Small Ruminant Research 140 (2016) 27–31

Contents lists available at ScienceDirect

Small Ruminant Research

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

Impaired antibody response and phagocytosis in goats fed a diet low in cobalt

Eugene H. Johnson*, Khalid Al-Habsi, Rashid Al-Busaidi, Mohammed Al-Abri

Department of Animal and Veterinary Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34. Al-Khod 123, Oman

ARTICLE INFO

Article history:
Received 4 November 2015
Received in revised form 24 May 2016
Accepted 25 May 2016
Available online 26 May 2016

Keywords:
Goats
Antibodies
Phagocytosis
Mitogens
Cobalt deficiency

ABSTRACT

Cobalt (Co) is utilized by rumen microflora to produce vitamin B_{12}. The aim of the present study was to determine whether feeding newly-weaned goats a diet containing low levels of Co results in impairment of neutrophil function, antibody production and/or lymphocyte proliferative responses. Forty, ten week old goats were fed a commercially-prepared concentrate (150 g/day) and Rhodesgrass hay ad libitum. This diet contained Co at <0.1 mg/kg dry matter. The goats were divided into a control group and a treated group (both $n = 20$). Goats in the treated group were supplemented with bi-monthly subcutaneous injections of 2 mg of hydroxycobalamin. Two months after weaning, the goats were immunized with keyhole limpet haemocyanin (KLH). A luminol-dependent chemiluminescence (CL) assay was employed to measure the oxidative respiratory burst of neutrophils during phagocytosis of zymosan A. The lymphocyte proliferative response to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) was assessed. The controls goats demonstrated a significantly lower antibody (IgG) response to KLH compared with the Co-supplemented goats that was apparent 1 week post immunization and was maintained for the 8 week observation period. Neutrophils from the Co-restricted group demonstrated significantly lower CL responses to opsonized zymosan A. Lymphocytes from the control and treated goats did not differ in their mitogenic responses to PHA and PWM. These results demonstrate that goats fed a diet low in Co exhibited impaired antibody and phagocytic responses.

1. Introduction

Small ruminants, such as sheep develop serious clinical signs of vitamin B_{12} deficiency when fed diets containing low levels of cobalt (Co) (Kennedy et al., 1996). Although goats are assumed to have similar dietary requirements for Co compared with sheep, they have been reported to be fairly resistant to clinical disease related to vitamin B_{12} deficiency (Clark et al., 1987; Mburu et al., 1993). However, the detrimental effects of restricted dietary Co on the health and performance of goats remain to be fully established.

Previous research from our laboratories has demonstrated that when newly weaned goats were fed diets containing 0.1 ppm Co/kg DM, an amount considered adequate to meet the minimum daily requirements of sheep (National Research Council, 1985), they exhibited serum concentrations of vitamin B_{12} significantly below normal reference values (Johnson et al., 2010b). Furthermore, goats receiving Co-restricted diets developed clinical signs of vitamin B_{12} deficiency, including anaemia (Al-Habsi et al., 2007), poor weight gains (Al-Habsi et al., 2007), decreased bone lengths (Kadim et al., 2006) and hepatic lipidosis (Johnson et al., 2004).

Goats with low serum vitamin B_{12} concentrations have been shown to exhibit higher coccidia counts, compared with those receiving supplementation (Al-Zadjali et al., 2004). Similarly, Patterson and Mac Pherson (1990) provided some evidence that cattle fed diets depleted with Co were more susceptible to the parasite *Ostertagia ostertagia* and Mac Pherson et al. (1976) alluded to the possibility that Co deficiency might be associated with sheep being more susceptible to some types of infections. Lambs born to ewes with Co deficiency have been reported to have higher mortality rates than those with adequate Co intake (Fisher and MacPherson, 1991). In a more recent study we reported preliminary results demonstrating that goats fed diets restricted in Co exhibited impaired neutrophil function compared to goats receiving parenteral injections of hydroxycobalamin (Johnson et al., 2010b) and it was postulated that impairment of phagocytic function might represent an early sign of vitamin B_{12} deficiency.

The aim of the present study was to investigate innate and adaptive immune responses in goats exposed to low levels of dietary Co by studying the respiratory burst of neutrophils as an indicator

* Corresponding author.
E-mail address: ejohnson@squ.edu.om (E.H. Johnson).

http://dx.doi.org/10.1016/j.smallrumres.2016.05.013
0921-4488/

of phagocytic function, antibody production to immunisation and lymphocyte proliferative responses to mitogen stimulation.

2. Materials and methods

2.1. Experimental animals

Forty, ten-week-old newly weaned male Batinah goat kids bred at the Agricultural Experimental Station of the Sultan Qaboos University, were randomly allocated to either a control or treated group ($n = 20$ per group). The kids were fed a diet consisting of Rhodes grass hay *(Chloris gayana)* ad libitum and 150 g/day per head of a commercially prepared ruminant concentrate (Al-Dharia Animal Feed Co). Co levels were measured as previously described (Kadim et al., 2006) and both feeds were shown to contain <0.10 mg Co/kg dry matter. Goats in the treated group received subcutaneous injections of 2 mg of hydroxycobalamin (Prolaject B12 2000, Bomac Laboratories, New Zealand) at weaning and bi-monthly thereafter throughout the duration of the study. This treatment regimen has been shown to maintain serum vitamin B_{12} concentrations within established reference values (Johnson et al., 2010b). The experiments were conducted in compliance with the regulations outlined by the Sultan Qaboos University Animal Ethics Committee.

2.2. KLH immunisation and serological testing

KLH (Sigma, St. Louis, MO) in alum was prepared as previously described by Hudson and Hay (1980). Two months after weaning, all goats were subcutaneously immunized with 1 mg KLH. Blood samples (5 mL) were collected into plain Vacutainer tubes at day 0 and weekly thereafter for 8 weeks. Serum samples were frozen at −20 °C prior to serological testing.

Serum antibodies against KLH were measured by indirect ELISA, as described by Pollock et al. (1991), with slight modifications. Optimal concentrations of all reagents were determined by chequer-board titration. Briefly, 96 well plates (Ratiolab, Dreieich, Germany) were coated overnight with KLH (100 μg/mL in PBS). The wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 200 μL of freshly prepared 1% bovine serum albumin/PBST for 30 min. Sera were added at a predetermined optimal dilution (1:100). Each well received 100 μL of serum. The plates were incubated for two h at 37 °C, washed with PBST and incubated for an additional 60 min with 100 μL/well of a 1:50,000 dilution of mouse monoclonal anti-goat IgG, conjugated with alkaline phosphatase (clone GT-34, No, A8062, Sigma) before washing and developing the plates with 50 μL/well of substrate (Sigma fast *p*-nitrophenyl phosphate), at 37 °C for 30 min. The plates were analysed using an Ascent Multiskan spectrophotometer (Thermo Electron Corporation, Finland) at 405 nm.

2.3. Isolation of blood cell populations

Blood samples were collected by jugular venepuncture into heparinised tubes for isolation of peripheral blood mononuclear cells (PBMC) and granulocytes as previously described (Johnson et al., 1991), starting on the day of weaning (Day 0) and monthly thereafter for mitogen and chemiluminescence assays, respectively. Briefly, 6 mL of heparinized whole blood was layered over 3 mL of Ficoll-Histopaque, 1077 (Sigma) in conical siliconized 15 mL centrifuge tubes and centrifuged at 1500*g* for 30 min. The PBMC were recovered and washed three times in PBS. The resulting pellets, containing the granulocytes were re-suspended in 1 mL of RPMI-1640 (Sigma), and treated with erythrocyte lysing buffer (ice-cold 0.85% NH_4Cl, supplemented with 0.84 g/L $NaHCO_3$ and 0.0432 g/L disodium ethylene diamine tetracetic acid). The procedure was repeated three times to completely remove all red blood cells. The resulting pellets were washed three times in PBS and cells were re-suspended in 1 mL of RPMI-1640.

Cell counts were performed in haemocytometers and differential counts were determined manually from smears by counting 200 cells. Cell viability was determined by trypan blue exclusion and only samples with >95% viable cells were used for experiments. Cell numbers for mitogen assays were adjusted in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL) and dihydro-streptomycin (100 μg/mL) to give a total count of 1×10^6 lymphocyte/mL for the mitogen assays. Neutrophils for the CL assays were re-suspended to 5×10^6 cells/mL in RPMI-1640. All cell separations were performed within 1 h after their collection.

2.4. Mitogen assays

PBMC were cultured with previously determined optimal concentrations of 2 μg/mL phytohaemagglutinin (PHA; Sigma), 5 μg/mL pokeweed mitogen (PWM; Sigma) or with culture medium only (negative control) in 96 well flat bottomed microtitre plates (Corning, Wiesbaden, Germany) at 100 μL/well in triplicate. Cells were incubated at 37 °C for 72 h in a humidified 5% CO_2 incubator. Thereafter, cell proliferation was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) with a 4 h incubation period. The optical density of each well was measured spectrophotometrically at 450 nm (OD_{450}) in a Multiskan Spectrum spectrophotometer (Thermo Electron Corporation, Finland). Mean values were calculated for the triplicate wells and stimulation indices (SI) were calculated according to the formula: SI = mean OD_{450} of PWM or PHA stimulated cells/mean OD_{450} of unstimulated cells.

2.5. Preparation of opsonized zymosan A and chemiluminescence (CL) assays

One hundred mg of zymosan A particles (Sigma–Aldrich) were boiled in 2 mL of PBS for 60 min. After three washes in PBS the zymosan was resuspended to give a final concentration of 50 mg/mL. Opsonisation was achieved by incubating 0.1 mL of the zymosan particles with nine parts of pooled adult goat serum at 37 °C for 15 min with continuous mixing. The mixture was subsequently washed with PBS, centrifuged at 850*g* for 20 min and the resultant pellet was re-suspended in 4 mL of RPMI.

CL assays were performed as previously described (Johnson et al., 2010a). Briefly, reaction mixtures consisted of 100 μL granulocytes (5×10^6/mL), 100 μL of opsonized zymosan and 100 μL of RPMI, containing 10^{-5} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma–Aldrich). Assays were performed in triplicates in 96-well microtitre plates at 37 °C with intermittent shaking between readings. Measurements were made in an Ascent Luminoskan luminometer (Thermo Electron Corporation). The area under the curve (AUC), defined by relative light units and time, was calculated over a 60 min recording period. CL was recorded as relative light units (RLU). Prior to the commencement of the studies it was demonstrated that the background CL of PMN was negligible. Based on trypan blue exclusion it was determined that there were no relevant differences in neutrophil viability at the beginning and end of the CL assays.

2.6. Statistical methods

The Mann Whitney *U* test was used to compare the differences between treated and control groups for mitogen and CL assays using the Statistical Analytical System (SAS, 2009). P values < 0.05 were considered statistically significant.

The AUC was determined by the trapezoidal rule of approximating integrals using a custom script written in Mathematica