Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development

J. M. Lozano^{1*}, P. Lonergan^{2,3}, M. P. Boland^{2,3} and D. O'Callaghan^{1,3}

¹Faculty of Veterinary Medicine and ²Agriculture; and ³Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland

Two experiments were carried out to study the effect of nutrition on embryo development in two periods in superovulated ewes (Expt 1) and on oocyte developmental capacity during the late follicular phase (Expt 2). In Expt 1, a lower superovulation response in terms of animals ovulating ($P < 0.05$), ovulation rate per ewe ovulating ($P = 0.1$) and number of good quality embryos per animal treated ($P < 0.07$) was noted in ewes fed an *ad libitum* diet compared with ewes offered control (1.5 times the daily maintenance energy requirements, $1.5 \times M$) or low energy ($0.5 \times M$) diets. Nutrition also modified the morphological and functional quality of the oocytes and embryos recovered. Thus, 92% of day 4 embryos recovered from ewes offered the control diet were classified as good embryos, compared with 70 and 82% of those recovered from ewes offered the *ad libitum* and low diets, respectively ($P < 0.05$). Ewes offered the *ad libitum* diet had a greater percentage of poorly developed embryos compared with ewes offered the control or low diets ($P < 0.05$). Ewes fed the low diet tended to have more non-fertilized oocytes than ewes offered the control diet ($P = 0.09$). Diet of recipient ewes to which good quality embryos were transferred on day 4 did not affect embryo quality, when assessed 12 days later (day 16 of pregnancy).

However, recipient diet affected prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production *in vitro*, and uterine tissue that originated from recipient ewes on the low diet secreted more $PGF_{2\alpha}$ relative to uterine tissue that originated from recipients on the control diet ($P < 0.05$). In Expt 2, fewer total ($P < 0.05$) and good quality ($P < 0.01$) oocytes and a lower percentage of good quality oocytes ($P < 0.01$) were obtained from superovulated ewes offered the *ad libitum* diet compared with ewes offered the low diet. In addition, cleavage rate tended to be higher (51 versus 35%, $P = 0.09$) in ewes offered the low diet compared with ewes offered the *ad libitum* diet. In conclusion, changes in diet can affect the quality of the oocyte and embryo in superovulated sheep. A lower superovulation response and a decrease in the quality of oocytes and embryos indicate that *ad libitum* diets are highly detrimental for superovulatory programmes when compared with low and control diets. In addition, the results from the present study indicate that a low energy diet during early embryo development increased the uterine production *in vitro* of $PGF_{2\alpha}$ which could lead to a poor uterine environment thereby compromising the development of the embryo.

Introduction

Extremes of nutrition can increase embryo mortality or delay embryo development in ewes. Undernutrition has been shown to delay embryo development during the first 2 weeks after fertilization (Parr *et al.*, 1987; Abecia *et al.*, 1997) and increase embryo mortality in the first 2 weeks of pregnancy (Rhind *et al.*, 1989; Abecia *et al.*,

1995). Conversely, overfeeding can also lead to reduced pregnancy rates (Parr *et al.*, 1987) and a decrease in the rate of development *in vitro* and the viability of embryos collected on day 2 after fertilization (Creed *et al.*, 1994). However, it is not known whether the effects of diet on embryo development are solely confined to the period of embryo development or whether diet can also affect oocyte development before fertilization, which is subsequently manifested in poor embryo development.

Progesterone is the main hormone involved in the maintenance of pregnancy. At present, the relationship between the effect of nutrition on jugular blood

*Current address: Department of Veterinary Preclinical Studies, University of Glasgow Veterinary School, Bearsden Road, Glasgow G61 1QH, UK
Email: j.m.lozano@vet.gla.ac.uk

concentrations of progesterone and embryo mortality is not clear. It has been shown that there is an inverse relationship between peripheral progesterone concentrations and nutrition (Parr *et al.*, 1987; Rhind *et al.*, 1989; Creed *et al.*, 1994). This relationship is most likely a consequence of an increased catabolism of steroids by the larger livers often present in ewes fed a high energy diet (Parr *et al.*, 1993).

Although this finding may explain the increase in embryo mortality in ewes fed a high energy diet, embryo mortality can be increased in both underfed and overfed ewes. Local concentrations of progesterone, measured at the ovary and uterus, are not related to peripheral concentrations measured in jugular venous blood (Lozano *et al.*, 1998). However, concentrations of progesterone in circulation near the reproductive organs may be biologically more important for embryo survival. As such, it is of interest that Lozano *et al.* (1998) reported lower endometrial progesterone concentrations on day 5 of the luteal phase in ewes offered half maintenance energy requirements compared with ewes offered 1.5 times maintenance energy requirements. These concentrations were inverse to those found in plasma collected from the jugular vein (Lozano *et al.*, 1998).

The main hormone responsible for luteolysis is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), although interferon tau ($IFN-\tau$) is the main embryonic antiluteolytic signal in ruminants. $IFN-\tau$ suppresses transcription of the oestrogen receptor and oxytocin receptor genes in the ovine endometrium (Spencer and Bazer, 1996), which may transform the response to circulating oxytocin from stimulating $PGF_{2\alpha}$ to prostaglandin E (PGE) (Asselin *et al.*, 1997) and, thus, regulate luteolysis. $IFN-\tau$ is secreted by the trophoderm of sheep conceptuses between day 11 and day 20 of pregnancy with maximal production per cell on about day 15 in sheep (for a review, see Martal *et al.*, 1998). Therefore, this is a critical period for embryo survival. Changes in embryo quality that could affect $IFN-\tau$ secretion during this period could adversely affect pregnancy rate.

The objective of the present study was to investigate the effect of nutrition on oocyte and embryo quality near the time of fertilization and to examine the role of progesterone, insulin-like growth factor I (IGF-I) and insulin in mediating the effects of nutrition on embryo quality. The hypothesis tested was that overnourished and undernourished ewes would have decreased concentrations of IGF-I and insulin and endometrial content of progesterone, and they would produce lower quality embryos after superovulation than would ewes in a control plane of nutrition. In the first experiment, an effect of nutrition was observed as early as day 4 of pregnancy, and a second experiment was carried out to test the hypothesis that overnutrition results in a decrease in the quality of oocytes when compared with undernutrition.

Materials and Methods

Experiment 1

The experimental design for Expt 1 is summarized (Fig. 1).

Animals and treatments. Forty-four crossbred adult ewes were used as embryo donors and were maintained in individual pens with free access to water. After 5 days of adaptation to the pens and grass meal as their dietary source, animals were allocated randomly to one of three groups and offered a diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg^{-1} DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements ($0.5 \times M$; low group, $n=22$), 1.5 times the maintenance energy requirements ($1.5 \times M$; control group, $n=11$) or an *ad libitum* diet (*ad libitum* group, $n=11$, intake estimated by residues = 2.2 times the maintenance energy requirements). At the same time, 80 ewes were used as embryo recipients and allocated to diets of grass meal at $0.5 \times M$ (low recipient group, $n=20$) or $1.5 \times M$ (control recipient group, $n=60$). Control recipient ewes were maintained in communal pens with free access to mineral blocks, whereas low recipient ewes were maintained in individual pens. All the donors and the low recipient ewes received $20 g day^{-1}$ of a general purpose sheep mineral supplement (GP Sheep Mineral, C. Wynne Co. Ltd, Ireland; Vitamin A, Vitamin D3, α -tocopherol, Co, Mn, I, Se, Ca, Na, Mg).

All ewes were synchronized using a 60 mg medroxy progesterone acetate pessary (Veramix Sheep, Pharmica and Upjohn Ltd, Dublin) inserted 5 days after the start of dietary treatment and withdrawn 2 weeks later. Donor ewes were superovulated with 200 mg NIH-FSH-equivalent (Folltropin-V, Vetrephearm Inc., Ontario). This treatment was administered i.m. in six injections of equal dose starting 48 h before pessary withdrawal (day 0), with the final injection administered 12 h after pessary withdrawal. Rams were introduced 36 h after pessary withdrawal and onset of oestrus was examined at 8 h intervals. Pessary withdrawal was delayed for 12 h in donor ewes relative to recipient ewes. Recipient ewes received an injection of 500 iu equine chorionic gonadotrophin (eCG) (Folligon®, Intervet, Boxmeer) at the time of pessary withdrawal.

Weight and body condition score (BCS) were recorded on a weekly basis. Blood samples were collected twice each week in donor ewes by jugular venepuncture from the day before the nutritional treatment started until the end of the experiment. IGF-I and insulin were assayed in these samples to monitor the metabolic state of the ewes. IGF-I concentration is known to increase during oestrus (Spicer and Zavy, 1992); therefore, additional samples were collected each day from day 3 to the day of slaughter and assayed for insulin and IGF-I. These samples were also assayed for progesterone to determine

Fig. 1. Design of Expt 1. After synchronization and superovulation treatment (FSH treatment, shaded bar) day 4 embryos were recovered, classified and transferred to synchronized embryo recipients. After a further 12 days, day 16 embryos were recovered from recipients and embryo production of interferon- τ *in vitro* was analysed. Insulin-like growth factor I (closed arrowheads) and progesterone (open arrowheads) concentrations were measured in embryo donor ewes. Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg^{-1} DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

the increase in progesterone concentration during the luteal phase. Progesterone concentration was also a measure of ovulation in some ewes fed the low diet that did not undergo surgery.

On day 5 after pessary withdrawal, donor ewes fed the *ad libitum* or control diets that showed oestrous behaviour (seven and nine ewes, respectively) and 15 donor ewes fed the low diet were anaesthetized (Sagatal®, Merial Animal Health Ltd, Lyon) and, after laparotomy, blood samples were collected from the ovarian vein. Subsequently, all the ewes were killed with an overdose of sodium pentobarbitone (Euthatal, Rhône Mérieux, Hertfordshire). The uterus was removed and oviducts were flushed with PBS to collect the embryos. Uterine samples were frozen in dry ice and subsequently stored at -70°C until progesterone content was determined. The liver was collected immediately and weighed.

Ewes were decapitated immediately after slaughter. Pituitary glands were dissected and samples from the anterior pituitary gland were snap-frozen in liquid nitrogen and stored at -70°C until analysed for FSH and LH content.

Recovered embryos were classified according to their stage of development. On day 4 of pregnancy, the embryo would normally have progressed to at least

the eight-cell stage (Fahey *et al.*, 2001). Therefore, the embryos recovered were classified as: empty zones and one-cell embryos (non-fertilized embryos), up to eight-cell embryos (retarded embryos) and embryos of eight-cells or more (normal embryos).

Embryos that were collected from donor ewes on the three diets and were considered to be good quality ($\geq$ eight-cells and acceptable morphological characteristics) were transferred to recipient ewes on control diets by surgical laparotomy. Recipient ewes on the low diet received good quality embryos produced in ewes on the low diet only, as a positive control for the effect of nutrition before day 4 of pregnancy.

A maximum of two embryos was transferred to the uterine horn ipsilateral to the most recent ovulation and each ewe received embryos originating from ewes in only one nutritional group. One control and four low recipient ewes received one embryo.

At day 12 after transfer of embryos, all recipient ewes were killed. The uteri were flushed with PBS to recover the embryos and samples of endometrium were then collected.

Incubation of endometrial tissue. Approximately 100 mg pieces of endometrial tissue from recipient ewes

were washed in TCM-199 and placed into individual wells of a 24-well tissue culture plate containing 500 μ l of cell culture medium. Samples from each uterine horn were cultured in duplicate with or without oxytocin ($1 \mu\text{mol l}^{-1}$) and incubated for 24 h in 5% CO_2 in humidified air at 37°C .

The cell culture medium consisted of Ham's F-12 medium supplemented with antibiotics, L-glutamine (0.29 mg ml^{-1}), insulin ($5 \mu\text{g ml}^{-1}$), transferrin ($5 \mu\text{g ml}^{-1}$) and selenium (5 ng ml^{-1}) (Thibodeaux *et al.*, 1994). There were two replicates for each tissue sample collected. After culture, the medium was collected and stored at -20°C until $\text{PGF}_{2\alpha}$ and PGE_2 concentrations were determined.

In vitro culture of conceptuses. Conceptuses obtained from recipient ewes were transferred into Petri dishes containing 15 ml modified Eagle's minimum essential medium (MEM) supplemented with 2% penicillin and streptomycin, and incubated for 24 h in 5% CO_2 in humidified air at 39°C . After culture, the medium was collected and stored at -20°C until the IFN- τ concentration was determined.

Endometrial progesterone content. Uterine concentrations of progesterone were determined using the technique described by Lozano *et al.* (1998). Endometrial tissue samples (500 mg) were homogenized in 10 ml isotonic buffer ($102.5 \text{ g sucrose l}^{-1}$, $0.375 \text{ g EDTA l}^{-1}$, $1.825 \text{ g Tris l}^{-1}$, pH 7.4). The supernatant was removed after centrifugation at 1000 g for 10 min. Progesterone was extracted from the supernatant, using 2 ml petroleum spirit each time. The extraction was repeated four times. The resulting extract was reconstituted in 300 μ l PBS and assayed for progesterone.

The proportion of progesterone recovered was estimated by measuring the recovery of tritiated progesterone added to a duplicate of the homogenate and the appropriate correction factor was then applied to the measured concentrations (Lozano *et al.*, 1998).

Hormone assays. For the IFN- τ anti-viral assay, culture medium was analysed for anti-viral activity using a cytopathic effect. The antiviral effect was quantified using Madin-Darby bovine kidney cell line (MDBK), Vesicular Stomatitis Virus Indiana strain as challenge virus and calibrated using GA 23-902-530 reference sample (NIH, Bethesda, MD) as described by La Bonnardière and Laude (1981). Results were expressed in international units (iu) as the reciprocal of the last dilution capable of preventing virus-induced cytopathic effect.

Culture media from wells were analysed for $\text{PGF}_{2\alpha}$ and PGE_2 using $\text{PGF}_{2\alpha}$ and PGE_2 enzymeimmunoassay kits (Cayman Chemical Co., Ann Arbor, MI). The kits were used in accordance with the manufacturer's instructions. Intra- and interassay coefficients of variation

for quality control samples were 8.2 and 10.7% for $\text{PGF}_{2\alpha}$ ($105.8 \pm 3.9 \text{ pg ml}^{-1}$) and 10.8 and 12.4% for PGE_2 ($30.0 \pm 1.3 \text{ pg ml}^{-1}$), respectively. The sensitivity of the assay was 7.8 and 3.9 pg ml^{-1} for $\text{PGF}_{2\alpha}$ and PGE_2 respectively.

Serum and endometrial concentrations of progesterone were measured using the AutoDelfiaTM fluoroimmunoassay (Wallac Ltd, Dublin). The kit was used in accordance with the manufacturer's instructions. Mean intra- and interassay coefficients of variation were 23.3 and 24.2% for low ($0.48 \pm 0.05 \text{ ng ml}^{-1}$), 15.0 and 13.1% for medium ($1.42 \pm 0.09 \text{ ng ml}^{-1}$) and 9.8 and 13.0% for high ($2.24 \pm 0.13 \text{ ng ml}^{-1}$) standards, respectively. The sensitivity of the assay was 0.05 ng ml^{-1} .

Serum IGF-I concentration was measured using a two-site immunoenzymometric assay, the OCTEIA[®] IGF-I ELISA kit (IDS Ltd, Boldon). Mean intra- and interassay coefficients of variation were 8.6 and 23.7% for low ($0.29 \pm 0.02 \mu\text{g l}^{-1}$) and 5.2 and 13.8% for high ($0.70 \pm 0.02 \mu\text{g l}^{-1}$) quality control samples, respectively. The sensitivity of the assay was $1.9 \mu\text{g ml}^{-1}$.

Serum concentrations of insulin were measured using a fluoroimmunoassay (AutoDELFA[®] kit, Wallac Ltd, Turku). Mean intra- and interassay coefficients of variation were 1.4 and 1.9% for low ($4.63 \pm 0.03 \mu\text{iu ml}^{-1}$) and 1.9 and 1.2% for high ($49.20 \pm 0.39 \mu\text{iu ml}^{-1}$) quality control samples, respectively. The sensitivity of the assay was $0.5 \mu\text{iu ml}^{-1}$.

LH pituitary content was measured by radioimmunoassay (Sweeney *et al.*, 1997). Mean intra- and interassay coefficients of variation were 11.4 and 10.5% for low ($0.44 \pm 0.05 \text{ ng ml}^{-1}$) and 5.5 and 9.9% for high ($6.01 \pm 0.34 \text{ ng ml}^{-1}$) quality control samples, respectively. The sensitivity of the assay was 0.1 ng ml^{-1} .

FSH pituitary content was measured by radioimmunoassay (Crowe *et al.*, 1997). Samples were run in a single assay with mean intra-assay coefficients of variation for low ($0.39 \pm 0.02 \text{ ng ml}^{-1}$), medium ($0.84 \pm 0.02 \text{ ng ml}^{-1}$) and high ($3.19 \pm 0.05 \text{ ng ml}^{-1}$) quality control samples of 9.2, 6.3 and 4.1%, respectively. The sensitivity of the assay was 0.06 ng ml^{-1} .

Experiment 2

Animals and treatments. Eighteen crossbred ewes were maintained in individual pens and offered the same grass meal as in Expt 1. After 5 days of adaptation and 2 days before the start of the same synchronization treatment as in Expt 1, they were divided into two nutritional groups that received either *ad libitum* ($n=9$) or $0.5 \times \text{M}$ diets (low, $n=9$). All ewes were superovulated with 1500 iu eCG administered 48 h before pessary withdrawal. At pessary withdrawal, all the ewes were killed. The ovaries were recovered and placed in PBS at approximately 35°C .

Table 1. Liver mass (kg) in ewes fed an *ad libitum* diet, or 1.5 (control) or 0.5 (low) times maintenance energy requirements

	<i>Ad libitum</i>	Control	Low
Number of ewes	6	9	15
Liver mass (kg)	1.28 ± 0.04 ^a	1.07 ± 0.06 ^b	0.78 ± 0.02 ^c
Liver:body weight ratio	1.8 × 10 ⁻² ± 7.0 × 10 ^{-4a}	1.6 × 10 ⁻² ± 6.8 × 10 ^{-4b}	1.32 × 10 ⁻² ± 3.7 × 10 ^{-4c}

Values are mean ± SE.

Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg⁻¹ DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

^{abc}Within the same row, values with different superscripts are significantly different ($P < 0.01$).

Oocytes were aspirated from all follicles that were > 2 mm in diameter. The oocytes were graded from one to four with regard to the morphology of the cumulus cell layers (Ward *et al.*, 2000): grade 1: oocytes with a compact multilayered cumulus investment; grade 2: intermediate oocytes; grade 3: denuded oocytes; and grade 4: oocytes had an expanded cumulus. All the oocytes recovered were matured and fertilized *in vitro* and embryos were cultured as described by Carolan *et al.* (1995). The number of oocytes cleaved was assessed at 48 h after insemination and the number of oocytes developing to the blastocyst stage was assessed on days 5, 6 and 7 after insemination.

Statistical analysis

The main effects of food intake on liveweight, body condition, and serum concentrations of IGF-I and insulin were analysed using ANOVA with repeated measures. Liver mass, IFN- τ secretion by the conceptus, endometrial secretion of PGF_{2 α} and PGE₂ *in vitro*, progesterone concentrations in the endometrium, ovarian and jugular veins, and FSH and LH pituitary content were compared using ANOVA. Duncan's post-test was performed when necessary in Expt 1. Data that were not normally distributed were logarithmically transformed before statistical analysis. Ovulation rate, classification of the embryos and oocytes recovered, cleavage rate, blastocyst formation rate, stage of development of embryo and embryo survival were compared using chi-squared tests.

Results

Experiment 1

Weight and body condition. Underfed ewes showed a significant decrease ($P < 0.001$) in body weight, whereas ewes fed the *ad libitum* diet showed a significant increase ($P < 0.001$) in body weight during the period in which the nutritional treatments were applied. Ewes that received the control diet showed significant changes ($P < 0.05$) in body weight, although initial body weight and weight at the time that the animals were killed were similar (67.9 ± 2.3, 65.8 ± 2.2, 64.6 ± 1.8 kg at

the start and 73.5 ± 2.4, 68.9 ± 6.8, 61.5 ± 1.8 kg at the end of the experiment for ewes offered the *ad libitum*, control and low diets, respectively). Ewes fed the *ad libitum* or control diets maintained their BCS throughout the experiment. However, underfed ewes showed a significant decrease ($P < 0.001$) in BCS during the experiment (2.59 ± 0.10, 2.48 ± 0.06, 2.49 ± 0.06 at the start 2.60 ± 0.06, 2.48 ± 0.08, and 2.42 ± 0.04 at the end of the experiment for ewes offered the *ad libitum*, control and low diets, respectively).

There were significant differences in liver mass among all the groups, and ewes had higher liver mass and ratio of liver to body weight at higher food intakes ($P < 0.001$) (Table 1).

Hormone and metabolite concentrations. There was an inverse relationship between level of nutrition and jugular progesterone concentration. Jugular concentrations of progesterone in ewes fed the low and control diets increased significantly from day 2 to day 5 after pessary withdrawal ($P < 0.05$). No significant changes were observed in the ewes fed *ad libitum* in the same period. Therefore, on day 4 and day 5, jugular blood progesterone concentrations were highest in ewes on the low diet, lowest in ewes on the *ad libitum* diet and intermediate in ewes on the control diet (Fig. 2a).

Mean serum progesterone concentrations in the ovarian vein ipsilateral to the corpus luteum tended to be higher in ewes offered the low diet and lower in ewes offered the *ad libitum* diet with intermediate values in the control ewes ($P = 0.07$) (Fig. 2b). Ovarian vein and jugular progesterone concentrations were correlated with the number of corpora lutea recorded at slaughter ($r = 0.677$; $P < 0.05$). The endometrial content of progesterone was not different among groups (Fig. 2b).

On day 5 after pessary withdrawal, pituitary LH content was lower in ewes offered the low diet compared with ewes offered the control diet (4.3 ± 0.8 and 2.4 ± 0.3 $\mu\text{g mg}^{-1}$ for ewes offered control and low diets, respectively, $P < 0.05$), but no differences were found between ewes offered the *ad libitum* diet (3.7 ± 0.8 $\mu\text{g mg}^{-1}$) and the other two groups. Pituitary FSH content was not significantly different among groups (0.20 ± 0.04, 0.38 ± 0.11, 0.25 ± 0.15 $\mu\text{g mg}^{-1}$ for ewes offered the *ad libitum*, control and low diets,

Table 2. Comparison between serum concentrations of insulin-like growth factor I (IGF-I; $\mu\text{g l}^{-1}$) before treatment and at day 13 after the start of nutritional treatment; and between serum concentrations of IGF-I ($\mu\text{g l}^{-1}$) at days 1, 2, 3 and 4 after pessary withdrawal in superovulated ewes fed an *ad libitum* diet, or 1.5 (control) or 0.5 (low) times maintenance energy requirements

Treatment group	IGF-I at					
	Start of treatment	Day 13 after start of treatment	Day 1 after pessary withdrawal	Day 2 after pessary withdrawal	Day 3 after pessary withdrawal	Day 4 after pessary withdrawal
<i>Ad libitum</i>	70.8 $\pm$ 3.5	76.0 $\pm$ 3.8	111.7 $\pm$ 16.8	104.5 $\pm$ 13.7	79.0 $\pm$ 11.7	67.6 $\pm$ 7.2
Control	82.8 $\pm$ 8.5	92.4 $\pm$ 12.2	132.7 $\pm$ 6.8	122.0 $\pm$ 30.2	93.5 $\pm$ 17.4	76.6 $\pm$ 16.7
Low	76.0 $\pm$ 13.0	95.0 $\pm$ 12.7	144.5 $\pm$ 51.5	83.2 $\pm$ 15.8	60.0 $\pm$ 10.1	54.8 $\pm$ 11.2
Mean	76.5 $\pm$ 5.1	87.8 $\pm$ 6.0	127.8 $\pm$ 12 ^a	103.1 $\pm$ 12.7 ^a	75.9 $\pm$ 8.3 ^b	66.3 $\pm$ 7.6 ^b

Values are mean $\pm$ SE.

Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg^{-1} DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

^{ab}Within rows, values with different superscripts are significantly different ($P < 0.05$).

Fig. 2. (a) Change in the serum concentration of progesterone in the jugular vein on days 2–5 after pessary withdrawal and (b) serum concentrations of progesterone in the ovarian vein (ng ml^{-1}) and endometrial progesterone content (ng mg^{-1}) on the day the sheep were killed (day 4 and day 5) in synchronized, superovulated ewes fed an *ad libitum* diet (AD), 1.5 (C) or 0.5 (L) times maintenance energy requirements. ^{a,b} $P < 0.05$; ^{a,c} $P = 0.07$. Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg^{-1} DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

respectively). Pituitary content of FSH and ovulation rate tended to be positively correlated ($r = 0.322$, $P = 0.09$).

Serum concentrations of IGF-I and insulin were not different among ewes in the different nutritional groups either at the start of the nutritional treatments or at 3 or 14 days later (Table 2). Serum concentrations of IGF-I increased during the periovulatory period, and there was

a higher concentration at days 1 and 2 than at days 3 and 4 after ovulation ($P < 0.05$) (Table 2).

Response to superovulation and synchronization. Sixty-four per cent (7 of 11 ewes), 82% (9 of 11) and 86% (19 of 22) of the embryo donor ewes offered the *ad libitum*, control and low diets, respectively, showed oestrous

behaviour. The percentage of ewes ovulating was lower ($P < 0.05$) in ewes offered the *ad libitum* diet (45%, 5 of 11) compared with ewes offered the control or low diets (73%, 8 of 11; 86%, 19 of 22, respectively) ($P < 0.05$).

The interval from pessary withdrawal to onset of oestrus was longer in ewes offered the *ad libitum* diet (44.6 ± 5.9 h) compared with ewes offered the control (36 ± 0 h) or low diets (36.6 ± 2.8 h) ($P < 0.05$).

Ewes in the *ad libitum* dietary group tended to have a lower ovulation rate than ewes offered the control or low diets (6.8 ± 2.3 , 11.4 ± 2.6 and 11.4 ± 1.6 in ewes offered the *ad libitum*, control and low diets, respectively, $P = 0.1$). Embryo recovery rate (embryos recovered divided by number of corpora lutea) was not different among groups (79.4, 80.2 and 79.4% per ewes offered the *ad libitum*, control and low diets, respectively).

When all treated ewes were considered (including those that failed to ovulate), a trend for fewer embryos recovered per ewe on nutritional treatment was observed in ewes offered an *ad libitum* diet compared with ewes offered control or low diets (2.5 ± 1.2 in the ewes offered the *ad libitum* diet, compared with 6.6 ± 2.0 and 7.1 ± 1.4 in the ewes offered control and low diets, respectively, $P = 0.08$). Similarly, when all superovulated ewes were considered, the mean number of embryos with eight or more cells with acceptable morphology tended to be lower ($P = 0.09$) in ewes offered the *ad libitum* diet (1.7 ± 0.7), compared with ewes offered the control or low diets (6.1 ± 1.9 and 5.8 ± 1.5 for ewes offered the control and low diets, respectively).

Embryo quality on day 4. Twelve animals (six *ad libitum*, three control and three low) that did not ovulate and five ewes offered the low diet that did not undergo surgery were removed from the experiment for the statistical analysis of embryo quality. Ewes fed the control diet had the highest percentage of good embryos (92% of embryos morphologically acceptable and with eight or more cells) compared with 70 and 82% in the *ad libitum* and low diet groups, respectively ($P < 0.05$) (Table 3).

Ewes offered the low diet tended to have more unfertilized oocytes compared with ewes offered the control or *ad libitum* diets ($P = 0.09$). A delay in embryo development was observed in embryos recovered from ewes offered *ad libitum* diets (26% of embryos recovered with fewer than eight cells) compared with 4 and 7% in ewes on the control and low diets, respectively ($P < 0.05$) (Table 3).

Embryo quality on day 16. Only embryos with eight cells or more and acceptable morphology were transferred to recipients on day 4 of pregnancy. The diet of the embryo donor ewe did not modify any parameter in the embryos recovered 12 days later from the recipient (day 16 of pregnancy). Thus, no differences in recovery

Table 3. Ovulation rate (corpora lutea), number of embryos recovered and classification of these embryos at day 4 after mating in superovulated ewes fed an *ad libitum* diet, or 1.5 (control) or 0.5 (low) times the maintenance energy requirements

	<i>Ad libitum</i>	Control	Low
Treated ewes*	11	11	22
Ewes responding†	5	8	14
Corpora lutea	34	91	160
Embryos recovered	27	73	127
One-cell or empty zones	1 (0.04) ^a	3 (0.04) ^a	14 (0.11) ^c
Two- to eight-cells	7 (0.26) ^a	3 (0.04) ^b	9 (0.07) ^b
Eight-cells or more	19 (0.70) ^b	67 (0.92) ^a	104 (0.82) ^b
Eight-cells or more/corpora lutea	0.54	0.73	0.65

Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg⁻¹ DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements. Values in parenthesis are the proportion with respect to the total number of embryos.

*Number of ewes undergoing superovulation.

†Number of ewes from which embryos were recovered.

Different superscripts indicate significant differences within rows (^{a,b} $P < 0.05$; ^{a,c} $P = 0.09$).

rate (embryos recovered/embryos transferred) or IFN- τ production *in vitro* were found in any of the groups (Table 4).

Neither the addition of oxytocin to the culture medium nor the presence of conceptus in the uterine horn modified the endometrial production of PGF_{2 α} or PGE₂ *in vitro* (Table 4). For subsequent statistical analysis, results from culture with and without oxytocin were pooled and only responses in uterine horns containing a conceptus were considered.

The diet of the embryo donor ewe did not modify production of PGF_{2 α} and PGE₂. The diet of the embryo recipient ewe modified production of PGF_{2 α} . Samples of endometrium collected from recipient ewes offered the low diet produced more PGF_{2 α} after culture *in vitro* than did those collected from recipient ewes offered the control diet ($P < 0.05$). However, there was no difference in the production of PGE₂ *in vitro*. Thus, the ratio of PGF_{2 α} :PGE₂ was also higher in recipient ewes offered the low diet ($P < 0.05$).

Experiment 2

Body weight and body condition score. There was no difference in initial body weight (67.5 ± 1.5 and 68.6 ± 1.9 kg) or BCS (2.58 ± 0.08 and 2.5 ± 0.09) for ewes offered the *ad libitum* and low diets, respectively. After 3 weeks of nutritional treatment, body weight was higher ($P < 0.01$) in ewes offered the *ad libitum* diet

Table 4. Recovery rate of embryos transferred on day 4 and collected on day 16 of pregnancy from superovulated ewes fed an *ad libitum* diet, or 1.5 (control) or 0.5 (low) times maintenance energy requirements to recipients in low and control diets, and PGF_{2α} and PGE₂ endometrial *in vitro* and IFN-τ embryonic *in vitro* secretion in uteri and embryos collected from recipients

Recipient group	Control			Low	
Donor group	<i>Ad libitum</i>	Control	Low	Total	Low
Embryos transferred	22	26	36	84	37
Embryos recovered (recovery rate)	9 (41)	13 (50)	15 (42)	37 (44)	17 (46)
IFN-τ (iu ml ⁻¹ × 10 ³)	25 ± 13	16 ± 5	27 ± 12	23 ± 5.7	9 ± 5.1
PGF _{2α} (ng mg ⁻¹)	1.00 ± 0.32	1.71 ± 0.74	0.92 ± 0.24	1.21 ± 0.28 ^a	1.65 ± 0.32 ^b
PGE ₂ (ng mg ⁻¹)	0.26 ± 0.06	0.42 ± 0.15	0.31 ± 0.05	0.33 ± 0.05	0.43 ± 0.09

Values are mean ± SE.

Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg⁻¹ DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

IFN-τ: interferon tau; PGF_{2α}: prostaglandin F_{2α}; PGE₂: prostaglandin E₂.

^{ab}Within rows, values with different superscripts are significantly different ($P < 0.05$).

Table 5. Oocytes recovered and oocytes classified as grade 1 per ewe (mean ± SE), total oocytes cleaved/total oocytes cultured and blastocysts developed in day 7 of culture in oocytes collected from synchronized, superovulated ewes offered an *ad libitum* diet or 0.5 (low) times maintenance energy requirements

	<i>Ad libitum</i> (%)	Low (%)	Significance
Number of ewes	9	9	
Total oocytes recovered per ewe	5.8 ± 1.1	10.3 ± 1.6	$P < 0.05$
Total grade 1 oocytes per ewe	3.4 ± 0.8	7.6 ± 1.4	$P < 0.05$
Grade 1 oocytes per total oocytes	31/52	68/93	NS
Oocytes cleaved per oocytes cultured	18/52 (34.6)	43/84 (51.1)	$P = 0.09$
Blastocysts developed per oocytes cultured	14/52 (26.9)	24/84 (28.6)	NS
Blastocysts developed per oocytes cleaved	14/18 (77.8)	24/43 (55.8)	NS

Low diet: diet of grass meal (90% dry matter (DM), 10.75 MJ metabolizable energy kg⁻¹ DM, 16% crude protein in DM) calculated to provide 0.5 times the maintenance energy requirements; control diet: 1.5 times the maintenance energy requirements; *ad libitum* diet: intake estimated by residues = 2.2 times the maintenance energy requirements.

Values in parenthesis are percentages.

NS: not significant.

(68.7 ± 1.2 kg) compared with ewes offered the low diet (60.9 ± 1.8 kg). BCS was also higher ($P < 0.05$) in ewes offered the *ad libitum* diet (2.70 ± 0.07) compared with ewes offered the low diet (2.33 ± 0.13).

As in the first experiment, liver mass (1.20 ± 0.06 and 0.77 ± 0.05 kg for ewes offered the *ad libitum* and low diets, respectively, $P < 0.05$) and the liver:body weight ratio ($1.7 \times 10^{-2} \pm 6.8 \times 10^{-4}$ and $1.3 \times 10^{-2} \pm 6.1 \times 10^{-4}$, for ewes offered the *ad libitum* and low diets, respectively, $P < 0.05$) were higher in the ewes offered the *ad libitum* diet than in those offered the low diet.

Oocyte collection and culture. Results of oocyte recovery and culture are shown (Table 5). More total oocytes and oocytes classified as grade 1 were recovered from animals offered the low diet than from those offered the *ad libitum* diet ($P < 0.05$). The cleavage rate (number of oocytes cleaved/total oocytes cultured)

tended to be higher in the oocytes collected from ewes offered the low diet compared with those from ewes offered the *ad libitum* diet ($P = 0.09$) but the effect was not significant. Nutritional treatments did not modify blastocyst formation rate at day 5 or day 7, when either total oocytes cultured or only oocytes that cleaved were considered.

Discussion

The experiments reported here show that short-term (up to 24 days) nutritional treatment modifies embryo quality, and that the effect is evident as early as day 4 of pregnancy. Moreover, results from the second experiment show an effect of nutrition on oocyte morphology and developmental capacity *in vitro*. In addition to effects on embryo quality, the results from the present study demonstrate that *ad libitum* diets are also detrimental

for the superovulatory treatment as fewer ewes fed the *ad libitum* diet showed oestrous behaviour and ovulation rates were lower than in ewes fed the control or low diets.

The *ad libitum* diet clearly reduced the performance of the superovulatory treatment. In Expt 1, ewes offered the *ad libitum* diet had a lower ovulation rate than ewes offered the low or control diets and in Expt 2, fewer follicles and, thus, fewer oocytes were recovered from such ewes relative to those offered the low diet. Therefore, these results are similar to those of studies in superovulated beef heifers which also demonstrated a positive effect of low compared with high nutrition on production of large (7–10 mm in diameter) follicles (Nolan *et al.*, 1998) and ovulation rate (Yaakub *et al.*, 1999). Moreover, an excess in energy supply decreased response to a superovulatory FSH treatment and embryo quality in Holstein dairy heifers (Humboldt *et al.*, 1997). However, other reports concluded that low nutrition intake was associated with a decrease in ovulation rate when compared with a control nutrition intake in synchronized non-superovulated ewes (Rhind *et al.*, 1989). This outcome is most likely due to a lack of responsiveness of follicles in the last stages of folliculogenesis to gonadotrophins and, as a result, the degree of atresia increases. However, the gonadotrophic treatment associated with the superovulation in the current experiments may have maximized the individual potential of each ewe and overcome the negative effects of low energy intakes (McEvoy *et al.*, 1995), resulting in no differences in ovulation rate between ewes offered low and control diets. A different experiment showed that more follicles (≥ 3 mm in diameter) were recovered in superovulated sheep offered 2.0 or 1.0 $\times$ maintenance diet compared with those offered 0.5 $\times$ maintenance diet. The differences with the present experiment could be due to variations in the diets and to the fact that the follicles were counted before the LH surge (O'Callaghan *et al.*, 2000).

Probably the most relevant result in the present study was the detrimental effect of the *ad libitum* diet on the quality of embryos and oocytes recovered after superovulation. The percentage of normal embryos (eight-cells or more and acceptable morphological classification) collected from ewes fed the *ad libitum* diet was lower compared with those collected from ewes fed the control diet. This difference was due to a delay in development in a higher percentage of the embryos recovered from ewes offered the *ad libitum* diet than in those fed the control or low diets. The delay in the onset of oestrus and, thus, ovulation, could partially explain the delay in embryo development. However, in Expt 2, *ad libitum* intakes had a negative effect on the morphological classification and cleavage rate of oocytes. Previous experiments have shown a detrimental effect of high energy diets on the development *in vitro* of embryos recovered as early as day 2 of pregnancy (Creed *et al.*, 1994). This finding indicates that the detrimental

effect of high energy intakes on embryo development are exerted, at least partially, before fertilization, during the acquisition of oocyte developmental competence.

A lower percentage of good embryos was collected from ewes offered the low diet than from ewes fed the control diet. However, unlike the *ad libitum* group, fertilization failure and not a delay in embryo development seems to be the most common cause, as the number of empty zonae pellucidae and unfertilized oocytes recovered tended to be higher in ewes fed the low diet compared with those fed the control diet. Rhind *et al.* (1989) reported an increase in embryo mortality in ewes fed the low diet after mating compared with those fed the control diet, but no effect was found when the low diet was fed exclusively before mating. Thus, the authors suggested that the negative effect of low energy intakes on embryo development is exerted during the period of embryo development and not during the period of acquisition of oocyte developmental competence. The results from Expt 2 support this theory, as a greater percentage of oocytes obtained from ewes offered the low diet were classified as grade 1 and they had a higher cleavage rate compared with those collected from ewes offered the *ad libitum* diet.

Progesterone, the main hormone sustaining pregnancy, may play an important role in mediating the effect of nutrition on embryo development. The results from Expt 1 show an inverse relationship between energy intake and jugular progesterone concentration, confirming a result found in previous experiments in sheep (Parr *et al.*, 1987; Rhind *et al.*, 1989; Creed *et al.*, 1994). The low concentrations of progesterone in ewes fed the *ad libitum* diet is likely to be due to fewer corpora lutea (Thorburn *et al.*, 1969) and to a faster clearance rate of progesterone as result of a higher liver mass (Parr *et al.*, 1993). However, jugular and endometrial concentrations of progesterone were not related at all and this result is in agreement with a report by Lozano *et al.* (1998). There is a countercurrent mechanism of transport of progesterone from the ovarian vein to the ovarian artery, before it enters general circulation (Hunter, 1987) so the concentration of progesterone in the uterine environment is several times higher than that in the peripheral circulation. These results highlight the need for examining the progesterone concentration in the local environment of the embryo (at the ovary and uterus) instead of peripherally (at the jugular vein) when studying embryo mortality.

Although diet can markedly affect insulin and related hormones, this experiment failed to demonstrate a role for IGF-I and insulin in the effect of nutrition on embryo development. Other studies have shown no effect of diet on plasma or follicular fluid concentrations of IGF-I (Spicer *et al.*, 1991), although short-term fasting for 48 h decreased circulating IGF-I concentrations (Spicer *et al.*, 1992). Differences with this experiment could be due to the time of collection of blood relative to the start of the nutritional treatment, as in the present

experiment IGF-I was measured 13 days after the start of the nutritional treatment. However, even without a difference in systemic IGF-I, the bioavailability of IGF-I could be different as a result of different concentrations of IGF binding proteins (IGFBP). In steers, nutrition modifies serum concentrations of IGFBP 2, 3 and 4 (Lee *et al.*, 2000). Thus, it is quite possible that nutrition regulates the bioavailability of IGF-I through the control of IGFBPs and a study of IGF-I concentration only is insufficient when analysing its role on the effect of nutrition on embryo or oocyte development.

When only good embryos were transferred on day 4 of pregnancy, the nutritional status of the embryo donor ewe did not modify the embryo quality 12 days later, on day 16 of pregnancy. It was concluded that, although nutrition influences early embryo development, good embryos transferred at the correct stage of development have the same likelihood to survive, regardless of the nutritional status of the embryo donor ewe. This effect has been observed before at an earlier stage of development, as oocytes obtained from ewes that were offered different diets had the same likelihood to survive once they cleaved, regardless of the nutritional treatment of the donor (Papadopoulos *et al.*, 2001).

The nutritional status of the recipient ewe did not affect the quality of the embryo at day 16 of pregnancy, as measured by embryo recovery rate at day 16 and production of IFN- τ *in vitro*. However, the endometrial secretion of PGF $_{2\alpha}$ *in vitro* was higher when the cultured endometrium came from recipient ewes offered the low diet compared with ewes offered control diet. In goats, an increase in progesterone concentration immediately after ovulation shortens the duration of the luteal phase (Menchaca and Rubianes, 2001) and the authors suggest a negative effect of progesterone in luteotrophic support or a premature release of luteolysin. Thus, the increase in plasma progesterone secretion in ewes fed the low diet could lead to an increase in endometrial secretion of PGF $_{2\alpha}$ *in vitro*. This adverse uterine environment could be detrimental to the survival or the further development of the embryo, even during postnatal life. Thus, some experiments have shown a negative effect of undernutrition on fetal development (Borwick *et al.*, 1997) and on offspring fertility (Gunn *et al.*, 1995; Wallace *et al.*, 1999).

In summary, the *ad libitum* diet has a highly detrimental effect on the performance of a superovulatory treatment when compared with both control and low diets, not only in the number of oocytes or embryos yielded, but also in the quality of the oocytes and day 4 embryos recovered. The superovulatory response of ewes offered the low diet was similar to that in ewes offered the control diet, but with a higher percentage of fertilization failures, probably as a result of postovulatory events.

Finally, although nutritional treatments had no effect on embryo survival or embryo production of IFN- τ *in vitro* at day 16, the low diets induced a poorer uterine

environment for embryo development as measured by the endometrial production of PGF $_{2\alpha}$ *in vitro*. This result confirms the effect of low energy intake on embryo quality through postovulatory events.

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