Direct Effects of Nerve Growth Factor-β, Purified From Bull Seminal Plasma,on Steroidogenesis and Angiogenic Markers of Thebovine Pre-ovulatory Follicle

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Short report

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Abstract

Nerve growth factor-β (NGF) is critical for ovulation in the mammalian ovary and is luteotrophic when administered systemically to camelids and cattle. This study aimed to assess the direct effects of purified bovine NGF on steroidogenesis and angiogenic markers in the bovine pre-ovulatory follicle. A chort of Holstein heifers were synchronized with a standard protocol and heifers with the preovulatory follicle (≥ 12 mm) had an the ovary containing the dominant follicle removed via colpotomy. Pre-ovulatory follicles were dissected in 24 pieces containing theca and granulosa cells that were randomly allocated to receive either cultured in media supplemented with purified bovine NGF (100 ng/mL) or untreated (control) for 72 h. The supernatant media was harvested for determination of progesterone, testosterone, and estradiol, whereas explants were used for mRNA analyses for steroidogenesis and angiogenic markers. Treatment of follicle tissue with NGF upregulated gene expression of steroidogenic enzyme HDS17B ($P = 0.04$) and increased testosterone production ($P < 0.01$). However, NGF treatment did not alter production of progesterone ($P = 0.81$) or estradiol ($P = 0.14$). Consistently, gene expression of steroidogenic enzymes responsible for producing these hormones (STAR , CYP11A1 , HSD3B , CYP17A1 , CYP19A1) were unaffected by NGF treatment ($P \geq 0.31$). Treatment with NGF downregulated gene expression of the angiogenic enzyme FGF2 ($P = 0.02$) but did not alter PGES ($P = 0.63$), VEGFA ($P = 0.44$), and ESR1 ($P = 0.77$). Collectively, these results demonstrate that NGF from seminal plasma may interact directly with the bovine pre-ovulatory follicle to alter downstream steroidogenesis and luteal development.

Background

Nerve growth factor-β (NGF) is a member of the neurotrophin family that has a critical role in mammalian follicle development and ovulation (1, 2). Expression of NGF has been localized to ovarian granulosa cells prior to the formation of the first primordial follicles in neonatal mice (2) and rats (3) and is involved in initiating folliculogenesis (4). Though present during late fetal development, expression of NGF and its receptor, TrkA, in the ovary decreases postnatally between 24 and 48 h after birth and remains low until puberty in rats (3). At the time of the first pre-ovulatory luteinizing hormone (LH) surge, transient activation of NGF/TrkA occurs and is integral to the process of follicular cytodifferentiation proceeding the first ovulation (1). In bovine theca cells, NGF induced prostaglandin E_2 (PGE) synthesis (5), which facilitates follicular rupture at ovulation (6). Additionally, NGF/TrkA signaling in theca cells contributes to the loss of gap junctions within the follicular wall that precedes ovulation (7). The presence of both LH and follicle-stimulating hormone (FSH) *in vitro* were necessary to stimulate NGF secretion from medium to large follicles in ewes, suggesting a synergistic role with gonadotropins during the pre-ovulatory cascade (8). Consistently, follicular NGF concentrations increased following the gonadotropin peaks in ewes, and *in vitro* treatment with NGF induced marked cumulus expansion and progressive cumulus-oocyte uncoupling (9), which functions to reinitiate meiotic progression of the oocyte at ovulation.

Our understanding of the role of NGF in promoting mammalian ovulation became even more complex when it was identified as the ovulation-inducing factor in camelid seminal plasma (10, 11). Though the

ability of the ovary to produce NGF in spontaneously ovulating species is well documented, few studies have evaluated whether the introduction of NGF from seminal plasma at time of breeding may have a role within the bovine hypothalamic-pituitary-ovarian axis (12–15). In camelids, intrauterine absorption of seminal plasma NGF into systemic circulation occurs within 15 min of copulation, after which it stimulates the preovulatory LH peak from the anterior pituitary gland and exerts a dose-dependent luteotropic effect on the developing corpus luteum (CL) (16). Studies have shown that NGF retains its luteotropic properties when administered systemically to cattle (12–14). While the luteotropic effect in camelids is attributed to the prolonged LH secretion from the pituitary, in cattle, there is evidence that NGF from the seminal plasma may act directly on the ovary (5).

Similar to camelids, bull seminal plasma contains NGF, which is concentrated into the sperm-rich fraction of the ejaculate, facilitating its transit into the cow reproductive tract (17). The bovine uterus maintains a local countercurrent exchange between the uterine venous drainage and the ovarian artery that allows for direct transport of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus to the ovary during luteolysis (18, 19). This anatomical mechanism could also provide a potential route for NGF to travel to and interact directly with the ovary (20). A previous study demonstrated that treating bovine theca cells with recombinant NGF *in vitro* resulted in increased androstenedione and progesterone release, PGE production, and theca cell proliferation when compared to hCG-treated controls (5). However, the signaling cascade within the ovary involves a complex interaction between the theca and granulosa cells, which has yet to be elucidated. This study aimed to assess the direct effects of NGF, purified from bull seminal plasma, on steroidogenesis and angiogenic markers in theca and granulosa cells of the bovine pre-ovulatory follicle. We hypothesize that NGF administration would stimulate steroidogenesis and angiogenic markers in thecal and granulosa cells from the bovine pre-ovulatory follicle.

Materials And Methods

Heifer synchronization and ovariectomy

A cohort of cyclic heifers (presence of corpus luteum > 16 mm and pre-ovualtory follicle > 12 mm) had their estrous cycle synchronized. The heifers received an intravaginal progesterone-releasing device (1.38 g progesterone; Eazi-Breed CIDR, Zoetis, Parsippany-Troy Hills, NJ, USA) and an injection of GnRH agonist (100 µg Factrel®, Zoetis, Parsippany-Troy Hills, NJ, USA) intramuscularly. The intravaginal device was removed 5 days later, and heifers were given $PGF_{2\alpha}$ analog (25 mg dinoprost tromethamine, Lutalyse, Zoetis, Parsippany-Troy Hills, NJ, USA) intramuscularly at the time of the intravaginal device removal and again 24 h later. Heifers were examined daily by transrectal ultrasonography to assess response to the synchronization program. Heifers that initiate follicle development and ovulate after the first GnRH treatment and had luteolysis after $PGF_{2\alpha}$ were eligible to have an ovariectomy. The procedure to remove the ovaries was performed at 48 h after the second $PGF_{2\alpha}$ injection when the dominant follicles reached at least 12 mm in diameter. Ovariectomy was performed via colpotomy in the standing position under sedation with 15 mg intramuscular xylazine (AnaSed® Injection, Lloyd, Inc., Shenandoah,

IA, USA) and caudal epidural anesthesia with 5 mL of 2% (w/v) lidocaine HCl (VetOne®, Boise, Idaho, USA) performed. Heifers were treated pre-operatively with ceftiofur crystalline free acid (6.6 mg/kg; Excede®, Zoetis, Parsippany-Troy Hills, NJ, USA) injected subcutaneously in the base of the ear to prevent infection. Flunixin meglumine (2.2 mg/kg; Norbrook® Inc., Overland Park, KS, USA) was administered intravenously daily for two to control inflammation and pain. An incision was made in the dorsolateral aspect of the vaginal fornix, and the peritoneum was manually punctured after blunt dissection through the adventitia. The mesovarium was manually compressed, and the ovary containing the dominant follicle was removed using a chain écraseur. Ovaries were placed in ice-cold phosphate-buffered saline solution containing 2% antibiotic-antimycotic mixture (25 µg/mL amphotericin B, 10,000 units/mL penicillin, 10,000 µg/mL streptomycin; Gibco, Gaithersburg, MD, USA) for transport to the laboratory.

Isolation of follicular wall cells and treatment allocation

The pre-ovulatory follicle was identified and dissected away from each ovary for use in the tissue culture system (Fig. 1). Follicular fluid was aspirated to facilitate further dissection and frozen at − 80 °C. The follicles were dissected into quarters, and the theca interna with adherent granulosa cells was peeled from the theca externa and surrounding stromal tissue. The remaining follicle wall preparations (theca interna and granulosa cells) were cut into 26 pieces (average weight: 5.3 ± 0.7 mg), 24 of which were transferred to a costar 24-well plate (1 piece/well; Cambridge, MA, USA) for tissue culture, as previously described (21, 22). The remainder of the tissue was flash-frozen in liquid nitrogen and maintained at − 80 °C until mRNA extraction.

The dissected 24 follicle wall pieces containing theca and granulosa cells were randomly allocated to receive a culture medium that was either supplemented with purified bovine NGF (100 ng/mL, n = 12) or left untreated (control, n = 12). The single plate was incubated at 37 °C in a humidified incubator gassed with 5% CO_2:95% air for 72 h. The NGF used was purified from bovine seminal plasma, as described previously (13). The follicle wall pieces were cultured in 0.5 mL of medium consisting of Eagle's MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% L-glutamine (Gibco, Gaithersburg, MD, USA), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin (Sigma-Aldrich), 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich), 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 40 ng/mL cortisol (Sigma-Aldrich), 4 ng/mL human recombinant LH (Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor- UCLA Medical Center, Torrance, CA, USA), and 4 ng/mL human recombinant FSH (Dr. A. F. Parlow, National Hormone and Peptide Program).

Hormone assays

The aliquots (0.5 mL) of culture medium at 3, 6, 12, 24, 48, and 72 h of culture were collected and preserved at − 20 °C for subsequent steroid assays. Progesterone, testosterone, and estradiol-17β secretions by granulosa and theca cells into the culture media were assessed using immunoassays (Immulite 2000 XPi platform; Siemens Medical Solutions, Malvern, PA, USA, Inc.). Total hormone production for each well was calculated by multiplying the measured concentration by the volume of

media (0.5 mL) and then dividing by tissue weight (mg). Intra-assay coefficient of variations were 4.0% (testosterone), 2.4% (progesterone), and 3.1% (estradiol-17β). Inter-assay coefficient of variations were 12% (testosterone), 19% (progesterone), and 15% (estradiol-17β). The progesterone assay had a detection range of 0.2 to 40 ng/mL and a sensitivity of 0.1 ng/mL. The testosterone assay had a detection range of 20 to 1600 ng/mL and a sensitivity of 15 ng/dL. The estradiol-17β assay had a detection range of 20-2000 pg/mL and a sensitivity of 15 pg/mL.

Quantitative real-time PCR analyses

At the completion of the 72-h culture period, follicle wall pieces were weighed and flash-frozen and kept at -80 °C until RNA extraction. Follicular tissue mRNA expression was determined for LH/choriogonadotropin receptor (*LHCGR*), FSH receptor (*FSHR*), PGE synthase (*PGES*), vascular endothelial growth factor A isoform 121 (*VEGFA121*), fibroblast growth factor 2 (*FGF2*), estrogen receptor 1 (*ESR1*), steroidogenic acute regulatory protein (*STAR*), cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), hydroxyl-delta-5-steroid dehydrogenase 3-beta (*HSD3B*), and hydroxysteroid 17-beta dehydrogenase (*HSD17B*). Primers were designed for the constitutively expressed mRNAs, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L 15 (*RPL15*), and ribosomal protein L 19 (*RPL19*), with the expression value of each gene normalized to the mean values of these genes (Table 1). Relative expression values were obtained by determining the PCR amplification efficiency (E = 2) to the power of the delta-delta threshold cycle (ΔΔCt) obtained from the ΔCt least square mean differences of pairwise comparisons between initial and cultured tissue (23).

Table 1
List of genes and primers used for quantitative real-time PCR

Target genes and abbreviations	NCBI Sequence	Primer	Primer Sequence
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_001034034	Forward	5’-GGCGCCAAGAGGGTCAT-3’
		Reverse	5’-ACGCCCATCACAAACATGG-3’
Ribosomal protein L 15 (*RPL15*)	AY786141	Forward	5’-TGGAGAGTATTGCGCCTTCTC-3’
		Reverse	5’-CACAAGTTCCACCACACTATTGG-3’
Ribosomal protein L 19 (*RPL19*)	NM_001040515	Forward	5’-CAGACGATACCTGAATCTAAGAAGA-3’
		Reverse	5’-TGAGAATCCGCTTGTTTTTGAA-3’
Follicular stimulating hormone receptor (*FSHR*)	NM_174061	Forward	5'-CGACTCTGTCACTGCTCTAACGG-3'
		Reverse	5'-CGTCAATTCCTTTGGCATAGGTGG-3'
Luteinizing hormone/ choriogonadotropin receptor (*LHCGR*)	NM_174381	Forward	5’-CAGTCCCCCGCTTTCTCAT-3’
		Reverse	5’-GTAGAGCCCCATGCAGAAGTCT-3’
Steroidogenic acute regulatory protein (*STAR*)	XR_083945	Forward	5’-GGATTAACCAGGTTCGGCG-3’
		Reverse	5’-CTCTCCTTCTTCCAGCCCTC-3’
Cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*)	NM_176644	Forward	5’-GCCACATCGAGAACTTCCAGAAG-3’
		Reverse	5’-CTGGTGTGGAACATCTTGTAGACG-3’
Hydroxyl-delta-5-steroid dehydrogenase 3-beta (*HSD3B*)	NM_174343	Forward	5'-TGTTGGTGGAGGAGAAGGATCTG-3'
		Reverse	5'-TGGGTACCTTTCACATTGACGTTC-3'
Hydroxysteroid 17-beta dehydrogenase (*HSD17B*)	NM_001102365	Forward	5'-TTGTGCGAGAGTCTGGCGATTCT-3'
		Reverse	5'-AGGAATCGCTCGGTGGTGAAGTA-3'
Cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*)	NM_174304	Forward	5’-TGTGGCCCCTACGCTGAT-3’
		Reverse	5’-CGCCAATGCTGGAGTCAAT-3’

Target genes and abbreviations	NCBI Sequence	Primer	Primer Sequence
Cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*)	NM_174305	Forward	5'-GTCCGAAGTTGTGCCTATTGCCAGC-3'
		Reverse	5'-CCTCCAGCCTGTCCAGATGCTTGG – 3'
Estrogen receptor 1 (*ESR1*)	NM_001001443	Forward	5’-AGGGAAGCTCCTATTTGCTCC-3’
		Reverse	5’-CGGTGGATGTGGTCCTTCTCT-3’
Fibroblast growth factor 2 (*FGF2*)	NM_174056	Forward	5’-GAACGGGGGCTTCTTCCT-3’
		Reverse	5’-CCCAGTTCGTTTCAGTGCC-3’
Prostaglandin E synthase (*PGES*)	NM_174443	Forward	5’-AGGACGCTCAGAGACATGGA-3’
		Reverse	5’-TTCGGTCCGAGGAAAGAGTA-3’
Vascular endothelial growth factor A isoform 121 (*VEGFA121*)	NM_174216	Forward	5’- CCGTCCCATTGAGACCCTG-3’
		Reverse	5’-CGGCTTGTCACAATTTTTCTTGTC-3’

Follicle tissue lysis and RNA extraction were conducted according to the manufacturer's recommendations (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA, USA). Isolated RNA was evaluated for concentration and purity using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A maximum of 2 µg of mRNA was used to synthesize complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA) supplemented with RNase inhibitor (RNase Inhibitor, human placenta, New England BioLabs, Ipswich, MA, USA). Complementary DNA was used for quantitative real-time reverse transcription PCR using a 7500 Real-Time PCR Detection System (Applied Biosciences) with PowerUp™ SYBR™ Green Master Mix (2X; Applied Biosciences). All assays were carried out in triplicate for each target mRNA. The amplification conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

Statistical analyses

Data are presented as percentage mean ± SEM. All statistical analyses were performed using R Version 3.4.3 (https://www.r-project.org/). Normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data were transformed using Tukey’s Ladder of Powers. If transformation did not result in a normalized population, a Kruskal-Wallis rank-sum test was performed. Analysis of variance was applied to parametric data using a general linear mixed model with repeated measures applied for hormone data. The covariance structure that resulted in the smallest Bayesian information criterion was selected from the mixed models. Significance was declared at $P \leq 0.05$.

Results

Progesterone production from follicular tissue (Fig. 2A) changed over time in the culture system ($P < 0.01$) but was not altered by NGF treatment ($P = 0.81$) or treatment by time interactions ($P = 0.54$). Consistently, there were no changes in gene expression of steroidogenic enzymes responsible for the conversion of cholesterol to pregnenolone *(STAR, CYP11A1*; $P \geq 0.34$; Table 2*)* or pregnenolone to progesterone *(HSD3B;* $P = 0.60$, Fig. 2B) after treatment with NGF for 72 h. Testosterone production in NGF-treated follicular tissue (Fig. 2C) was higher than in untreated controls ($P < 0.01$), but not affected by time ($P = 0.54$) or treatment by time interactions ($P = 0.62$). While there was no change in follicular *CYP17A1* expression ($P = 0.31$; Table 2), whose enzyme converts progesterone to androstenedione, NGF treatment upregulated follicular *HSD17B* expression ($P = 0.04$; Fig. 2D), whose enzyme converts androstenedione to testosterone in the theca cells (Fortune, 1986). Testosterone produced by theca cells is typically converted to estradiol in the granulosa cell via the aromatase enzyme (CYP19A1) (21). The results of the current study found that follicular estradiol production (Fig. 2E) was unaffected by NGF treatment ($P = 0.14$), time ($P = 0.60$), or treatment by time interactions ($P = 0.73$). Consistently, follicular *CYP19A1* expression was also unaffected by NGF treatment ($P = 0.53$; Fig. 2F).

Table 2
Fold change in mRNA expression of gonadotropin receptors, angiogenic enzymes, and steroidogenic enzymes in bovine follicle wall tissue preparations treated with 100 ng/mL NGF vs. untreated (Control) for 72 h. Data is presented as mean ± SEM.

Gene names and abbreviations	**Control**	**NGF**	***P*-value**
Steroidogenic acute regulatory protein (*STAR*)	0.17 ± 0.02	0.29 ± 0.1	0.34
Cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*)	17.81 ± 9.2	8.67 ± 5.0	0.40
Cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*)	0.01 ± 0.00	0.01 ± 0.00	0.31
Luteinizing hormone/ choriogonadotropin receptor (*LHCGR*)	0.07 ± 0.01	0.09 ± 0.03	0.41
Follicular stimulating hormone receptor (*FSHR*)	Undetected	Undetected	N/A
Estrogen receptor alpha (*ESR1*)	0.04 ± 0.01	0.05 ± 0.02	0.77

The cultured tissue was derived from follicles destined to ovulate within 24 h based on the synchronization protocol used. Therefore, by 72 h, we expected to see changes consistent with the post-ovulatory follicular to luteal transition. Following ovulation, the LH-receptor-bearing theca cells luteinize and become small luteal cells that secrete progesterone in response to LH signaling (24, 25). On the other hand, FSH-receptor-bearing granulosa cells luteinize and become large luteal cells, losing their FSH receptors (26). Follicular expression of *LHCGR* was unaltered by treatment after 72 h culture ($P = 0.41$), whereas *FSHR* was undetectable by the assay (Table 2). Another change commonly found during this transitional period is increased expression of *ESR1* during follicular growth and early luteal phase (d 1

through 4 of the estrous cycle) (27). We found no increase in the expression of *ESR1* following the 72-h culture period in either treatment group ($P = 0.77$; Table 2).

In cattle, ovarian function depends on a complex remodeling of the vascular system between ovulation and CL development that involves the temporal expression of vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2) (28). Another factor crucial to ovulation and CL formation is PGE, which can be stimulated from bovine theca cells with NGF treatment and is thought to be derived from PGE synthase (PGES) activity (5, 29). The results herein demonstrated that NGF treatment down-regulated expression of *FGF2* ($P = 0.02$; Fig. 3A) but did not alter *VEGFA121* (P = 0.44; Fig. 3B) or *PGES* ($P = 0.63$; Fig. 3C) expression in bovine follicle tissue.

Discussion

The current study evaluated the direct effects of NGF, purified from bull seminal plasma, on bovine pre-ovulatory follicle tissue. In a previous study, androstenedione and progesterone production was increased in isolated bovine theca cells treated with recombinant NGF (5). Androstenedione is converted to testosterone in the theca cells by the enzyme hydroxysteroid 17-beta dehydrogenase (HSD17B) (21), both of which were increased in the current study. Despite the increased production of its testosterone precursor, estradiol concentrations and aromatase enzyme gene expression (*CYP19A1*) were unaffected by NGF treatment. Previously, the highest expression of *ESR1* was observed in the theca interna cells of growing pre-ovulatory follicles in the bovine ovary and thought to provide a local feedback loop, where estradiol produced by granulosa cells could be used to further stimulate theca cell androgen production (27). The current study did not find an effect of NGF treatment of *ESR1* expression in bovine follicular tissue, further suggesting that this feedback loop is not the potential signaling mechanism for NGF-induced androgen production. However, NGF stimulated proliferation of theca cells from bovine pre-ovulatory follicles (5). Therefore, the increase in testosterone production observed herein could be due to an increase in theca cell number, since estradiol production by the granulosa cells was unaffected.

Treatment of follicle tissue with NGF did not alter the expression of other steroidogenic enzymes or progesterone production, which serves as a precursor for androstenedione production in the preovulatory follicle (21). This is not in agreement with a previous observation of increased theca cell progesterone production in response to NGF treatment (5). One difference between studies is that our culture media contained gonadotropins (LH/FSH) in both control and NGF wells, attempting to emulate the pre-ovulatory cascade, whereas the previous study treated only the control wells with gonadotropins (5). Supplementing culture media with LH and FSH stimulated endogenous NGF secretion from medium to large follicles in ewes (8). Therefore, the inclusion of gonadotropins in the control medium, but not in the NGF-treated medium, may confound the outcomes of exogenous NGF supplementation since the ovary is capable of endogenous NGF production in response to gonadotropin signaling. It is also worth noting that human chorionic gonadotropin (hCG) was used to treat control samples previously rather than LH (5). While hCG shares the same receptor with LH, hCG also stimulates different intracellular signaling pathways (30), which could alter its downstream effects.

Interestingly, there was a downregulation in gene expression of *FGF2* and no change in gene expression of *VEGFA121* in follicle tissue treated with NGF. Both VEGFA and FGF2 promote vascular supply growth during follicular to luteal transition in the cow ovary, with resulting changes in their localization patterns (31, 32). Follicular *FGF2* mRNA and FGF2 protein increased around 4 h after GnRH administration in cows, corresponding with the LH surge (33). Immediately following the LH surge, FGF2 stimulates the migration and proliferation of endothelial cells that help to establish luteal blood flow (28). During early CL formation, FGF2 concentrations decrease while the capillary beds are reconstructed to establish blood flow (28). In contrast, VEGFA concentrations remain high throughout ovulation and CL development to support endothelial cell survival (28). Given that the assays were performed after 72 h in culture, it is possible that the timing of this reconstruction phase was hastened by NGF treatment, which may account for the observed decrease in *FGF2* expression. Future studies assessing the temporal expression of these angiogenic enzymes are warranted to clarify exactly how NGF could alter the follicular to luteal transition.

Previously, NGF treatment stimulated PGE release for up to 8 h in theca cells extracted from bovine preovulatory follicles (5). Prostaglandin E_2 is synthesized by PGES and acts as a pro-angiogenic molecule in vascular endothelium by recruiting the paracrine-autocrine mechanism characteristic of endothelium cells, resulting in vascular remodeling (34). Prostaglandin E_2 also supports luteal progesterone production in cattle (35), potentially through increased CL vascularity (36). Consistently, one study observed higher *PGES* mRNA and PGES protein levels in the CL of early pregnancy (days 20 to 30) than in the luteal phase (days 8 to 12 of the estrous cycle) or after 40 days gestation of artificially inseminated cows (37). To our surprise, NGF treatment did not enhance follicular expression of *PGES* in the current study. However, HSD17B enzymes have also been found to play a role in the synthesis of arachidonic acid and its downstream eicosanoid metabolites, such as PGE (38). Additionally, HSD17B-knockout female mice failed to initiate pseudopregnancy after being mated by sterile males despite exhibiting normal cycles (39), suggesting a crucial role of this enzyme in CL development. Therefore, NGF may influence ovulation and CL development indirectly through its effects on ovarian HSD17B enzyme activity.

After ovulation, theca cells differentiate into small luteal cells that, in response to LH binding, produce an early rise in progesterone that is essential for supporting initial embryonic growth (40). Though we found no differences in expression of *LHCGR* in the current study, it would be worthwhile to evaluate its expression *in vivo* to determine if there are downstream effects on the presence of small luteal cells in the mature bovine CL. This finding may explain how systemic administration of NGF can improve CL development and function in cattle (12, 14, 15, 41).

Concluding Remarks

In conclusion, the results of the current study demonstrated that purified bovine NGF could act directly on the theca and granulosa cells of the bovine pre-ovulatory follicle to stimulate testosterone production, which may be secondary to theca cell proliferation. Additionally, decreased *FGF2* expression in NGF-

treated tissue suggests hastened onset of tissue remodeling that occurs during early luteal development. Further studies are warranted to unravel the putative roles of NGF in ovulation and luteal formation in cattle.

Abbreviations

CL = Corpus luteum

CYP11A1 = Cytochrome P450 family 11 subfamily A member 1

CYP17A1 = Cytochrome P450 family 17 subfamily A member 1

CYP19A1 = Cytochrome P450 family 19 subfamily A member 1

ESR1 = Estrogen receptor

FSH = Follicle stimulating hormone

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

HSD3B = Hydroxyl-delta-5-steroid dehydrogenase 3-beta

HSD17B = Hydroxysteroid 17-beta dehydrogenase

LH = Luteinizing hormone

LHCGR = LH/choriogonadotropin receptor

NGF = Nerve growth factor-β

PGE = Prostaglandin E2

PGES = PGE synthase

$PGF_{2\alpha}$= Prostaglandin $F_{2\alpha}$

RPL15 – Ribosomal protein L15

RPL19 – Ribosomal protein L19

StAR = Steroidogenic acute regulatory protein

VEGFA121 = Vascular endothelial growth factor A isoform 121

Declarations

Ethics approval

All experimental procedures in the present study were conducted from July to August 2018 at the College of Veterinary Medicine, University of Illinois Urbana-Champaign. The Institutional Animal Care approved all the animal procedures and Use Committees of the University of Illinois at Urbana-Champaign, USA (Protocol #18223).

Consent for publication

Not applicable.

Availability of data and materials

All data is fully present in the manuscript and any details will be made available per request.

Competing interest

The authors declare no competing interests.

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Author's contribution

JLS, JAF, IFC, and FSL contributed to conception and design of the work. JLS, IFC, and FSL contributed to sample colelction. JLS and LG contributed to culture theca and granulosa cells. VRGM and NWD contributed to hormonal assay analysis. JLS contributed to real-time PCR analysis. JLS, IFC and FSL contributed to data analysis and interpretation. JLS prepared the manauscript. All author read and contributed to final version submitted for publication.

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Figures

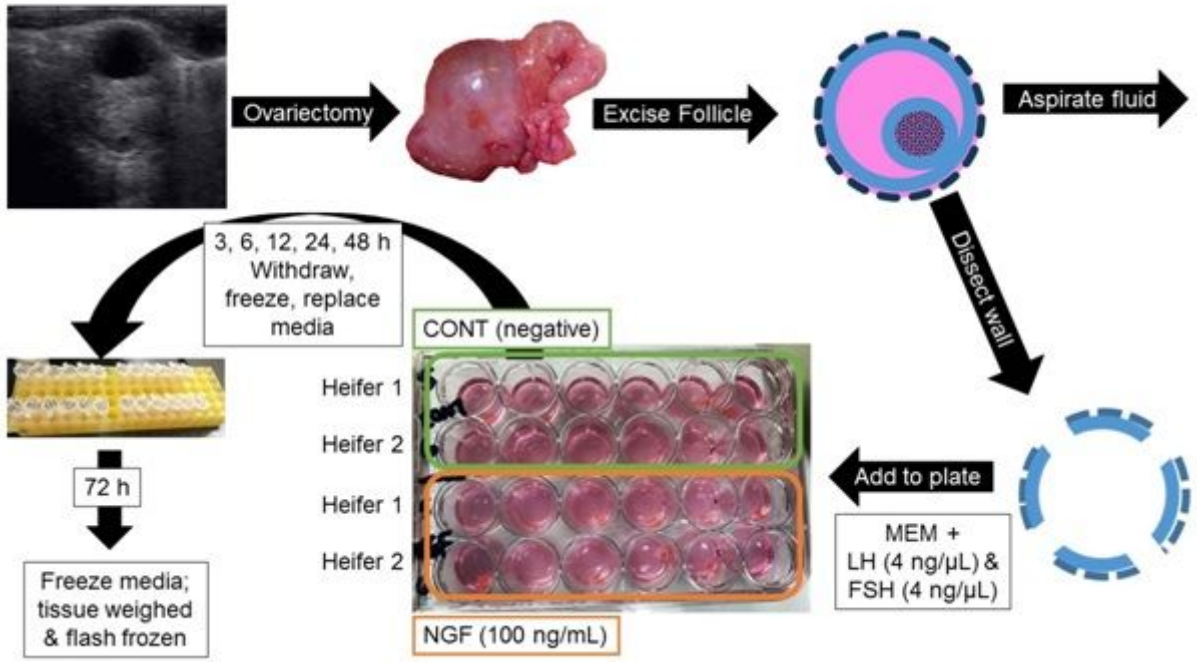

Figure 1

Graphical depiction of the methodology used to assess the effects of NGF, purified from bull seminal plasma, on the bovine pre-ovulatory follicle.

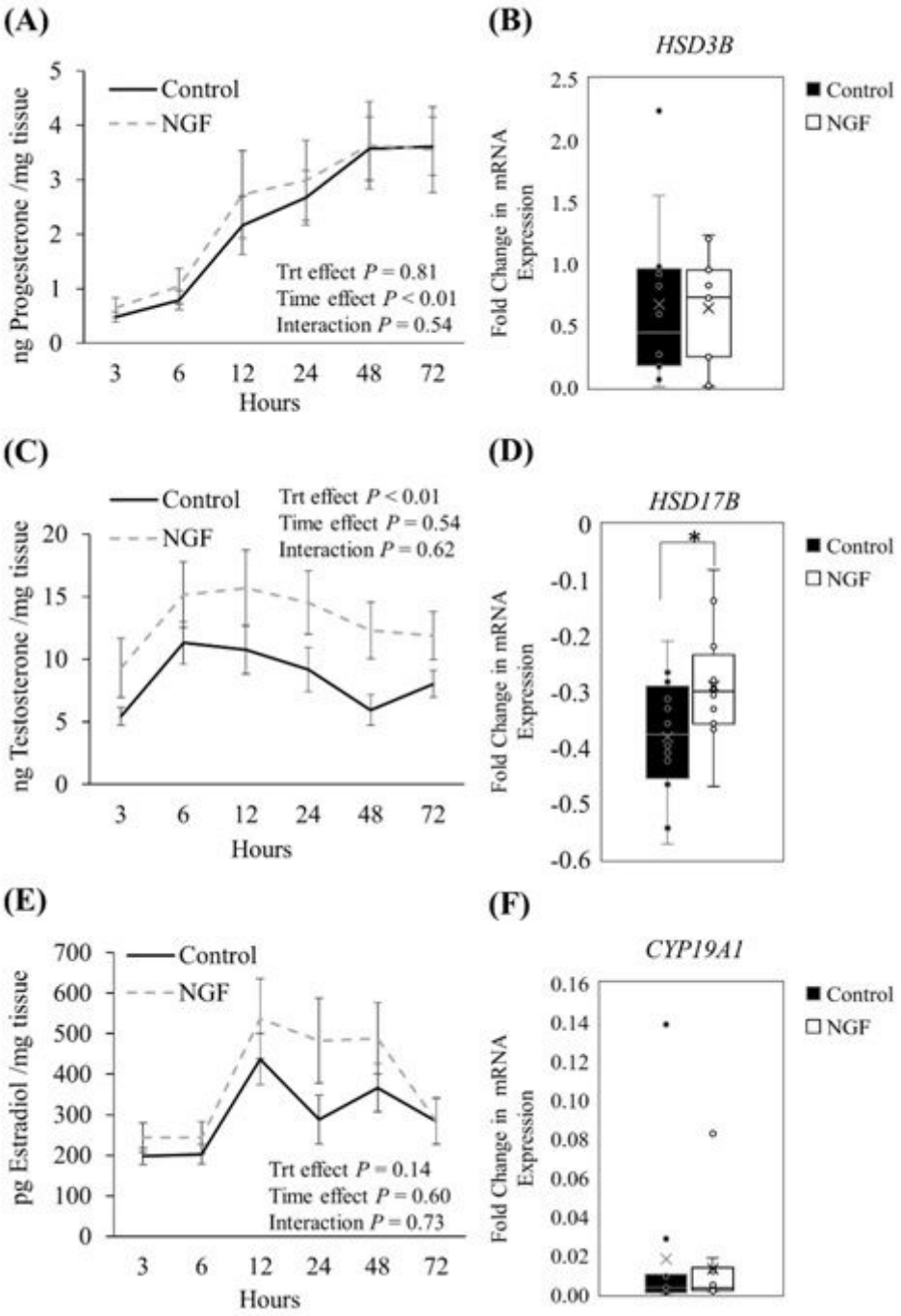

Figure 2

Steroid hormone production (A, C, E) and steroidogenic enzyme gene expression (B, D, F) in follicle tissue untreated (control) or treated with 100 ng/mL NGF for 72 h. Hormones are presented as mean ± SEM, whereas genes are presented as box-and-whisker plots. * P ≤ 0.05.

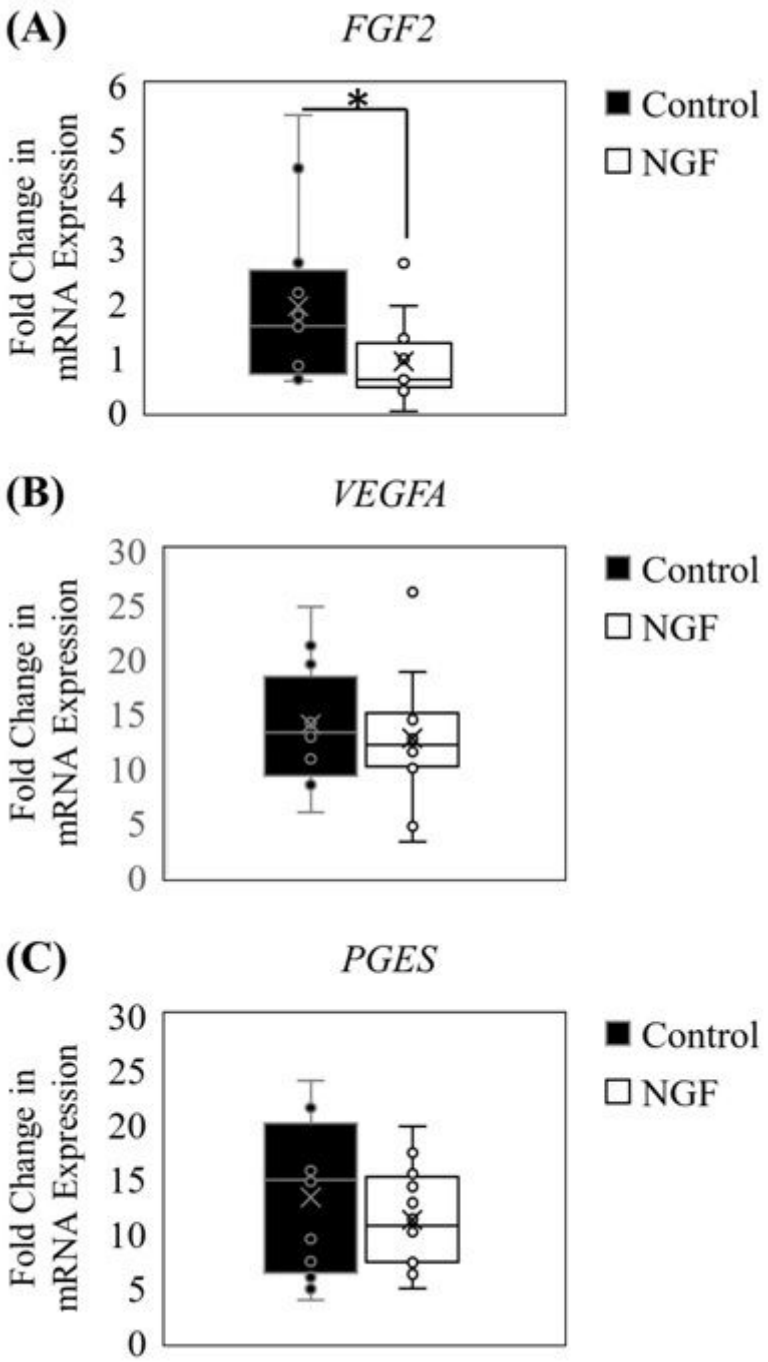

Figure 3

Box-and-whisker plots demonstrating gene expression of angiogenic enzymes in follicle tissue untreated (control) or treated with 100 ng/mL NGF for 72 h. * $P \leq 0.05$.