Cellular proliferation and vascularization in ovine fetal ovaries: effects of undernutrition and selenium in maternal diet

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Abstract

Sheep were fed a maintenance (M) diet with adequate (A) Se or high (H) Se concentration from 21 days before breeding to day 135 of pregnancy. From day 50 to day 135 of pregnancy (tissue collection day), a portion of the ewes from ASe and HSe groups were fed restricted (R; 60% of M) diet. Fetal ovarian sections were stained for: 1) the presence of proliferating cell nuclear antigen (a marker of proliferating cells) to determine the proportion of proliferating primordial follicles, or the labeling index (LI; percentage of proliferating cells) for primordial, primary, secondary and antral follicles, stromal tissues, and blood vessels; 2) factor VIII (a marker of endothelial cells) or 3) a presence of apoptotic cells/bodies. The number of proliferating primordial follicles and the LI of primordial follicles was decreased by R and/or HSe diets. The LI was similar for theca and granulosa cells, and for secondary or antral follicles, but was greater in secondary and antral than in primordial and primary follicles. R diet and/or Se affected the LI in all follicle types, in stromal tissues and blood vessels. A dense network of blood vessels was detected in the areas containing secondary to antral follicles, medulla, and hilus, but areas containing primordial follicles were poorly vascularized. The number of apoptotic cells was minimal. These results demonstrate that nutrient restriction and/or Se level in the maternal diet affected cellular proliferation in follicles, blood vessels, and stromal tissues in fetal ovaries. Thus, plane of nutrition and Se in the maternal diet may impact fetal ovarian development and function.

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Introduction

Hypertrophy and hyperplasia are the major processes for tissue growth and development (Baserga 1985). The fetal ovaries represent a type of tissue that expresses high cellular proliferation (Juengel *et al.* 2002, Sawyer *et al.* 2002, Van der Hurk & Zhao 2005). In sheep, by day 75 of pregnancy, ovaries containing primordial follicles are fully formed, and by the end of pregnancy fetal ovaries contain primordial to secondary or antral follicles (Lundy *et al.* 1999, McNatty *et al.* 2000, Erickson 2001, Juengel *et al.* 2002, van der Hurk & Zhao 2005).

It has been demonstrated that several factors including FSH, LH, estrogens, activin, kit ligand, epidermal growth factor, and many others affect growth and differentiation of follicles in fetal ovaries (McNatty *et al.* 2000, Tanaka *et al.* 2001, Byskov & Westergaard 2004, Van der Hurk & Zhao 2005, Pepe *et al.* 2006). Stromal tissues and blood vessels provide the scaffold to support gonadal development, which includes cell migration, organization, differentiation, and function (McIntush & Smith 1998, Augustin 2000, Robinson *et al.* 2001). However, the process of oocyte, granulosa and theca cell, stromal

tissue and blood vessel growth and/or differentiation, and their interactions during fetal folliculogenesis, are poorly understood.

In addition to the factors synthesized and secreted by fetal organs, the growth and differentiation of fetal ovaries can be affected by factors of maternal origin. It has been clearly demonstrated that environmental factors, including nutrition, can affect growth, development, and physiology in the fetal and postnatal life of mammals (Borwick *et al.* 1997, Wright *et al.* 2002, Wu *et al.* 2006, Barker 2007, Fowler *et al.* 2008). Both undernutrition and overnutrition of pregnant ewes negatively affect fetal growth and development (Redmer *et al.* 2004, Luther *et al.* 2005, 2007). In fact, cellular proliferation in fetal ovaries is regulated by numerous fetal and maternal factors including factors originating from maternal diet (Lea *et al.* 2006). Moreover, Se has been demonstrated to diminish cellular proliferation, neo-angiogenesis, and other cellular functions in several tissues including cancerous tissues and digestive tract (Clark *et al.* 1996, Ganther 1999, Combs 2001, Combs & Lu 2001, Neville *et al.* 2008, Zeng & Combs 2008).

We hypothesized that undernutrition and level of Se in maternal diet will affect cellular proliferation in fetal ovaries. Therefore, the objective of this experiment was to determine: 1) if maternal dietary restriction and differing levels of Se in diet impact cellular proliferation in fetal ovarian follicles, stromal tissue and blood vessels, and 2) incidence of apoptosis and blood vessel distribution in fetal ovaries obtained from sheep in late pregnancy.

Results

Fetal weight tended ($P<0.1$) to be lower in groups fed R diet (3427 ± 192 g) than those fed M diet (3860 ± 165 g). However, levels of Se in diet did not affect fetal weight, and there were no interactions between plane of nutrition and Se levels in diet.

Dietary restriction in maternal diet affected fetal ovarian weight, which was lower ($P<0.01$) for groups fed R diet (66 ± 4 mg) than for those fed M diet (93 ± 9 mg). However, levels of Se in diet did not affect fetal ovarian weight, and there were no interactions between plane of nutrition and Se levels in diet. Fetal ovarian weight in groups fed R diet with ASe or HSe was lower ($P<0.03$) than in group fed M diet with HSe but similar to group fed M diet with ASe (Table 1).

All fetal ovaries contained primordial, primary, and secondary follicles. Presence of antral follicles was detected in 72% ovaries (23 out of 32 ovaries analyzed). Maternal diet did not have any effect on incidence of antral follicles in fetal ovaries. Proliferating cells were detected in primordial, primary, secondary, and antral follicles, in stromal tissues and blood vessels of fetal ovaries (Fig. 1A–F).

The effects of dietary restriction ($P<0.08$) and Se ($P<0.07$) levels, but no interaction between plane of nutrition and Se in diet, on proportion of proliferating primordial follicles were observed. In addition, the effects of dietary restriction ($P<0.08$), but not Se or interactions between plane of nutrition and Se in diet, on the labeling index (LI) of primordial follicles were observed. The proportion of proliferating primordial follicles was lower ($P<0.08$) in groups fed R diet than groups fed M diet (7.4 ± 1.2 vs $11.3 \pm 1.7\%$), and was lower ($P<0.07$) in groups fed HSe than ASe (7.0 ± 1.0 vs 10.9 ± 1.6). The proportion of proliferating primordial

follicles was greater ($P<0.05$) in the group fed M diet with ASe than in the other treatment group (Fig. 2). The LI of primordial follicles tended ($P<0.08$) to be lower in groups fed R than M diet (2.9 ± 0.4 vs 4.9 ± 0.9). The LI tended ($P=0.1$) to be greater in group fed M diet with ASe than in any other group (Fig. 2).

Overall, the LI was the greatest ($P<0.001$) in granulosa cells of secondary and antral follicles, lower in primary follicles, and the lowest in primordial follicles (19.4 ± 0.6 and 19.5 ± 0.6 vs 10.4 ± 0.4 vs $4.6 \pm 0.5\%$; Figs 2 and 3). The LI in granulosa and theca cells of secondary (granulosa, $21.1 \pm 0.8\%$ vs theca, $17.8 \pm 0.7\%$) and antral (granulosa, $19.4 \pm 0.8\%$ vs theca, $19.7 \pm 1\%$) follicles was similar. Therefore, data for granulosa and theca cells were combined and analyzed together within secondary and antral follicles.

For primary follicles, interactions ($P<0.02$) between plane of nutrition and Se levels in diet on the LI were observed. The LI in granulosa layer of primary follicles was lower ($P<0.04$) in the group fed R diet with HSe than in groups fed M diet with HSe or R diet with ASe (Fig. 3). For secondary follicles, the interactions ($P<0.09$) between plane of nutrition and Se levels in diet and the effects of level of Se in diet ($P<0.005$) on the LI were observed. The LI was lower ($P<0.005$) in groups fed diet with HSe than with ASe (17.3 ± 0.8 vs $21.0 \pm 0.8\%$). The LI was lower ($P<0.03$) in the group fed M diet with HSe than in the groups fed M diet with ASe or fed R diet with ASe, but it was similar to group fed R diet with HSe (Fig. 3). For antral follicles, the LI was affected by the level of Se in diet, which was lower ($P<0.01$) in groups fed with HSe than with ASe (17.3 ± 0.9 vs $20.9 \pm 0.8\%$). The LI was lower ($P<0.002$) in group fed M diet with HSe than in any other treatment group (Fig. 3).

For stromal tissues, interactions ($P<0.09$) between plane of nutrition and Se levels and the effects of Se level in diet ($P<0.03$) on the LI were observed. The LI was lower ($P<0.03$) in groups fed a diet with HSe than with ASe (3.6 ± 0.8 vs $6.9 \pm 1.1\%$). The LI was greater ($P<0.05$) in the group fed M diet with ASe than in any other treatment group (Fig. 4).

For blood vessels, the effects of plane of nutrition ($P<0.09$) and Se level ($P<0.01$) in diet on the LI were observed. The LI tended ($P<0.09$) to be lower in groups fed R than M diet (7.9 ± 2.2 vs $11.6 \pm 2.7\%$), and the LI was lower ($P<0.01$) in groups fed a diet with HSe than with ASe (6.2 ± 1.5 vs $13.7 \pm 0.7\%$). The LI in blood vessels was greater ($P<0.04$) in the group fed M diet with ASe than any other treatment group (Fig. 5).

In the majority of fetal ovarian tissue sections, apoptotic cells were not detected. In only a few tissue sections (5 out of 32), apoptotic cells were present occasionally in a granulosa layer or stromal tissues in fetal ovaries (Fig. 6). Apoptotic cells were also detected in ovine luteal tissues serving as a control (data not shown; see Vonnahme *et al.* 2006, Grazul-Bilska *et al.* 2008).

Table 1 Effects of maternal maintenance (M) and restricted (R) diet with adequate (A) or high (H) Se concentrations on weight of fetal ovaries (mg) in sheep.

Treatment group	Number of ewes	Fetal ovarian weight (mg)
M ASe	8	87 ± 9^{ac}
M HSe	8	98 ± 15^{ab}
R ASe	10	66 ± 4^c
R HSe	6	66 ± 7^c

^{abc} $P<0.03$; Means $\pm$ S.E.M. with different superscripts differ.

Figure 1 Representative micrographs of PCNA staining in (A) primordial, (B) primary, (C) secondary and (D) early antral follicles, (E) in stromal tissues (F) and blood vessels in ovine fetal ovaries. Dark staining indicates proliferating cells (arrows), and pink staining indicates cell nuclei of non-proliferating cells. Inset represents control staining (no primary antibody). In (A), arrows indicate primordial follicles with at least one proliferating cell, and arrowheads indicate primordial follicles without visible proliferating cells. In (B), arrow indicates secondary follicle with proliferating cells. In (C, D, E, and F), arrows indicate proliferating cells in secondary follicles, antral follicle, stromal tissues, and blood vessels respectively. Bar = 25 μ m.

Appearance of apoptotic cells in fetal ovaries was not affected by nutritional treatment.

Blood vessels marked by factor VIII staining were detected in fetal ovaries (Fig. 7). Smaller size ($\leq 30 \mu$ m) blood vessels were present in the areas containing

primordial to antral follicles (Fig. 7A), and larger ($> 30 \mu$ m) and small blood vessels were detected in ovarian medulla and hilus (Fig. 7B). For antral follicles, a network of blood vessels was detected in the theca layer (Fig. 7A). However, areas containing primordial follicles were rather poorly vascularized (Fig. 7A). The distribution of blood vessels in the ovaries seemed not to be affected by nutritional treatment.

Figure 2 Proportion of proliferating primordial follicles/area (black bars) and labeling index (open bars) in primordial follicles in ovine fetal ovaries in four treatment groups fed maintenance (M) or restricted (R) diet with adequate (A) Se or high (H) Se concentration. ^{a,b} $P < 0.05$ and ^{A,B} $P < 0.1$; means $\pm$ S.E.M. with different superscripts differ (unprotected *F*-test) for proportion of proliferating follicles and the LI respectively.

Discussion

The results of this experiment show that both maternal dietary restrictions and level of Se in the diet differentially affected cell proliferation depending on fetal ovarian tissue compartment. This indicates that plane of nutrition and Se in the maternal diet are involved in the regulation of early folliculogenesis and ovarian tissue growth. In fact, dietary restriction tended to suppress cellular proliferation in primordial follicles and blood vessels, but high Se levels suppressed cellular proliferation in primordial, secondary, and antral follicles,

Figure 3 The labeling index in antral, secondary, and primary follicles of ovine fetal ovaries in four treatment groups fed maintenance (M) or restricted (R) diet with adequate (A) Se or high (H) Se concentration. ^{a,b,c} $P < 0.005$ – 0.04 ; means $\pm$ S.E.M. with different superscripts differ (unprotected F -test).

stromal tissues, and blood vessels. These observations are novel, since very limited information is available concerning maternal diet on cellular processes within specific compartments of fetal ovaries including developing follicles and supporting tissues such as stroma and blood vessels. In addition, these data emphasize importance of maternal diet for fetal tissue and organ growth, which is a central concept of fetal/developmental programming (Nathanielsz 2006, Barker 2007).

Dietary restrictions, but not Se, in the maternal diet tended to affect fetal weight of the female lambs in our study. However, inconsistent results of the effects of nutrient restriction on total fetal weight (i.e., differences due to fetal sex were not reported) at the end of pregnancy in sheep have been reported, showing a decrease or no effect on fetal weight (Rae *et al.* 2001, Osgerby *et al.* 2002, Redmer *et al.* 2004, Lea *et al.* 2006, Luther *et al.* 2007). Similar to our findings, a lack of effect of maternal dietary restriction or environmental pollutants on ovine fetal ovarian weight has been reported by others (Rae *et al.* 2001, Osgerby *et al.* 2002, Murdoch *et al.* 2003, Fowler *et al.* 2008). Thus, the effects of maternal diet restriction on fetal growth seem to depend on the level and/or length of restriction, and the time of restriction implementation. In addition, it seems that level of Se in maternal diet composition does not affect fetal ovarian growth.

Figure 4 The labeling index in stromal tissues of ovine fetal ovaries in four treatment groups fed maintenance (M) or restricted (R) diet with adequate (A) Se or high (H) Se concentration. ^{a,b} $P < 0.05$; means $\pm$ S.E.M. with different superscripts (unprotected F -test).

Figure 5 The labeling index in blood vessels of ovine fetal ovaries in four treatment groups fed maintenance (M) or restricted (R) diet with adequate (A) Se or high (H) Se concentration. ^{a,b} $P < 0.001$; means $\pm$ S.E.M. with different superscripts differ (unprotected F -test).

By the end of pregnancy, fetal ovaries may contain all types of follicles including primordial, primary, secondary, and antral follicles, as observed in this and other studies on sheep and cattle (Wandji *et al.* 1992, Lundy *et al.* 1999, Bodensteiner *et al.* 2000, Rae *et al.* 2001). Growth and development of fetal ovaries is regulated by numerous factors of fetal and maternal origin including FSH, LH, estrogens, activin, c-kit and its ligand stem cell factor, enzymes controlling steroidogenesis, growth differentiation factor 9, epidermal growth factor, and many others (McNatty *et al.* 2000, Tanaka *et al.* 2001, Juengel *et al.* 2002, Sawyer *et al.* 2002, Byskov & Westergaard 2004, Van der Hurk & Zhao 2005, Pepe *et al.* 2006). Since we rarely detected apoptotic cells and atretic follicles in fetal ovaries, it seems that by the end of pregnancy, selected ovarian compartments are rather in the growing phase, and the apoptotic process is minimal. In fact, our observations are in agreement with previously published data, demonstrating very low apoptosis in ovine fetal ovaries on day 99 of pregnancy, but enhanced apoptosis in the earlier stages (days 58–73)

Figure 6 Representative micrograph of TUNEL staining in ovine fetal ovaries. Arrows indicate apoptotic cells/bodies (dark brown). Control sections did not show any positive staining (not shown because they were similar to unstained areas shown in this micrograph). Note numerous primordial (pr) and a few primary (p) follicles in this section. Bar = 25 μ m.

Figure 7 Representative micrograph of factor VIII staining (black color) in ovine fetal ovaries in the areas containing primordial (p), secondary (s), and antral (a) follicles (A), and in ovarian medulla (B). Inset represents control staining (no primary antibody). Note in (A), poor staining in the area containing primordial follicles, and a dense network of blood vessels (arrows) in theca layer of secondary and antral follicles, and stromal tissues. Note in (B), presence of smaller (arrowheads) and large (arrows) blood vessels. Bar = 50 µm.

of pregnancy (Murdoch *et al.* 2003, Qi *et al.* 2008). Although, altered expression of genes *BAX* and *MCL1*, which are associated with apoptosis, in fetal ovaries on day 110 of pregnancy has been reported for underfed sheep, it is unclear whether changes in gene expression were accompanied by the appearance of apoptotic cells/bodies (Lea *et al.* 2006). Thus, these data indicate that apoptosis in fetal ovaries is negligible during late pregnancy.

In our study, 8–13% of primordial follicles had at least one granulosa cell that stained positively for proliferating cell nuclear antigen (PCNA). By contrast,

Lundy *et al.* (1999) demonstrated that 98.7% primordial follicles contained at least one proliferating granulosa cell in fetal ovaries on day 135 of gestation in sheep. These differences are likely owing to different techniques used to determine the proportion of proliferating cells even though both studies used PCNA as a marker of proliferating cells. We used 5 µm of histological sections to determine differences in proportion of proliferating primordial follicles in between nutritional treatments. However, Lundy *et al.* (1999) used a nucleator technique to determine a proportion of proliferating follicles within a follicle type, which allowed for determination of the absolute number of granulosa cells in the individual follicle.

The present study demonstrated that the LI was lower in primordial than primary follicles, and than in secondary and antral follicles (5 vs 10 vs 19%). Similar to our study, the LI was lower in primary follicles than secondary and antral follicles in adult rat ovaries (Gaytan *et al.* 1996). Since a longer period of time is required for a new growing follicle to reach the preantral stage than for a transition from the preantral stage to antral follicle (Gougeon 2004), we hypothesize that differences in the LI in follicle types may reflect the timing of growth from primordial to primary, from primary to secondary follicle stage, or from secondary to antral follicle stage. However, this subject requires additional study, since very little information is available concerning the timing of follicular growth in fetal ovaries.

The proliferation in granulosa and theca cells of antral fetal follicles (17–20%) was within a range of LI (17–22%) reported for small follicles from adult ovine ovaries (Jablonka-Shariff *et al.* 1994, 1996). This indicates that cellular proliferation within small antral follicles remains at similar level in fetal ovaries and ovaries from adult animals. In addition, the LI in stromal cells (3–9%) and in blood vessels (9–20%) were also very high. These great levels of cellular proliferation clearly demonstrate that growth of ovarian follicles is accompanied by growth of surrounding stromal tissues and angiogenesis in fetal ovaries at the end of pregnancy. In addition to providing the scaffold for growing follicles and blood vessels, stromal tissues are the source of matrix metalloproteinases and tissue inhibitors, which likely play a role in fetal gonadal development (Robinson *et al.* 2001). Stromal tissues also express estrogen, androgen, and progesterone receptors, which are likely involved in the regulation of fetal ovarian growth (Juengel *et al.* 2002). Vasculogenesis and angiogenesis, marked in this study with high proliferation in blood vessels and a dense network of blood vessels in medulla, hilus, and areas containing primordial to antral follicles are critical for supporting fetal organ growth (Augustin 2000). In addition, vascular endothelial growth factor protein, the potent angiogenic factor, is expressed in ovine fetal ovaries (AT Grazul-Bilska, unpublished observations). Although angiogenesis in adult ovaries

has been studied extensively (Redmer & Reynolds 1996, Grazul-Bilska *et al.* 2001, Fraser 2006), little is known about the angiogenic process in fetal ovaries, and therefore it needs further investigation. Nevertheless, a high density of blood vessels in ovarian medulla and hilus and the high proliferation in blood vessels and stromal tissues observed in this study indicates that fetal ovaries are quickly growing and differentiating. In fact, it seems that by the end of pregnancy, fetal ovaries are approaching a very fast growth stage, as ovarian weight increases more than 10-fold (from ~80 mg to 1 g), while offspring weight only increases 3-fold (from ~3.5 to 10 kg) from day 135 of gestation to day 20 *post partum* (AT Grazul-Bilska, KA Vonnahme & JS Caton, unpublished observations). Thus, it is reasonable to postulate that the high cellular proliferation in stroma and blood vessels observed in this study is critical for ovarian growth support. Moreover, a complex vascular network in mouse fetal ovaries has been recently described by Bullejos *et al.* (2002), which emphasizes the importance of blood supply to developing organs. Cellular proliferation in stromal tissues and blood vessels in fetal ovaries is likely controlled by growth and other factors. However, very little information is available concerning the expression and function of growth factors in fetal ovaries (McNatty *et al.* 2000), and the mechanism of regulation of fetal ovarian growth remains to be elucidated.

The present results clearly demonstrate that maternal diet affects cellular proliferation in fetal ovaries. A similar pattern of cellular proliferation decrease in primordial, secondary or antral follicles, stromal tissues and blood vessels was observed in groups fed R diet or in groups fed high Se comparing with groups fed M diet with ASe in our study. However, differential effects of maternal diet on cellular proliferation in follicle types (e.g., primary versus secondary or antral) were observed in this study. These differences are likely due to a specific stage of follicular development regulation by factors controlling ovarian growth and function (McNatty *et al.* 2000, Erickson 2001, Sawyer *et al.* 2002, Byskov & Westergaard 2004, van der Hurk & Zhao 2005). In fact, the diverse effects of maternal diet on ovine fetal ovaries including ovarian weight, delayed ovarian growth and development, and altered ovarian cell proliferation and apoptosis have been reported by others (Borwick *et al.* 1997, Rae *et al.* 2001, Osgerby *et al.* 2002, Da Silva *et al.* 2002, 2003). In addition, maternal undernutrition has been demonstrated to reduce the number of follicles beyond the primordial stage in sheep (Rae *et al.* 2001). This may be associated with reduced cellular proliferations in primordial follicles observed in our study, which likely progressed at slower rates to primary and secondary follicle stages in underfed animals compared with normally fed ones.

In contrast to our observations, Lea *et al.* (2006) reported enhanced cellular proliferation in the granulosa

layer of fetal follicles on day 110 of gestation in underfed sheep. In addition, undernutrition did not change cellular proliferation in primordial follicles (Lea *et al.* 2006). These differences are likely due to the different lengths of nutritional restriction and different gestational time points for tissue collection.

In our study, high Se in the maternal diet decreased cellular proliferation in primordial, secondary, and antral follicles, stromal tissues and blood vessels. Interestingly, high Se in the maternal diet also decreased jejunal proliferation in female and male fetuses in a similar study (Carlson *et al.* 2008). In fact, it has been demonstrated that Se is involved in the regulation of cell proliferation (Zeng 2002) and angiogenesis inhibition in mammary cancer (Jiang *et al.* 1999). Furthermore, high Se concentration can decrease cell proliferation through inhibition of DNA synthesis and induction of apoptosis (Salbe *et al.* 1990, Yeh *et al.* 2006, Zeng & Combs 2008). Because we rarely detected apoptotic cells in fetal ovaries in this study, we hypothesize that high Se in the maternal diet suppresses cell proliferation through affecting DNA synthesis and cell cycle events. However, additional studies should be undertaken at the cellular and molecular levels to determine the mechanism of Se regulation of cell proliferation in fetal ovaries.

The role of Se in regulation of ovarian function in the fetus is unclear. However, for adult ovaries in animal models, it has been demonstrated that Se may modulate granulosa cell proliferation and estradiol-17 β synthesis *in vitro*, and Se administration may affect the ovulation process, implantation, and the number of live embryos (Parshad 1999, Basini & Tamanini 2000).

We have clearly demonstrated that maternal diet affected fetal ovarian function marked in this study by changes in cellular proliferation. In fact, the effects of maternal diet and other environmental factors on fetal and postnatal organ growth and function have been investigated for several models including humans (Rhind *et al.* 2003, Redmer *et al.* 2004, Luther *et al.* 2005, Barker 2007, Gardner *et al.* 2008). It has been demonstrated that environmental factors, including maternal diet composition, affect fetal development through many different mechanisms including alternations of gene expression or changes in organ structure and physiology (Rhind *et al.* 2003). However, the mechanism of the effects of undernutrition and Se in the maternal diet on fetal ovarian function remains to be elucidated.

In summary, maternal dietary restriction and/or a high level of Se in the diet decreased cell proliferation in primordial, secondary and/or antral follicles, stromal cells, and blood vessels in fetal ovaries. Moreover, the LI was greater in secondary and antral than in primordial and primary follicles, and the LI was similar for granulosa and theca cells. Thus, dietary restriction and Se in the maternal diet affect fetal ovarian growth at the end of pregnancy. Furthermore, apoptosis was minimal and a

dense network of blood vessels was present in fetal ovaries by the end of pregnancy. The results of this study will help us to understand the role of maternal dietary restrictions and Se level in diet in regulation of cell proliferation in fetal tissues and how maternal diet may affect growing tissues. In addition, these results emphasize importance of vascularization in folliculogenesis and ovarian growth. Thus, maternal diet may impact fetal and likely postnatal/adult ovarian development and function, emphasizing the importance of maternal diet on reproductive and overall health of the offspring. On the other hand, it is currently unknown whether changes in fetal ovaries at the cellular level induced by maternal factors may affect fertility of the offspring. Since very limited and inconclusive data are available concerning maternal effects on offspring fertility (Gardner *et al.* 2008), further investigation is required.

Materials and Methods

Animals and treatments

The Institutional Animal Care and Use Committee at NDSU approved all animal procedures in this study. Animal feeding, maintenance, and management are described in detail by Reed *et al.* (2007). Following breeding, ewes were assigned randomly to either an adequate (A) or high (H) dietary Se treatment. Ewes were pen-fed a basal diet (2.04 kg/ewe per day) that contained (dry matter basis) 47% alfalfa hay, 20% corn, 20% sugarbeet pulp pellets, 8% malt barley straw, and 5% concentrated separator byproduct. In addition to the basal diet, ewes assigned to the ASe treatment were fed 100 g/day of a control pellet that was balanced to contain 0.30 p.p.m. Se (6 µg/kg body weight), whereas HSe ewes were fed 100 g/day of a high-Se pellet balanced to contain 47.5 p.p.m. Se (80 µg/kg body weight), provided as Se-enriched yeast (Sel-Plex, Alltech, Nicholasville, KY, USA). The control (ASe) and HSe pellets were formulated using similar ingredients to maintain similar concentrations of metabolizable energy (ME), crude protein, acid detergent fiber, neutral detergent fiber, Ca, and P. Selenium-enriched yeast replaced soybean meal and partially replaced ground corn relative to the control pellet. The approach by which dietary Se was supplemented to pregnant, primigravid ewes has been used previously by our laboratory (Reed *et al.* 2007, Carlson *et al.* 2008).

On day 50 of gestation, ewes within each Se treatment were stratified by average breeding date and assigned to one of two distinct plane of nutrition treatments. The ewes were offered diets that were balanced to meet either 100% (maintenance (M)) or 60% (restricted (R)) of predicted ME requirements of pregnant ewe lambs (NRC 1985). The plane of nutrition treatments were applied from day 50 to 135 of pregnancy, which resulted in four distinct treatment combinations designated by the following: M ASe ($n=8$ ewes), M HSe ($n=8$), R ASe ($n=10$), and R HSe ($n=6$). Ewes ($n=32$) with female singleton fetuses from which fetal ovaries were collected were included in this study.

Tissue collection and immunohistochemistry

On day 135 of the pregnancy, fetal weight was recorded, and fetal ovaries were collected and weighed. One ovary was fixed in Carnoy's solution and the other ovary in 10% formalin solution, and after dehydration the ovaries were embedded in paraffin. The ovaries ($n=6$ –10/treatment) were sectioned (one section/ovary along the longitudinal axis) at 5 µm and mounted onto a glass slide.

Detection of PCNA (a marker of proliferating cells) and factor VIII (a marker of endothelial cells and thus vascularization) was performed as previously described in Carnoy's fixed tissues (Grazul-Bilska *et al.* 2007, 2008). Briefly, ovarian tissue sections were deparaffinized, rehydrated, and incubated with 3% H₂O₂ in methanol to eliminate endogenous peroxidase activity. Then, the sections were rinsed several times in PBS containing Triton-X100 (0.3%, vol/vol) and treated for 20 min with PBS containing normal horse serum (3%, vol/vol; ABC kit, Vector Laboratories, Burlingame, CA) to block non-specific binding of antibodies. The sections were incubated overnight at 4 °C in PBS containing a primary monoclonal mouse antibody against PCNA (1:500 dilution; MAB24R, Chemicon International, Temecula, CA, USA) or rabbit polyclonal antibody against factor VIII (1:100 dilution; Sigma). Primary PCNA or factor VIII antibodies were detected by using biotin-labeled secondary anti-mouse or anti-rabbit antibodies (Vector Laboratories) respectively, and the ABC method. For color development, SG substrate was used as described previously (Grazul-Bilska *et al.* 2007, 2008). For controls, the primary antibody was replaced with normal mouse IgG (4 µg/ml) or rabbit serum. After immunostaining of PCNA, the tissue sections were counterstained with nuclear fast red to visualize nuclei.

To localize apoptotic cells in fetal ovarian sections fixed in formalin, TdT-FragEL, DNA fragmentation detection kit was used according to the manufacturer's protocol (Oncogene Research Products, San Diego, CA, USA). As a positive control, tissues sections from the corpora lutea undergoing luteolysis were used as described previously (Vonnahme *et al.* 2006).

Image analysis

For all ovaries, images of stained sections (0.025 mm² per field) were taken for each of the four types of follicles (i.e., primordial, primary, secondary, and antral), for stromal tissue within medulla not containing follicles or blood vessels greater than 10 µm in diameter, and for all visible blood vessels (>10 µm in diameter) within medulla and hilus (total 5–30 images/ovary). The images were then used for quantitative image analysis using the Image Pro-plus software (Media Cybernetics Inc., Silver Spring, MD, USA) to determine the proportion (%) of proliferating primordial follicle of the total number of primordial follicles in the area, the LI for granulosa cells of the primordial and primary follicles, the LI for granulosa and theca cells of the secondary and antral follicles, stromal tissues, and blood vessels for the four treatment groups. The primordial follicle was considered as proliferating when at least one granulosa cell was PCNA-positive. For secondary and antral follicles, the LI was determined for granulosa and theca layers separately. The LI for thecal cells included all labeled

Table 2 The number of primordial, primary, secondary, and antral follicles analyzed for each treatment group.

Treatment group	Follicle type			
	Primordial	Primary	Secondary	Antral
M ASe	699	83	120	144
M HSe	464	85	92	92
R ASe	542	105	142	54
R HSe	537	47	92	34
Total number of follicles analyzed	2242	320	446	325

cells, and no attempt was made to distinguish between cell types within thecal layer. The number of primordial, primary, secondary, and antral follicles analyzed for each treatment group is presented in Table 2. For blood vessels, both endothelium and smooth muscle cell layers were analyzed together, and blood vessels were not classified as arterioles or venules. The LI was calculated as a percentage of proliferating cells out of the total cells per marked area of follicle, stromal tissue or blood vessel. Due to heterogeneous distribution of factor VIII staining, and thus vascularization, using image analysis was not performed (Fig. 7).

Statistical analyses

Data are expressed as mean $\pm$ S.E.M.. Data were analyzed as a completely randomized design with a 2 $\times$ 2 factorial arrangement of treatments using PROC GLM (SAS Inst. Inc., Cary, NC, USA 2005). The model contained effects for plane of nutrition (M and R), level of Se (ASe and HSe), and the plane of nutrition $\times$ Se interactions. When the *F*-test was significant ($P < 0.05$ or 0.1), differences among means were evaluated by using the least square means procedure (Kirk 1982). Means were considered different when $P < 0.05$ unless otherwise stated.

Declaration of interest

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

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