1,67) = 0.026$ $P = .87$	$F(1,67) = 0.113$ $P = .74$	$F(1,67) = 1.025$ $P = .32$
GM-CSF (pg/mL)			
PBS	$F(1,67) = 1.192$ $P = .28$	$F(1,67) = 2.024$ $P = .16$	$F(1,67) = 0.368$ $P = .55$
LPS	$F(1,67) = 0.576$ $P = .45$	$F(1,67) = 1.258$ $P = .27$	$F(1,67) = 1.340$ $P = .25$
LTA	$F(1,67) = 0.171$ $P = .68$	$F(1,67) = 0.020$ $P = .89$	$F(1,67) = 2.178$ $P = .15$
IL-6 (pg/mL)			
PBS	$F(1,67) = 1.120$ $P = .29$	$F(1,67) = 1.757$ $P = .19$	$F(1,67) = 0.811$ $P = .37$
LPS	$F(1,67) = 0.169$ $P = .68$	$F(1,67) = 1.516$ $P = .22$	$F(1,67) = 2.942$ $P = .09$
LTA	$F(1,67) = 0.015$ $P = .90$	$F(1,67) = 0.588$ $P = .45$	$F(1,67) = 0.113$ $P = .74$
TNF-α (pg/mL)			
PBS	$F(1,67) = 0.491$ $P = .49$	$F(1,67) = 0.798$ $P = .10$	$F(1,67) = 1.099$ $P = .30$
LPS	$F(1,67) = 0.377$ $P = .54$	$F(1,67) = 0.088$ $P = .77$	$F(1,67) = 2.464$ $P = .12$
LTA	$F(1,67) = 1.020$ $P = .36$	$F(1,67) = 1.613$ $P = .21$	$F(1,67) = 0.233$ $P = .63$
IL-10 (pg/mL)			
PBS	$F(1,67) = 0.750$ $P = .39$	$F(1,67) = 0.091$ $P = .77$	$F(1,67) = 0.609$ $P = .44$
LPS	$F(1,67) = 3.135$ $P = .08$	$F(1,67) = 1.645$ $P = .19$	$F(1,67) = 3.684$ **$P = .02$**
LTA	$F(1,67) = 0.031$ $P = .86$	$F(1,67) = 0.781$ $P = .51$	$F(1,67) = 2.193$ $P = .10$

Data are presented as means and SD. Immunologic variables included phagocytosis of opsonized *E coli* (percent of cells performing phagocytosis and the mean number of bacteria phagocytosed per cell [MFI]), opsonized *E coli*–induced oxidative burst (percent cells performing reaction and intensity of reaction per cell [MFI]), and supernatant concentrations of cytokines after leukocyte exposure to LPS, LTA, or PBS.

Bolded *P* values were significant.

The linear mixed-effect models identified a significant difference in the percentage of GM exhibiting oxidative burst over time, independent of the intervention ($P = .007$; Table 2). Multiple comparisons indicated that oxidative burst percentage was greater at T2 than T0 ($P = .01$) as well as at T2 compared with T1 ($P = .002$; **Supplementary Table S2; Supplementary Figure S1**). The models also found that the mean number of *E coli* phagocytized per cell was different based on intervention ($P = .04$) and time ($P = .02$) without an interaction between the factors. However, no subsequent pairwise comparisons were significant.

Association between plasma methadone concentrations and immunologic variables

Correlation analysis of immunologic variables tested with plasma methadone concentrations showed only a significant linear relationship between oxidative burst percentage and methadone concentration. This correlation was moderately negative,

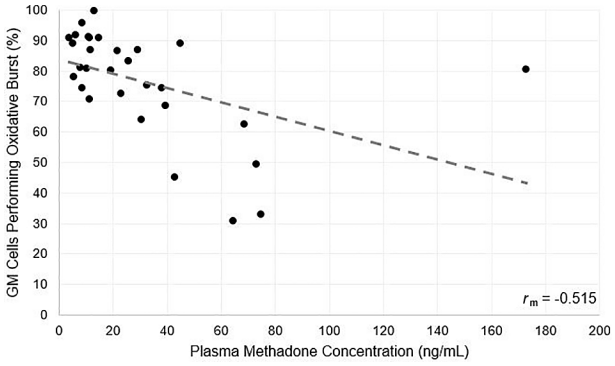

Figure 2—Scatter plot illustrating the linear relationship between plasma methadone concentrations and the percentage of granulocytes and monocytes (GMs) performing oxidative burst. Black circles represent individual dog data. The line of best fit highlights a significant trend ($P = .02$).

with the percentage of GM cells performing oxidative burst decreasing as the concentration of methadone increased (r = −0.52 [95% CI, −0.78 to −0.11], r^2 = 0.27, P = .02; **Figure 2**). The strength of the correlation between the percentage of GM cells performing oxidative burst and plasma methadone concentration increased after the analysis was repeated with the removal of an outlier data point (methadone concentration of 172.7 ng/mL; r = −0.88, r^2 = 0.77, P < .001). No other correlations between immunologic variables and plasma methadone concentrations were significant.

Discussion

Here we evaluated the in vivo effects of a clinically relevant dose of methadone on leukocyte cytokine production as well as GM phagocytosis and oxidative burst and analyzed whether any of these immune parameters were associated with plasma methadone concentrations. While LPS-stimulated leukocyte IL-10 concentration appeared to increase significantly in the methadone group, this significance did not hold up after adjusting for multiple comparisons. Likewise, the intensity of phagocytic function increased with respect to time and was blunted in the methadone group; however, following multiple pairwise comparisons, neither of these findings proved to be significant. This indicates that these observations were only marginally significant and that methadone's effects on these immune parameters were weak.

There was a moderate inverse linear relationship between plasma methadone concentrations and the percentage of GM performing oxidative burst. This means that as plasma methadone concentrations increased, the overall proportion of cells having performed oxidative burst decreased. These results may have clinical implications as the negative effects on oxidative burst seem to occur as concentrations increase with successive dosing over time. Take, for example, a dog with a septic abdomen recovering from an exploratory laparotomy. Results from our study suggest that methadone may not be the best analgesic for a case like that because of the possible negative effects on immune function. Of course, this hypothesis must be investigated in sick dogs before declarative statements regarding methadone administration in a clinical setting can be made. In addition, it is unknown whether similar effects on oxidative burst would occur with alternative dosing strategies in clinical practice. The dosage and frequency of methadone administration were specifically chosen to be representative of what is commonly used by veterinarians.[16]

While these results support our hypothesis that methadone would affect at least 1 immune parameter tested, they also contribute to the discordance within the literature regarding the effect that methadone has on the immune system. Previous studies have generally found methadone to be less immunosuppressive than morphine, yet the only other in vivo study[13] examining opioids' immune effects in dogs found that neither morphine nor buprenorphine had significant effects, including on oxidative burst.

To the authors' knowledge, studies investigating the impact of methadone on oxidative burst in other species are limited and inconclusive. Methadone was shown to enhance the oxidative burst response of macrophages from mice,[17] but an in vitro study[18] on swine peripheral blood mononuclear cells showed methadone suppressed oxidative burst in a dose-dependent manner. An in vitro study[20] on human peripheral blood mononuclear cells found no effect of methadone on oxidative burst, yet another similar study[19] found that it did suppress oxidative burst. Study design may account for some of these discrepancies, as in vitro models do not account for those immune effects mediated by the activation of the μ-opioid receptor in the CNS. It is also important to note that given methadone's role in opiate addiction treatment, many immune studies evaluating this opioid have been performed in the context of "methadone maintenance treatment." One such in vivo study[21] examining the immune effects of methadone treatment for heroin addiction found that methadone overall has a normalizing effect on immune responses in these patients, including oxidative burst. Curiously, this study also observed the differential impact of methadone on the proportion of cells performing oxidative burst and their intensity. One month after starting methadone treatment, an increased proportion of patients' cells were performing oxidative burst, but their intensity did not increase until after 6 months of methadone treatment.[21] Taken with our findings, this indicates methadone's in vivo effects on immune function, especially oxidative burst, remains underexplored and may be species specific.

Our results also showed that oxidative burst percentage increased over time regardless of intervention. Possible explanations for this observation center around kennel stress, eg, perhaps extended confinement in the hospital triggered cortisol or other hormones in the dogs to mediate this increase. Cortisol has been positively correlated with oxidative burst in juvenile salmon and dairy cows, but this is another area requiring further study.[22,23]

This study had several limitations that must be considered. First, phagocytosis and oxidative burst were explored in the context of only opsonized *E coli*. Therefore, our results cannot be extrapolated as an overall representation of phagocytic function and respiratory burst in dogs. Future studies utilizing other substrates for phagocytosis and activators of respiratory burst would be useful to provide a more comprehensive assessment. Next, our study used a more global approach to evaluating the innate immune response to methadone rather than individual immune cells. Specifically, phagocytic function and oxidative burst were evaluated only in GMs, and whole blood cultures were performed to assess leukocyte cytokine production. These methodologies were intentionally broad so that our results may have improved clinical relevance. For example, evaluating the effect of methadone on phagocytosis and oxidative burst collectively in GMs, the primary cellular mediators in an innate immune response, may

arguably be more clinically relevant than individual cell types. Similarly, whole blood cultures allow cellular reactions to occur in a more physiologic milieu rather than extracting singular cell types to measure cytokine production. Additional studies that focus on specific immune cell types are needed to understand whether methadone may have varying immunomodulatory effects based on specific leukocytes. Our study included healthy dogs that were not given any medications so as to avoid any confounding effects on immune function. It is unclear whether these results would be similar in systemically unwell dogs or in cases with comorbid disorders. Finally, the sample population in our study was conservative and may have contributed to type II error. Notably, methadone yielded significant effects on LPS-stimulated IL-10 concentrations and mean number of *E coli* phagocytized per cell initially in our model, but significance was lost after adjustment for multiple comparisons, likely because of reduced power.

In conclusion, our findings suggest it is unlikely that methadone causes substantial detriment to cytokine production and phagocytosis in canines, although our study was also likely underpowered to detect differences in both the initial and subsequent pairwise comparisons. Further research with larger sample populations is needed to confirm the putative effects of methadone on canine immune function and to assess the translation of our findings to nonhealthy dogs.

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References

1. Grubb T, Sager J, Gaynor JS, et al. 2020 AAHA anesthesia and monitoring guidelines for dogs and cats. *J Am Anim Hosp Assoc.* 2020;56(2):59–82.
2. Selley DE, Cao C-C, Sexton T, Schwegel JA, Martin TJ, Childers SR. μ Opioid receptor-mediated G-protein activation by heroin metabolites: evidence for greater efficacy of 6-monoacetylmorphine compared with morphine. *Biochem Pharmacol.* 2001;62(4):447–455.
3. Kerr CL, Swanton WE. Anesthesia update — incorporating methadone into companion animal anesthesia and analgesic protocols: a narrative review. *Can Vet J.* 2023;64(11):1058–1065.
4. Menegheti TM, Wagatsuma JT, Pacheco AD, et al. Electrocardiographic evaluation and degree of sedation with three doses of methadone in healthy dogs [corrected]. *Vet Anaesth Analg.* 2014;41(1):97–104.
5. Monteiro ER, Figueroa CDN, Choma JC, Campagnol D, Bettini CM. Effects of methadone, alone or in combination with acepromazine or xylazine, on sedation and physiologic values in dogs. *Vet Anaesth Analg.* 2008;35(6):519–527.
6. Shah MD, Yates D, Hunt J, Murrell JC. A comparison between methadone and buprenorphine for perioperative analgesia in dogs undergoing ovariohysterectomy. *J Small Anim Pract.* 2018;59(9):539–546.
7. Amon T, Kästner SBR, Kietzmann M, Tünsmeyer J. Plasma levels of a methadone constant rate infusion and their corresponding effects on thermal and mechanical nociceptive thresholds in dogs. *BMC Vet Res.* 2021;17(1):35.
8. Keating S, Fries R, Kling K, Graham L, Clark-Price S, Schaeffer DJ. Effect of methadone or hydromorphone on cardiac conductivity in dogs before and during sevoflurane anesthesia. *Front Vet Sci.* 2020;7:573706.
9. van der Laan JW, Krajnc EI, Krajnc-Franken MA, van Loveren H. Immunotoxicological screening of morphine and methadone in an extended 28 day study in rats. *Int J Immunopharmacol.* 1995;17(6):535–543.
10. Pacifici R, Di Carlo S, Bacosi A, Zuccaro P. Macrophage functions in drugs of abuse-treated mice. *Int J Immunopharmacol.* 1993;15(6):711–716.
11. DeClue AE, Yu D-H, Prochnow S, et al. Effects of opioids on phagocytic function, oxidative burst capacity, cytokine production and apoptosis in canine leukocytes. *Vet J.* 2014;200(2):270–275.
12. Axiak-Bechtel SM, Tsuruta K, Amorim J, et al. Effects of tramadol and *O*-desmethyltramadol on canine innate immune system function. *Vet Anaesth Analg.* 2015;42(3):260–268.
13. Monibi FA, Dodam JR, Axiak-Bechtel SM, et al. Morphine and buprenorphine do not alter leukocyte cytokine production capacity, early apoptosis, or neutrophil phagocytic function in healthy dogs. *Res Vet Sci.* 2015;99:70–76.
14. Jaffey JA, Amorim J, DeClue AE. Effects of calcitriol on apoptosis, toll-like receptor 4 expression, and cytokine production of endotoxin-primed canine leukocytes. *Am J Vet Res.* 2018;79(10):1071–1078.
15. Jaffey JA, Amorim J, DeClue AE. Effects of calcitriol on phagocytic function, toll-like receptor 4 expression, and cytokine production of canine leukocytes. *Am J Vet Res.* 2018;79(10):1064–1070.
16. Papich MG. Methadone hydrochloride. In: Papich MG, ed. *Papich Handbook of Veterinary Drugs.* 5th ed. W.B. Saunders; 2021:581–583.
17. Filipczak-Bryniarska I, Nowak B, Sikora E, et al. The influence of opioids on the humoral and cell-mediated immune responses in mice. The role of macrophages. *Pharmacol Rep.* 2012;64(5):1200–1215.
18. Molitor TW, Murtaugh MP, Click RE, Gekker G, Chao C, Peterson PK. Functional alterations of swine peripheral blood mononuclear cells by methadone. *J Leukoc Biol.* 1992;51(2):124–128.
19. Boland JW, Foulds GA, Ahmedzai SH, Pockley AG. A preliminary evaluation of the effects of opioids on innate and adaptive human in vitro immune function. *BMJ Support Palliat Care.* 2014;4(4):357–367.
20. Peterson PK, Gekker G, Brummitt C, et al. Suppression of human peripheral blood mononuclear cell function by methadone and morphine. *J Infect Dis.* 1989;159(3):480–487.
21. Ciobanu A-M, Manda G, Neagoe I, et al. The immune status of heroin addicts during treatment with methadone. *Farmacia.* 2023;71(6):1189–1196.
22. Herron CL, Cogliati KM, Dolan BP, Munakata A, Schreck CB. Stress up-regulates oxidative burst in juvenile Chinook salmon leukocytes. *Fish Shellfish Immunol.* 2018;80:655–659.
23. Chaveiro A, Moreira da Silva F. In vitro effect of the reproductive hormones on the oxidative burst activity of polymorphonuclear leucocytes from cows: a flow cytometric study. *Reprod Domest Anim.* 2010;45(5):e40–e45.

Supplementary Materials

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