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The effects of short term dietary restriction on haematological responses and leukocyte gene expression of anovulatory and ovulatory beef heifers

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ABSTRACT

The study objective was to characterise the impact of negative energy balance (NEB) on immune-stress responsiveness in beef heifers. A short term (18-day) dietary restriction model was used. Dietary restriction (0.4 maintenance (Mn) energy requirements) induced abrupt onset of anoestrus in nine heifers (Restricted Anovulatory; RA) while nineteen heifers maintained oestrous cyclicity (Restricted Ovulatory; RO). In addition a control (C) group of 12 heifers received a higher level of feeding (1.2 Mn). Haematological related biomarkers of husbandry stress, leukocyte gene expression of seven cytokine genes and five immunological biomarkers were investigated. After 18 days of differential feeding of the heifers alterations in eosinophil and monocyte numbers and altered expression of *CXCL8*, *IL2* and *TNFα* could be attributed to diet restriction. More specifically, changes in these five variables were found in heifers that became anovulatory (RA) and are therefore considered to be more sensitive biomarkers to an energy deficit.

1. Introduction

Negative energy balance (NEB) impairs normal reproductive function in the cow. Thus a cow in NEB is unable to meet the energy demands of maintenance and lactation from diet alone. The animal's response to NEB is to mobilise body reserves in an attempt to maintain energy homeostasis and also channel available energy toward survival and away from processes such as reproduction (Hill et al., 2008). Consequently, normal ovarian function is delayed in animals experiencing NEB and cows may have increased susceptibility to disease (Lucy et al., 1992).

In human studies, fasting is reported to increase plasma concentrations of the stress hormone cortisol (Fichter et al., 1986) which in turn has a negative impact on reproduction (Ferin, 1999). There is limited information in the literature on the effect of dietary restriction on immune response variables in the beef heifer. We and others have reported variation in the tolerance to NEB between individuals where animals respond divergently for reproductive performance (Konigsson et al., 2008; Lucy and Crooker, 2001; Mackey et al., 1999; Walsh et al., 2012). However, understanding of the immune system response to NEB remains to be elucidated. Measures of immunocompetence can be used as an indicator of the presence of a stress response (Moberg, 1987a, 1987b, 1999, 2000). In particular, cytokine (immune-derived, hormone-like mediators), lymphocyte, neutrophil, leukocyte and eosinophil release are initiated to instigate phagocytosis, antibody formation and activation of the lymphatic system (Chrousos, 2009; Goehler et al., 2000). Research measuring stress related immune function in cattle has primarily focused on husbandry practices, namely, weaning (Hotzel et al., 2010, 2011; Lynch et al., 2010), castration (Earley and Crowe, 2002; Earley et al., 2011), transport (Earley and Murray, 2010; Earley et al., 2010) and housing (Hickey et al., 2003) with limited or no research elucidating the effect of an energy deficit in beef heifers on the stress-immune response. Leukocytes are known to carry gene expression signatures characteristic of other physiological (Burton et al., 2005; Weber et al., 2006) and disease states (Coussens et al., 2004a, 2004b; Hill et al., 2005; Meade et al., 2006; O'Loughlin et al., 2011, 2012). Therefore, haematological profiles and leukocyte expression of targeted immune response genes may provide potential biomarkers of NEB. Therefore to gain new insights into nutrition induced NEB the objective of the study was to characterise the effect of dietary restriction on haematological responses and leukocyte

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immune response gene expression and to investigate if anovulatory heifers, that are less tolerant to NEB, experience a greater degree of stress.

2. Materials and methods

All animal procedures performed in this study were conducted under experimental licence from the Irish Department of Health and Children (licence number B100/846). Protocols were in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC and were sanctioned by the Research Ethics Committee, University College Dublin (UCD), Ireland.

2.1. Experimental model

A short term (18-day) dietary restriction model was developed which induced abrupt onset of anoestrus in some animals while others maintained oestrous cyclicity. Previous work from our group has found that following short term severe dietary restriction, heifers become anoestrus within approximately two weeks, whereas others will continue normal oestrous cyclicity (Mackey et al., 1999). The authors reported the failure of the dominant follicle to ovulate in 60% of heifers after 13–15 days of restriction. In the present study, Charolais crossbred heifers (n = 40) exhibiting regular oestrous cycles with an initial mean weight of 395 ((s.e.) 3.7) kg and body condition score (BCS), of 2.99 ((s.e.) 0.04) were used. Oestrus was synchronised using an 8-day combined CIDR and prostaglandin $F_{2\alpha}$ regimen. During oestrous synchronisation all heifers were fed a diet supplying 1.2 estimated maintenance energy requirements (Mn). One day before CIDR removal (day (d) 0), heifers were allocated randomly to either a diet supplying 0.4 Mn (n = 28) or retained on 1.2 Mn (Control (C); n = 12). Following CIDR removal ovarian follicular growth and ovulation were monitored using transrectal ultrasonography. On d 11 after diet allocation, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered to induce luteolysis, oestrus and ovulation. In the 0.4 Mn treatment group, heifers were classified as either restricted ovulatory (RO) or restricted anovulatory (RA) depending on whether the dominant follicle (DF) ovulated or failed to ovulate, respectively. On d 18 of the study, all heifers were slaughtered in a commercial abattoir and hypothalamic and anterior pituitary tissue was recovered.

2.2. Blood sample collection

Heifers were blood sampled via jugular venepuncture on day (d) -2, 0, 5, 9, 13 and 18. The heifers were led gently to a holding pen, with a squeeze chute facility and were blood sampled with minimal restraint. Blood sampling was carried out by the same experienced operator on each occasion and the time taken to collect the blood samples was less than 90 s/heifer. Blood samples were collected into 1 × 6 ml K_3Ethylenediaminetetraacetic acid (K_3EDTA) tubes (Vacuette, Cruinn Diagnostics, Ireland) for haematological analysis and into 5 × 9 ml acid citrate dextrose (ACD) tubes (Vacuette, Cruinn Diagnostics, Ireland) for leukocyte isolations.

2.3. Haematology

Unclotted whole K_3EDTA blood samples were analysed using an ADVIA haematology analyser (AV ADVIA 2120, Bayer Healthcare, Siemens, UK) equipped with software for bovine blood analysis. Total leukocyte, neutrophil, lymphocyte, eosinophil and monocyte number, red blood cell (RBC) number, haemoglobin (HGB), mean corpuscular haemoglobin concentration (MCHC), haematocrit (HCT) percentage and platelet (PLT) number were measured. The N:L ratio was also calculated.

2.4. Leukocyte isolation from whole blood

Thirty-six ml of blood from the ACD tubes was pooled from each animal within three hours of collection and split into three 12 ml aliquots. Red blood cells were lysed for 90 s using 24 ml of a hypotonic solution (10 mM Na_2HPO_4, 2 mM NaH_2PO_4; pH 7.2) followed by restoration using 12 ml of a hypertonic solution (10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 461 mM NaCl; pH 7.2). The tubes were then centrifuged at 1000 g for 5 min at 4 °C to collect the leukocyte pellet. The leukocyte pellet was washed twice by resuspending in Dulbecco's phosphate buffered saline (DPBS) and centrifuged for 5 min. The isolated leukocytes were resuspended in 1 ml of TRI Reagent (Sigma-Aldrich Ireland Ltd., Dublin, Ireland), pooled by animal and stored in a sterile tube at –80 °C until RNA extractions were performed.

2.5. RNA extraction and cDNA synthesis

A modified TRI Reagent extraction method (Simms et al., 1993) was used to extract total RNA from leukocytes via homogenization of the pellet in TRI Reagent and the subsequent addition of chloroform followed by precipitation using isopropanol and ethanol. RNA was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed on an Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Dublin, Ireland) and only RNA samples with a RNA Integrity Number (RIN) of greater than 8.0 were used. Samples were DNase treated and purified using an RNeasy mini kit (Qiagen Ltd., Crawley, UK). One μg of total RNA per animal was reverse transcribed into cDNA using random hexamers and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Ireland) in a 20 μl reaction and stored at –20 °C.

2.6. Primer design

Primers were designed using the Primer3 online software programme (Rozen and Skaletsky, 2000) based on a bovine mRNA sequence located in the GenBank database from NCBI (http://www.ncbi.nlm.nih.gov/Genbank/), or using primer-BLAST software provided by PubMed (http://www.ncbi.nlm.nih.gov/tools/primerblast/). Where possible, primers were designed to span exon-exon junctions to limit the amplification of non-mRNA products. Primers were validated using BLAST.

The stability of five reference genes was calculated in geNorm version 3.5 (Vandesompele et al., 2002) using expression data from quantitative real-time PCR (RT-qPCR). These were β-actin (*ACTB*), succinate dehydrogenase complex subunit A (*SDHA*), ribosomal protein S9 (*RPS9*), ubiquitin (*UBQ*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The software calculates the intra- and intergroup variation and combines both figures to give a stability value, a lower value implying a higher stability in gene expression. A combination of *ACTB, SDHA* and *RPS9* exhibited the greatest stability, with an M value of 0.21. Therefore, this panel of genes were selected as standard reference genes for the relative measurement of gene expression.

The relative expression of 12 genes on d 0, d 5, d 9, d 13, and d 18 were measured by RT-qPCR in a subset of 21 heifers (6 RA, 8 RO, 7 C). Gene specific primer sequences were as previously described (Buckham Sporer et al., 2007; Neuvians et al., 2004; O'Loughlin et al., 2011; Pang et al., 2011) or designed specifically for this study (Table 1). Primers used were commercially synthesized (Sigma-Aldrich Ireland Ltd. Dublin, Ireland). The PCR products obtained by RT-qPCR were sequenced to verify their identity (Biochemistry DNA Sequencing Facility, University of Cambridge, UK.). In the case of all genes examined in this study, DNA sequences were 100% identical to published sequences.