Effects of overnutrition and undernutrition on in vitro fertilization (IVF) and early embryonic development in sheep

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ABSTRACT

Nutrition has been shown to influence several reproductive functions including hormone production, oocyte competence and fertilization, and early embryonic development. To determine effects of maternal diet on in vitro fertilization (IVF) and early embryonic development, ewes (n = 48; 58.4 ± 1.7 kg initial body weight [BW]; 2.3 ± 0.1 initial body condition score [BCS]) were divided into control, overfed (ad libitum feeding) and underfed (60% of control) nutritional planes for 8 weeks before oocyte collection. Ewes were individually fed once daily with pelleted diets containing 2.4 Mcal of ME/kg and 13% CP (DM basis). Control ewes were fed to maintain BW and offered 760 g/day/50 kg. Synchronization of estrus was performed using progestagen sponges for 14 days. Follicular development was induced by twice daily injections of follicle stimulating hormone (FSH) on days 13 and 14 of the estrous cycle. During the 8 week experiment, control ewes lost 1.0 ± 0.9 kg, overfed ewes gained 11.8 ± 1.1 but underfed ewes lost 14.2 ± 0.9 kg, and BCS increased by 0.7 ± 0.2 and by 2.0 ± 0.1for control and overfed ewes, respectively, but decreased by 0.7 ± 0.1 for underfed ewes to compare with initial BCS. Oocytes were collected from all visible follicles on day 15 of the estrous cycle. After IVF, the proportion of developing embryos was evaluated throughout the 8 day culture period. Overnutrition and undernutrition decreased ($P < 0.0001$) rates of cleavage, and morula and blastocyst formation (from 85% to 51% and 48%; from 63% to 18 and 28% and from 40% to 5% and 6% for control, overfed and underfed ewes, respectively). However, number of visible follicles (large and small), total number of oocytes, number of healthy oocytes and percentage of healthy oocytes were similar for control and underfed ewes. These data indicate that overnutrition and undernutrition of donor ewes resulting in lower BW and BCS, has a negative effect on oocyte quality which results in lower rates of cleavage, and morula and blastocyst formation. These data demonstrate that nutrition level substantially affect IVF and early embryonic development.

Key words: *overnutrition, undernutrition, assisted reproduction, IVF, embryo, sheep*

INTRODUCTION

Assisted reproductive technologies (ART) have many applications in agriculture. Research directed toward improved quality of oocytes and in vitro embryo production has predominantly focused on optimization of culture conditions (Thompson, 1997; Guler et al., 2000; Rizos et al., 2002) and/or diet manipulation of donor ewes (O'Callaghan et al., 2000; Lozano et al., 2003; Peura et al., 2003) and cows (Yaakub et al., 1999; Sinclair et al., 2000; Armstrong et al., 2001).

Nutritional status is a major factor influencing an animal's ability to reproduce (Robinson, 1990; Webb et al., 1999; O'Callaghan et al., 2000). Nutrition has a significant impact on

numerous reproductive functions including hormone production, fertilization and early embryonic development (Boland et al., 2001; Armstrong et al., 2003; Boland and Lonergan, 2005). Nutritional status has been correlated with embryo survival and is a key factor influencing efficiency in ART (Armstrong et al., 2003; Webb et al., 2004). Conflicting results have been reported for the effects of low or high energy diets on oocyte quality and early embryonic development in ruminants (Kendrick et al., 1999; Boland et al., 2001; Papadopoulos et al., 2001). For example in sheep, low energy diets decreased cleavage rates compared with high energy diets (Papadopoulos et al., 2001). In contrast, a higher proportion of ova from ewes on low calorie diets were considered viable compared with those from ewes on high calorie diets (McEvoy et al., 1995). For cows, positive (Nolan et al., 1998; Kendrick et al., 1999; Boland et al., 2001), negative (Yaakub et al., 1999; Armstrong et al., 2001) or no effects (Tripp et al., 2000) of plane of nutrition (high or low energy diets) on oocyte quality, fertilization rate and early embryonic development have been reported. Therefore, additional study should clarify the effects of nutritional plane on oocyte quality and early embryonic development.

We hypothesized that overfeeding or underfeeding of donor ewes would alter oocyte quality as measured by the rates of fertilization and early embryonic development in vitro. Therefore, the aim of the present study was to evaluate the effects of nutritional plane (control vs. overfed or underfed) on follicular development, in vitro fertilization (IVF), and early embryonic development in FSH-treated ewes.

MATERIALS AND METHODS

Treatment of animals

Western range (predominantly Targhee and Rambouillet) 2-3 years old ewes were standardized for live weight and body condition score (BCS). Ewes were housed and fed in individual pens (0.86 x 1.47 m) at the Animal Nutrition and Physiology Center under 14 h of darkness and 10 h of light at 12°C with free access to water and mineral supplements. Ewes were divided into three groups: control (n = 13 ewes) received a maintenance diet (see below), overfed (n = 18 ewes) fed ad libitum, and underfed (n = 17 ewes) received 60% of the maintenance diet for two months before oocyte collection. Once a week, during the duration of the experiment ewes were weighed and BCS was evaluated. Estrus was synchronized by insertion of chrono-gest sponges (Intervet, UK) to the uterus for 14 days. By using vasectomized rams, estrus was detected 40-48 hours after sponge withdrawal. Ewes received twice daily (morning and evening) injections with FSH-P (Sioux Biochemical, Sioux Center, IA, USA) on days 13 (5 mg/injection) and 14 (4 mg/injection) following estrus (day 0) as described before (Stenbak et al., 2001). On day 15 of the estrus cycle ewes were ovariectomized (Luther et al. 2005). The study was initiated during the normal breeding season in August and finished in November. All procedures were performed at the animal experimental facilities of North Dakota State University (NDSU) located in Fargo, ND, USA (approximately 46.9° latitude and -96.8° longitude) and were approved by the Institutional Animal Care and Use Committee of NDSU.

Nutritional management

After arrival and a 3 day adaptation to individual pens and pelleted diets, ewes were allocated randomly to three nutritional groups as described above. The diet contained:

dehydrated beet pulp, 36.5%; dehydrated alfalfa, 20.3%; corn, 24.2%; soy hulls, 16%; soybean meal, 3.0% (% of dietary DM). The pelleted (0.48 cm diameter) diet, which was prepared and analyzed on site, supplied 2.4 Mcal/ME and 130 g crude protein (13%) per kg of diet DM basis and was offered in one portion daily. Dietary management procedures for both groups were similar to those described by Scheaffer et al., 2004a,b. The maintenance diet, which was prepared and analyzed on site on a weekly basis, supplies 2.4 Mcal/kg of metabolizable energy and 130 g crude protein (13%) per kilogram diet and was offered in one equal ration daily. The control group received 760 g/day/50 kg (100%), overfed group was fed ad libitum (200% or more of control) and the underfed group received 456 g/day/50 kg (60%) of the maintenance diet (dry matter basis).

Oocyte collection

Following ovariectomy, ovaries were immersed in PBS and transported to the laboratory in an incubator at 39°C. The number of visible small (≤ 3mm) and large (> 3mm) follicles on each ovary was determined, and cumulus oocyte complexes (COC) were isolated by opening each visible follicle with a scalpel blade and flushing it two to three times with oocyte collection medium (Grazul-Bilska et al., 2003, 2005; Luther et al., 2005). Under a stereomicroscope, COC were recovered from each dish and transferred to a petri dish containing fresh collection medium without heparin. Cumulus oocyte complexes were then evaluated and categorized as healthy or atretic based on their morphology (Thompson et al., 1995). All COC were then washed three times in maturation medium (TCM-199 containing 10% fetal bovine serum, ovine FSH [5 μg/mL; oFSH-RP-1; NIAMDD-NIH, Bethesda, MD, USA], ovine LH [5 μg/mL; oLH-26; NIADDK-NIH], estradiol -17β [1 μg/mL; Sigma St. Louis, MO, USA], glutamine [2 mM; Sigma], sodium pyruvate [0.25 mM; Sigma], epidermal growth factor [10 ng/mL; Sigma,] and penicillin/streptomycin [100 units/mL penicillin and 100 μg/mL streptomycin; Gibco, Grand Island, NY, USA]; Grazul-Bilska et al. 2003, 2005; Luther et al. 2005). Total number of oocytes used for IVF was 162 for control, 264 for overfed and 232 for underfed ewes.

In vitro maturation

Oocytes were matured in vitro in maturation medium for 24 h at 39°C in 5% CO_2 and 95% air followed by cumulus cell removal using 1% (wt/vol) hyaluronidase (Type I; Sigma) in PBS. The oocytes were again evaluated for health based on morphology. Oocytes classified as healthy were used for IVF and were transferred to equilibrated fertilization medium consisting of synthetic oviductal fluid (SOF) prepared in our laboratory (Stenbak et al., 2001) and 2% heat-inactivated sheep serum collected from sheep on day 0-1 of the estrous cycle (Grazul-Bilska et al., 2003, 2005; Luther et al., 2005).

In vitro fertilization and embryo culture

Frozen capacitated semen pooled from 4 Hampshire rams was thawed and viable sperm were separated using the swim up technique (Grazul-Bilska et al., 2005). The sperm (0.5 to 1.0 x 10^6 sperm/mL) were added to the IVF medium containing oocytes and incubated for 18 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. The presumptive zygotes were then washed three times with culture medium without glucose (SOF supplemented with BSA, glutamine, MEM non-essential

amino acids, BME amino acids [Sigma] and penicillin/streptomycin) and cultured in the same medium for 24 h at 39ºC, 5% O_2, 5% CO_2 and 90% N_2 (Grazul-Bilska et al., 2003, 2005). The dishes were then evaluated to determine the number of cleaved oocytes. The embryos were transferred to culture medium containing glucose (1.5 mM). After 48 h, the developmental stage was evaluated and embryos were transferred to fresh culture medium with glucose. The rate of cleavage (number of cleaved vs. non-cleaved oocytes), and the rate of early embryonic development (time and percentage reaching stage of morula or blastocyst) were evaluated every second day during 8 day culture.

Statistical analysis

To compare changes in BW, average daily gain (ADG) and BCS during the experiment, number of follicles and oocytes, and oocyte quality variables (e.g., the rates of cleavage, and morula and blastocyst formation) for control and underfed ewes, data were analyzed statistically by using the GLM program of SAS (SAS Inst., Inc., Cary, NC). Means were separated using the method of least significant difference. In addition, data for the proportion of overfed and underfed ewes providing oocytes which developed to blastocyst stage were analyzed by Chi-square.

RESULTS

At the time treatment was initiated, BW was similar for control, overfed and underfed groups (56.7± 2.1, 60.7± 1.2 and 57.8 ± 1.8 kg respectively; Figure 1A). At the end of 8 week the experiment, BW of overfed ewes was greater ($P<0.01$) than control or underfed ewes, and BW of underfed ewes was lower ($P < 0.05$) than control ewes (Figure 1A). Changes in BW were significantly different ($P < 0.001$) for nutrition groups at week 8 (Table 1). Average daily gains were greater or lower ($P < 0.001$) for overfed or underfed compared with control ewes during the during 8 week of experiment, respectively (Table 1). When compared to initial BW, control ewes lost 1.0 ± 0.9 kg, overfed ewes gained 11.8 ± 1.1 but underfed ewes lost 14.2 ± 0.9 kg over the 8 week experiment.

At the beginning of treatment, BCS was similar for control, overfed and underfed ewes (2.3 ± 0.1, 2.3 ± 0.1 and 2.4 ± 0.1 respectively; Figure 1B). Body condition score of overfed ewes was greater ($P < 0.001$) than control or underfed ewes at week 8 of the experiment (Figure 2B). Changes in BCS were significantly different ($P < 0.001$) for nutrition groups at week 8 (Table 1). During 8 week experiment, BCS increased by 0.7 ± 0.2 and by 2.0 ± 0.1 for control and overfed ewes, respectively, but decreased by 0.7 ± 0.1 for underfed ewes to compare with initial BCS.

Mean number of visible follicles, number of large and small follicles, total number of oocytes, number of healthy oocytes, percentage of healthy oocytes, and the number of oocytes used for IVF per ewe were similar for control, overfed and underfed groups (Table 1). The number of oocytes cleaved, cleavage rates, number of morula and blastocysts and the rates of morula and blastocyst formation were greater ($P < 0.0001$ to 0.057) for control compared with overfed or underfed ewes (Table 1). Proportion of ewes providing oocytes which developed to blastocyst stage tended ($P<0.1$) to be greater in underfed than overfed ewes.

Table 1. Effects of nutrition on the follicular development, the number collected oocytes, the number of healthy oocytes, the rates of cleavage and morula and blastocyst formation, and BW, ADG and BCS

Parameter	Control	Overfed	Underfed	*P* value
Number of ewes	13	18	17	
Total follicles (n)	27.5 ± 2.7	26.7 ± 2.2	25.8 ± 2.2	0.885
Large follicles (n)	12.9 ± 1.1	14.8 ± 1.5	14.5 ± 1.4	0.638
Small follicles (n)	14.5 ±2.3	11.9 ± 1.3	11.3 ± 1.6	0.402
Total Oocytes (n)	26.1 ± 2.8	25.9 ± 2.2	23.3 ± 2.4	0.651
Healthy oocytes (n)	22.5 ± 2.7	24.3 ± 2.0	21.5 ± 2.2	0.641
Healthy oocytes (%)	86.1 ± 4.5	94.7± 1.4	92.5 ± 1.6	0.063
Oocytes used for IVF	12.5 ± 0.9	14.7 ± 1.2	13.6 ± 1.4	0.490
Cleaved oocytes (n)	10.5 ± 0.9	7.5 ± 1.2	6.6 ± 1.1	0.057
Cleavage rate (%)	84.8 ± 2.5	51.1 ± 7.0	48.0 ± 5.1	0.0001
Morula (n)	6.5 ± 0.5	1.7 ± 0.5	1.7 ± 0.4	0.0001
Morula (%)	63.2 ± 4.8	17.6 ± 4.8	27.6 ± 6.5	0.0001
Blastocyst (n)	4.2 ± 0.4	0.4 ± 0.2	0.4 ± 0.1	0.0001
Blastocyst (%)	40.1 ± 3.6	5.0 ± 3.4	6.4 ± 3.2	0.0001
Initial BW (kg)	56.7 ± 2.1	60.7 ± 1.2	57.8 ± 1.8	0.232
Final BW (kg)	55.7 ± 1.6	72.5 ± 1.1	43.7 ± 1.2	0.0001
Difference in BW (kg)	-1.0 ± 0.9	11.8 ± 1.1	-14.2 ± 0.9	0.0001
ADG (kg)	-0.02 ± 0.01	0.20 ± 0.02	-0.24 ± 0.01	0.0001
Initial BCS	2.3 ±0.1	2.3 ± 0.1	2.4 ± 0.1	0.299
Final BCS	3.0 ±0.2	4.2 ± 0.1	1.8 ± 0.1	0.0001
Difference in BCS	0.7 ± 0.2	2.0 ± 0.1	-0.7 ± 0.1	0.0001

*All values (mean ± SEM) are expressed per ewe.

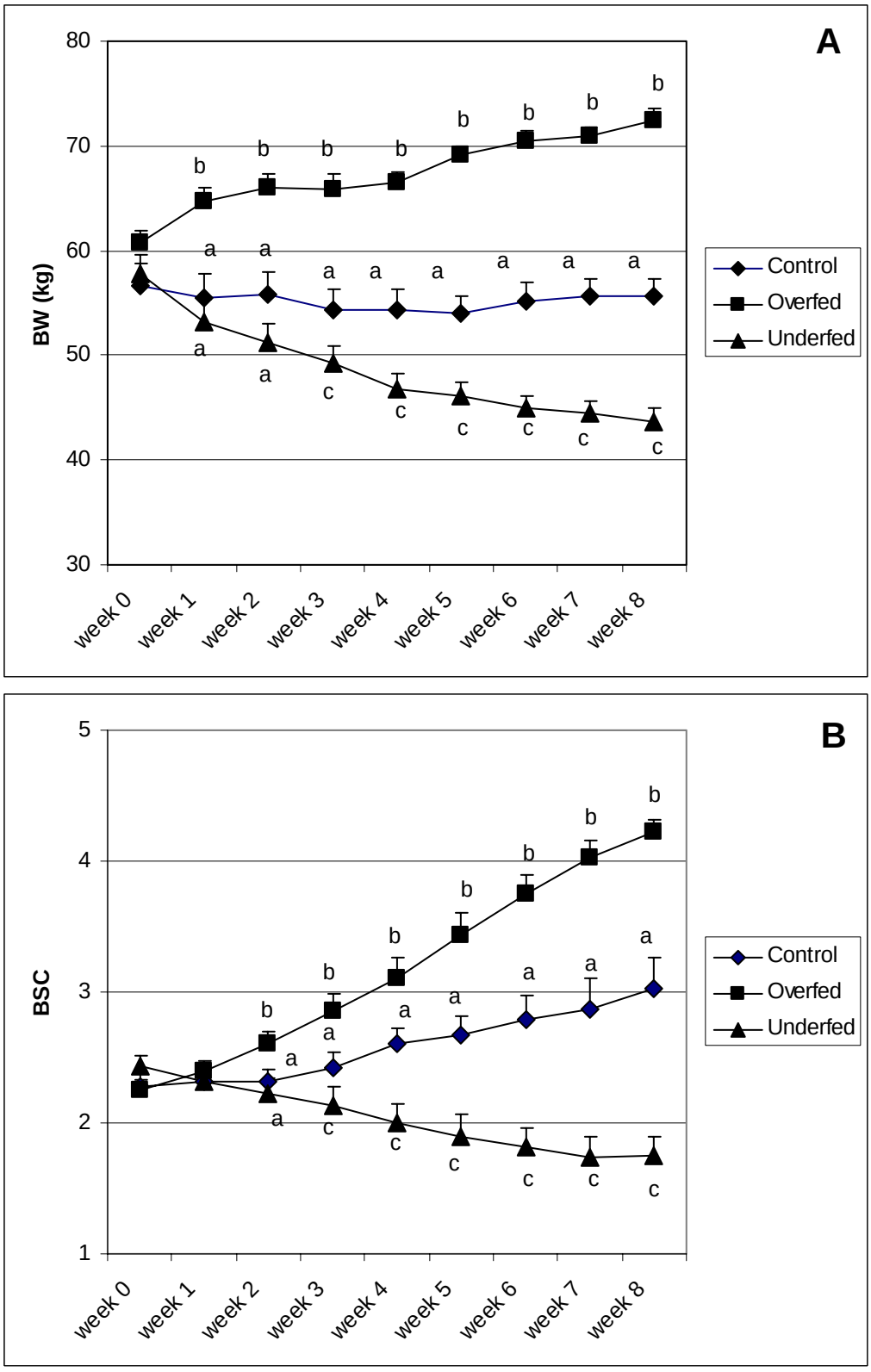

Figure 1. Body weight (A) and BCS (B) in control (black bars), overfed (grey bars) and underfed (open bars). [a,b,c]$P < 0.0001$; Means ± SEM with different superscripts differ within a specific week.

DISCUSSION

The present study demonstrated that overfeeding and underfeeding of ewes resulted in lower BW and BCS when compared to control ewes. Furthermore, oocytes derived from overfed and underfed ewes yielded fewer zygotes, morulas and blastocysts and had lower rates of cleavage, and morula and blastocyst formation compared with oocytes derived from control ewes.

In previous studies, for mature ewes fed a low energy diet (approximately 0.5 - 0.6 times maintenance energy requirement) for 3 to 4 week, decreased BCS (from 2.61 to 2.1 and from 2.5 to 2.33; Abecia et al., 1999 and Lozano et al., 2003, respectively) was observed along with decreased cleavage rates, number of good quality embryos and the rates of pregnancy. On the other hand, ewes ad libitum feeding for approximately 3 week resulted in enhanced BCS (from 2.58 to 2.7), but lower superovulation responses, lower number of good quality oocytes and embryos, and a greater percentage of poorly developed embryos in mature ewes (Lozano et al., 2003). In addition, Snijders et al. (2000) demonstrated that rates of cleavage and blastocyst formation from oocytes derived from cows with BCS 1.5-2.5 were lower (70.4 vs. 77.4%, and 6.8% vs. 11.4%, respectively) compared with those from cows with BCS 3.3-4.0 during the first or third lactation. These data and also our data indicate that decreased or increased BCS may be associated with decreased oocyte quality measured by the rates of in vitro fertilization and early embryonic development in sheep. This suggests that BCS can be used to predict successful embryonic development in sheep. However, determination of BCS is relatively subjective and may vary from study to study.

In the present experiment, nutritional plane had no effect on the number of ovarian follicles. Peura et al. (2003) reported for adult ewes that low (0.7 x) or high (1.3 x) maintenance diets for 3 to 5 months before FSH-induced superovulation did not affect ovulation rates. Moreover, superovulatory responses after FSH-treatment were not affected by 0.5 x, 1.0 x or 1.5 x maintenance diet fed during peri-conception period in adult ewes (Kakar et al., 2005). These data indicates that these specific nutritional treatments did not affect follicular development measured by the number of follicles or ovulations in sheep. The number of follicles was similar in lactating dairy cows (not treated with FSH) receiving low (1.52 Mcal NE_l /kg DM) or high (1.78 Mcal NE_l /kg DM) energy diet for approximately 25 week postpartum (Kendrick et al., 1999) and in yearling beef heifers (not treated with FSH) fed ad libitum or 0.75 x ad libitum for 100 d (Tripp et al., 2000). On the other hand, FSH-treated beef heifers fed a low energy (9.6 Mcal/kg ME/d) diet for 17 to19 days had more follicles than cows fed a high energy (28.6 Mcal/kg ME/d) diet (Nolan et al., 1998). Thus, these results show that nutritional treatments had no effect on the number of visible follicles in sheep but not in cows. Moreover, number of follicles in the present study was similar to that previously reported for FSH-treated mature ewes fed a maintenance diet during the normal breeding season and seasonal anestrus (Stenbak et al., 2001; Grazul-Bilska et al., 2003; Luther et al., 2005).

The cleavage rates were lower for overfed (51%) or underfed (48%) compared with control (85%) ewes in our study. Papadopoulos et al. (2001) have also shown that cleavage rates were decreased (from 88% to 66%) in ewes fed a low energy (0.5 x maintenance energy requirements) diet in comparison with a high energy (2 x maintenance energy requirements) diet for 28 days. Similar to our results for overfed or underfed ewes, low cleavage rates, 51% and 35%, were observed for ewes underfed (0.5 x maintenance energy requirements) and overfed (ad libitum intake) for approximately 24 days, respectively (Lozano et al., 2003). This indicates that

inadequate diet (e.g., underfeeding or feeding ad libitum) affects oocyte quality measured by IVF rates in sheep. In addition, rates of cleavage similar to cleavage rates in our control group were reported for mature sheep fed a maintenance diet (Watson et al., 1994; Ledda et al., 1997; O'Brien et al., 1997). Thus, the rates of fertilization may be influenced by different nutritional regimens under which oocytes were developed in the maternal environment.

In the present study, the number of morulas and blastocysts and the rate of morula and blastocyst formation were lower for overfed and underfed ewes compared with control ewes. In contrast, Lozano et al. (2003) demonstrated that restricted diets (0.5 x of maintenance diet for about 24 days) did not affect the rates of blastocyst formation in mature sheep. Moreover, supplementation with urea to the diet with low energy (0.5 x of maintenance) did not affect the blastocyst cell number and blastocyst hatching rate in sheep (Papadopoulos et al., 2001). Studies of McEvoy (1995) demonstrated that embryos in the early stage of development, produced in vivo and then cultured in vitro collected from mature ewes fed a low calories (0.6 x maintenance) diet for about 2 week, were considered more viable, had a greater protein synthesis index and number of nuclei in the blastocyst compared with those produced in ewes fed a high calorie diet. In addition, the greater number of cells in blastocysts produced in vivo was observed for ewes fed low calories diet (0.5 x maintenance diet) to compare with ewes fed 1 x or 1.5 x maintenance diet during peri-conception period (Kakar et al., 2005). Data from these two studies indicate that ewe is able to respond to acute changes in nutrition during peri-conception period, resulting in changes of embryonic development. For yearling beef heifers, restriction of dietary energy (75% of ad libitum fed) did not affect the rate of blastocyst formation (Tripp et al., 2000). Therefore, it seems that the level of overfeeding or feed restriction and/or length of specific feeding reported in some studies were not severe enough to induce a negative impact on the rate of blastocyst formation, which was observed in the current study. Moreover, the effects of nutritional treatment on blastocyst formation may also depend on specific diet composition and breed.

Numerous experiments indicate that nutrition has direct effects on some reproductive function by affecting hormonal production (O'Callaghan and Boland, 1999; Lucy, 2003; Hunter et al., 2004). For example, for underfed or overfed mature ewes with enhanced or decreased blood progesterone concentrations, respectively, altered oocyte and embryo quality was observed (Lozano et al., 2003). This indicates that effects of nutrition on oocyte and embryonic development may be indirectly linked through regulation of hormone secretion. In the present study, we have not evaluated the level of hormones in peripheral blood. Therefore, future studies should be undertaken to further define association between hormone levels and oocyte quality.

The effects of nutrition on oocyte and embryonic development may reflect the general energy balance (e.g., maintenance diet vs. low or high energy diets) but also can be attributed to the specific nutrients in diets, such as vitamins, minerals and other supplements (Wrenzycki et al., 2000). For example, Tarin et al. (1998) observed that supplementation with a mixture of vitamins C and E to the maternal diet enhanced the number of ovulations but did not affect the rates of cleavage or blastocyst formation in mice. Moreover, McEvoy et al. (1997) demonstrated that high concentration of urea in diet fed for 12 week increased embryo mortality and decreased pregnancy rates after embryo transfer in mature sheep. Additional studies should be undertaken to determine which nutritional factors affect oocyte quality.

In conclusion, our results demonstrate that overnutrition and undernutrition did not affect number of developing follicles, number of recovered oocytes, number and percentage of healthy oocytes per ewe, but decreased number of cleaved oocytes, and the rates of fertilization and

morula and blastocyst formation. These data indicate that donor animals likely require specific nutritional management procedures to provide the highest quality oocytes for ART. Nutrition of donor animals seems to be a key component affecting development of oocytes and the preimplantation embryo. We manipulated total dietary intake in the present study, but future investigations that address specific dietary nutrient composition should provide insight into the underlying mechanisms associated with changes in efficiency of in vitro embryo production.

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