Animal Reproduction Science 209 (2019) 106138

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effects of N-carbamylglutamate and arginine on steroidogenesis and proliferation of pig granulosa cells *in vitro*

T. Feng[a], A.A. DeVore[b], M.C. Perego[b], B.C. Morrell[b], L.J. Spicer[b,*]

[a] Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences, Beijing, 100097, China
[b] Department of Animal and Food Sciences, Oklahoma State University, Stillwater, OK 74078, USA

ARTICLE INFO

Keywords:
N-Carbamylglutamate
Arginine
Granulosa Cell
Proliferation
Steroidogenesis

ABSTRACT

Results of *in vivo* studies indicate dietary N-carbamylglutamate (NCG) and arginine (ARG) can enhance reproductive performance in gilts. It was hypothesized that both NCG and ARG will alter hormone-induced estradiol (E2) production by granulosa cells (GC), explaining why these compounds could improve reproductive performance in pigs. The objective of these studies, therefore, was to evaluate the direct effects of NCG and ARG on porcine GC proliferation and steroidogenesis, using an *in vitro* cell culture system. The GC from small (SM; 1–5 mm) and large (LG; > 5 mm) pig follicles were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 2 days in serum-free medium containing 500 ng/mL of testosterone (as an E2 precursor), and NCG or ARG at various doses in the presence of either follicle-stimulating hormone (FSH; 30 ng/mL), insulin-like growth factor-1 (IGF1; 30 ng/mL), or both. Numbers of GC were determined at the end of the experiment and concentrations of progesterone (P4) and E2 in culture medium were determined. Results indicated that LG-follicle GC were more responsive to NCG and ARG than SM-follicle GC. Specifically, in LG-follicle GC, NCG inhibited ($P < 0.05$) basal and FSH-induced P4 and E2 production but stimulated cell numbers; whereas ARG inhibited FSH-induced E2 production and cell numbers. In SM-follicle GC, treatment with NCG and ARG decreased IGF1 plus FSH induced P4 production, but E2 production and cell proliferation were not affected. These studies indicate that NCG and ARG may directly affect follicular function in pigs.

1. Introduction

In the swine industry, reproductive performance has a pivotal role in gilt and sow productivity. The number of piglets produced per sow has increased as technological advances have been implemented into raising animal for food production. This has been accomplished through artificial insemination, controlled environment and nutrient supplementation (Hazeleger et al., 2005; Peltoniemi and Virolainen, 2006; Kraeling and Webel, 2015; Knox, 2016).

The N-carbamylglutamate (NCG) compound, is a structural analogue of N-acetylglutamate, a primary substrate of N-acetylglutamate synthase in one of the many parts of the L-arginine (ARG)-synthetic pathway (Wu et al., 2004), allowing pigs to synthesize ARG more efficiently. The amino acid, ARG, is an essential amino acid particularly important for the growth of piglets (Wu and Morris, 1998). Dietary supplementation of ARG has a multifaceted benefit in treating numerous health and developmental problems in pigs (Wu et al., 2009). Results of recent studies indicate NCG-supplementation from gestation to parturition improves

* Corresponding author at: 114 Animal Science Bldg., Department of Animal and Food Sciences, Oklahoma State University, Stillwater, OK, USA.
E-mail address: leon.spicer@okstate.edu (L.J. Spicer).

https://doi.org/10.1016/j.anireprosci.2019.106138
Received 8 April 2019; Received in revised form 24 June 2019; Accepted 25 July 2019
Available online 26 July 2019
0378-4320/

pregnancy outcome in gilts and is associated with increased concentrations of ARG in plasma and placental angiogenic factors (Zhang et al., 2014), whereas in late gestating sows, ARG supplementation had no effect on litter size, number born alive, or birth weights of live piglets (Bass et al., 2017). Results of previous studies indicate NCG supplementation during gestation in sows increased litter size, number born alive, and birth weights of piglets (Wu et al., 2012; Zhu et al., 2015; Feng et al., 2018a) and increased litter size and number born alive in rats (Zeng et al., 2012). Similarly, in Awassi sheep (Al-Dabbas et al., 2008) and rats (Pau and Milner, 1982), ARG supplementation increased ovulation rate. In Suffolk ewes, ARG supplementation increased fertility but did not alter ovulation rate (de Chavez et al., 2015). In women, ARG supplementation decreased the number of follicles and pregnancy rates after *in vitro* fertilization (IVF) (Battaglia et al., 2002). In mares, ARG supplementation increased follicle diameter but did not affect inter-ovulatory interval (Kelly et al., 2014), but whether ARG or NCG directly or indirectly affects follicular function is uncertain. To the authors' knowledge, no study has been conducted to evaluate effects of ARG and NCG on porcine granulosa cell (GC) function. It was hypothesized that both NCG and ARG will alter hormone-induced E2 production by GC. Objectives, therefore, were to determine if ARG and NCG directly affect steroidogenesis and proliferation of GC of pigs.

2. Materials and methods

2.1. Reagents and hormones

The reagents used in cell culture were: Dulbecco's modified Eagle medium (DMEM), hyaluronidase, pronase, DNase, collagenase, sodium bicarbonate, ARG, NCG, and trypan blue obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO); pig serum (PS) and fetal calf serum (FCS) were obtained from Atlanta Biologicals (Flowery Branch, GA). Purified ovine follicle-stimulating hormone (FSH) (FSH activity: 15 x NIH-FSH-S1 U/mg) from the National Hormone and Pituitary Program (Torrance, CA), testosterone from Steraloids (Wilton, NH) and recombinant human insulin-like growth factor-1 (IGF1) from R&D Systems (Minneapolis, MN).

2.2. Cell culture

Ovaries from non-pregnant gilts were collected at a local slaughterhouse where humane slaughter practices were followed, according to USDA guidelines. Three separate ovarian collections at the slaughterhouse provided three biological replicates for these studies. Based on surface diameter, GC were collected from small (SM; 1 to 5 mm) or large (LG; > 5 mm) follicles as described previously (Ranzenigo et al., 2008). The GC were re-suspended with a basal DMEM medium (containing 0.12 mM of gentamicin and 38.5 mM of sodium bicarbonate) containing collagenase at 1.25 mg/mL and DNase at 0.5 mg/mL to prevent cell clumping. Viability of GC was determined *via* Trypan blue exclusion method, and averaged 36.5%, which is within the range previously reported for pig GC (Cortinovis et al., 2014; Evans et al., 2014).

On average 2.6×10^5 viable cells were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA). Cells were cultured in an environment of 5% CO_2 and 95% air at 38.5 °C in 5% FCS and 5% PS for the first 48 h with medium changed every 24 h. Cells were then washed twice with 0.5 mL of serum-free medium and the various treatments applied in serum-free medium for 48 h. Medium was changed after 24 and 48 h, medium was collected for steroid radioimmunoassays (RIA) and cells were enumerated.

2.3. Steroid radioimmunoassays (RIA) and cell counting

Progesterone (P4) and estradiol (E2) RIA were conducted as previously described (Spicer and Chamberlain, 1998; Evans et al., 2014). Inter- and intra-assay coefficients of variation averaged 12.0% and 7.1%, respectively for the P4, and 7.9% and 8.6%, respectively for the E2 RIA. Sensitivity of the P4 RIA, defined as 90% of total binding averaged 1.6 ng/mL. Sensitivity of the E2 RIA, defined as 95% of total binding averaged 4.8 pg/mL.

To determine cell numbers, cells were washed with saline, treated with trypsin solution for 30 min at 37 °C, and counted using a Coulter counter (Z2 Coulter® Particle Count and Size Analyzer; Beckman Coulter, Hialeah, FL) as previously described (Ranzenigo et al., 2008; Evans et al., 2014).

2.4. Experimental design

Experiment 1 was designed to evaluate the effects of NCG and ARG on proliferation and steroidogenesis by pig GC from SM follicles. Cells were cultured as stated before and the following treatments were applied for 48 h: NCG (0 or 4 mM) and ARG (0, 0.4, or 4 mM) in the presence of IGF1 (30 ng/mL) and testosterone (500 ng/mL) with or without FSH (30 ng/mL). The doses of IGF1 and FSH were based on previous studies (Ranzenigo et al., 2008; Spicer et al., 2002). The doses of NCG and ARG were selected based on previous studies (Lamanna et al., 2007; Greene et al., 2013; Feng et al., 2018b). Cells were counted and RIA was conducted to quantify P4 and E2 concentrations in medium.

Experiment 2 was designed to evaluate the effect of NCG and ARG on proliferation and steroidogenesis of pig GC from LG follicles. Cells were cultured as stated before and the following treatments were applied for 48 h: NCG (0 or 4 mM) and ARG (0 or 4 mM) in the presence of IGF1 (30 ng/mL) and testosterone (500 ng/mL) with or without FSH (30 ng/mL). Dosages of NCG and ARG were selected based on the results from Experiment 1.

2.5. Statistical analysis

For each experiment, three different biological pools of GC (*i.e.*, biological replicate) were used as experimental replicates, and each treatment was replicated two or three times in each experiment and biological replicate. The GC in each pool were from a total volume of 1 to 6.5 mL of follicular fluid (ovaries from 2 to 5 gilts) per pool. Cell numbers from each experiment were calculated to determine steroid production as ng or pg /10^5 cells per 24 h. Treatment effects on the dependent variables were determined using an ANOVA and the general linear models (GLM) procedure of SAS for Windows (Version 9.3, SAS Institute Inc., Cary, NY). Data from Experiment 1 were analyzed as a one-way ANOVA. Data from Experiment 2 were analyzed as a 2 × 3 factorial ANOVA. Mean differences were determined using the Fisher's protected least significant differences test (Ott, 1977) only if significant main effects in the ANOVA were detected. Data are presented as least square means ± SEM.

3. Results

3.1. Experiment 1: effect of NCG and ARG on SM-follicle GC steroidogenesis and cell proliferation

In SM-follicle GC, treatment with 4 mM of NCG and ARG decreased ($P < 0.05$) IGF1 plus FSH-induced P4 production by 31.5% and 19.8%, respectively (Fig. 1A). In SM-follicle GC treated with IGF1 alone, treatment with ARG and NCG had no effect ($P > 0.10$) on P4 production whereas treatment with FSH increased ($P < 0.05$) IGF1-induced P4 production by 3.1-fold (Fig. 1A).

In SM-follicle GC, treatment with NCG and ARG did not affect ($P > 0.10$) FSH plus IGF1-induced E2 production or E2 production in SM-follicle GC treated with IGF1 alone (Fig. 1B). The IGF1-induced E2 production was increased ($P < 0.05$) by 2.4-fold as a result of FSH treatment (Fig. 1B).

Numbers of SM-follicle GC were not affected by NCG or ARG. Treatment with FSH increased ($P < 0.05$) cell numbers by 1.3-fold in IGF1-treated SM-follicle GC (Fig. 1C).

3.2. Experiment 2: effects of NCG and ARG on LG-follicle GC steroidogenesis and cell proliferation

In LG-follicle GC treated with IGF1 alone, treatment with NCG decreased ($P < 0.05$) P4 production by 36%, whereas treatment with FSH increased ($P < 0.05$) P4 production by 18%, and ARG had no effect ($P > 0.10$) on P4 production (Fig. 2A). Similarly, in LG-follicle GC treated with FSH and IGF1, treatment with NCG decreased ($P < 0.05$) P4 production by 46% whereas ARG had no effect ($P > 0.10$) on P4 production (Fig. 2A).

In LG-follicle GC treated with IGF1 alone, treatment with NCG decreased ($P < 0.05$) E2 production by 46% whereas ARG had no significant effect on E2 production, and treatment with FSH increased ($P < 0.05$) E2 production by 1.7-fold (Fig. 2A). In LG-follicle GC treated with FSH and IGF1, treatment with ARG and NCG decreased ($P < 0.05$) E2 production 26% and 63%, respectively (Fig. 2B).

In LG-follicle GC treated with IGF1 alone, treatment with NCG increased ($P < 0.05$) cell numbers by 1.4-fold and FSH increased ($P < 0.05$) cell numbers by 1.5-fold, whereas treatment with ARG had no significant effect (Fig. 2C). In LG-follicle GC treated with FSH plus IGF1, treatment with ARG decreased ($P < 0.05$) cell numbers by 20% whereas treatment with NCG had no effect ($P > 0.10$; Fig. 2C).

4. Discussion

The results of this study provide evidence for the first time of the effects of ARG and NCG on steroidogenesis and cell proliferation in ovarian GC of a multi-ovular species. Formerly, the effects of ARG and NCG on ovarian cell steroidogenesis and proliferation had been minimally studied. Recently, pregnant ewes that were fed ARG and NCG as a dietary supplement had lesser blood concentrations of P4 and E2 (Zhang et al., 2016). Similarly, results from *in vitro* studies using SM-follicle GC of cattle indicated that both NCG and ARG inhibited IGF1- and FSH-induced E2 production but only NCG inhibited P4 production (Feng et al., 2018b). In the present study, only FSH plus IGF1-induced P4 production was reduced when treated with ARG and NCG in SM-follicle GC without affecting E2 production. In contrast, P4 production was inhibited by NCG (not ARG) and E2 production was inhibited by both ARG and NCG in LG-follicle GC, suggesting that ARG and NCG effects are more pronounced in more differentiated LG-follicle GC. In testicular tissue of pigs, ARG (1 mM) treatment resulted in a decrease in basal testosterone production (Lamanna et al., 2007), supporting the thought that ARG may be inhibitory to steroidogenesis in both male and female pigs. Why NCG and ARG may suppress steroidogenesis of highly differentiated cells more than less differentiated cells will require further study. Production of E2 and P4 increase as GC undergo differentiation and is a result of increased FSH-induced cAMP production (Hsueh et al., 1983). Previously, a combined treatment of FSH and IGF1 increased production of E2 and P4 in cultured pig GC (Ranzenigo et al., 2008), and results of the present study are consistent with this previous finding. Perhaps with amplified steroidogenesis in LG-follicle GC, inhibitory factors such as ARG and NCG have a more marked effect on steroidogenesis.

For the first time, cell proliferation of pig GC treated with NCG and ARG has been studied. Specifically, results of the present study indicate there is no effect of NCG and ARG on proliferation of SM-follicle GC of pigs, whereas treatment with NCG increased numbers of LG-follicle GC in the absence of FSH treatment. In the presence of IGF1, FSH increased numbers of both SM-follicle GC and LG-follicle GC. Consistent with the results of the present study, in previous studies there have been reports that FSH increases proliferation of cattle GC in the presence of IGF1 (Khamsi and Armstrong, 1997; Spicer et al., 2002, 2006; Dentis et al., 2017) and has no

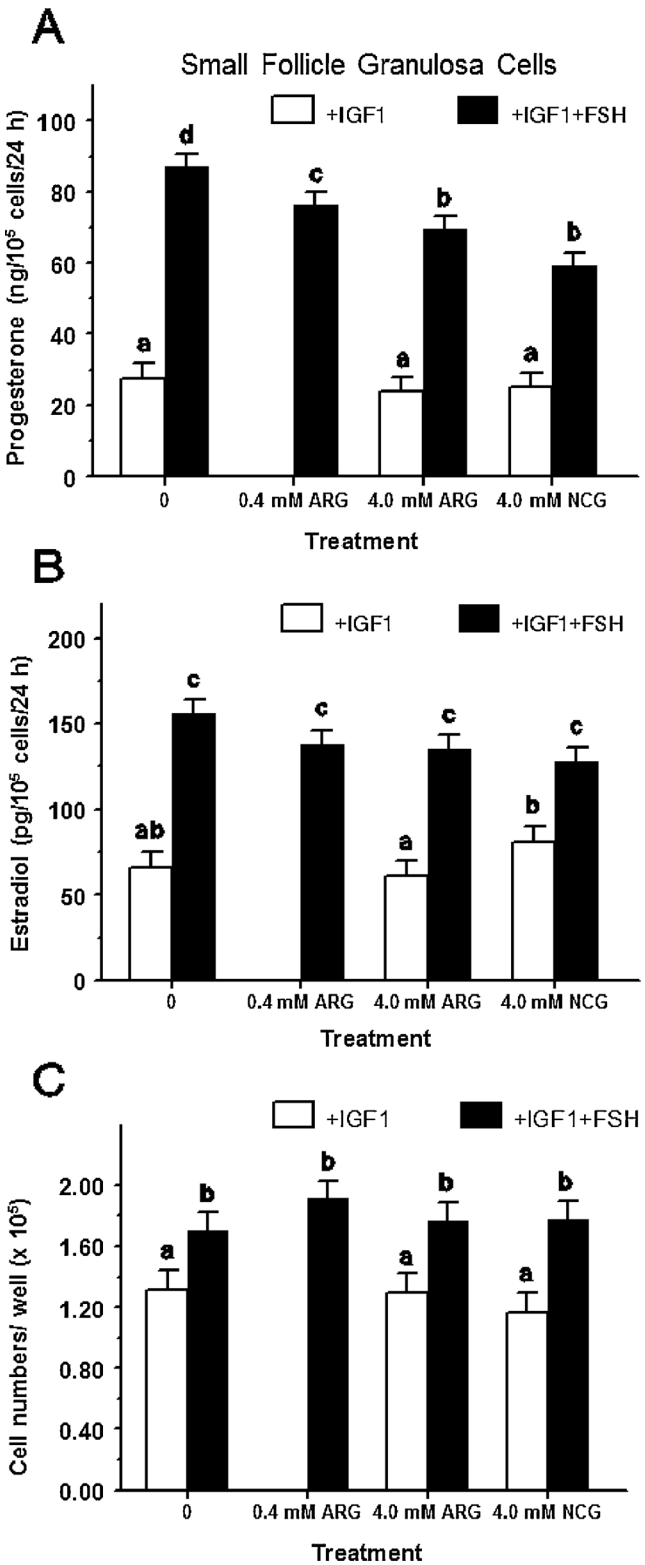

Fig. 1. Effects of arginine (ARG) and N-carbamylglutamate (NCG) on pig granulosa cell (GC) numbers and estradiol (E2) and progesterone production by small-follicle GC; GC from small follicles were treated with NCG (4 mM) or ARG (0.4 or 4 mM) for 48 h and concomitantly treated with testosterone (500 ng/mL; as an E2 precursor) and IGF1 (30 ng/mL), with or without FSH (30 ng/mL); Medium was changed every 24 h; [abcd]Within a panel, means (n = 9) without a common letter differ ($P < 0.05$); Note: 0.4 mM of ARG was not tested in the IGF1-treated cells.

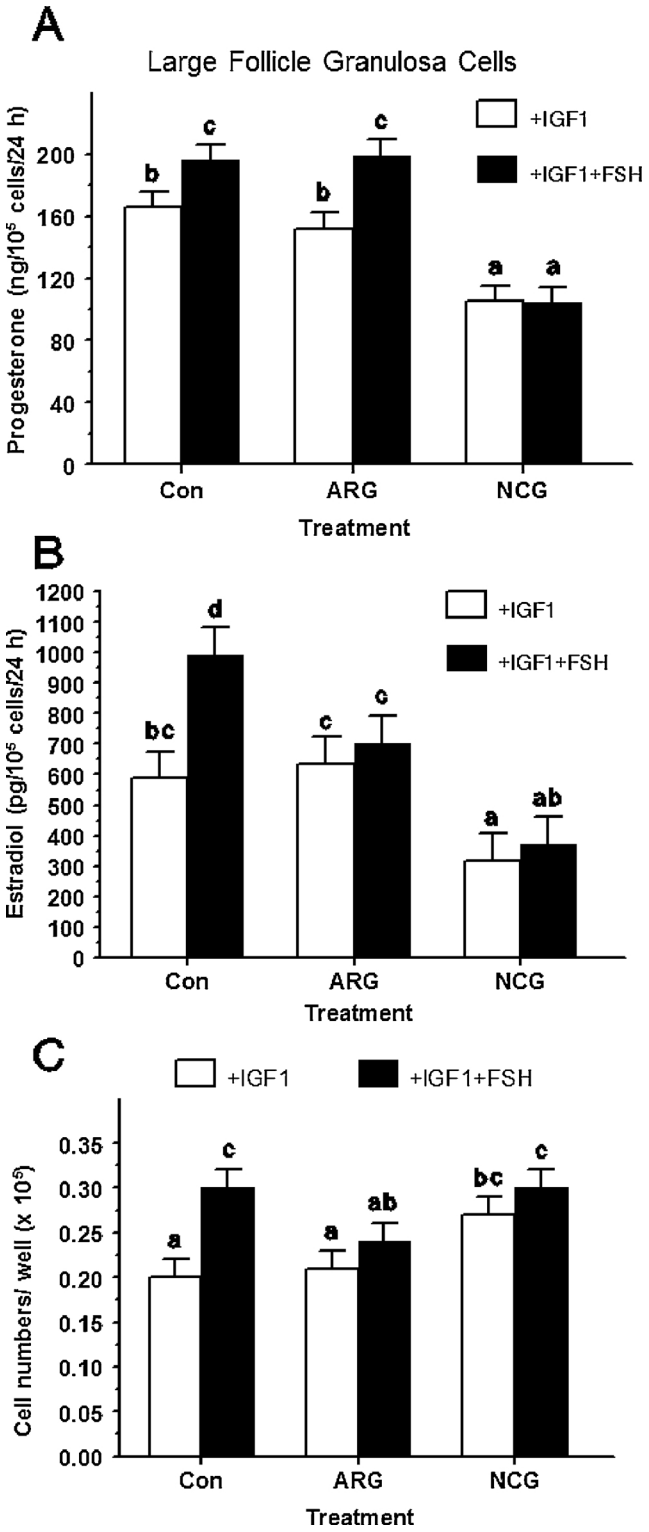

Fig. 2. Effects of arginine (ARG) and N-carbamylglutamate (NCG) on pig granulosa cell (GC) numbers and estradiol (E2) and progesterone production by large-follicle GC; GC from large follicles were treated with NCG (4 mM) or ARG (4 mM) for 48 h and concomitantly treated with testosterone (500 ng/mL; as an E2 precursor) and IGF1 (30 ng/mL), with or without FSH (30 ng/mL); Medium was changed every 24 h; [abcd]Within a panel, means ($n = 6$) without a common letter differ ($P < 0.05$).

effect in the absence of IGF1 (Langhout et al., 1991; Spicer et al., 2002). Similarly, treatment with FSH had no effect on basal proliferation of pig GC (Hammond and English, 1987). Previously, treatment with NCG increased measures of intestinal epithelium cell proliferation in weanling piglets (Wu et al., 2010), and the absence of ARG in medium reduces both follicle survival and ovulation rate in mouse ovaries cultured *in vitro* (Mitchell et al., 2004). In a previous study, results indicated there was a function of NCG and ARG on microRNA gene expression and the effects on the size and volume flow of the umbilical vein of piglets (Liu et al., 2012), supporting a biological effect for these two amino acid derivatives. Furthermore, results of a previous study indicate dietary ARG and NCG supplementation may affect placental vascular function (Wu et al., 2012). Results from a recent study indicate NCG and ARG increased cell numbers induced by IGF1 and FSH in SM-follicle GC of cattle (Feng et al., 2018b). Results of the present study indicate that treatment with NCG and ARG affect differentiation of pig GC (*i.e.*, steroid production) more than numbers of GC. Why ARG and NCG had opposing effects on cell numbers of highly differentiated pig GC will require further study. Considering ARG blocked the FSH-induced increase in E2 production by LG-follicle GC and reduced the FSH-induced increase in cell numbers, perhaps ARG is in some way blocking the FSH-induced intracellular signaling cascade. One possible mechanism of ARG inhibition of cell proliferation and steroidogenesis may be through ARG induction of nitric oxide (NO) production (Thippeswamy et al., 2006) because NO is known to inhibit FSH-induced steroidogenesis in rat GC (Ishimaru et al., 2001). In contrast, treatment with NCG increased cell numbers in LG-follicle GC treated with IGF1 alone and suggests the mechanism of action of NCG differs from that of ARG. Because NCG is a N-acetylglutamate synthase analog (Jones et al., 2008; Nissim et al., 2011) and actions of this enzyme are important in the synthesis of polyamines (Nissim et al., 2011), and polyamines induce proliferation of pig and mice GC (Veldhuis and Hammond, 1979; Lee and Dias, 1988), perhaps NCG is stimulating polyamine synthesis resulting in polyamine-induced cell proliferation. Additional studies will be required to verify these suggestions regarding potential mechanisms of action of ARG and NCG.

5. Conclusion

Results of the present studies indicate that NCG and ARG may directly affect follicular function in pigs, and imply that ARG and NCG could be used as nutritional supplements to help regulate ovarian function in pigs by inhibiting P4 production in SM-follicle GC and E2 production in LG-follicle GC. Understanding the mechanism of action of NCG and ARG through further research will allow for understanding how these nutritional supplements can improve reproduction in pigs and other species.

Declaration of Competing Interest

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

Acknowledgements

The authors thank the Amber Hemple and Jacqueline Nichols for their laboratory assistance; Dr. A. F. Parlow, National Hormone & Pituitary Program (Torrance, CA) for purified FSH; and the Oklahoma State University Food and Agricultural Products Center for their generous donation of pig ovaries. This research was supported in part by: National Natural Science Foundation of China (31501950), the Oklahoma State University Agricultural Experiment Station (OKL02970), Beijing Nova Program (Z141105001814046) and State Scholarship Fund of China (201509110025).

References

Al-Dabbas, F.M., Hamra, A.H., Awawdeh, F.T., 2008. The effect of arginine supplementation on some blood parameters, ovulation rate and concentrations of estrogen and progesterone in female awassi sheep. Pak. J. Biol. Sci. 11, 2389–2394.

Bass, B.E., Bradley, C.L., Johnson, Z.B., Zier-Rush, C.E., Boyd, R.D., Usry, J.L., Maxwell, C.V., Frank, J.W., 2017. Influence of dietary -arginine supplementation of sows during late pregnancy on piglet birth weight and sow and litter performance during lactation. J. Anim. Sci. 95, 248–256.

Battaglia, C., Regnani, G., Marsella, T., Facchinetti, F., Volpe, A., Venturoli, S., Flamigni, C., 2002. Adjuvant L-arginine treatment in controlled ovarian hyper-stimulation: a double-blind, randomized study. Hum. Reprod. 17, 659–665.

Cortinovis, C., Caloni, F., Schreiber, N.B., Spicer, L.J., 2014. Effects of fumonisin B1 alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroid production. Theriogenology 81, 1042–1049.

de Chavez, J.A., Guzman, A., Zamora-Gutierrez, D., Mendoza, G.D., Melgoza, L.M., Montes, S., Rosales-Torres, A.M., 2015. Supplementation with rumen-protected L-arginine-HCL increased fertility in sheep with synchronized estrus. Trop. Anim. Health Prod. 47, 1067–1073.

Dentis, J.L., Schreiber, N.B., Burress, A.M., Spicer, L.J., 2017. Effects of angiogenin on granulosa and theca cell function in cattle. Animal 11, 811–819.

Evans, J.R., Schreiber, N.B., Williams, J.A., Spicer, L.J., 2014. Effects of fibroblast growth factor 9 on steroidogenesis and control of FGFR2IIIc mRNA in porcine granulosa cells. J. Anim. Sci. 92, 511–519.

Feng, T., Bai, J.H., Xu, X.L., Guo, Y., Huang, Z., Liu, Y., 2018a. Supplementation with N-carbamylglutamate and vitamin C: improving gestation and lactation outcomes in sows under heat stress. Anim. Prod. Sci. 58, 1854–1859.

Feng, T., Schutz, L.F., Morrell, B.C., Perego, M.C., Spicer, L.J., 2018b. Effects of N-carbamylglutamate and L-arginine on steroidogenesis and gene expression in bovine granulosa cells. Anim. Reprod. Sci. 188, 85–92.

Greene, J.M., Feugang, J.M., Pfeiffer, K.E., Stokes, J.V., Bowers, S.D., Ryan, P.L., 2013. L-arginine enhances cell proliferation and reduces apoptosis in human endometrial RL95-2 cells. Reprod. Biol. Endocrinol. 11, 15–20.

Hammond, J.M., English, H.F., 1987. Regulation of deoxyribonucleic acid synthesis in cultured porcine granulosa cells by growth factors and hormones. Endocrinology 120, 1039–1046.

Hazeleger, W., Soede, N.M., Kemp, B., 2005. The effect of feeding strategy during the pre-follicular phase on subsequent follicular development in the pig. Domest. Anim. Endocrinol. 29, 362–370.

Hsueh, A.J., Jones, P.B., Adashi, E.Y., Wang, C., Zhuang, L.Z., Welsh Jr., T.H., 1983. Intraovarian mechanisms in the hormonal control of granulosa cell differentiation

in rats. J. Reprod. Fertil. 69, 325–342.
Ishimaru, R.S., Leung, K., Hong, L., LaPolt, P.S., 2001. Inhibitory effects of nitric oxide on estrogen production and cAMP levels in rat granulosa cell cultures. J. Endocrinol. 168, 249–255.
Jones, S., Reed, C.A., Vijay, S., Walter, J.H., Morris, A.A., 2008. N-carbamylglutamate for neonatal hyperammonaemia in propionic acidaemia. J. Inherit. Metab. Dis. 31 (Suppl. 2), S219–S222.
Kelly, D., LeBlanc, M.M., Warren, L.K., Montensen, C.J., 2014. Influence of L-arginine supplementation on reproductive blood flow and embryo recovery rates in mares. Theriogenology 81, 752–757.
Khamsi, F., Armstrong, D.T., 1997. Interactions between follicle-stimulating hormone and growth factors in regulation of deoxyribonucleic acid synthesis in bovine granulosa cells. Biol. Reprod. 57, 684–688.
Knox, R.V., 2016. Artificial insemination in pigs today. Theriogenology 85, 83–93.
Kraeling, R.R., Webel, S.K., 2015. Current strategies for reproductive management of gilts and sows in North America. J. Anim. Sci. Biotechnol. 6, 3.
Lamanna, C., Assisi, L., Vittoria, A., Botte, V., Di Fiore, M.M., 2007. D-aspartic acid and nitric oxide as regulators of androgen production in boar testis. Theriogenology 67, 249–254.
Langhout, D.J., Spicer, L.J., Geisert, R.D., 1991. Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. J. Anim. Sci. 69, 3321–3334.
Lee, C.Y., Dias, J.A., 1988. Cellular origin of prolonged induction of ornithine decarboxylase in the rat ovary. Proc. Soc. Exp. Biol. Med. 187, 350–354.
Liu, X.D., Wu, X., Yin, Y.L., Liu, Y.Q., Geng, M.M., Yang, H.S., Blachier, F., Wu, G.Y., 2012. Effects of dietary L-arginine or N-carbamylglutamate supplementation during late gestation of sows on the miR-15b 16, miR-221 222, VEGFA and eNOS expression in umbilical vein. Amino Acids 42, 2111–2119.
Mitchell, L.M., Kennedy, C.R., Hartshorne, G.M., 2004. Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. Hum. Reprod. 19, 30–40.
Nissim, I., Horyn, O., Nissim, I., Daikhin, Y., Caldovic, L., Barcelona, B., Cervera, J., Tuchman, M., Yudkoff, M., 2011. Down-regulation of hepatic urea synthesis by oxypurines: xanthine and uric acid inhibit N-acetylglutamate synthase. J. Biol. Chem. 286, 22055–22068.
Ott, L., 1977. An Introduction to Statistical Methods and Data Analysis. Duxbury Press, North Scituate, MA, pp. 384–388.
Pau, M.Y., Milner, J.A., 1982. Dietary arginine and sexual maturation of the female rat. J. Nutr. 112, 1834–1842.
Peltoniemi, O.A., Virolainen, J.V., 2006. Seasonality of reproduction in gilts and sows. Soc. Reprod. Fertil. Suppl. 62, 205–218.
Ranzenigo, G., Caloni, F., Cremonesi, F., Aad, P.Y., Spicer, L.J., 2008. Effects of fusarium mycotoxins on steroid production by porcine granulosa cells. Anim. Reprod. Sci. 107, 115–130.
Spicer, L.J., Aad, P.Y., Allen, D., Mazerbourg, S., Hsueh, A.J., 2006. Growth differentiation factor-9 has divergent effects on proliferation and steroidogenesis of bovine granulosa cells. J. Endocrinol. 189, 329–339.
Spicer, L.J., Chamberlain, C.S., 1998. Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. Endocrine 9, 153–161.
Spicer, L.J., Chamberlain, C.S., Maciel, S.M., 2002. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest. Anim. Endocrinol. 22, 237–254.
Thippeswamy, T., McKay, J.S., Quinn, J.P., Morris, R., 2006. Nitric oxide, a biological double-faced janus–is this good or bad? Histol. Histopathol. 21, 445–458.
Veldhuis, J.D., Hammond, J.M., 1979. Role of ornithine decarboxylase in granulosa-cell replication and steroidogenesis in vitro. Biochem. Biophys. Res. Commun. 91, 770–777.
Wu, G., Morris Jr, S.M., 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336, 1–17.
Wu, G., Knabe, D.A., Kim, S.W., 2004. Arginine nutrition in neonatal pigs. J. Nutr. 134, 2783S–2790S.
Wu, G., Bazer, F.W., Davis, T.A., Kim, S.W., Li, P., Marc Rhoads, J., Carey Satterfield, M., Smith, S.B., Spencer, T.E., Yin, Y., 2009. Arginine metabolism and nutrition in growth, health and disease. J. Amino Acids 37, 153–168.
Wu, X., Ruan, Z., Gao, Y., Yin, Y., Zhou, X., Wang, L., Geng, M., Hou, Y., Wu, G., 2010. Dietary supplementation with L-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. Amino Acids 39, 831–839.
Wu, X., Yin, Y.L., Liu, Y.Q., Liu, X.D., Liu, Z.Q., Li, T.J., Huang, R.L., Ruan, Z., Deng, Z.Y., 2012. Effect of dietary arginine and N-carbamoylglutamate supplementation on reproduction and gene expression of eNOS, VEGFA and PlGF1 in placenta in late pregnancy of sows. Anim. Reprod. Sci. 132, 187–192.
Zhang, B., Che, L.Q., Lin, Y., Zhuo, Y., Fang, Z.F., Xu, S.Y., Song, J., Wang, Y.S., Liu, Y., Wang, P., Wu, D., 2014. Effect of dietary N-carbamylglutamate levels on reproductive performance of gilts. Reprod. Domest. Anim. 49, 740–745.
Zhang, H., Sun, L., Wang, Z., Deng, M., Nie, H., Zhang, G., Ma, T., Wang, F., 2016. N-carbamylglutamate and L-arginine improved maternal and placental development in underfed ewes. Reproduction 151, 623–635.
Zeng, X., Huang, Z., Mao, X., Wang, J., Wu, G., Qiao, S., 2012. N-carbamylglutamate enhances pregnancy outcome in rats through activation of PI3K/PKB/mTOR signaling pathway. PLoS One 7, e 41192.
Zhu, J., Zeng, X., Peng, Q., Zeng, S., Zhao, H., Shen, H., Qiao, S., 2015. Maternal N-carbamylglutamate supplementation during early pregnancy enhances embryonic survival and development through modulation of the endometrial proteome in gilts. J. Nutr. 145, 2212–2220.