Effects of maternal diet on expression of gap junctional connexins in fetal ovaries in sheep[1]

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SUMMARY

Our objective was to determine if maternal diet impacts expression of connexin (Cx) 26, 37 and 43 in fetal ovaries. Sheep were fed a maintenance (M) diet with adequate (A) selenium (Se) or high (H) Se levels from 21 days before breeding to day 135 of pregnancy. From day 50 to 135 of pregnancy (tissue collection day), a portion of the ewes from ASe and HSe groups was fed restricted (R; 60% of M) diet. Sections of fetal ovaries were immunostained for the presence of Cx26, Cx37 and Cx43 followed by image analysis. All four connexins were detected, but the distribution pattern differed. Connexin 26 was immunolocalized in the oocytes from primordial, primary, secondary and antral follicles, in granulosa and theca layers of secondary and antral follicles, stroma and blood vessels; Cx37 was expressed on the borders between oocyte and granulosa/cumulus cells of primordial, primary, secondary and antral follicles, and in endothelium; and Cx43 was expressed on cellular borders in granulosa and theca layers, and between oocyte and granulosa/cumulus cells of primordial, primary, secondary and antral follicles. Connexin 26 expression in antral follicles was decreased by R diet. The expression of Cx43 in granulosa cells of primary and granulosa and theca layers of antral follicles was increased by HSe in M diet. Thus, maternal diet affected Cx26 and Cx43 expression, and connexins may be differentially involved in regulation of fetal ovarian function in sheep, which emphasizes the importance of maternal diet in fetal growth and development.

INTRODUCTION

Fetal ovaries represent a type of tissue that expresses high tissue growth and differentiation, which are controlled by factors of fetal and maternal origin (Juengel et al. 2002, Sawyer et al. 2002, Van der Hurk & Zhao 2005; Grazul-Bilska et al. 2009). In fact, numerous factors of fetal and maternal origin, including nutrition, FSH, LH, estrogens, activin, c-kit with its ligand stem cell factor, enzymes controlling steroidogenesis, growth differentiation factor 9, epidermal growth factor and many others, may affect the

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growth, development, and physiology of the fetal and postnatal life of mammals (Borwick et al. 1997, McNatty et al. 2000, Wright et al. 2002, Pepe et al. 2006, Wu et al. 2006, Fowler et al. 2008). Additionally, our previous study demonstrated that maternal diet affected fetal body and ovarian weight, and ovarian cell proliferation in sheep (Grazul-Bilska et al. 2009).

Regulation of organ growth and function can be mediated by contact-independent and contact-dependent mechanisms (Grazul-Bilska et al. 1997). Contact-dependent cellular interactions are mediated by gap junctional pathways which facilitate and regulate the exchange of regulatory molecules (Grazul Bilska et al. 1997). Gap junctions have a hexameric structure known as a connexon that is composed of specific gap junctional proteins termed connexins (Holder et al. 1993, Grazul-Bilska et al. 1997, Kidder & Mhawi 2002, Sohl & Willecke 2003 2004, Wei et al. 2004). It has been well established that connexins not only form gap junctional channels, but also have additional functions including signal transduction, secretory function, paracrine signaling and control of growth and development of organs and tissues in physiological and pathological conditions (Moorby & Patel 2001, Serre-Beinier et al. 2002, Ebihara 2003; Goodenough & Paul 2003, Gittens et al. 2005, Borowczyk et al. 2007).

Gap junctions and/or gap junctional proteins or mRNA are expressed in the adult ovaries of several species (Grazul-Bilska et al. 1997, 1998, Johnson et al. 1999, Nuttinck *et al.* 2000, Metlon et al. 2001, Wright et al. 2001, Borowczyk et al. 2006a,b, Gershon et al. 2008). However, for fetal ovaries, expression of mRNA for several connexins and Cx43 protein has been only demonstrated in mice (Juneja 2003, Pérez-Armendariz et al. 2003). In ovaries, connexins exhibit a unique pattern of expression that can be metabolically, hormonally or developmentally regulated (Grazul-Bilska et al. 1997, Johnson et al. 1999, Gershon et al. 2008).

We hypothesized that Cx26, Cx37 and Cx43 proteins are expressed in fetal ovaries, and that expression of these connexins will be affected by maternal diet. Therefore, the objective of this experiment was to immunolocalize Cx26, Cx37 and Cx43 in ovarian compartments, and to determine if maternal plane of nutrition and Se level in the diet impacts expression of connexin proteins in fetal ovaries in sheep.

PROCEDURES

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 Grazul-Bilska et al. (2009). Briefly, following breeding, pen-fed a basal diet (2.04 kg/ewe daily) ewes were assigned randomly to either an adequate (A) or high (H) dietary Se treatment. In addition, ewes were fed 100 g/day of a control pellet that contained 0.3 ppm ASe or a high-Se pellet balanced to contain 47.5 ppm Se provided as Se-enriched yeast (Sel-Plex, Alltech, Nicholasville, KY). This approach provided 6 μg/kg of ewe body weight in the ASe and 80 μg/kg of ewe body weight in the HSe treatments, respectively, The 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. The approach by which dietary Se was supplemented to pregnant, primigravid ewes has been used previously by our laboratory (Carlson et al. 2008, Grazul-Bilska et al. 2009).

On day 50 of gestation, ewes within each Se treatment were stratified by average breeding date and assigned to distinct planes of nutrition treatments. 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 dietary restriction treatment was accomplished by reducing intake of restricted ewes to 60% of control treatment and therefore represents a global nutrient reduction. The plane of nutrition treatments were applied from day 50 to 135 of pregnancy, which resulted in 4 distinct treatment combinations designated by the following; MASe (n=8 ewes), MHSe (n=8), RASe (n=10) and RHSe (n=6). Only ewes with female singleton fetuses were included in this study.

Tissue Collection and Immunohistochemistry. On day 135 of the pregnancy, fetal ovaries were collected. One ovary was fixed in Carnoy's solution and the other ovary in 10% formalin solution, and after dehydration, ovaries were embedded in paraffin. 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 connexins in Carnoy's fixed tissues was performed as previously described (Grazul-Bilska et al. 1998, Borowczyk et al. 2006a,b). Briefly, ovarian tissue sections were deparaffinized, rehydrated, and incubated with 3% H_2O_2 in methanol to eliminate endogenous peroxidase activity. Then, 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 nonspecific binding of antibodies. Sections were incubated overnight at 4°C in PBS containing a primary rabbit polyclonal antibody against Cx26 (1:50; Zymed Laboratories Inc., San Francisco, CA), Cx37 (1:50; Alpha Diagnostics, San Antonio, TX) or Cx43 (1:500; as described by Grazul-Bilska et al. 1998). Primary antibody was detected using biotin-labeled anti-rabbit secondary antibody and the ABC method (Vector Laboratories). For color development of Cx26, Cx37 and Cx43, SG substrate was used as described before (Grazul-Bilska et al. 1998). For controls, the primary antibody was replaced with rabbit serum.

Image analysis. For all ovaries, images of stained sections were taken for each of the four types of follicles (e.g., primordial, primary, secondary and antral), and for stromal tissue within the medulla and hilus not containing follicles (total 5-15 images/ovary). The images were then used for quantitative image analysis using the Image Pro-plus software (Media Cybernetics Inc., Silver Spring, MD) to determine the proportion (%) of area stained positively for a specific connexin out of the total area of granulosa or theca layer. Image analysis was performed for Cx26 and Cx43 in primary, secondary and/or antral follicles. However, quantification of Cx37 expression using image analysis was not performed due to the heterogeneous distribution within the ovary. The number of primordial, primary, secondary and antral follicles analyzed for each connexin expression using image analysis is presented in Table 2.

Statistical Analyses. Data were analyzed as a completely randomized design with a 2 x 2 factorial arrangement of treatments using PROC GLM (SAS Inst. Inc. Cary, NC 2010). The model contained effects for plane of nutrition (M and R), level of Se (ASe and HSe), and the plane of nutrition x Se interactions. When the F-test was significant ($P < 0.05$), differences among means were separated by using the least square means procedure (Kirk 1982). Means were considered different when $P < 0.05$ unless otherwise stated. Data are expressed as mean ± SEM.

RESULTS

All three connexins were detected in fetal ovaries, but the pattern of localization of these

Table 1. Number of follicles used for image analysis of Cx26 and Cx43 expression

Follicle type	Total number of follicles analyzed		Number of follicles analyzed per nutrition group
	Cx26	Cx43	
Primary	0 (no positive staining)	33	6-11
Secondary	126	121	19-43
Antral	108	183	12-97

Table 2. Localization of Cx26, Cx37 and Cx43 in ovine fetal ovaries

Ovarian compartment	Protein
Primordial follicles	Cx37 and Cx43
Primary follicles	Cx37 and Cx43
Secondary follicles	Cx26, Cx37 and Cx43
Antral follicles	Cx26 and Cx43
Blood vessels	Cx26 and Cx37
Stromal tissues	Cx26

connexins differed (Fig. 1-3; Table 1). Connexin 26 was localized on the cellular borders and/or in the cytoplasm of cells in theca layer of secondary and antral follicles, in stromal tissues and blood vessels, and also was detected in cumulus cells of approximately 10% of antral follicles (Fig. 1). Connexin 37 was expressed as linear and punctate staining on the borders between oocyte and granulosa/ cumulus cells of primordial, primary, secondary and antral follicles, and as punctate and cytoplasmic staining in endothelium (Fig. 2). Connexin 43 was expressed as punctate staining on the cellular borders and cytoplasm in the granulosa layer of primordial, primary, secondary and antral follicles, in the theca layer of antral follicles, and on borders between the oocyte and granulosa/cumulus cells (Fig. 3).

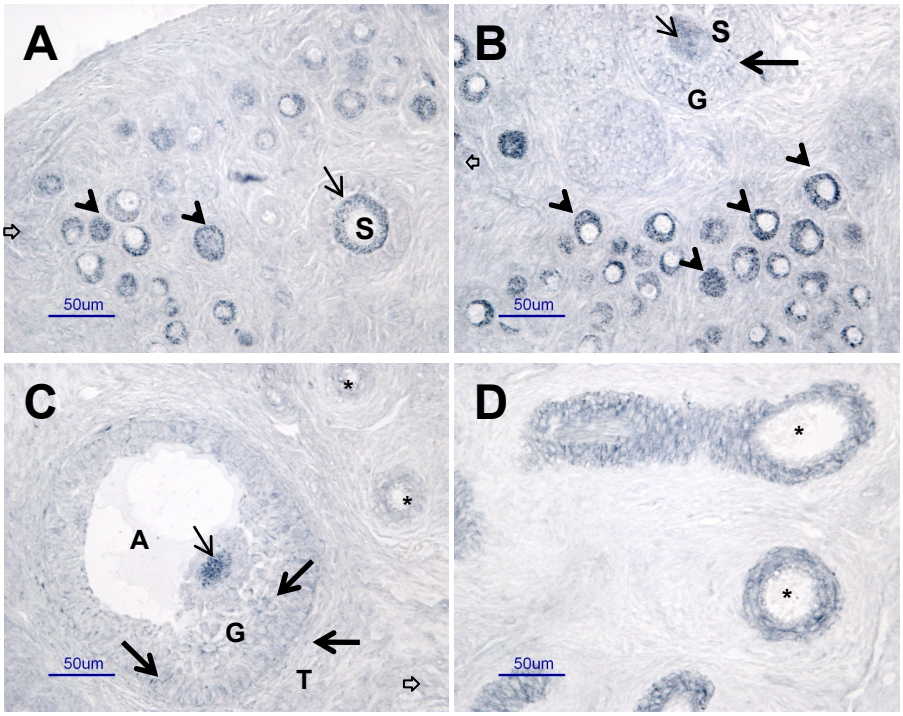

Figure 1. Representative micrograph of Cx26 immunolocalization in ovine fetal ovaries. Arrowheads indicate positive staining (dark color) in oocytes of primordial and primary follicles (A, B); small arrows indicate positive staining in oocytes of secondary (S) and antral (An) follicles (A, B, C), large arrows indicate positive staining in granulosa (G) cells of S and An follicles (B, C), and theca (T) cells of An follicle (C), open arrows indicate positive staining in stromal tissues (A, B, C), and asterisks indicate blood vessels (C, D). Control staining (no primary antibody) did not show any positive staining and was similar to the images' background (data not shown). Bar = 50 μm.

Expression of Cx26 in secondary follicles was not affected by nutritional treatments, but Cx26 expression in antral follicles was decreased (P<0.01) by high level of selenium in M and R diet (Fig. 4A). Overall, Cx26 expression in granulosa cells of antral follicles was greater (P<0.0001) than in secondary follicles (0.35±0.04 vs. 0.12±0.01%).

Expression of Cx43 in the granulosa layer of primary follicles was greater (P<0.05) in the group fed M diet with HSe than in groups fed M diet with ASe or R diet with HSe (Fig. 4B). For secondary follicles, Cx43 expression was similar in all treatment groups (data not shown). For antral follicles, Cx43 expression in granulosa and theca layers was greater (P<0.01) in the group fed M diet with HSe than in any other treatment group (Fig. 4C). Overall, Cx43 expression was greater (P<0.001) in granulosa than in the theca layer of antral follicles (1.5±0.1 vs 0.6±0.05%), and expression of Cx43 in granulosa cells was greater (P<0.001) in antral than in secondary or primary follicles (1.5±0.1 vs 0.6±0.05 or 0.5±0.06%, respectively).

DISCUSSION

The results of this experiment show that Cx26, Cx37 and Cx43 are expressed in fetal ovaries and that distribution of these connexin within ovarian compartments differs. In addition, maternal dietary restrictions and/or level of Se in the diet differentially affected Cx26 and Cx43 expression depending on fetal ovarian compartment. This suggests that maternal plane of nutrition and Se level in the maternal diet may be involved in the control of gap junction expression. These observations are novel, since very limited information is available concerning the expression of connexin proteins in fetal ovaries and regulation of their expression. In addition, these data emphasize that maternal diet may have the major impact on normal fetal ovarian growth and development, which

is a central concept of fetal/ development programming also known as developmental origins of health and disease (Nathanielsz 2006, Barker 2007).

The pattern of Cx26 localization in the thecal layer of secondary and antral follicles, in stromal tissues and blood vessels in fetal ovaries resembled the pattern observed in adult ovine ovaries (Grazul-Bilska et al. 1998). However, in contrast to adult ovine and bovine ovaries, in ovine fetal ovaries Cx26 was not expressed in surface epithelium, in the oocytes of primordial follicles, or in the granulosa layer of antral follicles (Grazul-Bilska et al. 1998, Johnson et al. 1999). Moreover, Cx26 seems to be developmentally regulated in fetal and adult ovaries since Cx26 expression was the greatest in antral follicles in this study, and in large antral follicles in adult cows (Johnson et al. 1999). The presence of Cx26 in the thecal layer of secondary and antral follicles stromal tissue and blood vessels suggests its role in the regulation of follicular development, and stromal and blood vessel function in fetal ovaries. In addition, Cx26 is likely involved in maintenance of oocyte health, since decreased Cx26 expression in cumulus oocyte complex was associated with a decreased quality of oocytes in the diabetic mouse model (Ratchford et al. 2008). However, in the present study, Cx26 was expressed in cumulus cells only in a small portion of the antral follicles present in fetal ovaries. Therefore, additional studies should be undertaken to determine what role Cx26 may have in maintenance of oocyte health in fetal ovaries in sheep.

In the present study, Cx37 protein was uniformly and consistently distributed on oocyte-cumulus/granulosa borders from primordial to antral follicles and in endothelium. Similarly, in adult ovine ovaries, Cx37 protein was detected on the oocyte/ cumulus border and in endothelial cells (Borowczyk et al. 2006b). In addition, Cx37 was present in granulosa cells of adult ovine and bovine antral follicles and murine oocytes (Nuttinck et al. 2000, Teilmann 2005, Borowczyk et al. 2006b, Ratchford et al. 2008). For the mouse, it has been clearly demonstrated that Cx37 is critical for normal oogenesis/ folliculogenesis (Simon et al. 1997; Gittens & Kidder 2005). Localization of Cx37 on the border between oocyte and cumulus/granulosa cells in all follicular stages in fetal ovaries in the present study, suggests that Cx37 may be as important for ovine oocyte/follicle development as for mice.

Connexin 26 and Cx37, but not Cx43, were expressed in blood vessels in fetal ovaries in this study. In fact, a dense network of blood vessels in the medulla and hilus and a less dense network in the cortex is present in ovine fetal ovaries (Grazul-Bilska et al. 2009). It has been demonstrated that several connexins including Cx37, Cx40, Cx43 and Cx45 are involved in the regulation of vascular function and angiogenesis in several organs in adults (Haefliger et al. 2004, Schmidt et al. 2008, Johnstone et al. 2009). It seems that type of connexin expressed in blood vessels in fetal ovaries

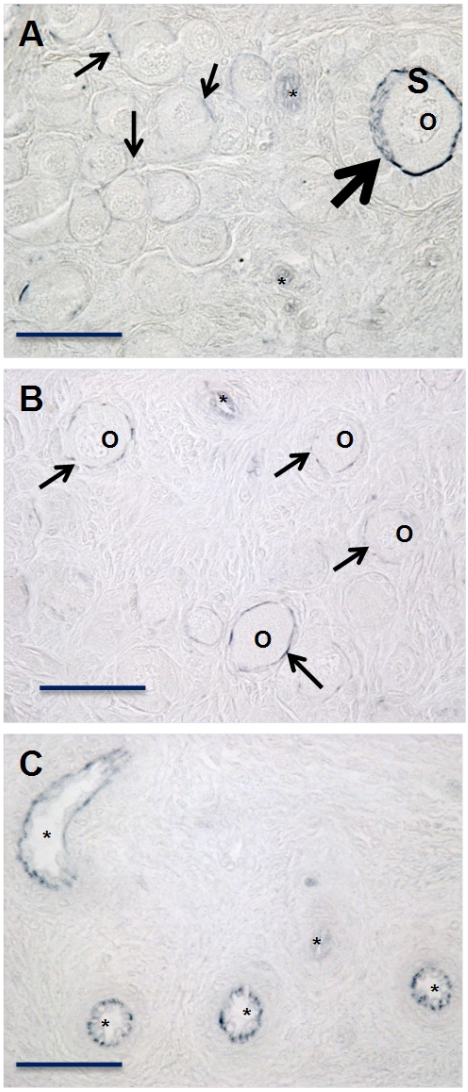

Figure 2. Representative micrograph of Cx37 immunolocalization in ovine fetal ovaries. Small arrows indicate positive Cx37 staining (dark color) on the border between oocyte (O) and granulosa (G) layer of primordial and primary follicles (A, B). Large arrow indicates positive Cx37 staining on the border between O and G layer of secondary (S) follicle (A). Asterisks indicate positive staining in blood vessels (C). Control staining (no primary antibody) did not show any positive staining and was similar to the images' background (data not shown). Bar = 50 μm.

differs from adult organs, since in fetal ovaries we detected the presence of Cx26 but not Cx43. This difference is likely due to the early developmental stage of fetal ovaries. Therefore in fetal ovaries, Cx26 and Cx37 are likely involved in regulation of blood vessel function and angiogenesis.

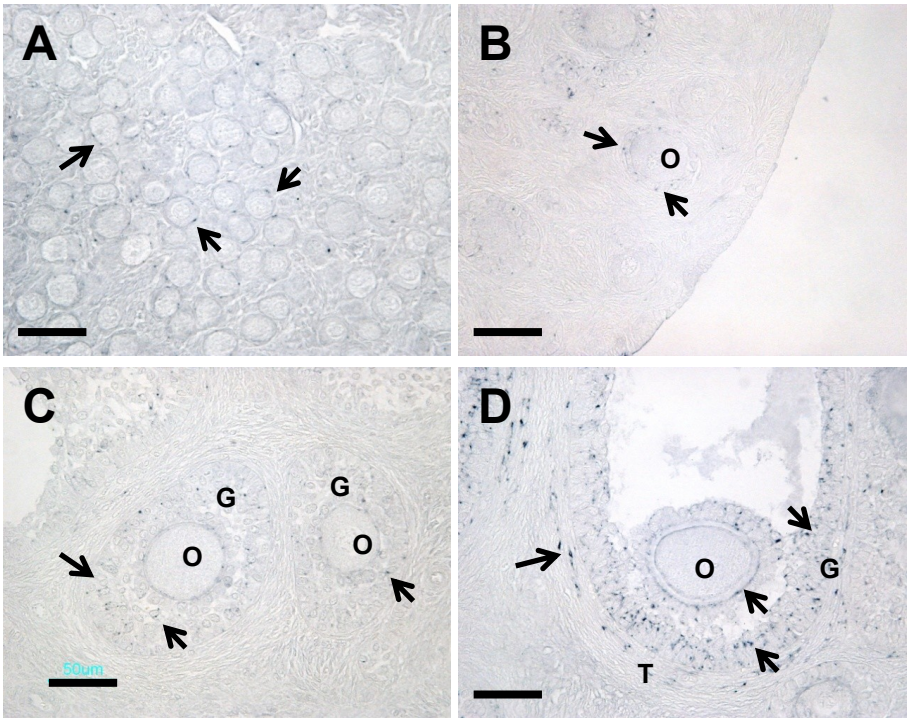

Figure 3. Representative micrograph of Cx43 immunolocalization (dark color) in primordial (A), primary (B), secondary (C) and antral (D) follicles in ovine fetal ovaries. Arrows indicate positive staining in flat granulosa (G) layer (A); in G layer of primary and secondary follicle (B, C); in G and theca (T) layers, and on the border between oocyte and G/cumulus cells in antral follicle (D). Bar = 50 μm.

Connexin 43 was exclusively expressed in granulosa and theca layer in all follicle types, and its expression level increased from the primary to antral stage of follicular development in the present study. Similarly, Cx43 was detected during fetal ovarian development in the mouse (Pérez-Armendariz et al. 2003). In addition, a similar spatio-temporal pattern of Cx43 expression was observed for adult ovaries in several species (Grazul-Bilska et al. 1998, Johnson et al. 1999, Nuttinck et al. 2000, Teilmann 2005). Thus, the Cx43 expression pattern suggests that Cx43 is involved in the regulation of folliculogenesis in ovine fetal ovaries. In addition, it has been clearly demonstrated that level of Cx43 expression in cumulus cells may serve as a marker of oocyte quality in humans (Wang et al. 2009) and that Cx43 is directly associated with steroidogenesis in the ovine ovary (Borowczyk et al. 2007). The importance of Cx43 for follicular development was emphasized in a mouse knockout model showing a lack of development beyond primary follicles (Juneja et al. 1999, Gershon et al. 2008). Thus, it is reasonable to postulate that in fetal ovaries similar to adult ovaries, Cx43 plays a major regulatory role during folliculogenesis.

In this study, Cx26 expression in antral follicles was decreased by HSe in M and R diet, and Cx43 expression in primary and antral follicles was increased by HSe level in M diet but not by maternal dietary restrictions. These differences may be due to a different localization of each connexin, to the specific stage of follicular development, and possibly to the different functions of Cx26 vs. Cx43 in fetal ovaries. Differential effects of maternal diet on fetal ovarian weight, delayed ovarian growth and development, and altered ovarian cell proliferation and apoptosis have been reported for sheep (Borwick et al. 1997, Rae et al. 2001, Osgerby et al. 2002, Da Silva et al. 2002, 2003, Grazul-Bilska et al. 2009). In addition, it has been demonstrated that high Se level in diet affects increased Cx43 dephosphorylation in rat heart which may contribute to arrhythmogenesis (Rakotovao et al. 2005). Recently, we clearly demonstrated that both maternal dietary restrictions and/or HSe level decreased cellular proliferation in primordial, secondary and/or antral follicles, stromal tissues and blood vessels in fetal ovaries (Grazul-Bilska et al. 2009). Interestingly, the enhanced Cx43 expression observed in the present study in primary and antral follicles in the group fed M diet with HSe corresponds to decreased cellular proliferation in the same compartments of fetal ovaries from the same nutrition-

treatment group (Grazul-Bilska et al. 2009). Since Cx43 is recognized as an important regulator of cell and tissue growth (Grazul-Bilska et al. 1997, Moorby & Patel 2001), we hypothesize that increased expression of Cx43 may be a part of a compensatory mechanism preventing an additional decrease of cell proliferation and maintaining follicle integrity in fetal ovarian follicles when cell proliferation is reduced by HSe in the diet. In fact, it has been demonstrated that Se is involved in regulation of several cellular functions such as cell proliferation and angiogenesis in many organs (Salbe et al. 1990, Jiang et al. 1999, Zeng 2002, Yeh et al. 2006, Carlson et al. 2008, Zeng & Combs 2008). This study suggests that Se is involved in the regulation of Cx43 expression. However, additional studies should be undertaken to determine the mechanism of the effects of the level of energy and Se in maternal diet on regulation of gap junction expression and function in fetal tissues.

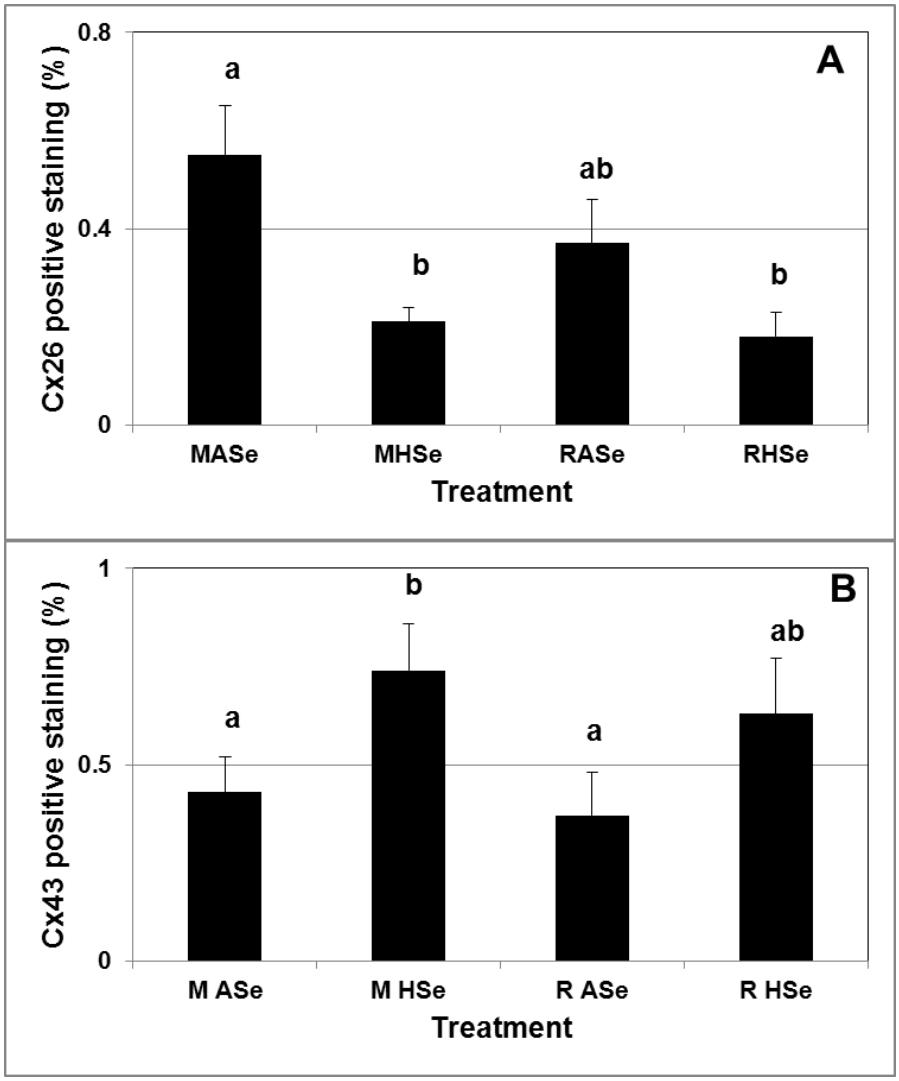

Figure. 4. Effects of maternal nutrition on expression of Cx26 in antral follicles (A) and Cx43 in primary (B) and antral follicles (C). $^{a,b}P < 0.01$ for Cx26 and 0.04 for Cx43; means ± SEM with different superscripts differ within follicle type.

In summary, Cx26, Cx37 and Cx43 are expressed in several ovarian compartments but the distribution of these connexin differed within fetal ovaries. The presence of these connexins in fetal ovaries suggests that they may play a differential role in gap junction mediated regulation of growth and function of fetal ovaries. Expression of Cx26 and Cx43 was affected by the stage of follicular development and by maternal diet; nutrient restriction decreased Cx26 expression in thecal layer of antral follicles, and high Se concentration in M diet increased Cx43 expression in granulosa cells of primary and granulosa and theca layers of antral follicles. Therefore, we postulate that Cx26 and Cx43 are involved in the regulation of folliculogenesis. Our results emphasize the importance of maternal diet in the regulation of fetal ovarian growth. The results of this study will help us begin to understand the role of maternal diet in regulation of gap junction function in fetal tissues.

Although we have demonstrated the effects of maternal diet on fetal ovarian development and suggested a role for connexins in the process, it is currently unclear if dietary supplements would affect oocyte quality and thus the reproductive function of the offspring. Therefore, more research is required to demonstrate if exposure of the fetus to specific environmental factors may affect reproductive function in the offspring (Gardner *et al.* 2008). Nevertheless, the knowledge generated

in this study will likely contribute to the concept of fetal programming/fetal origin of adult disease.

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