The Veterinary Journal 236 (2018) 31–36

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Original Article

Effect of calcitriol on in vitro whole blood cytokine production in critically ill dogs

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ARTICLE INFO

Article history:
Accepted 17 April 2018

Keywords:
Anti-inflammatory
APPLE fast score
Canine
Immune dysregulation
Vitamin D

ABSTRACT

Hypovitaminosis D has been identified as a predictor of mortality in human beings, dogs, cats and foals. However, the immunomodulatory effects of vitamin D in critically ill dogs has not been evaluated. The aim of this study was to evaluate the effect of calcitriol on cytokine production from whole blood collected from critically ill dogs in vitro. Twelve critically ill dogs admitted to a veterinary intensive care unit (ICU) were enrolled in a prospective cohort study. Whole blood from these dogs was incubated with calcitriol (2×10^{-7} M) or ethanol (control) for 24 h. Subsequent to this incubation, lipopolysaccharide (LPS)-stimulated whole blood production of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were measured using a canine-specific multiplex assay. Calcitriol significantly increased LPS-stimulated whole blood production of IL-10 and decreased TNF-α production without significantly altering IL-6 production. There was no significant difference in whole blood cytokine production capacity between survivors and non-survivors at the time of discharge from the ICU or 30 days after discharge. These data suggests that calcitriol induces an anti-inflammatory phenotype in vitro in whole blood from critically ill dogs.

Introduction

The classic paradigm of vitamin D function involves its role in calcium, phosphorus and skeletal homeostasis (Penna and Adorini, 2000; Szymczak and Pawliczak, 2016; Altieri et al., 2017). In order for vitamin D to exert biologic effects, its precursors must undergo two modifications. Cholecalciferol and ergocalciferol are converted to 25-hydroxycholecalciferol, the major circulating metabolite of vitamin D in the liver. This prohormone undergoes hydroxylation via 1α-hydroxylase in the kidney to form calcitriol, the biologically active form of vitamin D. Identification of vitamin D receptors and 1α-hydroxylase, the enzyme required to synthesize calcitriol, in tissues outside the kidney suggests that it has a wider role than previously thought (Altieri et al., 2017).

In addition to its classic role, vitamin D has a clinically relevant role in the immune system (Penna and Adorini, 2000; Szymczak and Pawliczak, 2016). In human beings, calcitriol enhances the chemotactic and phagocytic abilities of immune cells, and augments antimicrobial activity via increased transcription of cathelicidin and defensin B2 (Tokuda and Levy, 1996; Chandra et al., 2004; Gombart et al., 2005; Motlagh et al., 2015). Calcitriol attenuates inflammation without compromising antimicrobial functions of human polymorphonuclear cells (Villaggio et al., 2012; Harishankar et al., 2014; Neve et al., 2014; Grubczak et al., 2015). Similarly, calcitriol blunts pro-inflammatory cytokine production from canine whole blood without significant alteration in phagocytosis or toll-like receptor 4 expression in canine granulocytes and monocytes in vitro (Jaffey et al., 2018a).

Hypovitaminosis D in critically ill human patients has been associated with increased mortality (Braun et al., 2011; Venkatram et al., 2011; Braun et al., 2012; Higgins et al., 2012; Quraishi et al., 2014; Dickerson et al., 2016; Guan et al., 2017), as well as increased susceptibility to infections (Braun et al., 2011; Arnson et al., 2012; Flynn et al., 2012; Higgins et al., 2012; de Haan et al., 2014). Similarly, hypovitaminosis D has been associated with mortality in critically ill foals (Kamr et al., 2015) and cats (Titmarsh et al., 2015). While hypovitaminosis D has been identified in critically ill dogs (Jaffey et al., 2018c) and dogs with sepsis (Carver and Koenigshof, 2016), there is no information on the immunomodulatory effects of calcitriol on immune cells collected from critically ill dogs.

The primary aim of this study was to evaluate the effect of calcitriol on in vitro cytokine production from whole blood collected from critically ill dogs admitted to a veterinary intensive care unit (ICU). We hypothesized that calcitriol would decrease lipopolysaccharide (LPS)-stimulated and unstimulated (phosphate buffered saline, PBS) leukocyte production of tumor necrosis factor

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https://doi.org/10.1016/j.tvjl.2018.04.010
1090-0233/

(TNF)-α and interleukin-6 (IL-6), as well as increase production of interleukin-10 (IL-10), from whole blood of critically ill dogs. Our secondary aim was to determine if whole blood cytokine production capacity and severity of illness were significantly different in dogs that were alive at discharge and 30 days after discharge from those that were not alive.

Materials and methods

Animals and selection criteria

The study was performed as a prospective, serial admission, cohort study. The first 12 client-owned dogs presented to the Emergency and Critical Care Service at the University of Missouri Veterinary Health Center, Missouri, USA, that required ICU hospitalization in January 2017 were enrolled in this prospective study, unless they had one or more exclusion criteria. Exclusion criteria for dogs included pregnancy, lactation, hypercalcemia of malignancy, hyperparathyroidism, hypoparathyroidism, chronic renal failure and supplementation with vitamin D or calcium. The study protocol was approved by the University of Missouri Animal Care and Use Committee (protocol number 7334; date of approval 7th January 2017) and all owners provided informed written consent. The acute patient physiologic and laboratory evaluation (APPLE) fast scoring system was used to assess illness severity. The APPLE fast score for each dog was calculated using blood glucose, albumin, lactate, platelet count and mentation score obtained within 24 h of admission to the ICU (Table 1; Hayes et al., 2010). Criteria for mentation scoring are summarized in Table 2 (Hayes et al., 2010).

Sample collection and calcitriol treatment

Survival data were obtained from clinical records or follow-up telephone calls to clients or referring veterinarians for each dog for survival to discharge and at day 30 after discharge. Dogs were defined as being critically ill if they were hospitalized in the ICU because the primary clinician determined that the animal had illness of sufficient severity that there was a need for urgent therapeutic intervention. The same analyzers were used for hematology (XT-2000iV/XT-1800iV Hematology Analyzer, Sysmex) and biochemistry (AU400e Chemistry Analyzer, Beckman Coulter). Blood lactate concentration was measured using a handheld device (Lactate Plus, Nova Biomedical) previously validated in dogs (Nye et al., 2017). Whole blood was collected from dogs, via lateral saphenous venipuncture, into tubes with sodium heparin anti-coagulant. Blood was diluted 1:2 with RPMI 1640 culture medium containing 200 U/mL penicillin and 200 mg/mL streptomycin (Gibco Invitrogen). The blood-RPMI mixture was incubated with calcitriol (final concentration 2×10^{-7} M) or ethanol (13.5×10^{-2} M) as a negative control for 24 h at 37 °C in 5% CO_2 in the dark.

Table 1
Acute patient physiologic and laboratory evaluation (APPLE) fast score calculated by adding the values in the right column for each of the five parameters listed, with a maximum score of 50.

Parameter	Value	Score assignment
Glucose (mg/dL)	<84	7
	84–102	8
	103–164	9
	165–273	10
	>273	0
Albumin (g/dL)	<2.6	8
	2.6–3.0	7
	3.3–3.5	0
	3.1–3.2	6
	>3.5	2
Lactate (mg/dL)	<18.0	0
	18.0–72.1	4
	72.2–90.1	8
	>90.1)	12
Platelet count ($\times 10^9$ cells/L)	<151	5
	151–200	6
	201–260	3
	261–420	0
	>420	1
Mentation score	0	0
	1	4
	2	6
	3	7
	4	14

(Adapted from Hayes et al., 2010).

Table 2
Mentation score calculation adapted from Hayes et al. (2010.

Score	Definition
0	Normal
1	Able to stand unassisted, responsive but dull
2	Can stand only when assisted, responsive but dull
3	Unable to stand, responsive
4	Unable to stand, unresponsive

Whole blood cytokine production

Following incubation with calcitriol or control solution, stimulated leukocyte cytokine production capacity was determined using whole blood culture (DeClue et al., 2008; Deitschel et al., 2010; Fowler et al., 2011). Samples were placed in 24-well plates and stimulated again for an additional 24 h at 37 °C in 5% CO_2 in the dark with LPS from *Escherichia coli* O127:B8 (final concentration, 100 ng/mL; Sigma–Aldrich) or PBS (unstimulated control). Following incubation, plates were centrifuged ($400 \times g$ for 7 min at room temperature) and cell-free supernatant was collected and frozen at −80 °C for batch analysis (DeClue et al., 2008; Hoffman et al., 2018). Tumor necrosis factor, IL-6 and IL-10 were measured in the supernatant using a canine cytokine-specific multiplex bead-based assay (Milliplex; Millipore; Karlsson et al., 2012; DeClue et al., 2014). Each sample was analyzed in duplicate with appropriate controls and associated data analysis software to determine the median fluorescence intensity and cytokine concentration (pg/mL). As determined in our laboratory, the lower limit of detection of this assay for each cytokine was 49 ng/mL, the intra-assay coefficient of variation was <5% and the inter-assay coefficient of variation was <15%.

Statistical analysis

Statistical analysis was performed using SigmaPlot (Systat Software). A Shapiro–Wilk test was used to assess normality. Data with a non-normal distribution were assessed using the Mann–Whitney *U* test, and the median, first quartile (Q1) and third quartile (Q3) were determined. We compared cytokine production in LPS-stimulated or unstimulated (PBS) whole blood incubated with control (ethanol) or calcitriol using a Friedman repeated measures analysis of variance (ANOVA) with Ranks and Student–Newman–Keul's multiple comparison post-test. Whole blood cytokine production capacity was defined as the difference in cytokine production in blood stimulated with LPS and PBS, divided by cytokine production of PBS stimulated blood, i.e. (LPS–PBS)/(PBS)). *P* values <0.05 were considered to be statistically significant.

Results

Study subjects

Twelve dogs, comprising nine purebred and three mixed breed dogs, seven spayed females, three neutered males and two intact males, were enrolled serially in the study, with a mean ± standard deviation (SD) age of 7.6 ± 3.4 years and a mean ± SD weight of 21.6 ± 9.7 kg.

The 12 dogs were admitted to the ICU and diagnosed with acute intractable vomiting and diarrhea, gastrointestinal ulceration, intracranial disease, myelodysplasia and pyrexia, myeloid leukemia, nasal carcinoma, newly diagnosed diabetes mellitus with ketonuria, pancreatitis, pyrexia with concurrent progressive respiratory difficulty, respiratory distress related to brachycephalic syndrome, septic peritonitis or urinary obstruction. The APPLE fast scores were mean ± SD 26.7 ± 8.4. Three of the 12 dogs (25%) were euthanazed before discharge. Thirty days after hospital discharge, 5/12 (41.7%) dogs had died; all five were euthanazed because of clinical decline and poor prognoses.

Whole blood cytokine response

Exposing whole blood from critically ill dogs to LPS resulted in significant production of TNF-α ($P = 0.002$; Fig. 1) and IL-6