

# Unchanged interleukin 6 level of protein and energy restricted goats during late gestation: the role of elevated blood nitric oxide

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## Abstract

Twelve pregnant goats were assigned to three dietary treatments during late gestation, namely control (C: metabolizable energy, 5.75 MJ/kg; crude protein, 12.6% and dry matter basis), 40% protein restricted (PR) and 40% energy restricted (ER), to examine the effects of nutrient restriction on the immune status of pregnant goats. Plasma was sampled on day 90, 125 and 145 from pregnant goats to determine cytokine production (interleukin 2 (IL2), IL6) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). Peripheral blood mononuclear cells were obtained on day 145 and activated by lipopolysaccharide to determine cytokine production, and then exposed (PR and ER) to sodium nitroprusside (SNP), a nitric oxide (NO) donor, or control to NG-nitro-L-arginine methyl ester hydrochloride (L-NAME), an NO synthase

inhibitor to explore the role of NO in regulating cytokine production. Plasma IL2, IL6 and TNF $\alpha$  were not altered during gestation, but NO was increased ( $P < 0.05$ ) at gestation day 145 for PR and ER. *In vitro*, compared with control, NO was lower for PR and ER ( $P < 0.001$ ), but IL6 was higher for PR ( $P < 0.001$ ) and ER ( $P = 0.11$ ). The addition of SNP decreased IL6 ( $P < 0.001$ , PR;  $P = 0.12$ , ER) in the malnourished group, and L-NAME increased ( $P < 0.001$ ) IL6 in control compared to those treatments without SNP or L-NAME. The results indicate that plasma NO acted as a regulator of cytokine function exhibiting negative feedback to maintain steady plasma IL6 concentration in PR or ER goats during late gestation.

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## Introduction

It has been postulated that predisposition to some adult diseases, such as type 2 diabetes, cardiovascular diseases and syndrome X, is associated with a low-fetal birth weight (Barker 1994). It has been also proposed that adverse fetal environments, such as suboptimal supply of maternal nutrients, lead to permanent alternation in fetal cognitive functions and greater prevalence of metabolic syndromes (He *et al.* 2009). Bloomfield *et al.* (2003) demonstrated that maternal nutritional restriction of protein and energy resulted in a temporary effect on fetal weight but enduring changes in hypothalamic–pituitary–adrenal axis function in sheep. Thus, maternal nutrition might have long-lasting impacts on the growth and development of offspring.

In China, the nutritional status of flocks and herds is greatly influenced by conditions of the growing season (Glindermann *et al.* 2009). Winter (i.e. dry season) is the reproductive season of goats and sheep in Inner Mongolia, the yet nutritive value of pasture can be sub-optimal during this time.

For example, neutral detergent fibre and lignin contents of pasture during winter are highest (68 and 12% of dry matter (DM) respectively (Wang *et al.* 1997)), while crude protein (CP) content is lowest (reduced from 14% in summer and autumn to 5% of DM in spring and winter (Wang *et al.* 1997)). Low nutritive value of consumed pasture in winter results in low DM intake (DMI), and consequently the supply of metabolizable energy (ME, 5.5 MJ/kg) can be below the requirement for local breeds of pregnant goats and sheep (Wang *et al.* 1997).

Maternal protein and energy restriction of grazing ruminants in the dry season resulting from low nutritive value of pasture and low DMI have stimulated interest in studying the relationship between maternal nutrient restriction and physiological function of the fetus. To date, the study of fetal programming induced by maternal nutrient restriction has focused on the physiological function of the hypothalamic–pituitary–adrenal axis and the endocrine system in the offspring, such as hypothalamic–pituitary–adrenal dysfunction (Bloomfield *et al.* 2003), glucose intolerance

(Langley *et al.* 1994), insulin resistance (Petry *et al.* 2001), elevated blood pressure (Ozaki *et al.* 2001) and vascular dysfunction (Holemans *et al.* 1999). However, few experiments have investigated the effects of maternal nutrient restriction on maternal immune, biochemical and physiological conditions, and how these conditions may affect fetal programming.

The objective of the current study was to investigate the effects of maternal protein and energy restriction on the immune function of goats in late gestation. Concentrations of cytokines and other immune mediators (interleukin 2 (IL2), IL6) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) were measured as indices of immune function.

## Materials and Methods

### In vivo experiment

The experiment was conducted according to the Animal Care and the Use Guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, The Chinese Academy of Sciences (Changsha, China).

Twenty-five pregnant (second parity) female goats (Liuyang Blacks, local breed) of similar age ( $2.0 \pm 0.3$  years) and body weight ( $20.0 \pm 1.0$  kg) before pregnancy were obtained from the Liuyang Black Goat Reproduction Centre (Liuyang, Hunan Province, China). The goats had previously been synchronized for estrus and artificially inseminated to ensure consistency of pregnancy date. During early (0–30 days) and mid-gestation (30–82 days), the pregnant goats remained at pasture, each goat receiving 300 g/day of concentrate. The concentrate contained (DM basis): 74.0% corn meal, 20.3% soybean meal, 1.3% calcium bicarbonate, 1.6% calcium carbonate, 0.8% sodium chloride and 2% mineral–vitamin premix. The concentrate supplied 11.0 MJ/kg ME and 15.4% CP. Twelve goats carrying one kid (pregnancy examination by ultrasound at gestation day 60) were chosen as experimental subjects for the study. At day 83 of gestation, the goats were moved to an indoor facility with an average temperature of  $24 \pm 1$  °C and penned individually with free access to fresh water.

The experiment was a completely randomized design with 12 pregnant goats randomly allocated to one of three diets (four animals per group) at day 90 until parturition. The three diets were control diet (C: ME 9.34 MJ/kg, CP 12.5%), protein restricted diet (PR, 60% of the CP supplied by the control group: ME 9.28 MJ/kg, CP 7.5%) and energy restricted (ER) diet (60% of the energy supplied by the control group: ME 5.75 MJ/kg, CP 12.6%). The ingredients and composition of the three diets are given in Table 1. The control diet supplied 1.2 times the maintenance ME and CP requirement according to the Chinese feeding standards (Zhang & Zhang 1998) for pregnant goats. The level of restriction was chosen to represent the typical protein or energy status of goats during the dry season in this area of

**Table 1** Ingredients and composition of diets

|                               | Treatment     |       |       |
|-------------------------------|---------------|-------|-------|
|                               | Control group | PR    | ER    |
| Ingredients (% DM)            |               |       |       |
| Maize stover                  | 60.00         | 60.00 | 60.00 |
| Corn                          | 8.58          | 21.11 | 3.60  |
| Soybean                       | 8.11          | 1.28  | 4.78  |
| Whey                          | 0.00          | 0.00  | 2.00  |
| Wheat bran                    | 2.12          | 2.04  | 2.00  |
| Alfalfa meal                  | 0.00          | 0.00  | 20.64 |
| Fat powder                    | 10.53         | 11.60 | 0.00  |
| Corn gluten meal              | 8.56          | 1.58  | 4.80  |
| Calcium bicarbonate           | 0.25          | 0.72  | 0.61  |
| Calcium carbonate             | 0.75          | 0.56  | 0.47  |
| Sodium chloride               | 0.30          | 0.31  | 0.31  |
| Premix <sup>a</sup>           | 0.80          | 0.80  | 0.80  |
| Nutrient content <sup>b</sup> |               |       |       |
| ME (MJ/kg)                    | 9.34          | 9.28  | 5.75  |
| CP (%)                        | 12.5          | 7.5   | 12.6  |
| Ca (%)                        | 0.518         | 0.518 | 0.518 |
| P (%)                         | 0.34          | 0.34  | 0.34  |

PR, protein restricted group; ER, energy restricted group.

<sup>a</sup>Contained per kilogram: 119 g MgSO<sub>4</sub>·H<sub>2</sub>O, 2.5 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.8 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 3 g MnSO<sub>4</sub>·H<sub>2</sub>O, 5 g ZnSO<sub>4</sub>·H<sub>2</sub>O, 10 mg Na<sub>2</sub>SeO<sub>3</sub>, 40 mg KI, 30 mg CoCl<sub>2</sub>·6H<sub>2</sub>O, 95 000 IU vitamin A, 17 500 IU vitamin D and 18 000 IU vitamin E.

<sup>b</sup>CP, Ca and P were determined values, and ME was calculated according to the data of Zhang & Zhang (1998).

China. The goats were offered the experimental diets 1 week before starting the study (day 83) and were allowed to feed *ad libitum*, to allow them time to adapt. The amounts offered were 1–1.1 kg/day from day 90 to 120 and 1.1–1.2 kg/day from day 120 to 145. Feed was offered in two equal amounts at 0800 and 1800 h daily and feed offered and orts were recorded daily to measure feed intake. All animals were weighed before the morning feeding on day 90, 100, 120 and 140 of gestation, and had free access to water.

Blood samples were collected using sterile hypodermic syringes from the jugular vein of each goat on day 90, 125 and 145 of gestation into sterile tubes treated with sodium heparin or EDTA. The samples were centrifuged at 3000 g for 15 min, and plasma were separated into aliquots and stored at –20 °C for subsequent analysis.

### In vitro experiments

**Culture of peripheral blood mononuclear cells** The first assay was conducted to determine the effects of nutritional restriction of the goats on nitric oxide (NO), IL2, IL6 and TNF $\alpha$  secreted by peripheral blood mononuclear cells (PBMCs). The experiment was a completely randomized design using blood samples from day 145 from three goats for each of the dietary treatments (control, PR and ER). Blood samples preserved in EDTA were diluted in Hanks solution. The PBMCs were separated by density gradient centrifugation

at 2000 g for 15 min using lymphocyte separation media (Mediatec Inc., Herndon, VA, USA). The PBMCs were lifted from the interface and washed three times in RPMI 1640 medium (Invitrogen). The cells were plated at  $1 \times 10^6$  cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin, then treated with lipopolysaccharide (LPS; *Escherichia coli*, 0111:B4; Sigma). The LPS concentration was adjusted to 20 ng/ml according to previous study (Anstead *et al.* 2003). Later, the cells were incubated at 37 °C in 5% CO<sub>2</sub>. Suspensions were collected at 4 and 48 h in duplicate.

A subsequent assay was conducted to determine if NO displays negative feedback regulation on IL2, IL6 and TNFα secreted by PBMCs from goats affected by nutritional restriction. The assay was conducted as a completely randomized design using PBMCs from three goats per diet, with and without NO inhibitor in the case of control goats or with and without exogenous NO in the case of PR and ER goats. The inhibitor was NG-nitro-L-arginine methyl ester hydrochloride (L-NAME; Cayman Chemical Company, Ann Arbor, MI, USA). The L-NAME was dissolved in DMSO (Sigma) to make a stock solution of 5 mg/ml, from which final concentrations were prepared. Sodium nitroprusside (SNP; Beyotime, Haimen, Jiangsu, China) was directly dissolved in PBS. For control goats, L-NAME was added to the culture 15 min after the addition of LPS (20 ng/ml) to a concentration of 1 mM. For the PR and ER goats, SNP was added to the LPS-stimulated PBMC to a concentration of 0.1 mM. At the same time, appropriate concentrations of DMSO or PBS were added to cell suspensions as controls for L-NAME or SNP respectively. After 4 h, the suspensions were collected and assayed for NO, IL2, IL6 and TNFα.

**Measurement of NO, IL2, IL6 and TNFα** The NO production of plasma and PBMC suspensions was determined colorimetrically with a u.v. spectrophotometer (8500 II; Thermo Electron Corporation, Rochester, NY, USA). The assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

The IL2, IL6 and TNFα concentration of plasma and PBMC cell suspension were determined using ELISA kits (R&D System, Minneapolis, MN, USA). Briefly, 96 well plates coated with anti-goat IL2, IL6 and TNFα monoclonal antibodies were incubated for 30 min at 37 °C after plasma and cell suspension samples were added. Then the wells were washed five times using washing buffers and probed with HRP-tagged anti-goat IL2, IL6 and TNFα antibodies for 30 min at 37 °C. Later, the wells were washed five times using the washing buffers and further added with chromogen solution. The plates were incubated for 10 min at 37 °C in the dark, and the reaction was stopped by adding 1 M H<sub>2</sub>SO<sub>4</sub>. The optical density was measured at 450 nm by microplate reader (Labsystems, Helsinki, Finland). The intra- and inter-assay coefficient of variations determined NO, IL2, IL6 and TNFα according to the above-mentioned procedures were all below 10%.

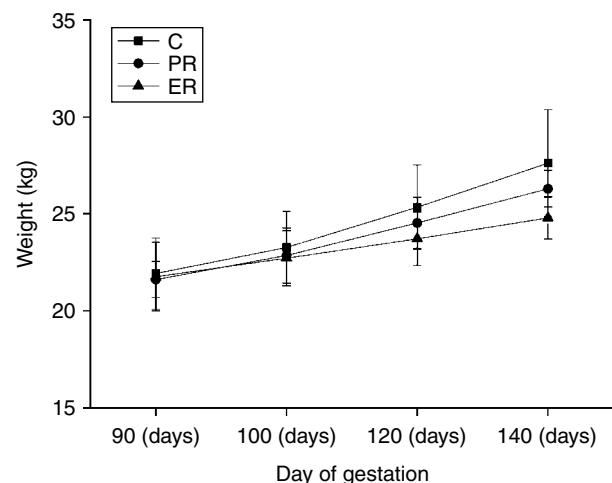
### Statistical analysis

For the *in vivo* experiment, data for body weight and cytokine analysis of plasma samples were analysed using the PROC MIXED procedure of SAS 9.2 (2002; SAS Inc., Cary, NC, USA). The model included the fixed effect of diet and diet × day, with day considered a repeated effect. Differences between diets were tested using a least square difference test using the Kenward–Roger adjustment. For the *in vitro* experiment the cytokine analysis was analysed by time (4 and 48 h) using the proc mixed procedure using a model that included the fixed effect of diet, replication and diet × replication. The PBMC suspensions were analysed by diet (control, PR and ER) using the PROC MIXED procedure with a model that included the fixed effect of treatment (control, LPS alone or LPS + L-NAME; PR and ER, LPS alone or LPS + SNP), replication, and their two-way interaction. Least square means are presented in the text and statistical significance was declared at  $P \leq 0.05$  with trends discussed at  $P \leq 0.15$ .

## Results

### Feed intake and body weight

Daily feed intake was not affected by protein or energy restriction during late gestation (control,  $1027 \pm 24$  g; PR,  $1025 \pm 15$  g; ER,  $1028 \pm 22$  g). Daily CP intake in PR and ME intake in ER were decreased 40.0% ( $76.9 \pm 1.19$  vs  $128.4 \pm 3.06$  g) and 38.3% ( $5.9 \pm 0.13$  vs  $9.6 \pm 0.23$  MJ respectively) compared to the control group. Body weight increased from an average of  $22.2 \pm 1.46$  kg at day 90 of gestation to  $26.7 \pm 1.78$  kg at day 140, but body weight was not affected ( $P = 0.91$ ) by diet (Fig. 1).



**Figure 1** Body weight change of pregnant goats fed three diets. Control, C; protein restricted group, PR; energy restricted group, ER. No difference among diets at day 90, 100, 120 and 140 ( $P > 0.05$ ).

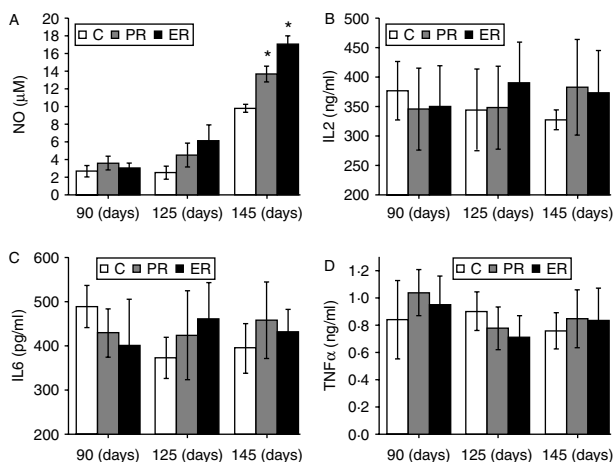
*Nutrient restriction promoted the release of plasma NO, but did not alter IL2, IL6 and TNF $\alpha$  concentrations*

Pregnant goats subjected to ER or PR had higher plasma concentrations of NO ( $P < 0.05$ ) than control goats by day 145 (Fig. 2A). However, plasma IL2, IL6 and TNF $\alpha$  concentrations were unaffected by protein and energy restriction from day 90 to 145 of gestation (Fig. 2B, C and D).

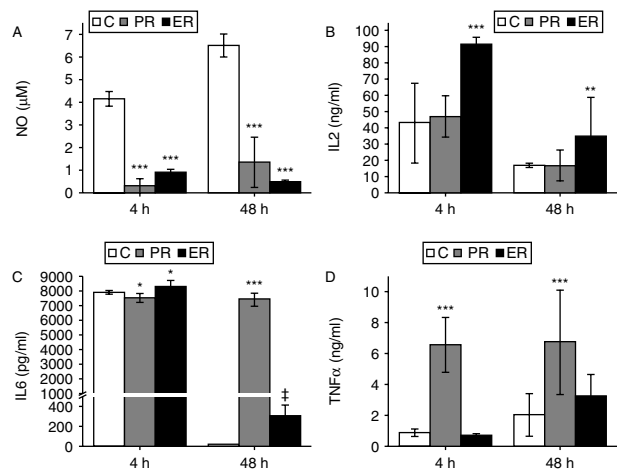
*PBMCs from nutrient restricted goats produced less NO and altered the pro-inflammatory response to LPS*

At both time points, PBMCs from PR and ER goats produced less NO than those from control goats ( $P < 0.001$ ) after stimulation with LPS (Fig. 3A). After 4 h of stimulation, the NO concentration produced by PBMCs from the control group was almost 13 and four times more than those from PR and ER groups respectively. By 48 h, the NO concentration produced by PBMCs from control goats was almost four and 13 times more than those from PR and ER groups respectively.

At both 4 and 48 h, LPS-stimulated PBMCs from ER produced ( $P < 0.05$ ) more IL2 than PBMCs from control (Fig. 2B). Protein restriction, however, had no effect on IL2. For IL6, the concentration secreted by LPS-stimulated PBMCs from PR goats was lower ( $P = 0.02$ ) than from control goats at 4 h, but it was substantially higher ( $P < 0.001$ ) at 48 h (Fig. 3C). Concentration of IL6 was only higher ( $P = 0.01$ ) for ER compared to control at 4 h. For TNF $\alpha$ , LPS-stimulated PBMCs were higher ( $P < 0.001$ ) for PR compared to control at both time points, but there was no difference between control and ER (Fig. 3D).



**Figure 2** Effects of protein and energy restriction on plasma (A) NO, (B) IL2, (C) IL6 and (D) TNF $\alpha$  concentration at gestation day 90, 125 and 145 in pregnant goats. \* $P < 0.05$ , ER and PR compared with C. Control, C; protein restricted group, PR; energy restricted group, ER.



**Figure 3** Effects of protein and energy restriction on secretion of (A) NO, (B) IL2, (C) IL6 and (D) TNF $\alpha$  by PBMCs from goats on day 145 of gestation stimulated with LPS and incubated for 4 and 48 h. The cells were obtained from three goats per group, with six replicates of every sample. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and † $P < 0.10$ ; ER and PR compared with C. Control, C; protein restricted group, PR; energy restricted group, ER.

*Exogenous NO resulted in less IL6 production in PBMCs from PR and ER goats*

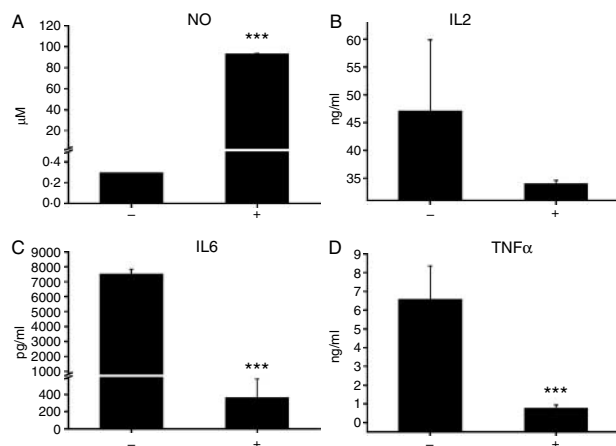
With SNP addition, NO concentration in PBMCs from PR and ER groups increased 316- and 98-fold, respectively (Figs 4A and 5A). For PBMCs from the PR goats, addition of SNP had no effect on IL2, but decreased ( $P < 0.001$ ) IL6 and TNF $\alpha$  (Fig. 4B, C and D). For PBMCs from the ER goats, addition of SNP decreased ( $P < 0.001$ ) IL2, tended ( $P = 0.12$ ) to decrease IL6 and increased ( $P < 0.001$ ) TNF $\alpha$  (Fig. 5B, C and D).

*Inhibition of NO synthase reduced IL6 production in PBMCs from well-nourished goats*

When L-NAME was added to the media of LPS-stimulated PBMCs from well-nourished goats, NO accumulation at 4 h decreased ( $P < 0.001$ ) 7.2-fold (Fig. 6A). The inhibition of NO production increased ( $P < 0.001$ ) IL6 by 74% (Fig. 6C) and there were numerical increases ( $P = 0.06$ ) of TNF $\alpha$  (Fig. 6D), but not ( $P = 0.35$ ) IL2 (Fig. 6B).

## Discussion

A 40% protein or energy restriction for 2 months during late gestation of goats was chosen as the animal model to explore the effects of malnutrition on maternal cytokine production. Restriction of energy or protein promoted the release of plasma NO, but did not alter IL2, IL6 and TNF $\alpha$  concentrations. In contrast to these results, rats fed a low-protein (2%) diet for 14 days showed increases in plasma



**Figure 4** Effects of exogenous NO on concentrations of (A) NO, (B) IL2, (C) IL6 and (D) TNF $\alpha$  released by PBMCs collected from PR goats on day 145 of gestation. Cultures of cells were supplemented with exogenous NO using SNP. The PBMCs were stimulated with LPS alone (-) or LPS + SNP (+) for 4 h of incubation. The PBMCs were obtained from three goats and six replicates were conducted. \*\*\* $P < 0.0001$ ; effects of SNP addition.

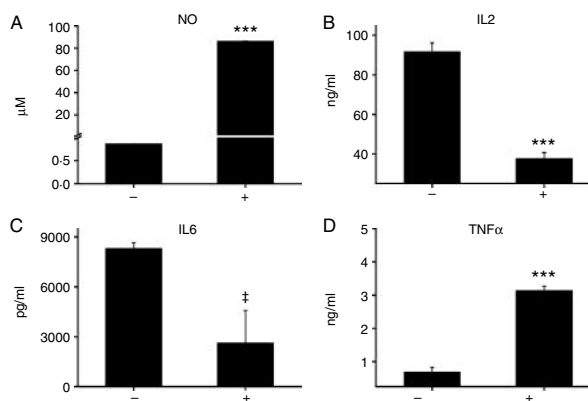
pro-inflammatory cytokines (including TNF $\alpha$  and IL6) compared to rats fed a 20% protein diet (Ling *et al.* 2004). These conflicting results may indicate that the extent of nutrient restriction might have a significant effect on cytokine production. While the level of restriction imposed in our study was about 40% below the protein and/or energy requirements of the goats, body weight was not affected, indicating that the level of restriction was moderate compared to the acute restriction level imposed by Ling *et al.* (2004). The lack of change in body weight might be due to the reduction of maintenance requirement of energy in PR or ER pregnant goats (Ryan *et al.* 1993). In previous experiments, there have been variable inflammatory immune responses to malnutrition. Decreased plasma TNF $\alpha$  production has been reported in rodent models of protein-energy malnutrition (Chan *et al.* 1996, Schaffer *et al.* 1997, Anstead *et al.* 2001), but increased plasma IL6 concentration has been observed in malnourished patients (Malave *et al.* 1998, Johann-Liang *et al.* 2000, de Martino *et al.* 2000). In addition, maternal dietary protein restriction decreased NO synthase (NOS) in endometrium and placenta of pigs during early gestation (Wu *et al.* 1998). These varying responses may indicate that variation of immune response to malnutrition depends on the animal model and duration and extent of nutrient restriction.

The *in vitro* model further explored the effects of dietary restriction on the cytokines produced by PBMCs. The PBMCs from both ER and PR nutrient goats produced less NO than control goats, which generally increased the pro-inflammatory response to LPS. Our results are in agreement with Anstead *et al.* (2003) who demonstrated that multi-nutrient undernutrition dysregulates the resident macrophage

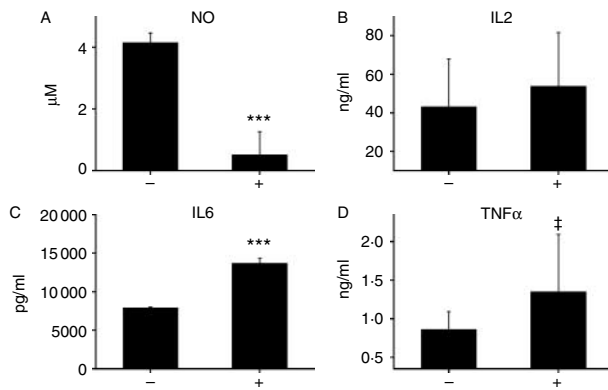
pro-inflammatory cytokine network in mice. The PBMCs separated from blood contain lymphocytes and monocytes, which mediate host defense of innate immunity when these cells are recognized pathogens, such as LPS (Beutler 1999). The production of pro-inflammatory cytokines, such as IL2, IL6 and IL10, is regulated by the transcription factor of NF $\kappa$ B (Blackwell & Christman 1997, Ghosh *et al.* 1998), which has been shown to be dysregulated by multi-nutrient undernutrition (Anstead *et al.* 2003). In a previous study, LPS-stimulated PBMCs from severely malnourished children were found to produce less TNF $\alpha$  (Doherty *et al.* 1994), which is not consistent with the higher concentrations of TNF $\alpha$  observed for PR goats in our study (Fig. 3D). These results indicate that other regulatory factors may play an important role in TNF $\alpha$  production during gestation.

The current results demonstrated that there was a discrepancy between the plasma NO and the NO released by PBMCs. The results suggested that inflammatory cells were not the source of the increased plasma NO in late pregnant goats under the condition of protein or energy restriction. For example, vascular endothelial cells can produce NO. Simultaneously, the conflicting results for cytokine production in relation to NO concentration *in vivo* and *in vitro* may indicate that there are other regulation factors in pregnant goats under the conditions of protein and energy restriction. It is possible that the cytokine network was regulated through the increasing NO concentration observed during protein and energy restriction in pregnant goats. Further, we confirmed that the negative feedback function of NO (Connelly *et al.* 2001) regulated the production of IL2 and IL6 in these goats.

In the subsequent *in vitro* experiments, PBMCs, representing lymphocytes and macrophages, were used to study the relationship between NO and cytokine production through



**Figure 5** Effects of exogenous NO on concentrations of (A) NO, (B) IL2, (C) IL6 and (D) TNF $\alpha$  released by PBMCs collected from ER goats on day 145 of gestation. Cultures of cells were supplemented with NO by adding SNP. The PBMCs stimulated with LPS alone (-) or LPS + SNP (+) for 4 h of incubation. The PBMCs were obtained from three goats and six replicates were conducted. \*\*\* $P < 0.0001$  and † $P < 0.10$ ; effects of SNP addition.



**Figure 6** Effects of NOS inhibition on (A) NO, (B) IL2, (C) IL6 and (D) TNF $\alpha$  concentrations released by PBMCs collected from well-nourished goats on day 145 of gestation. The NOS was inhibited using L-NAME. The PBMCs were stimulated with LPS alone (-) or LPS+L-NAME (+) for 4 h of incubation. The PBMCs were obtained from three goats and six replicates were conducted. \*\*\* $P < 0.001$  and ‡ $P < 0.10$ ; effects of L-NAME addition.

the addition of SNP and L-NAME to PBMC culture media. The increased exogenous NO concentration of PBMCs collected from PR and ER goats with SNP added to the culture medium and corresponding declines in IL2 (only for ER) and IL6 concentrations confirm the negative feedback role of NO in goats. Additionally, the higher IL6 concentration with addition of L-NAME to the culture medium of PBMCs collected from control goats further confirms the role of NO in regulating cytokines, and in particular IL6. It follows that plasma cytokine concentration (especially IL6) in malnourished pregnant goats remained constant throughout late gestation by elevated NO production, which down-regulated the increased cytokine production released by PBMC. However, with the limitation of animal numbers in the current study, the important finding on negative feedback function of NO regulating the cytokine production needs more experiments to testify for malnourished pregnant ruminants.

In this study, we found that the biosynthesis of NO increased with time of gestation in late pregnant goats. Similarly, biosynthesis of NO increased in rats and sheep throughout gestation, but NO status in human pregnancy is inconsistent according to the review of Sladek *et al.* (1997). NO is endogenously produced from L-arginine and is catalysed by two types of NOS: constitutive NOS and inducible NOS (iNOS). Under normal physiological conditions, only a small amount of NO is produced by constitutive endothelial NOS and neuronal NOS and it has several biological functions, including the regulation of blood vessel tone and neurotransmission. Increased NO in late gestation has been shown to regulate the uterine and fetoplacental blood flow, and it is involved in uterine quiescence prior to parturition (Sladek *et al.* 1997). The large amounts of NO produced by iNOS are known to be responsible for the inflammation, vasodilation and

hypotension observed in septic shock and cancer metastasis (Bogdan 2001, Guzik *et al.* 2003, Lange *et al.* 2009, Luiking *et al.* 2010). Additionally, large amounts of NO are toxic and pro-inflammatory, and are thought to play a central role in endotoxin-induced tissue damage (Kmiec 2001). High output of NO increases the risk of susceptibility to human disease because of NO-mediated cytotoxicity (Kroncke *et al.* 1998). Localized concentration of NO (approaching 4–5  $\mu$ M) in a site of inflammation is toxic to all cells in the vicinity (Marletta & Spiering 2003). In the current study, plasma NO concentrations of 40% PR and ER goats were increased when compared with control goats at day 145 of gestation. Increased plasma NO production in nutrient restriction groups could result in a detrimental impact on the uterus and placenta, thereby retarding placental and fetal growth. Similarly, protein or energy restriction might increase the plasma NO for human pregnancies. Given the cytotoxicity of high NO concentration, it would be interesting to further investigate whether the elevated NO production in the malnourished mother plays a role in fetal programming.

In summary, protein or energy restriction increased plasma NO production but did not affect the concentration of plasma IL2, IL6 and TNF $\alpha$  in pregnant goats during late gestation. Further *in vitro* studies using PBMCs from these goats demonstrated that protein or energy restriction decreased NO production but generally increased the secretion of IL2 and IL6. Finally, plasma NO was confirmed to act as a negative feedback regulator for IL2 and especially IL6 production released by lymphocytes and macrophages. Together, these findings indicate that malnutrition of pregnant goats during late gestation caused elevated NO levels in plasma, which inhibited cytokine production, particularly that of IL6. Consequently, cytokine concentrations in plasma remain relatively unchanged as a result of malnutrition of goats in late gestation.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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