Original Research

Choline supplementation influences ovarian follicular development

Xiaoshu Zhan[1,2], Lauren Fletcher[2], Serena Dingle[2], Enzo Baracuhy[2], Bingyun Wang[1,*], Lee-Anne Huber[2], Julang Li[2,*]

[1]*Department of Life Science and Engineering, Foshan University, 528231 Foshan, Guangdong, China,* [2]*Department of Animal BioSciences, University of Guelph, Guelph, ON N1G 2W1, Canada*

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Methods and materials
- *3.1 Animal trial*
- *3.2 Histological analysis of ovarian tissues*
- *3.3 Total RNA isolation and qRT-PCR*
- *3.4 Reverse transcription and miRNA expression quantification*
- *3.5 Hydrophilic interaction chromatography analysis of serum*
- *3.6 Statistical analysis*

4. Results
5. Discussion
6. Conclusions
7. Author contributions
8. Ethics approval and consent to participate
9. Acknowledgment
10. Funding
11. Conflict of interest
12. References

1. Abstract

Background: Female infertility is a health issue for both humans and animals and despite developments in medical interventions, there are still some conditions that cannot be treated successfully. It is important to explore other potential therapies or remedies that could improve reproductive health. Choline is an over-the-counter supplement and essential nutrient that has many health benefits. It has been suggested to be beneficial in various aspects of fertility, including fetal development and endocrine disorders like polycystic ovarian syndrome (PCOS). However, choline's impact on ovarian function has not been explored. **Methods**: To study the effects of choline on ovarian development, 36 female Yorkshire $\times$ Landrace pigs were fed the following four supplemented diets between 90 and 186 days of age: (1) Control (corn and soybean meal-based diet that met estimated nutrient requirements, n = 9); (2) Choline (additional 500 mg choline per 1 kg of control diet, n = 8); (3) Omega-3 (additional 5556 mg Omega-3 per 1 kg control diet by introducing fish oil); (4) Choline + Omega-3 (500 mg choline + 5556 mg Omega-3 per 1 kg control diet). Pigs fed the choline-supplemented diet were compared to the control group and those fed diets supplemented with Omega-3 as fertility-promoting agent. **Results**: It was found that the number of corpus luteum per ovary in the Choline (16.25 $\pm$ 2.88), Omega-3 (10.78 $\pm$ 1.71) and Choline + Omega-3 (14.89 $\pm$ 2.97) groups were all higher in comparison to that of the control group (5.56 $\pm$ 1.72, $p < 0.05$). The percentage of antral follicles in the Choline + Omega-3 group were higher compared to the control group ($p < 0.05$). To elucidate the potential molecular mechanism of choline on these improved ovarian phenotypes, the expression of a group of genes that are involved in ovarian development, including cytochrome P450 family 11 subfamily A member 1 (*CYP11A1)*, follicle stimulating hormone receptor *(FHSR)* and luteinizing hormone receptor (*LHR*), was analyzed using RT-qPCR. The expression of both *LHR* and *CYP11A1* was significantly upregulated in the choline-supplemented group ($p < 0.05$), while there are no differ-

Submitted: 15 August 2021 Revised: 24 November 2021 Accepted: 25 November 2021 Published: 30 December 2021

ences in *FSHR* expression among all the groups. Additionally, the expression of miR-21, -378, -574, previously found to be important in ovarian function, were examined. Our data showed that miR-574 was upregulated in the Choline group while miR-378 was upregulated in the Choline + Omega-3 group in comparison to the control group ($p < 0.05$). Further, serum metabolite analysis showed that 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine, a form of phosphatidylcholine metabolite, was significantly increased in all the treatment groups ($p < 0.05$), while testosterone was significantly increased in both Omega-3 and Choline + Omega-3 groups ($p < 0.05$) and tended to be reduced in the choline-supplemented group ($p = 0.08$) compared to the control group. **Conclusions**: Our study demonstrated choline's influence on ovarian function *in vivo*, and offered insights into the mechanisms behind its positive effect on ovarian development phenotype.

2. Introduction

Infertility is a global issue that affects approximately 8–12% of reproductively mature individuals, with 70% of cases being female [1]. Infertility is a disorder characterized by failure to become or remain pregnant after at least 12 months of unprotected sexual intercourse in humans, which may stem from ovulatory or hormonal disorders, diminished ovarian reserve (DOR), and anatomical, genetic or immunological abnormalities of the reproductive system [2]. In the livestock industry, infertility also causes productivity concerns and economic losses. The annual economic losses of infertility in beef cattle range from 700 to 1100 USD per cow, while seasonal infertility in swine results in approximately 420 million USD losses in the United States annually [3]. Therefore, addressing infertility and increasing reproductive capability is of great significance to both human health and livestock industries.

Research efforts have been focused on the relationship between diet and human fertility over the last decade. Specifically, "healthy" diets are associated with better fertility and higher live birth rate in assisted reproductive technology studies [4]. The majority of infertility cases caused by ovulation-related disorders may be preventable or improved by modifications in diet and lifestyle [5]. Long-chain Omega-3 fatty acids are one of the "healthy" dietary components that appear to improve female fertility [4, 6]. Specifically, lower dietary omega-6-to-Omega-3 ratio is more desirable in decreasing the risk of many diseases which may affect female fertility [7]. In a murine model, the lifelong consumption of an Omega-3 rich diet postponed ovarian aging and improved oocyte quality at advanced maternal age in comparison to the consumption of an Omega-6 rich diet [8]. Furthermore, women consuming more Omega-3 fatty acids are less likely to experience anovulation [9], which may be partly due to the alteration of sexual hormone production by Omega-3 fatty acids [9] and anti-inflammatory properties of the Omega-3 fatty acids, which modulate the immune response during ovarian follicular development and oocyte maturation [10]. Omega-3 fatty acids are not produced at a high enough quantity in the body and need to be obtained from dietary sources [11], similar to other essential nutrients. Hence, optimizing dietary composition is an ideal way to regulate female reproductive capacity, while being extremely cost-effective and safe for wide-spread application.

Choline is a lipotropic amine that carries out various functions in the body and has been described as an essential nutrient for humans [12]. The importance of choline in normal physiological function is noted in most mammals, where choline deficiency has been observed to lead to disorders of the liver, kidneys, pancreas, memory, and growth [13]. Choline is also important in the formation of acetylcholine, which is a neurotransmitter mainly involved in controlling smooth muscle contractions. It can be metabolized through the same pathway as folate and vitamin B12 that are involved in DNA methylation [14]. DNA methylation is important for regulating gene expression, as methylation in the promoter region and CpG islands of genes can influence transcription and subsequent cellular function [15]. Choline is also associated with fertility and fetal development. In utero, choline is pumped against a concentration gradient into the fetus, with the amniotic fluid containing ten times the concentration of choline compared to maternal blood [16]. Further, neural tube development defects are four times more likely to occur in fetuses of women who are in the lowest quartile of choline intake compared to the uppermost quartile [17]. Mother rats receiving choline-deficient diets in late pregnancy had offspring with lower numbers of progenitor cells and higher rates of apoptosis in the hippocampus, indicating poor fetal development [18]. Moreover, choline was significantly decreased in both plasma and follicular fluid of patients with PCOS [19, 20], a metabolic disorder that alters serum levels of a variety of metabolites, ultimately reducing the fertility of affected women [19]. This suggests that choline might have an overarching role in regulating ovarian function. However, the effects of dietary choline on ovarian development and ovulation are not known. *Sus scrofa* is a widely-used medical model due to its anatomical, physiological and metabolic similarities to humans [21]. Furthermore, studies show that the oocyte development patten, menstrual cycle length and hormonal cycle of female pigs are more similar to women compare to that of rodent species [22, 23], which makes it an idea model for human reproductive performance research. The objective of this study was to assess the effect of choline supplementation on ovarian follicular development and ovulation compared to the basic and Omega-3-supplemented diets. We hypothesized that, in a pig model, choline supplementation improves ovarian follicular development through regulation of genes and miRNA expression and altering levels of serum metabolites.

3. Methods and materials

3.1 Animal trial

Thirty-six Yorkshire × Landrace gilts were recruited at 90 days of age and randomly divided into four experimental groups based on litter, body weight and backfat thickness. Pigs were housed in individual pens and fed one of the following diets: (1) Standard commercial corn and soybean meal-based diet that met estimated nutrient requirements for growing pigs (the National Research Council, 2012; Control, n = 9); (2) Choline-supplemented diet composed of the control diet with additional choline to achieve 1000 mg choline per kg of diet, which was 2× the choline content versus Control (Choline, n = 8); (3) Omega-3 supplemented diet composed of the control diet with an additional 5556 mg/kg ω-3 by introducing 2.5% fish oil in the grower stage and then 2.0% fish oil in the finisher stage to achieve omega-6-to-Omega-3 ratios of 1.6 and 1.9, respectively (Omega-3, n = 9); (4) Choline + Omega-3 diet composed of the control diet supplemented with both choline and fish oil as for the previous two diets (Choline + Omega-3, n = 9). The diet ingredient composition and calculated nutrient contents are outlined in Table 1. The lower ratio of omega-6-to-Omega-3 fatty acids have been widely described to confer a variety of health benefits including the reproductive system and be essential in a number of biological pathways [24]. As the known fertility-promoting properties of Omega-3 fatty acids are well known, the effect of Omega-3 supplementation on ovarian development is not thoroughly discussed in this study. This study was conducted at the Arkell Swine Research Station, University of Guelph, ON, Canada. All experimental procedures were approved by the University of Guelph's Animal Care Committee (AUP #4068) and followed the Canadian Council of Animal Care Guidelines (CCAC, 2009).

Experimental feed was provided to pigs *ad libitum* between 90 and 186 days of age. Starting at 90 days of age, pigs were fed the grower diet. At 140 days of age, the pigs were administered PG600 (Intramuscular injection of 400 IU of Equine chorionic gonadotropin and 200 IU of Human chorionic gonadotropin) and boar exposure to synchronize estrous cycles. Feed was also switched to the experimental finisher diet at 140 days of age. Five days later, the pigs entered heat cycle 1, indicating that heat cycle 3 would occur at 187 days of age based on the approximate 21-day estrous cycle. During the grower stage, one of the pigs was culled due to lameness and 35 pigs successfully completed the trial.

Pigs were sacrificed at 186 days of age. Just prior to sacrifice, gilts were weighed, backfat was measured ultrasonically, and 10 mL of blood sample was collected from the orbital sinus. After collection of the whole blood in a 15 mL sterile centrifuge tube, blood was allowed to clot for 30 min at room temperature and the clot was removed by centrifuging at 3000 × g for 10 min. Serum was transferred, aliquoted and stored at –80 °C for later analysis. After sacrifice, the whole reproductive tract was removed from the carcass and ovarian weight, oviduct length, and vagina-cervix length were measured. Vagina-cervix length was measured from the vulva to the uterine body, including the length of vagina and cervix (method described by Tuz R *et al*.) [25]. The corpus luteum on both sides of the ovaries were counted. The left side of the ovaries was fixed in 4% formaldehyde, while the right side was kept in RNA later stabilization solution (Invitrogen, Cat#: AM7021, Carlsbad, CA, USA) and stored at –80 °C for later total RNA extraction and gene expression analysis. The timeline of the study design is shown in Fig. 1.

3.2 Histological analysis of ovarian tissues

The ovary samples were fixed for 24 h in 4% formaldehyde and then switched to 70% ethanol for long-term storage. Fixed ovarian samples were dehydrated and then processed by paraffin block embedding using standard techniques [26]. The embedded samples were trimmed and sliced into 5 μm-thick sections. General histology was visualized by Hematoxylin and Eosin (H&E) staining, and the ovarian follicles in different developmental stages were subsequently observed and counted under a microscope (Leica, DMR, Wetzlar, Germany).

3.3 Total RNA isolation and qRT-PCR

Total RNA was isolated from both ovarian and serum samples using the Norgen Total RNA purification kit (Norgen Biotek, Cat#: 17200, Thorold, ON, Canada). For the ovary samples, 10 mg of ovarian tissue from each pig was thawed, homogenized in the provided lysis buffer, and total RNA was extracted per manufacturer's directions. For serum samples, 750 μL TRIzol (TribioScience, Cat#: TBS6003, Sunnyvale, CA, USA) was added into 500 μL serum and vortexed. After vortexing, 150 μL of chloroform was added into each serum mix, vortexed again, and incubated for 15 min on ice. Followed by centrifugation for 15 min at 14,000 × g, the clear supernatant in the upper layer was transferred to a new RNase-free microfuge tube. An equal volume of 70% ethanol was added to stabilize the nucleic acids and the mixture was vortexed again. Manufacturer's directions were followed for the rest of the isolation. The concentration and 260/230 ratio of the collected total RNA was and assessed using a NanoDrop 8000 (Thermo Fisher, Cat#: ND8000-GL, Waltham, MA, USA) to ensure the samples were viable for reverse transcription. Total RNA sample was reverse transcribed into complementary DNA using iScript reverse transcription supermix kit (Bio-Rad, Cat#: 1708841, Berkeley, CA, USA) according to manufacturer's directions and conducted in a gradient Thermal cycler at the following conditions: 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min, and then held at 4 °C (Bio-Rad, T100, Berkeley, CA, USA). Quantitative PCR was performed using Ssoadvanced™ Universal SYBR

Table 1. Formulas of the experimental diets.

	Grower phase*				Finisher phase*			
Ingredient (%)	Control	Choline	Omega-3	Choline + Omega-3	Control	Choline	Omega-3	Choline + Omega-3
Corn	56.87	56.75	56.87	56.75	52.67	52.56	52.67	52.56
Wheat	6.89	6.89	6.89	6.89	20.00	20.00	20.00	20.00
Soybean meal	19.90	19.90	19.90	19.90	12.00	12.00	12.00	12.00
Canola meal	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
AV fat (Corn oil)	2.50	2.50	0.00	0.00	2.00	2.00	0.00	0.00
Vitamin and mineral premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	1.05	1.05	1.05	1.05	1.00	1.00	1.00	1.00
Mono-Ca phosphate	1.01	1.01	1.01	1.01	0.70	0.70	0.70	0.70
Sodium chloride	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Lys-HCl	0.49	0.49	0.49	0.49	0.39	0.39	0.39	0.39
Met	0.06	0.06	0.06	0.06	0.01	0.01	0.01	0.01
Thr	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.08
Vit B4 Choline Cl, 60%	0.00	0.11	0.00	0.11	0.00	0.11	0.00	0.11
Fish oil	0.00	0.00	2.50	2.50	0.00	0.00	2.00	2.00
Vitamin E	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
SUM (above)	100	100	100	100	100	100	100	100
Net energy (kcal/kg)	2447.4	2447.4	2447.4	2447.4	2452.69	2452.69	2452.69	2452.69
Protein (%)	19.51	19.51	19.51	19.51	17.16	17.16	17.16	17.16
SID-lysine (%)	1.16	1.16	1.16	1.16	0.99	0.99	0.99	0.99
Choline (mg/kg)	500	1000	500	1000	500	1000	500	1000
Omega-6 (%)	1.61	1.61	1.13	1.13	1.49	1.49	1.11	1.11
Omega-3 (%)	0.099	0.099	0.716	0.716	0.088	0.088	0.582	0.582
Omega-6: Omega-3	16.3	16.3	1.58	1.58	17	17	1.9	1.9

*Grower phase and finisher phase were fed between day 90 and 139, 140 and 186, respectively.

*Provided per kg of premix: vitamin A, 2,000,000 IU as retinyl acetate; vitamin D3, 200,000 IU as cholecalciferol; vitamin E, 8000 IU as dl-α-tocopherol acetate; vitamin K, 500 mg as menadione; pantothenic acid, 3000 mg; riboflavin, 1000 mg; choline, 100,000 mg; folic acid, 400 mg; niacin, 5000 mg; thiamine, 300 mg; pyridoxine, 300 mg; vitamin B12, 5 mg; biotin, 40 mg; Cu, 3000 mg from CuSO4 $\times$ 5H2O; Fe, 20,000 mg from FeSO4; Mn, 4000 mg from MnSO4; Zn, 21,000 mg from ZnSO4; Se, 60 mg from Na2SeO2 and I, 100 mg from KI (DSM Nutritional Products Canada Inc., Ayr, ON, Canada).

*SID-lysine: standardize ileal digestible (SID) lysine.

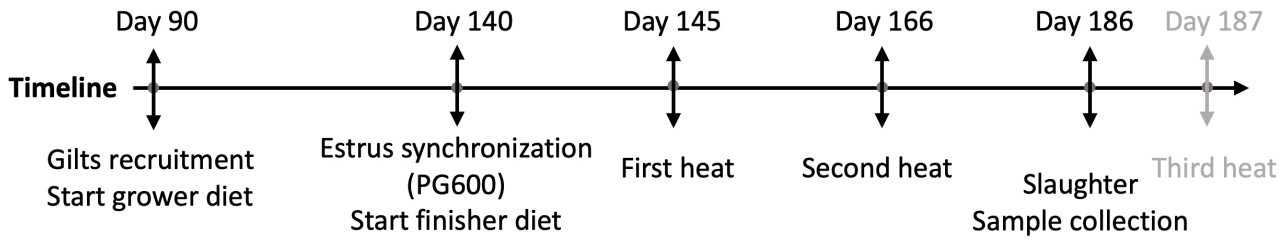

Fig. 1. Timeline of animal trial. Animals were recruited at 90 days of age and fed with experimental grower diet. At 140 days of age, PG600 was administrated for estrus cycle synchronization and feed was switched to the experimental finisher diet. At 186 days of age, one day before the expected third heat, gilts were sacrificed, and samples were collected for future analysis.

(Bio-Rad, Cat#: 1725275, Berkeley, CA, USA). Thermal cycling was performed for 2 min at 95 °C for enzyme activation, denaturation for 5 seconds at 95 °C, and annealing for 20 seconds at 60 °C. Real-time PCR was performed for 40 cycles in a Real-Time PCR System (Bio-Rad, CFX Connect, Berkeley, CA, USA). All the primer sets for gene expression were synthesized from Integrated DNA Technologies (IDT, Coralville, IA, USA). The primer sequence

Table 2. Primers for gene expression detection.

Gene	Product size	Forward 5′-3′	Reverse 5′-3′
GAPDH	147	TCGGAGTGAACGGATTTGGC	TGCCGTGGGTGGAATCATAC
UBB	127	GGTGGCTGCTAATTCTCCAG	TTTTGGACAGGTTCAGCTATTAC
FSHR	199	ATCACACATGCCATGCAACT	GTACGAGGAGGGCCATAACA
CYP11A1	501	TTCCAGAAGTATGGTCCCATTTA	TGAGCATGGGGACACTAGTGTGG
LHR	279	TGCTTTCCAAGGGATGAATAACG	ATCCTATTCTCTGAAAAAACTGCC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *UBB*, Ubiquitin B; *FSHR*, Follicle Stimulating Hormone Receptor; *CYP11A1*, cytochrome P450 family 11 subfamily A member 1; *LHR*, luteinizing hormone receptor.

Table 3. Primers for miRNA expression detection.

miRNAs	GeneGlobe ID	Forward 5′-3′
Ssc-miR-21-5p	YP00204230	UAGCUUAUCAGACUGAUGUUGA
Ssc-miR-378-3p	YP00205946	ACUGGACUUGGAGUCAGAAGGC
Ssc-miR-574-3p	YP00206011	CACGCUCAUGCACACACCCACA

and expected product size are listed in Table 2. Primer efficiency was calculated, and the product size were verified by agarose gel electrophoresis. For the gene expression normalization, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Ubiquitin B (UBB)were used as reference genes. Relative quantification was conducted using the $2^{-\Delta\Delta CT}$ method [27]. The changes in gene expression were presented as fold changes relative to the control group.

3.4 Reverse transcription and miRNA expression quantification

The total RNA was reverse transcribed into complementary DNA for miRNA detection using the miRCURY LNA RT kit (Qiagen, Cat#: 339340, Hilden, Germany) following manufacturer instructions and incubated in a Thermocycler at 95 °C for 5 min, then at 42 °C for 60 min and subsequent cooling to 4°C. Before qPCR, the miRNA cDNA was diluted 20 times with nuclease-free water. Quantitative PCR was performed using the miRCURY LNA^{TM} Universal RT microRNA PCR kit (Exiqon, Cat#: 203400, Vedbaek, Denmark) in accordance with manufacturer's directions. Primers for miRNA amplification were ordered from Qiagen (Cat#: 339306, Hilden, Germany) and the sequences were provided in Table 3. Samples were then amplified at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C and 1 min at 60 °C, with a ramp-rate of 1.6 °C/s[4] in a Real-Time PCR System (Bio-Rad, CFX Connect, Berkeley, CA, USA). The expression of target microRNAs was normalized to the housekeeping gene S5. Relative quantification was conducted using the $2^{-\Delta\Delta CT}$ method [27]. The changes in gene expression were presented as fold changes relative to the control group.

3.5 Hydrophilic interaction chromatography analysis of serum

A solution composed of 1:1 acetonitrile: methanol by volume was added to pig serum to create an 80% organic solvent by mixing 800 μL of solution and 200 μL of serum. To remove particulates and protein, the mixture was filtered through a 0.22 μm filter. Samples were analyzed using Hydrophilic Interaction Chromatography (HILIC) in cooperation with the Department of Chemical Engineering and Applied Chemistry, University of Toronto, ON. Targeted compounds are outlined in **Supplementary Table 1**. Untargeted compounds were also detected in the analysis, though only two showed significances. The untargeted compounds were identified by comparing the HILIC data with the The Human Metabolome Database (HMBD)and MetaCyt databases.

3.6 Statistical analysis

Statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) with each pig as the experimental unit, dietary treatment as the fixed effect, and block as the random effect. Statistical significance was measured with either an ANOVA post hoc Tukey test or independent two sample *t*-test. Differences at $p < 0.05$ were considered significant.

For analysis of metabolomic data, the HILIC data was analyzed using MetaboAnalyst 4.0 (McGill University, https://www.metaboanalyst.ca/). A one-way ANOVA was performed at the $p < 0.05$ level of significance followed by Tukey's Studentized Range test.

4. Results

To study the influence of choline supplementation on growth performance and reproductive tract development, backfat thickness, oviduct length, vagina-cervix length and the ovary weight data were collected and analyzed. Initial backfat thickness was not different among treatment groups, nor were feed intake in the growing and finishing phases, or final bodyweight (data not shown). Oviduct length and ovary weight had no significant differences among treatment groups (Table 4, Ref. [25]). Overall backfat gain was lowest in the control group, with the Omega-3 group having significantly higher backfat gain compared to the control group ($p < 0.05$, Table 4), while the other groups had intermediate backfat gain. The vagina-cervix length in the Choline group was ~14% longer than

Table 4. Reproductive organ development of gilts supplemented with choline or/and Omega-3 adding diet (mean ± SEM).

Item	Control	Choline	Omega-3	Choline + Omega-3	SEM[2]	*p* value
	N = 9	N = 8	N = 9	N = 9		
Overall backfat gain (mm)	7.43^{a}	8.65^{ab}	9.47^{b}	9.12^{ab}	0.63	$p = 0.019$
Oviduct length (L, cm)	27.78	30.13	30.83	30.10	4.72	$p = 0.250$
Oviduct length (R, cm)	32.16	34.43	34.28	33.34	5.43	$p = 0.398$
Vagina-cervix-length (cm)[1]	40.56^{a}	46.08^{b}	44.97^{ab}	42.56^{ab}	1.79	$p = 0.027$
Ovary weight (g)	6.94	7.26	7.72	7.87	2.35	$p = 0.808$

[1]Vagina-cervix length was measured from the vulva to the uterine body, including the length of vagina and cervix, described by Tuz R *et al*. [25].

[2]Maximum value of standard error of the means.

*Significance was measured using a one-way ANOVA post hoc Tukey test. Means assigned different letters (a, b) within a factor of are significantly different ($p < 0.05$).

that of the control group ($p < 0.05$, Table 4), while the Omega-3 and Choline + Omega-3 groups had no significant changes in comparison to the control group. There were no differences in both oviduct length and ovary weight among all the groups.

To investigate the effects of a choline-supplemented diet on ovarian development, the number of ovarian follicles at different stages and corpus luteum were counted. As shown in Fig. 2, the percentage of antral follicles (percentage of antral follicles = number of antral follicles/number of total follicles × 100% [28]) were significantly increased from ~24% in the control group to ~35% in the Choline + Omega-3 group ($p < 0.05$). However, there were no significant differences in the percentage of antral follicles observed in Choline and Omega-3 groups compared to control group. Additionally, the average number of corpus luteum per ovary was significantly increased from 5 per ovary in the control group to 15 per ovary in the Choline group ($p < 0.05$). A significant increase in number of corpus luteum per ovary was also observed in the Omega-3 and Choline + Omega-3 groups in comparison to the control group ($p < 0.05$; Fig. 3).

To test the hypothesis that choline influences ovarian function through the regulation of the expression of genes involved in ovarian development, qPCR assays were conducted on the steroidogenesis related genes: *LHR*, *FSHR* and *CYP11A1*. Out of all the measured genes, *CYP11A1* and *LHR* expression were significantly increased in the Choline-supplemented group in comparison to the control group ($p < 0.05$, Fig. 4A–B), while no significant differences in both *CYP11A1* and *LHR* expression were observed between the control, Omega-3, or Choline + Omega-3 groups. No significance between groups was observed for *FSHR* expression (Fig. 4C).

To examine if choline supplementation alters the expression of miRNAs known to play an important role in ovarian function, the expression level of miR-21, -378, -574 in both ovarian tissue and serum were detected via qPCR. As shown in Fig. 5, miR-21 expression was significantly increased in the Omega-3 group, miR-378 expression was significantly higher in the Choline + Omega-3 group, and miR-574 expression was significantly higher in both Choline and Omega-3 groups, all in comparison to the control group ($p < 0.05$). No change in the expression of the miRNAs in serum was observed among all the treatment groups (data not shown).

To study if choline supplementation influence serum metabolic profiles, Hydrophilic Interaction Chromatography analysis was performed. Of the 22 targeted (**Supplementary Table 1**) and 159 untargeted metabolites (**Supplementary Table 2**), only the levels of the untargeted compound, 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycerol-3-phosphocholine, a phosphatidylcholine, was significantly increased in the Choline group compared to control group ($p < 0.05$) (Fig. 6A). Testosterone, a relevant metabolite in the context of female fertility, showed a decreasing trend in the choline group compared to the control group ($p = 0.08$) (Fig. 6B).

5. Discussion

Although choline is known for its health benefits in adults, fetal development and other aspects of fertility [29], its specific role in the female reproductive tract and ovarian development has not yet been explored. Our findings demonstrate that choline increases reproductive tract development, which could increase future litter size, the number of live fetuses and the lifetime piglet production of female pigs [25, 30]. In humans, the cervical length is inversely proportional to the risk of spontaneous preterm births in which those with the shortest cervical length have the highest risk of prematurity [31]. How the length of the vagina-cervix is increased by choline supplementation is currently unclear. It is possible that increased levels of phosphatidylcholine, the dominant phospholipid component of eukaryotic membranes, was synthesized from the choline, which may have contributed to the further growth of the reproductive tract tissues in the Choline group [32].

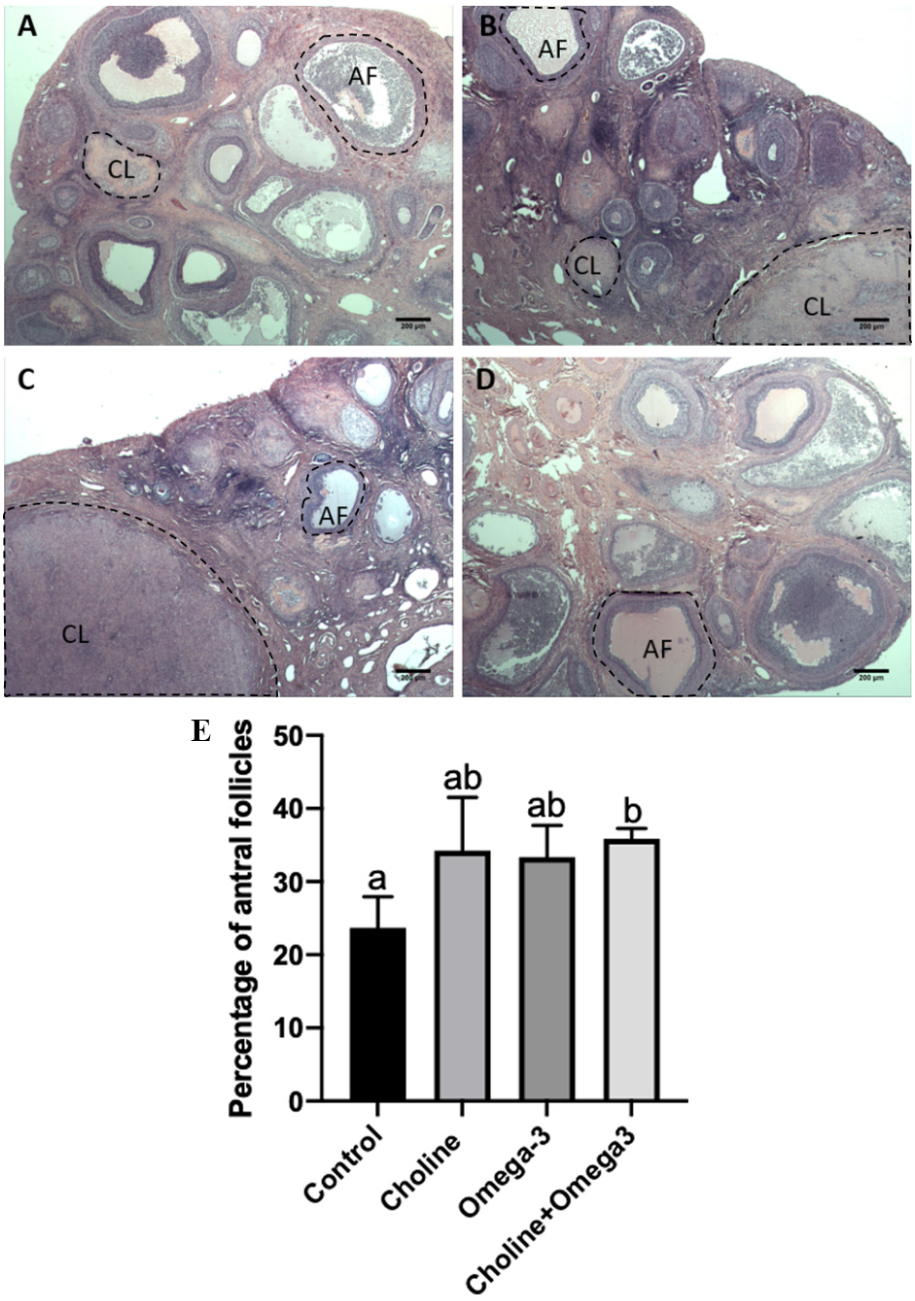

Fig. 2. The representative histological section of the ovary in each group (A–D, H&E staining, 25×) and the percentage of antral follicles in each group (E). (A) Control group. Most follicles were in the developmental stage, with many early antral follicles (eAF) and a small amount of degenerated corpus luteum (dCL). (B) Choline group. Compared with the control group, the ovaries in this group have a larger number of corpus luteum and a smaller number of antral follicles. (C) Omega-3 group. More fresh and degenerated corpus luteum with fewer antral follicles were shown in the sections. (D) Choline + Omega-3 group. More larger follicles were showed in this group compared to the control group, indicating that the pigs in this group were closer to ovulation. (E) Percentage of antral follicles in each group. Values are means ± standard error of mean for n = 6 gilts. Significance was measured using a one-way ANOVA post hoc Tukey test. Means assigned different letters (a, b) within a factor are significantly different ($p < 0.05$).

It has been described that different tissues express different levels of enzymes that catalyse phosphatidylcholine formation [33], and the results of the present study could suggest that the reproductive tract expresses higher levels of these enzymes relative to other tissues. Backfat thickness has also been described to positively correlate to reproductive potential in pigs [34, 35]. During reproductive periods, more nutrients are required for maintenance and lactation, leading to the mobilization of fat reserves [36]. Therefore, prenatal backfat reserves of the sows may contribute to later reproductive success. In our study, backfat thickness was numerically higher in all the supplementation groups, although the difference in comparison to the control was only significant in the Omega-3 group ($p < 0.05$). This result is consistent

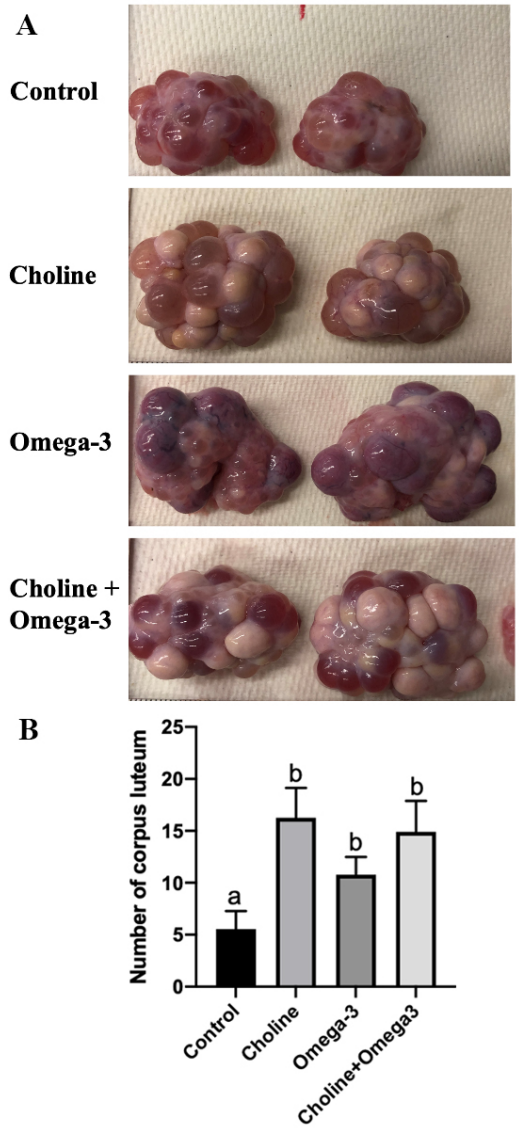

Fig. 3. Representative ovarian picture of each group (A) and The number of visible corpus lutea (B). Values are means ± standard error of mean for n = 8 to 9 gilts. Significance was measured using a one-way ANOVA post hoc Tukey test. Means assigned different letters (a, b) within a factor of are significantly different ($p < 0.05$).

with Omega-3's role as a fatty acid, and it has been well demonstrated to promote the formation of adipocytes [37]. Interestingly it is known that bodyfat reserve is positively related to women reproductive ability under healthy physiological conditions, as bodyfat is the main extragonadal source of estradiol which aromatized from androgen [38].

Ovarian developmental status and oocyte quality are the most important factors that affect female fertility [39]. During unassisted pregnancy in females, follicular development results in the release of oocytes from the ovary to the fallopian tube, where the oocytes can be fertilized by sperm. Moreover, ovulation rate is positively related to fecundity in various species such as sheep [40], cow [41], mice [42] and pig [43]. In this study, a higher percentage of antral follicles were found in Choline + Omega-3 supplemented group in comparison to controls, indicating that more follicles were recruited to form the antral follicle cohort. Follicle recruitment rate is positively correlated with reproductive ability and the oocyte quality in humans [44]. Furthermore, both Choline and Omega-3 groups had a significantly increased number of corpus luteum per ovary in comparison to the control, meaning more follicles had ovulated. Choline is also a substrate for the synthesis of betaine via choline dehydrogenase during oocyte *in vitro* maturation, and this betaine accumulation is reported to be required for oocyte meiotic maturation [45]. Taken together, this suggests that choline and/or Omega-3 supplementation promotes and enhances ovulation for successful fertilization and implantation. Additionally, choline is an essential component in forming phosphatidylcholines, a class of phospholipids that are a major element in forming the cellular membrane. Phosphatidylcholines accumulate during follicle growth, and their depletion significantly affects follicular development and antrum formation [46]. Thus, choline supplementation may have contributed to oocyte maturation and ovulation by providing additional substrate for the synthesis of these biomolecules.

During the estrus cycle, the level of luteinizing hormone acutely rises to trigger ovulation and generate corpus luteum. In response to the changes of the concentration of this gonadotropin, the density of its receptor, *LHR*, and related downstream catalytic enzyme expression in the ovary changes to ensure that the menstrual cycle progresses in an orderly manner [47–49]. Our finding that a significant increase of *LHR* expression in the ovary of the Choline-supplemented group is consistent with this notion, and agrees with the fact that more corpus luteum were observed. Furthermore, the expression of *CYP11A1*, a gene coding for the P450scc mitochondrial enzyme that is the first and rate-limiting catalytic enzyme in steroidogenesis for the synthesis of pregnenolone from cholesterol [48], was upregulated in the ovaries of pigs in the Choline group. This result is consistent with a previous finding that P450scc expression increases along with luteinizing hormone surge and corpus luteum formation [48]. These steroidogenesis-related gene expression results suggest that choline potentially plays a role in the regulation of the ovarian menstrual cycle and steroid hormone production.

MiR-21, -378, -574 are all important regulators of ovarian function. MiR-21 promotes cumulus expansion and oocyte maturation in vitro via targeting tissue inhibitor of metalloproteinase 3(*TIMP3*) [50], and miR-574 modulates phosphorylated extracellular-signal-regulated kinase (ERK) 1/2 level and increases estradiol production. MiR-378 regulates estradiol production and apoptosis rate in the ovary [51, 52]. Our findings that miR-21 expression was significantly increased in the Omega-3 group, and miR-574 expression was significantly increased in both the Choline and Omega-3-supplemented groups is consistent with the

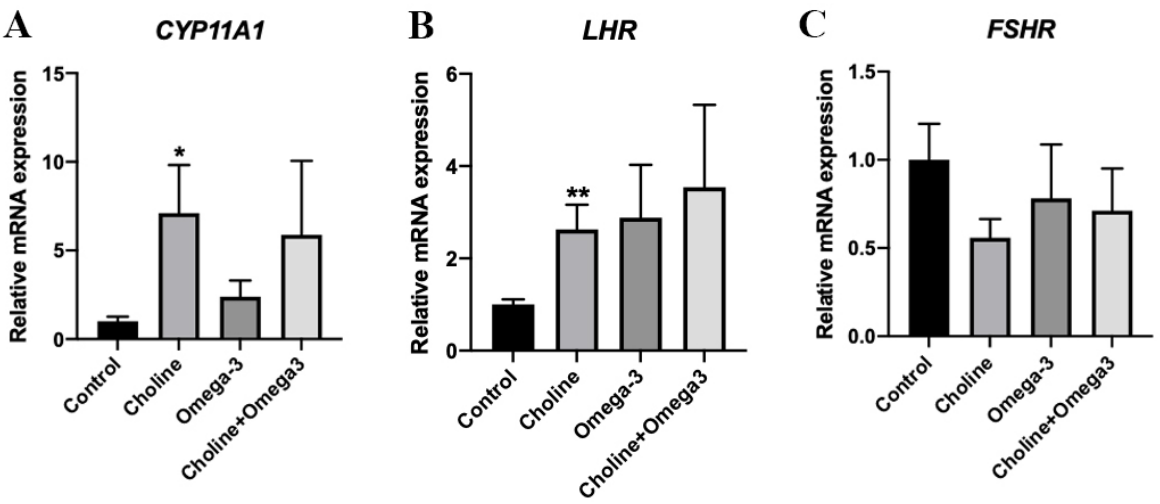

Fig. 4. Relative mRNA expression of steroidogenesis related genes in ovarian tissues. (A) *CYP11A1*. (B) *LHR*. (C) *FSHR*. Values are means ± standard error of mean for n = 8 or 9 gilts. Significance was measured using an independent two sample *t*-test. Astricts signify that the gene expression is significantly different in comparison to the control group (*$p < 0.05$ and **$p < 0.01$). mRNA levels of both *CYP11A1* and *LHR* were significantly higher in choline supplemented group compared to control, while mRNA levels of *FSHR* showed no differences between all groups.

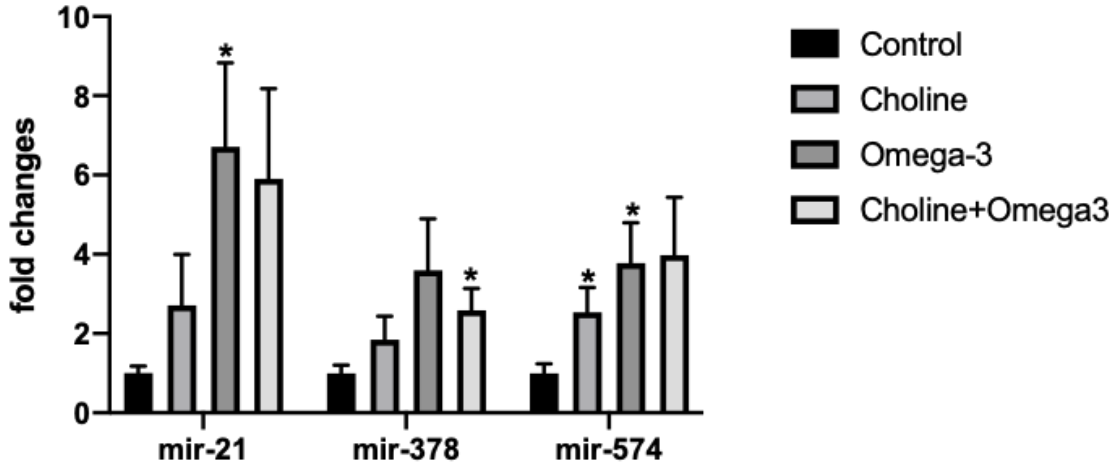

Fig. 5. miRNAs expression level in the ovarian tissues. Values are means ± standard error of mean for n = 6 gilts. Significance was measured using an independent two sample *t*-test. Astricts signify that the miRNAs expression is significantly different in comparison to the control group (*$p < 0.05$ and **$p < 0.01$).

observed enhanced follicular development and ovulation observed. Interestingly, others have also reported that miR-21 is highly expressed in the female goats with high ovulation rate [53]. Ma *et al.* [54] identified that miR-378 as the most differentially expressed miRNA in which its expression level increased over fivefold during luteal maturation. In our study, miR-378 expression was significantly higher in Choline + Omega-3 group, which is consistent with the previous results that more corpus luteum were formed in this group in comparison to the control group. These results suggest that Omega-3 and choline supplementation promote positive regulators of folliculogenesis and ovulation, possibly enhancing reproductive potential of the individual.

Lastly, serum analysis of metabolites taken from the pigs revealed a significant change in phosphatidylcholine phospholipids and trending changes in testosterone. Testosterone is an androgen that coordinates a variety of bodily processes including energy metabolism, muscle and bone mass, libido, and especially, fertility [55]. Polycystic ovarian syndrome (PCOS) is a reproductive metabolic syndrome particularly characterized by hyperandrogenism, a state of excess androgen production in which testosterone is included [56]. Currently, it is unknown how choline administration may impact metabolites related to infertility and the PCOS phenotype. However, the trending lower level of testosterone suggests that choline may have potential in the treatment or prevention of PCOS. Further, the metabolic

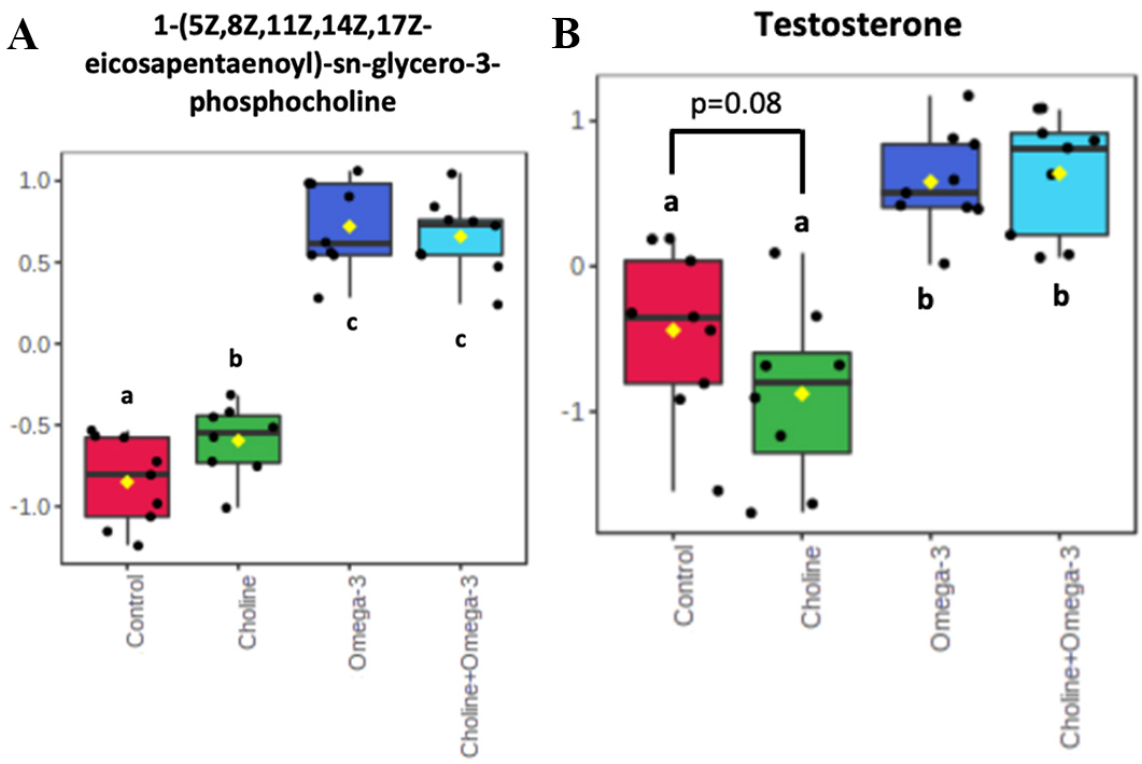

Fig. 6. Normalized concentrations of 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine (A) and testosterone (B) in serum. Only the levels of 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine were significantly increased ($p < 0.05$) in the choline group compared to control, while testosterone demonstrated trends towards a decrease ($p = 0.08$) in the choline group compared to control. Values are means ± standard error of mean for n = 8 or 9 gilts. Significance was measured using a one-way analysis of variance (ANOVA) post hoc Tukey test. Means assigned different letters (a, b) within a factor of are significantly different ($p < 0.05$).

analysis of pig serum in the Choline group showed a significant increase in the levels of 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine, a form of phosphocholine, which is reasonable as choline is a major element of its components, however, its specific role in follicular development remains unknown. It is reported that choline and phosphocholine were reduced in the follicular fluid from ovarian follicles where the fertilized oocyte failed to cleave [57], suggesting that phosphocholine may play a role in the oocyte competence development in which 1-(5Z, 8Z, 11Z, 14Z, 17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine may be involved.

6. Conclusions

In summary, we report that choline supplementation promotes reproductive tract development and ovulation which is accompanied by enhanced expression of steroidogenesis-related genes and miRNAs related to ovarian regulation. To our knowledge, this is the first *in vivo* examination of the influence of choline supplementation on ovarian development and function. Our findings support that the supplementation of this nutrient may have fertility enhancement benefits and offer insights into how choline supplementation could be applied to improve reproductive function.

7. Author contributions

XZ, BW, LAH and JL conceived and designed the experiments; XZ, LF, SD and EB performed the experiments; XZ, LF and EB analyzed the data; XZ, LF, EB, LAH and JL contributed manuscript writing.

8. Ethics approval and consent to participate

All experimental procedures were approved by the University of Guelph's Animal Care Committee (AUP #4068) and followed the Canadian Council of Animal Care Guidelines (CCAC, 2009).

9. Acknowledgment

We thank Douglas Wey and the staff at Arkell Swine Research Station for their assistance in the animal trial, Renee Hilker for her assistance in collecting animal samples.

10. Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC, grant number: 400259), and the Food from Thought Research program (grant number: 499093).

11. Conflict of interest

The authors declare no conflict of interest.

12. References

[1] Esteves SC, Humaidan P, Roque M, Agarwal A. Female infertility and assisted reproductive technology. Panminerva Medica. 2019; 61: 1–2.

[2] Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, *et al*. The International Glossary on Infertility and Fertility Care, 2017. Human Reproduction. 2017; 32: 1786–1801.

[3] Prevatt C, Lamb GC, Dahlen C, Mercadante VR, Waters K. What Is the Economic Impact of Infertility in Beef Cattle? EDIS. 2018; 2018.

[4] Gaskins AJ, Chavarro JE. Diet and fertility: a review. American Journal of Obstetrics and Gynecology. 2018; 218: 379–389.

[5] Chavarro JE, Rich-Edwards JW, Rosner BA, Willett WC. Diet and Lifestyle in the Prevention of Ovulatory Disorder Infertility. Obstetrics & Gynecology. 2007; 110: 1050–1058.

[6] Wise LA, Wesselink AK, Tucker KL, Saklani S, Mikkelsen EM, Cueto H, *et al*. Dietary Fat Intake and Fecundability in 2 Preconception Cohort Studies. American Journal of Epidemiology. 2018; 187: 60–74.

[7] Simopoulos AP. The importance of the ratio of omega-6/Omega-3 essential fatty acids. Biomedicine & Pharmacotherapy. 2002; 56: 365–379.

[8] Nehra D, Le HD, Fallon EM, Carlson SJ, Woods D, White YA, *et al*. Prolonging the female reproductive lifespan and improving egg quality with dietary Omega-3 fatty acids. Aging Cell. 2012; 11: 1046–1054.

[9] Mumford SL, Schisterman EF, Dasharathy S, Pollack AZ, Zhang C, Wactawski-Wende J. Omega-3 fatty acids and ovulatory function. Fertility and Sterility. 2011; 96: S15.

[10] Calder PC. Omega-3 Fatty Acids and Inflammatory Processes. Nutrients. 2010; 2: 355–374.

[11] Barsky M, Blesson CS. Oocytes, obesity, and Omega-3 fatty acids. Fertility and Sterility. 2020; 113: 71–72.

[12] Zeisel SH, Da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, *et al*. Choline, an essential nutrient for humans. The FASEB Journal. 1991; 5: 2093–2098.

[13] Zeisel SH, Blusztajn JK. Choline and Human Nutrition. Annual Review of Nutrition. 1994; 14: 269–296.

[14] Troen AM, Chao WH, Crivello NA, D'Anci KE, Shukitt-Hale B, Smith DE, *et al*. Cognitive Impairment in Folate-Deficient Rats Corresponds to Depleted Brain Phosphatidylcholine and is Prevented by Dietary Methionine without Lowering Plasma Homocysteine. The Journal of Nutrition. 2008; 138: 2502–2509.

[15] Moore LD, Le T, Fan G. DNA Methylation and its Basic Function. Neuropsychopharmacology. 2013; 38: 23–38.

[16] Sweiry JH, Page KR, Dacke CG, Abramovich DR, Yudilevich DL. Evidence of saturable uptake mechanisms at maternal and fetal sides of the perfused human placenta by rapid paired-tracer dilution: studies with calcium and choline. Journal of Developmental Physiology. 1986; 8: 435–445.

[17] Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. Periconceptional Dietary Intake of Choline and Betaine and Neural Tube Defects in Offspring. American Journal of Epidemiology. 2004; 160: 102–109.

[18] Albright CD, Tsai AY, Friedrich CB, Mar MH, Zeisel SH. Choline availability alters embryonic development of the hippocampus and septum in the rat. Developmental Brain Research. 1999; 113: 13–20.

[19] Sun L, Hu W, Liu Q, Hao Q, Sun B, Zhang Q, *et al*. Metabonomics Reveals Plasma Metabolic Changes and Inflammatory Marker in Polycystic Ovary Syndrome Patients. Journal of Proteome Research. 2012; 11: 2937–2946.

[20] Ersahin AA, Çalışkan E. Clomiphene citrate changes metabolite content of follicular fluid of PCOS women. European Review for Medical and Pharmacological Sciences. 2018; 22: 4359–4362.

[21] Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends in Microbiology. 2012; 20: 50–57.

[22] Mordhorst BR, Prather RS. Pig Models of Reproduction. In Heide Schatten, Gheorghe M. Constantinescu (eds.) Animal Models and Human Reproduction (pp. 213–234). John Wiley & Sons: Hoboken, New Jersey. 2017.

[23] Lorenzen E, Follmann F, Jungersen G, Agerholm JS. A review of the human vs. porcine female genital tract and associated immune system in the perspective of using minipigs as a model of human genital Chlamydia infection. Veterinary Research. 2015; 46: 116.

[24] Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and their Health Benefits. Annual Review of Food Science and Technology. 2018; 9: 345–381.

[25] Tuz R, Schwarz T, Małopolska M, Nowicki J. The Use of Vagina-Cervix Length Measurement in Evaluation of Future Reproductive Performance of Sows: A Preliminary Study under Commercial Conditions. Animals. 2019; 9: 158.

[26] Huynh E, Penney J, Caswell J, Li J. Protective Effects of Protegrin in Dextran Sodium Sulfate-Induced Murine Colitis. Frontiers in Pharmacology. 2019; 10: 156.

[27] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods. 2001; 25: 402–408.

[28] Yan K, Cheng L, Liu P, Liu Z, Zhao S, Zhu W, *et al*. Polyinosinic–Polycytidylic Acid Perturbs Ovarian Functions through Toll-Like Receptor 3-Mediated Tumor Necrosis Factor a Production in Female Mice1. Biology of Reproduction. 2015; 93: 11.

[29] Zeisel SH. Choline: Critical Role during Fetal Development and Dietary Requirements in Adults. Annual Review of Nutrition. 2006; 26: 229–250.

[30] Martin Rillo S, de Alba Romero C, Romero Rodriguez A, Cidoncha R, Ziecik AJ. Litter Size and Vagina-cervix Catheter Penetration Length in Gilts. Reproduction in Domestic Animals. 2001; 36: 297–300.

[31] McIntosh J, Feltovich H, Berghella V, Manuck T. The role of routine cervical length screening in selected high- and low-risk women for preterm birth prevention. American Journal of Obstetrics and Gynecology. 2016; 215: B2–B7.

[32] Ridgway ND. The role of phosphatidylcholine and choline metabolites to cell proliferation and survival. Critical Reviews in Biochemistry and Molecular Biology. 2013; 48: 20–38.

[33] Ridgway ND. Phospholipid Synthesis in Mammalian Cells. In Ridgway ND, McLeod RS (eds.) Biochemistry of Lipids, Lipoproteins and Membranes (pp. 209–236). 6th edn. Elsevier: Boston. 2016.

[34] McKay RM. Preweaning losses of piglets as a result of index selection for reduced backfat thickness and increased growth rate. Canadian Journal of Animal Science. 1993; 73: 437–442.

[35] Roongsitthichai A, Tummaruk P. Importance of Backfat Thickness to Reproductive Performance in Female Pigs. The Thai Journal of Veterinary Medicine. 2014; 44: 171.

[36] Houde AA, Méthot S, Murphy BD, Bordignon V, Palin MF. Relationships between backfat thickness and reproductive effi-

ciency of sows: a two-year trial involving two commercial herds fixing backfat thickness at breeding. Canadian Journal of Animal Science. 2010; 90: 429–436.
[37] Kuda O, Rossmeisl M, Kopecky J. Omega-3 fatty acids and adipose tissue biology. Molecular Aspects of Medicine. 2018; 64: 147–160.
[38] Frisch RE. Body fat, menarche, fitness and fertility. Human Reproduction. 1987; 2: 521–533.
[39] Farquhar CM, Bhattacharya S, Repping S, Mastenbroek S, Kamath MS, Marjoribanks J, *et al*. Female subfertility. Nature Reviews Disease Primers. 2019; 5: 7.
[40] García-Guerra A, Motta JCL, Melo LF, Kirkpatrick BW, Wiltbank MC. Ovulation rate, antral follicle count, and circulating anti-Müllerian hormone in Trio allele carriers, a novel high fecundity bovine genotype. Theriogenology. 2017; 101: 81–90.
[41] Echternkamp SE, Gregory KE, Dickerson GE, Cundiff LV, Koch RM, Van Vleck LD. Twinning in cattle: II. Genetic and environmental effects on ovulation rate in puberal heifers and postpartum cows and the effects of ovulation rate on embryonic survival. Journal of Animal Science. 1990; 68: 1877–1888.
[42] Clutter AC, Kirby YLK, Nielsen MK. Uterine capacity and ovulation rate in mice selected 21 generations on alternative criteria to increase litter size. Journal of Animal Science. 1994; 72: 577–583.
[43] Kemp B, Da Silva CLA, Soede NM. Recent advances in pig reproduction: Focus on impact of genetic selection for female fertility. Reproduction in Domestic Animals. 2018; 53: 28–36.
[44] Wallace WH, Kelsey TW. Human Ovarian Reserve from Conception to the Menopause. PLoS ONE. 2010; 5: e8772.
[45] McClatchie T, Meredith M, Ouédraogo MO, Slow S, Lever M, Mann MRW, *et al*. Betaine is accumulated via transient choline dehydrogenase activation during mouse oocyte meiotic maturation. Journal of Biological Chemistry. 2017; 292: 13784–13794.
[46] Lai FN, Liu XL, Li N, Zhang RQ, Zhao Y, Feng YZ, *et al*. Phosphatidylcholine could protect the defect of zearalenone exposure on follicular development and oocyte maturation. Aging. 2018; 10: 3486–3506.
[47] Puett D, Angelova K, da Costa MR, Warrenfeltz SW, Fanelli F. The luteinizing hormone receptor: Insights into structure-function relationships and hormone-receptor-mediated changes in gene expression in ovarian cancer cells. Molecular and Cellular Endocrinology. 2010; 329: 47–55.
[48] Davis JS, LaVoie HA. Molecular Regulation of Progesterone Production in the Corpus Luteum. In Leung PCK, Adashi EY (eds.) The Ovary (pp. 237–253). 3rd edn. Academic Press: Cambridge, MA. 2019.
[49] Narayan P, Puett D. Luteinizing Hormone Receptor Signaling. In Henry HL, Norman AW (eds.) Encyclopedia of Hormones (pp. 612–616). Academic Press: New York. 2003.
[50] Bo Pan, Julang Li. MicroRNA-21 up-regulates metalloprotease by down-regulating TIMP3 during cumulus cell-oocyte complex in vitro maturation. Molecular and Cellular Endocrinology. 2018; 477: 29–38.
[51] Sun XF, Li YP, Pan B, Wang YF, Li J, Shen W. Molecular regulation of miR-378 on the development of mouse follicle and the maturation of oocyte in vivo. Cell Cycle. 2018; 17: 2230–2242.
[52] Pan B, Toms D, Shen W, Li J. MicroRNA-378 regulates oocyte maturation via the suppression of aromatase in porcine cumulus cells. American Journal of Physiology-Endocrinology and Metabolism. 2015; 308: E525–E534.
[53] Zi X, Lu J, Ma L. Identification and comparative analysis of the ovarian microRNAs of prolific and non-prolific goats during the follicular phase using high-throughput sequencing. Scientific Reports. 2017; 7: 1921.
[54] Ma T, Jiang H, Gao Y, Zhao Y, Dai L, Xiong Q, *et al*. Microarray analysis of differentially expressed microRNAs in non-regressed and regressed bovine corpus luteum tissue; microRNA-378 may suppress luteal cell apoptosis by targeting the interferon gamma receptor 1 gene. Journal of Applied Genetics. 2011; 52: 481–486.
[55] Kelly DM, Jones TH. Testosterone: a metabolic hormone in health and disease. Journal of Endocrinology. 2013; 217: R25–R45.
[56] Atiomo W, Khalid S, Parameshweran S, Houda M, Layfield R. Proteomic biomarkers for the diagnosis and risk stratification of polycystic ovary syndrome: a systematic review. BJOG: an International Journal of Obstetrics abd Gynaecology. 2009; 116: 137–143.
[57] Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. Fertility and Sterility. 2012; 97: 1078–1084.e8.

Supplementary material: Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/26/12/10.52586/5046.

Keywords: Infertility; Ovarian development; Female reproductive; Choline; Follicle maturation

Send correspondence to:

Julang Li, Department of Animal BioSciences, University of Guelph, Guelph, ON N1G 2W1, Canada, E-mail: jli@uoguelph.ca

Bingyun Wang, Department of Life Science and Engineering, Foshan University, 528231 Foshan, Guangdong, China, E-mail: bywang63@163.com