EFFECTS OF 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 the effects of maternal diet on in vitro fertilization (IVF) and early embryonic development, ewes (n=18) were divided into control and underfed groups and received 100% and 60% maintenance diet, respectively, for 8 weeks before oocyte collection. Once a week ewes were weighted and the body condition score (BCS) was determined. Estrous synchronization was performed using progestagen sponges for 14 days. Follicular development was induced in ewes by twice daily injections of follicle stimulating hormone **(**FSH) on day 13 and 14 of the estrous cycle. Oocytes were collected from all visible follicles on day 15 of the estrous cycle. After IVF, the rate of early embryonic development was evaluated throughout the 8 day culture period. An average initial body weight was 53.8 ± 1.2 kg and 49.4 ± 0.6 kg at the end of experiment weight was 55.4 ± 1.5 kg and 44.4 ± 0.9 kg for control and underfed ewes, respectively. An average initial BCS was 3.4 ± 0.04 at the end of the experiment BCS was 3.7 ± 0.1 and 3.1 ± 0.1 for control and underfed ewes, respectively. Both, the weights and BCS were different ($P<0.01$) for control and underfed ewes at the end of experiment. Undernutrition decreased ($P<0.001$) the number of blastocysts per ewe and the rate of blastocyst formation. However, number of visible follicles (large and small), the total number of oocytes, the number of healthy oocytes, the percentage of healthy oocytes and cleavage rates per ewe were similar for control and underfed ewes. These data indicate that undernutrition of donor ewes resulting in lower weight and BCS, has a negative effect on oocyte quality which results in a lower rate of blastocyst formation.

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

INTRODUCTION

Assisted reproductive technologies (ART), over last 20 years, have found many applications in agriculture use. Since then, continuous progress was made in modern embryology in order to improve production of good quality embryos. There are two main ways leading to the improvements in in vitro embryo production systems: one by improvement of in vitro conditions, and other by improvements of conditions of donor animals by diet manipulation. Thus, numerous experiments have been performed to improve in vitro culture conditions for embryo production (Thompson, 1997; Guler et al. 2000; Stenbak et al. 2001; Rizos et al., 2002; Galli et al., 2001, 2003; Hoshi, 2003; Grazul-Bilska et al. 2003). In addition, several studies have been performed to optimize a diet to maintain high reproductive efficiency for several species including sheep (McEvoy et al., 1995; O'Callaghan et al., 2000; Boland et al., 2001; Papadopoulos et al., 2001;

Lozano et al., 2003; Peura et al. 2003) and cows (Kendrick et al., 1999; Yaakub et al., 1999; Sinclair et al., 2000; Wrenzycki et al., 2000; Tripp et al., 2000; Armstrong et al., 2001).

Nutritional status is a major factor influencing the ability of an animal to reproduce (Robinson, 1990; O'Callaghan and Boland, 1999; Robinson et al. 1999; Webb et al, 1999a,b; O'Callaghan et al. 2000). Nutrition has significant impact on numerous reproductive functions including hormone production, fertilization and early embryonic development. Improved nutritional status is positively correlated with embryo survival and is a key factor influencing efficiency in ART (Armstrong et al. 2003). However, the effects of nutrition on embryo production systems have not been fully evaluated and characterized in detail.

The aim of the present study was to evaluate the effects of undernutrition on follicular development, IVF, and early embryonic development in (FSH)-treated sheep.

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). Then, they were divided into two groups (n=9/each): Control received a maintenance diet and Underfed 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 ovariectomized (Luther et al. 2005). The study was initiated during the normal breeding season in October and finished in December. 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 (IACUC) of NDSU.

Nutritional management

Ewes were kept in individual pens under 14 h of darkness and 10 h of light at 50-55°C with free access to water and mineral supplements. After a 3 day adaptation to pens and pelleted diet, ewes were allocated randomly to two nutritional groups as described above. Dietary management procedures for both groups were similar to those described by Arnold et al., 2001 and 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 1000 g/day (100%) and the underfed group received 600 g/day (60%) of the maintenance diet (dry matter basis). After first 4 weeks, the diet was modified to decrease energy and protein to 720 g/day (100%) and 432 g/d (60%). The diet contained: dehydrated beet pulp, 36.5%; dehydrated alfalfa, 20.3%; corn, 24.2%; soy hulls, 16%; soybean meal, 3.0% (% of dietary dry matter).

Oocyte collection

Following ovariectomy, ovaries were transported to the laboratory in an incubator at 39°C. The number of visible follicles on each ovary was determined and oocytes 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; Luther et al. 2005). Under a stereomicroscope, oocytes were recovered from each dish and transferred to a petri dish containing fresh collection medium without heparin. Oocytes were then evaluated and categorized as healthy or atretic based on their morphology (Thompson et al. 1995). All oocytes were then washed three times in maturation medium (TCM-199 containing 10% FBS, ovine FSH [oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine luteinizing hormone [oLH-26; NIADDK-NIH], estradiol [Sigma St. Louis, MO], glutamine [Sigma], sodium pyruvate [Sigma], epidermal growth factor [Sigma] and penicillin/streptomycin; Grazul-Bilska et al. 2003; Luther et al. 2005).

In vitro maturation

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

In vitro fertilization

Frozen capacitated semen pooled from 4 Hampshire rams, was thawed and viable sperm were separated using the swim up technique (Stenbak et al., 2001). The sperm ($0.5\text{-}1.0 \times 10^6$ 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 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). The dishes were then evaluated to determine the number of cleaved oocytes. The embryos were transferred to culture medium containing glucose. After 48 hours, the developmental stage was evaluated and embryos were transferred to the fresh culture medium with glucose. The rate of cleavage (number of cleaved vs. non-cleaved oocytes), and the rate of morula and blastocyst formation (time and percentage reaching each stage) were evaluated every second day during 8 day culture. Data was compared for Control and Underfed ewes.

Evaluation of maturation status of oocytes.

Three days after IVF all unfertilized oocytes were separated from embryos and sperm was removed by repeated pipetting with a micropipette of diameter 150 µm. Naked oocytes were then fixed in methanol followed by DAPI staining (Luther et al. 2005, Pant et al. 2005).

Statistical analysis

All data are reported as mean ± S.E.M. Data was analyzed statistically by using the general linear models program of the Statistical Analysis System (SAS User's Guide, 1985). When the F-tests were significant, differences between specific means were evaluated by using Least Significant Difference test (Kirk, 1982).

RESULTS

At the beginning of treatment, weight of ewes was 53.8 ± 1.2 kg and 49.4 ± 0.6 kg for Control and Underfed groups, respectively, and BCS was 3.4 ± 0.02 for both groups. After 8 weeks of duration of the experiment, weight and BCS were grater ($P<0.001$) for control than underfed ewes (55.5 ± 1.5 kg vs. 44.4 ± 0.9 kg and 3.6 ± 0.1 vs. 3.1 ± 0.1, respectively). To compare with initial weights, control ewes gained 1.7 ± 1.2 kg, but underfed ewes lost 5.0 ± 0.5 kg. Mean number of all visible follicles, the number of large and small follicles, the total number of oocytes, the number of healthy oocytes and the percentage of healthy oocytes, the number of cleaved oocytes and cleavage rates per ewe were similar for control and underfed group (Table 1). Total number of oocytes used for IVF was 93 for control ewes and 79 for underfed ewes. However, number of blastocysts per ewe and the rate of blastocyst formation based on total number of oocytes used for IVF or based on number of cleaved oocytes were greater ($P<0.001$) for control than underfed ewes (Table 1). Maturation rate evaluated for non-fertilized oocytes was similar for control and underfed ewes (Table 1). Overall, out of the total number of oocytes used for evaluation of maturation status (6.4± 1.1), the mean number and the percentage of matured oocytes were 4.7± 0.55 and 75.1 ± 7.1%, respectively.

Table 1. Effects of nutrition on the follicular development, the number collected oocytes, the number of healthy oocytes, the rate of cleavage and blastocyst formation and oocyte maturation*.

Parameter	Control	Underfed	P value
Total follicles (n)	22.0 ± 2.64	21.0 ± 3.13	0.810
Large follicles (n)	10.0 ± 1.28	10.56 ± 1.34	0.768
Small follicles (n)	11.55 ± 1.99	10.44 ± 2.55	0.735
Total Oocytes (n)	21.34 ± 2.85	20.56 ± 3.27	0.859
Healthy oocytes (n)	13.66 ± 1.39	13.78 ± 2.27	0.967
Healthy oocytes (%)	67.78 ± 3.95	67.89 ± 3.72	0.983
Cleavage of oocytes (n)	10.33 ± 1.54	8.77 ± 1.59	0.248
Cleavage rate (%)	76.0 ± 6.49	64.55 ± 7.01	0.494
Blastocyst (n)	3.33 ± 0.55	0.77 ± 0.36	**0.001**
Blastocyst developed per oocytes used for IVF (%)	23.77 ± 1.95	6.09 ± 2.98	**0.0001**
Blastocyst developed per oocytes cleaved (%)	32.23 ± 2.86	7.85 ± 3.70	**0.0001**
Oocytes used for evaluation of maturation status (n)	5.66 ± 0.83	7.25 ± 2.15	0.484
Matured oocytes (%)	75.56 ± 11.12	74.61 ± 9.26	0.949

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

DISCUSSION

The present study demonstrated that 60 days of underfeeding resulted in lower body weight and BCS when compared to control ewes. Furthermore, oocytes derived from underfed ewes yielded fewer blastocysts and had lower rates of blastocyst formation than oocytes derived from control ewes.

In previous studies Snijders et al. (2000) demonstrated that the rate of cleavage and blastocyst formation from oocytes derived from cows with the lower BCS were significantly decreased compared with those from cows with the higher BCS. For ewes underfed for 3 or 4 weeks, decreased BCS was observed along with decreased cleavage rates or the rates of pregnancy, respectively (Abecia et al., 1999; Lozano et al., 2003). On the other hand, ewes fed ad libitum diet with enhanced BCS had a lower superovulation responses, lower number of good quality oocytes and embryos, and a greater percentage of poorly developed embryos (Lozano et al., 2003). This indicates that BCS can be used to predict successful embryonic development. Thus, production of good quality embryos may be highly influenced by different nutritional regiments under which oocytes were developed in the maternal environment.

In the present experiment, nutrition had no effect on the number of ovarian follicles. Similar, unchanged number of follicle was reported in lactating dairy cows receiving a high energy compared with a limit-fed group (Kendrick et al. 1999; Tripp et al. 2000, Peura et al. 2003). Thus, these results show that nutrition had no effect on the follicular development. In addition, number of follicles in the present study was similar to previous reports for FSH-treated 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 (overall 70%) were similar for control and underfed group of ewes. Similar rates of cleavage were reported for sheep, which were a fed maintenance diet (Slavik et al. 1992; Watson et al. 1994; Ledda et al. 1997; O'Brien et al. 1996, 1997; Nolan et al. 1998; Stenbak et al. 2001; Grazul-Bilska et a. 2003). However, studies of Papadopoulos (2001) show that cleavage rates were decreased in ewes fed allow energy diet in comparison with a high energy diet. Lower cleavage rates that reported in the present experiment were also observed for underfed (51%) and overfed ewes (35%; Lozano et al., 2003). In addition, studies of McEvoy (1995) show that a higher proportion of ova from ewes on low calorie diet were considered viable compared with those produced in ewes on high calorie diet. These differences may be due to different experimental conditions and breed.

In the present study, the number of blastocyst and the rate of blastocyst formation were lower for underfed ewes than for control ewes. In contrast, it was demonstrated that restriction of dietary energy (75% of libitum fed) did not affect the rate of blastocyst formation in cows (Tripp et al., 2000). Also in sheep, supplementation with urea to the diet with low energy did not affect the blastocyst cell number and blastocyst hatching (Papadopoulos et al., 2001). Therefore, it seems that the level of feed restriction was not always severe enough to induce a negative impact on the rate of blastocyst formation, but it may depend on specific diet composition and breed.

Numerous experiments strongly indicate that nutrition has direct effects on reproductive function by affecting hormonal production (O'Callaghan et al., 2000; Wrenzycki et al., 2000; Armstrong et al., 2001, 2002; Boland et al., 2001; Lucy, 2003). For example, for underfed or overfed ewes with enhanced or decreased blood progesterone concentrations, respectively, altered oocyte and embryo quality was observed (Lozano et al., 2003). This indicates that the effects of nutrition on oocyte and embryonic development may be indirect through regulation of

hormone secretion. In the present study, we have not evaluated level of hormones in peripheral blood. Therefore, future studies should be undertaken to determine association between hormone levels and oocyte quality.

The effects of nutrition on oocyte and embryonic development may reflect the general energy balance and also can be attributed to the specific nutrients in diets, such as vitamins, minerals and others (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 number of ovulations but did not affect the rates of cleavage or blastocyst formation in mice. Additional study should be undertaken to determine which nutritional factors affect oocyte quality.

In conclusion, the results from this study demonstrate that undernutrition did not affect follicular development and fertilization rates but decreased the rates of blastocyst formation. These data indicate that donor animals require a special diet to provide good quality oocytes for ART. Maternal nutrition seems to be a key component affecting development of oocytes and the preimplantation embryo. We restricted the level of energy in the present diet, but the specific composition of the diet should be considered when developing nutrition-based solutions for improving reproductive efficiency through procedures such as in vitro embryo production or embryo transfer.

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LITERATURE CITED

Abecia JA, Forcada F, Lozano JM. A preliminary report on the effect of dietary energy on prostaglandin F2 alpha production in vitro, interferon-tau synthesis by the conceptus, endometrial progesterone concentration on days 9 and 15 of pregnancy and associated rates of embryo wastage in ewes. Theriogenology 1999; 52:1203-1213.

Armstrong DG, Gong JG, Webb R. Interactions between nutrition and ovarian activity in cattle: physiological, cellular and molecular mechanisms. Reproduction Supplement 2003; 61: 403-414.

Armstrong DG, Gong JG, Gardner JO, Baxter G, Hogg CO, Webb R. Steroidogenesis in bovine granulosa cells: the effect of short-term changes in dietary intake. Reproduction 2002; 123:371-378.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol. Reprod. 2001; 64:1624-1632.

Arnold DR, Scheaffer AN, Redmer DA, Caton JS, Reynolds LP. Effects of nutrient restriction on placental vascularity and fetal growth in sheep. Biol. Reprod. 2001; 64 (Suppl. 1):352 (Abstr #625).

Boland MP, Lonergan P, O'Callaghan D. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology 2001; 55:1323-40.

Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. Theriogenology 2001; 55:1341-1357.

Galli C, Duchi R, Crotti G, Turini P, Ponderato N, Colleoni S, Lagutina I, Lazzari G. Bovine embryo technologies. Theriogenology 2003; 59:599-616.

Grazul-Bilska AT, Choi JT, Bilski JJ, Weigl RM, Kirsch JD, Kraft KC, Reynolds LP, Redmer DA. Effects of epidermal growth factor on early embryonic development after in vitro fertilization of oocytes collected from ewes treated with follicle stimulating hormone. Theriogenology 2003; 59:1453-1461.

Guller A, Poulin N, Mermillod P, Terqui M, Cognie Y. Effects of growth factors, EGF and IGF-1, and estradiol on in vitro maturation of sheep oocytes. Theriogenology 2000; 54:209-18.

Hoshi H. In vitro production of bovine embryos and their application for embryo transfer. Theriogenology 2003; 59:675-685.

Kendrick KW, Bailey TL, Garst AS, Pryor AW, Ahmadzadeh A, Akers RM, Eyestone WE, Pearson RE, Gwazdauskas FC. Effects of energy balance of hormones, ovarian activity, and recovered oocytes in lactating Holstein cows using transvaginal follicular aspiration. J. Dairy Sci. 1999; 82:1731-1741.

Kirk RE. 1982. Experimental Design: Procedures for the Behavioral Sciences, 2nd Edn., Brooks/Cole, Belmont, CA.

Ledda S, Boglio L, Calvia P, Leoni G, Naitana S. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J. Reprod. Fertil. 1997; 109:73-78.

Lozano JM, Lonergan P, Boland MP, O'Callaghan D. Influence of nutrition on the effectiveness of superovulation programs in ewes: effect on oocyte quality and post-fertilization development. Reproduction 2003; 125:543-553.

Lucy MC. Mechanisms linking nutrition and reproduction in postpartum cows. Reproduction Suppl. 2003; 61:415-427.

Luther JS, Redmer DA, Reynolds LP, Choi JT, Pant D, Navanukraw Ch, Arnold DR, Scheaffer AN, Borowicz P, Kirsch JD, Weigl RM, Kraft KC, Grazul-Bilska AT. Ovarian follicular development and oocyte quality in anestrous ewes treated with melatonin, and controlled internal drug release (CIDR) device and follicle stimulating hormone. Theriogenology 2005 (in press).

McEvoy TG, Robinson JJ, Aitken RP, Findlay PA, Palmer RM, Robertson IS. Dietary-induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. Anim. Reprod. Sci. 1995; 38:89-107.

Nolan R, O'Callaghan D, Duby RT, Lonergan P, Boland MP. The influence of short-term nutrient changes on follicle growth and embryo production following superovulation in beef heifers. Theriogenology 1998; 50:1263-1274.

O'Brien JK, Catt SL, Ireland KA, Maxwell WMC, Evans G. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology 1997; 47:433-1443.

O'Brien JK, Dwarte D, Ryan JP, Maxwell WMC, Evans G. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. Reprod. Fertil. Dev. 1996; 8:1029-1037.

O'Callaghan D, Boland MP. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Animal Science 1999; 68:299-314.

O'Callaghan D, Yaakub H, Hyttel P, Spicer LJ, Boland MP. Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. J. Reprod. Fertil. 2000; 18:303-313.

Pant D, Reynolds LP, Luther JS, Borowicz PP, Stenbak T, Bilski JJ, Weigl RM, Lopes F, Johnson ML, Redmer DA, Grazul-Bilska AT. Expression of connexin 43 and gap junctional intercellular communication in cumulus oocyte complex in sheep. Reproduction 2005 (in press).

Papadopoulos S, Lonergan P, Gath V, Quinn KM, Evans AC, O'Callaghan D, Bolan MP. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology 2001; 55:1059-1069.

Peura TT, Kleemann DO, Rudiger SR, Nattrass GS, McLaughlan CJ, Walker SK. Effect of nutrition of oocyte donor on the outcomes of somatic cell nuclear transfer in the sheep. Biol. Reprod. 2003; 68:45-50.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev. 2002; 61:234-248.

Robinson JJ, Sinclair KD, McEvoy TG. Nutritional effects on foetal growth. Animal Science 1999; 68; 315-322/

Robinson JJ. Nutrition in the reproduction of farm animals. Nutrition Research Reviews 1990; 3:961-966.

SAS. 1985. User's Guide, Statistics, 5th Edn., Statistical Analysis System Institute, Cary, NC.

Scheaffer AN, Caton JS, Redmer DA, Reynolds LP. The effect of dietary restriction, pregnancy, and fetal type in different ewe types on fetal weight, maternal body weight, and visceral organ mass in ewes. J. Anim. Sci. 2004a; 82:1826-1838.

Scheaffer AN, Caton JS, Redmer DA, Arnold DR, Reynolds LP. Effect of dietary restriction, pregnancy, and fetal type on intestinal cellularity and vascularity in Columbia and Romanov ewes. J. Anim. Sci. 2004b; 82:3024-2033.

Sinclair KD, Kuran M, Gebbie FE, Webb R, McEvoy TG. Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. J. Anim. Sci. 2000; 78:2670-2680.

Slavik T, Fulka J, Goll I. Pregnancy rate after the transfer of sheep embryos originated from randomly chosen oocytes matured and fertilized in vitro. Theriogenology 1992; 38:749-756.

Snijders SEM, Dillon P, Callaghan DO, Boland MP. Effect of genetic merit, milk yield, body condition and lactation number on in vitro oocyte development in dairy cows. Theriogenology 2000; 53(4):981-989.

Stenbak TK, Redmer DA, Berginski HR, Erickson AS, Navanukraw C, Toutges MJ, Bilski JJ, Kirsch JD, Kraft KC, Reynolds LP, Grazul-Bilska AT. Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrous. Theriogenology 2001; 56:51-64.

Tarin J, Ten J, Vendrell FJ, De Oliveira MNM, Cano A. Effects of maternal ageing and dietary antioxidant supplementation on ovulation, fertilization and embryo development in vitro in the mouse. Reprod. Nutr. Dev. 1998; 38:499-508.

Thompson JG. Comparison between in vitro-derived and in vitro-produced pre-elongation embryos from domestic ruminants. Reprod. Fertil. Dev. 1997; 9:341-354.

Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol. Reprod. 1995; 53:1385-1391.

Tripp MW, Ju JC, Hoagland TA, Riesen JW, Yang X, Zinn SA. Influence of somatotropin and nutrition on bovine oocyte retrieval and in vitro development. Theriogenology 2000; 53:1581-1590.

Watson AJ, Watson PH, Warnes D, Walker SK, Armstrong DT, Seamark RF. Preimplantation development of in vitro-matured and in vivo-fertilized ovine zygotes: comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere. Biol. Reprod. 1994; 50:7150724.

Webb R, Garnsworthy PC, Gong JG, Robinson RS, Wathes DC. Consequences for reproductive function of metabolic adaptation to load. Metabolic Stress in Dairy Cows Occasional Publication No 24, British Society of Animal Science 1999a.

Webb R, Gosden RG, Telfer EE, Moor RM Factors affecting folliculogenesis in ruminants. Animal Science 1999b; 68:257-284.

Wrenzycki C, De Sousa P, Overstrom EW, Duby RT, Herrmann D, Watson AJ, Niemann H, O'Callaghan D, Boland MP. Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos. Journal of Reproduction and Fertility 2000; 0022-4251:69-78.

Yaakub H, O'Callaghan D, Boland MP. Effect of type and quantity of concentrates on superovulation and embryo yield in beef heifers. Theriogenology 1999; 51:1259-1266.