Short-Term Undernutrition Affects Final Development of Ovulatory Follicles in Sheep Synchronized for Ovulation

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Contents
The objective of this study was to determine, in sheep, the effect of a short-term undernutrition on growth dynamics and competence of pre-ovulatory follicles. Synchronization of sexual cycles and induction of ovulation were performed, with progestagens and gonadotrophins, in 14 adult female sheep. Morphological characteristics and developmental competence of ovarian follicles to achieve ovulation were determined by imaging techniques (ultrasonography and laparoscopy) and blood sampling. All the animals ovulated and mean ovulation rates were similar between groups (2.0 ± 0.6 corpora lutea in control ewes and 2.2 ± 0.8 in undernourished sheep). However, nutritional restriction, even during a short period, was related to the presence of large follicles in static growing phase which, despite reaching ovulation, persisted static during the induced follicular phase and evidenced functional alterations as there was no inhibition of the development of subordinate follicles. Thus, this study suggests the existence of deleterious effects from short-term undernutrition on functionality of pre-ovulatory follicles, which can compromise fertility.

Introduction
In sheep, like in other species, undernutrition is linked to decreased pregnancy rates (Abecia et al. 2006). Early studies related this effect with deficiencies in early embryo development (Lozano et al. 2003), possibly as a result of inappropriate oocyte competence, abnormalities of the embryo, luteal inadequacy, failure of the supply of progesterone to the uterus or malfunction of the mechanisms involved in maternal recognition of pregnancy (Martin et al. 2004; Abecia et al. 2006). Competence of the oocyte to resume meiosis and become fertilized is determined by the pre-ovulatory development and functionality of its follicle. The previous studies report on the effect of undernutrition on follicle characteristics evidenced differences in late stages of follicle development (Rhind and McNeilly 1998; O’Callaghan et al. 2000).

However, most of the studies on the effect of undernutrition are focused in long periods of deprivation and, to our knowledge, the effects on follicle development of deficiencies for a short period has not been previously evaluated. Thus, the specific objective of this study was to determine, in the sheep, the effect of a short-term undernutrition on dynamics and competence to ovulate of ovulatory-sized follicles growing during the follicular phase.

Materials and Methods
Animals and treatments
This experiment was carried out at the experimental farm of the University of Zaragoza, Spain (latitude 41°41’N), under the supervision of the Ethical Committee of the University and accordingly to the requirements of the European Union for Scientific Procedure Establishments. Fourteen Rasa Aragonesa ewes, 3–4 years old, with a mean (±SEM) body weight of 62.9 ± 0.5 kg and a mean body condition of 3.3 ± 0.1 (body condition is a reliable and widely used system for discerning amount of stored fat, which is scaled from 0, emaciated, to 5, obese; Gordon 1975) were used during the breeding season. The animals belonged to the experimental herd of the University and had no previous evidence of reproductive or health problems; throughout the experimental period, were housed in individual pens and, for allowing adaptation to experimental conditions, offered a diet formulated to fulfil their maintenance requirements (AFRC, 1993) for 1 month before the onset of the experimental procedure. The diet comprised 0.42 kg of pellets and 0.70 kg of barley straw per day, providing 7.8 MJ of metabolizable energy per ewe. The pelleted diet consisted of barley (85%) and soy bean (15%). The animals had unrestricted access to water and mineral supplement.

Reproductive cycle and ovulation was synchronized in all the females as usually performed in this species. In brief, intravaginal progestagen pessaries (40 mg of fluorogestone acetate, Chronogest; Intervet International, Boxmeer, the Netherlands) were inserted 1 month after the onset of the adaptation period and maintained for 12 days. At pessary insertion, sheep were allocated to two groups and fed diets that provided either 1 (Control group, n = 7) or 0.5 times (underfed group, n = 7), the daily requirements for maintenance; these regimens were maintained until the end of the experiment. At pessary withdrawal, the ewes were injected with gonadotrophins (300 IU of eCG; Foligon International, Boxmeer, the Netherlands) for inducing ovulation. Appearance of oestrous signs (considered as day 0 for experimental purposes) was detected every 8 h, from 12 h after pessary removal, with trained males. Body weight and body condition were recorded at insertion and withdrawal of pessaries, as well as at day 14, when ovulation rate was determined.
Morphometric evaluation of follicle dynamics

Individual assessment of every ≥2 mm follicle was performed daily from progestagen withdrawal to day of oestrous detection. Ovaries were examined by trans-rectal ultrasonography using a real-time B-mode scanner (Aloka SSD 500; Aloka Co., Tokyo, Japan) fitted to a 7.5-MHz linear-array probe. Scanning was performed as previously described (Schrick et al. 1993) and validated in our laboratory (Gonzalez-Bulnes et al. 1994). In brief, observations were conducted with the sheep placed in dorsal recumbence on a metallic cradle as used for laparoscopy. After introducing a hydro soluble contact gel into the rectum, the transducer was introduced perpendicularly to the abdominal wall. When the urinary bladder was surpassed and the uterine horns were located, the probe was rotated laterally 90° clockwise and 180° counter-clockwise to observe both ovaries and their structures. Each ovary was scanned several times from different angles to image all follicles ≥2 mm. The largest diameter of each of these follicles was measured and its position was recorded on a diagram of each ovary.

Assessment of ovulation and luteal function

Effects of undernutrition on ovulation rate were evaluated by determining, using laparoscopy, the number of corpora lutea at day 14 after the onset of oestrous behaviour. Luteal function was assessed by determining plasma progesterone concentration in samples obtained, every day from day −1 (0 = oestrus) to 13, by using vacuum blood evacuation tubes containing heparin (Vacutainer® Systems Europe; Becton Dickinson, Meylan Cedex, France). Immediately after collection, the blood samples were centrifuged at 1500 g for 10 min and plasma was removed and stored at −20°C until assayed. Plasma progesterone was determined using a direct solid-phase RIA (Coat-A-Count Progesterone®; Diagnostic Products Corporation, Los Angeles, CA, USA). The sensitivity of the assay was 0.1 nmol/l. The intra-assay coefficients of variation for low (10 nmol/l), medium (47.7 nmol/l) and high (95.4 nmol/l) concentrations were 6.5%, 5.2% and 5.6% respectively. The corresponding inter-assay coefficients of variation were 7.8%, 6.7% and 5.3%.

Statistical analysis

Evaluation of body weight and condition from the beginning to the end of the undernutrition period was performed using analysis of variance (ANOVA). Ultrasonographic data were summarized to characterize patterns of follicular development; these data were normalized to the day of appearance of oestrous behaviour (day 0). All follicles recorded by ultrasonography were classified by their largest diameter for assessment of possible effects of time of treatment on the number of follicles of various size categories. Four groups were categorized: total follicles ≥2 mm in size, large (≥6 mm), medium (4–5 mm) and small follicles (2–3 mm). After this, during follicular phase, the number of new follicles (not previously detected), growing follicles (those that increased in size with respect to the previous day) and decreasing follicles (those that decreased in diameter with respect to the previous day or disappeared) was also considered. The size of the largest (LF1) and the ovulatory follicles (OF) were recorded every day. Effects of treatment on the number and size of follicles and characteristics of pre-ovulatory follicles throughout the entire period of study were assessed by split-plot ANOVA, followed by Kruskal–Wallis test, when Levene’s test showed non-homogeneous variances and by Pearson correlation analysis and linear regression procedures, followed by Spearman non-parametric correlation tests where appropriate for non-homogeneous variables. Effect of treatment on ovulation rate and plasma progesterone concentrations was estimated by the analysis of variance (ANOVA). All results were expressed as the mean ± SEM and the statistical significance was accepted from p < 0.05.

Results

Body weight and condition were maintained, during the experimental period, in control sheep (63.1 ± 0.5–63.2 ± 0.5 kg and 3.29 ± 0.07–3.22 ± 0.07 respectively; Table 1). On the other hand, both parameters decreased in undernourished sheep (63.0 ± 0.5–58.6 ± 0.5 kg, p < 0.0001, and 3.36 ± 0.07–2.86 ± 0.07, p < 0.0001 respectively). Thus, both at days −1 and 14, body weight and condition were higher in the control group than in the undernourished sheep (p < 0.05).

Table 1. Mean body condition and weight (± pooled standard errors) at the beginning of the nutritional treatment and pessary insertion (day 13), pessary withdrawal (day 1) and slaughter (day 14) in ewes fed 1 time (control) and 0.5 times (underfed) the maintenance requirements

<table>
<thead>
<tr>
<th>Body weight (kg)</th>
<th>Control</th>
<th>Underfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 13</td>
<td>3.29 ± 0.07⁺</td>
<td>3.36 ± 0.07⁺</td>
</tr>
<tr>
<td>Day 1</td>
<td>3.25 ± 0.07⁺</td>
<td>3.07 ± 0.07⁺</td>
</tr>
<tr>
<td>Day 14</td>
<td>3.22 ± 0.07⁺</td>
<td>2.86 ± 0.07⁺</td>
</tr>
</tbody>
</table>

Within the same variable, different superscripts between groups indicate significant differences, p < 0.05.

Effects of short-term undernutrition on oestrus, ovulation and luteal activity

All the animals showed oestrous behaviour and ovulation after progestagen removal; there were no significant differences, either in timing of oestrous appearance or in ovulation rate, among the groups. Sheep showed sign of oestrous behaviour during the first 48 h after sponge removal; four of seven control ewes (57%) and five of seven undernourished ewes (71%) showed oestrous behaviour at 24 h after pessary withdrawal. The mean ovulation rate was similar in both groups (2.0 ± 0.6 corpora lutea in control ewes and 2.2 ± 0.8 in undernourished sheep).

Analysis of plasma progesterone levels showed that most of the animals, both in control and underfed groups, showed normal luteal phases in terms of
duration and progesterone concentration (Fig. 1a). However, there were three females showing luteal dysfunction. In two of them, one undernourished and one control, the rise in plasma progesterone levels was delayed until day 8 after oestrus (Fig. 1b, the control ewe is presented as example). The third sheep, from the control group, showed a short cycle with very low levels of progesterone for 4 days (Fig. 1c). These animals were not considered for the follicle dynamics comparison. In all the sheep developing active corpora lutea, independently of the group, plasma progesterone concentrations increased linearly (p < 0.0001) from day 3 after oestrus; reaching maximum values at day 13 of the cycle (Fig. 1a). There was no effect of nutritional treatment on plasma progesterone concentrations, except for day 11, when undernourished sheep presented higher levels than the control group (p < 0.05).

**Effects of short-term undernutrition on pre-ovulatory follicle dynamics**

The analysis of pre-ovulatory follicular development in sheep with adequate luteal support showed that the OF and subsequent corpora lutea (control: 2.0 ± 0.6; underfed: 2.2 ± 0.8) arose, in all the animals, from antral follicles present in the ovary at progestagen withdrawal. A total of 17 of the 21 OF (80.9%) were the largest follicles present in the ovaries at sponge removal. This percentage tended to be higher in underfed ewes (control: 70.0%; underfed: 90.9%; p = 0.07) as 85% of the underfed ewes had large follicles ≥6 mm in size, which were found in only 50% of the control females. Thus, at sponge removal, there were a higher number of ≥6 mm follicles in undernourished sheep (control: 0.2 ± 0.2; underfed: 1.0 ± 0.6; p < 0.05; Fig. 2a).

Thereafter, during the induced follicular phase, no significant increase in the number of ≥6 mm follicles was found in underfed group while the increase was significant in the control group (p < 0.01; Fig. 2a). The evolution in size of the largest follicles during the follicular phase was also different between groups; the two largest follicles (LF1 and LF2) increased in size in the control group (p = 0.07 for LF1 and p = 0.09 for LF2), but did not in underfed females (Fig. 2b).

The analysis of the relationship between the OF and the other remaining follicles showed that, in the control sheep, the number of medium follicles (4–5 mm in size) decreased during the period of growth of the pre-ovulatory follicles (p < 0.05), mainly by an inhibitory effect on follicles with 5 mm in diameter (p < 0.05). On the other hand, the mean number of 5-mm follicles was maintained towards oestrus in undernourished sheep (Fig. 3). These differences between groups were determined by differences in the pattern of growth of remaining follicles. No differences were found in the
number of new and decreasing follicles between groups; however, the number of remaining follicles in growing phase tended to increase in underfed sheep and to decrease in the control group (Fig. 4). A similar observation was found when considering the evolution of only those follicles growing from gonadotrophin-responsive to gonadotrophin-dependant stages (i.e. smaller to medium sizes, Fig. 4, upper panel).

Discussion

Most of the studies performed for ascertaining possible links between nutrition and reproduction have been based in positive inputs (Scaramuzzi et al. 2006) through moderate overfeeding but without reaching obesity. In brief, influence of body weight and condition on ovulation rate may be classified as long term or 'static effect', in which heavier ewes have higher ovulation rates than light ewes, and short term or 'dynamic effect', through increases in body weight and condition by an augmented feeding over 3–4 weeks before mating (Smith and Stewart 1990). Positive changes in body weight and condition act through positive influence on follicular growth and development to ovulation (Downing and Scaramuzzi 1991). Thus, it would be expected that a negative change in body weight and condition, even during a short period, would exert a negative influence on follicular development, as found in studies with a long-term nutritional deprivation (Rhind and McNeilly 1998; O'Callaghan et al. 2000).

In this study, underfed sheep with a negative energy balance evidenced a deviation in the patterns of final development of OF when compared with the control. In control sheep, coincidentally with the previous studies with different treatments for ovulation induction (Gonzalez-Bulnes et al. 2001, 2004a), most of the OF arose from antral follicles present in the ovaries at treatment withdrawal, grew during the induced follicular phase to reach ovulatory size and inhibited the development of the remaining follicles to gonadotrophin-dependent stages. In underfed sheep, most of the OF were large follicles in static phase at treatment withdrawal, persisted static during the induced follicular phase and evidenced functional alterations as there was no inhibition of the development of subordinate follicles.

Follicular dynamics in control sheep was similar to the dynamics classically described (Schrick et al. 1993; Souza et al. 1997; Tsonis et al. 1984). In brief, most of the pre-ovulatory follicles arise from antral follicles, being usually the largest growing follicle present in the ovaries; in those ewes in which the largest follicle is in regressing or early atretic phase, the pre-ovulatory follicles arise from the pool of antral growing follicles. These pre-ovulatory follicles exert dominance over the remaining follicles during its period of terminal growth; in sheep, conversely to other mammals (Fortune 1994), the dominant follicles decrease but does not suppress the emergence of new follicles, although it inhibits their subsequent growth to larger sizes by an increased atresia (Gonzalez-Bulnes et al. 2001, 2004b).

On the other hand, a high number of pre-ovulatory follicles were present at sponge removal in underfed sheep, despite the suppressor effect of progestagens on final follicle development (Noël et al. 1994; Leyva et al. 1998). Elucidation of possible causes would need the development of specific studies, but a possible hypothesis would arise from the fact that fasting depresses the hypothalamic expression of Kiss1 (Castellano et al. 2005). Kisspeptins are currently recognized as the most potent stimulators of GnRH and LH (Navarro et al. 2005a, b) and hypothalamic neurons expressing Kiss1 are crucial for adequate gonadotrophins secretion and for stimulatory effects of oestrogen (Estrada et al. 2006; Smith et al. 2006; Adachi et al. 2007). Dysfunction in the positive-feedback regulation of LH secretion by oestradiol is identified to be the initial factor inducing growth of follicles to larger than ovulatory size and even to formation of follicular cysts (Kaneko et al. 2002); moderate undernutrition and/or suckling (when Kiss signalling is inhibited; Yamada et al. 2007) can cause this condition, while defects in follicle growth to large sizes are only found in severe undernutrition (Wiltbank et al. 2002).

The large follicles found in underfed sheep of current study were likely to be in static, or early atretic, phase at progestagen withdrawal and exhibited a lack of

Fig. 3. Mean number of medium follicles (4–5 mm; main panel) and follicles with 5 mm in size (insert) during the follicular phase of control (solid bars) and underfed ewes (open bars; hour 0 = oestrus)

Fig. 4. Mean number of growing follicles (main panel) and follicles growing from smaller to medium (insert) during the follicular phase of control (solid bars) and underfed ewes (open bars; hour 0 = oestrus)
dominance, similarly to static follicles previously described by our group using a gonadotrophin-stimulated model (Veiga-Lopez et al. 2006, 2008a,b). Absence of dominance effects is likely to be related to an alteration of stereiodogenesis in these follicles, reinforcing hypothesis of the previous paragraph, as dominance is caused by a depression in the plasma FSH levels in response to an increased secretion of oestradiol and inhibit by dominant follicles (Tsonis et al. 1988; Gonzalez-Bulnes et al. 2004b). Usually, static, aged, follicles regress and a growing follicle ovulates; however, in the sheep with restricted nutrition in this study, the turnover was minimal and these large follicles reach ovulation.

However, we have to recognize that, whilst some of the results are consistent with a deleterious effect of subnutrition, some of the statistical analyses only indicate a trend for differences between underfed and control sheep although it is necessary to bear in mind that this trend was reached even with a rather reduced number of animals. On the other hand, the lack of greater differences may be related to the hormonal treatment elected for ovulation induction; progestagens are the most used method in practice. However, it is widely recognized that progestagens lead to lower conception rates than natural service, due to alterations in patterns of LH release (Gordon 1975; Scaramuzzi et al. 1988) and in the quality of ovulations (Killian et al. 1985). In a previous study developed by our group, a similar progestagen treatment affected the functionality of OF when compared with natural cycles (Evans et al. 2001); thus, the follicle development in the control sheep of this study may have been affected by the treatment, thereby masking some differences.

These considerations may also explain the lack of differences between groups in the ability of follicles to ovulate and in the capacity of induced corpora lutea to secrete progesterone during the subsequent luteal phase; although current results evidenced presence of aged follicles in underfed sheep. It has been described that aged follicles, even ovulating and developing in a functional corpus luteum, led to lower fertility (Ungerfeld and Rubianes 1999; Viñoles et al. 2001), as its ovulation has been related to alterations in the developmental competence of the oocyte (Revah and Butler 1996; Mihm et al. 1999), fertilization and early embryo development (Greve et al. 1995). Ovulation of defective follicles has also been related to alterations of the oviductal secretory patterns (Binelli et al. 2004b). Usually, static, aged, follicles regress and a growing follicle ovulates; however, in the sheep with restricted nutrition in this study, the turnover was minimal and these large follicles reach ovulation.

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Author contributions

All the authors participated equally to the research and its publication.

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