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Granulosa cell-conditioned medium enhances steroidogenic competence of buffalo (*Bubalus bubalis*) theca cells

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Abstract

Granulosa cells (GCs) and theca cells (TCs) are the main components of follicles, and the interactions between GCs and TCs play a significant role in steroidogenesis, follicular growth, and atresia. However, the effects of GCs in the form of conditioned medium on steroidogenesis in buffalo TCs remain unclear. In the present study, the impacts of GC-conditioned medium (GCCM) on androgen synthesis in buffalo TCs were examined. The results showed that GCCM collected at 48 h promoted both the expression levels of androgen synthesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) and the secretion levels of testosterone in TCs. The treatment time of 48 h in GCCM improved both the expression levels of androgen synthesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) and the secretion levels of testosterone in TCs. Furthermore, GCCM that was collected at 48 h and applied to TCs for 48 h (48 h and 48 h) promoted the sensitivity of buffalo TCs to LH. This study indicated that GCCM (48 h and 48 h) enhanced the steroidogenic competence of TCs mainly through facilitating the responsiveness of TCs to LH in buffalo. This study provides a basis for further exploration of interactions between GCs and TCs for steroidogenesis in the ovary.

Keywords Buffalo · Granulosa cells · Conditioned medium · Theca cells · Steroidogenesis

Introduction

Oocytes, granulosa cells (GCs), and theca cells (TCs) are the main components of follicles, and the interactions among them play an essential role in steroidogenesis, follicular development, and atresia (Orisaka *et al.* 2009). Oocyte-granulosa cell communications, largely carried out by gap junctions, are necessary for follicular growth and oocyte maturation in vivo. Some paracrine factors such as natriuretic peptide type C (CNP), brain-derived neurotrophic factor (BDNF), and insulin-like growth factors-I (IGF-I) are specifically secreted by GCs (Zhang *et al.* 2010; Oberlender *et al.* 2013; Zhao *et al.* 2019; Rouhollahi Varnosfaderani *et al.* 2020). These factors regulate follicular development and contribute to the modulation of oocyte maturation. However, unlike that of GCs and oocytes, the interactions between GCs and TCs in follicles have not been extensively explored. Therefore, it is important to understand more about the relationship between GCs and TCs in follicular development.

It is generally accepted that TCs provide androgen for estrogen biosynthesis in GCs, which stimulates the early follicular development (Wang *et al.* 2001; Stocco *et al.* 2007; Tajima *et al.* 2007; Gervasio *et al.* 2014). Previous studies have also shown that TCs played a crucial role in proliferation, differentiation, and apoptosis of GCs during follicle maturation (Kotsuji *et al.* 1990; Kotsuji and Tominaga 1994). TCs regulated the fate of GCs throughout follicular growth process by secreting factors that suppressed apoptosis (Yada *et al.* 1999; Tajima *et al.* 2002). However, the effects of GCs on TCs, especially on androgen production, are not known very well. Therefore, the effects of GCs on androgen synthesis in TCs need to be further studied in the future.

In vitro studies have shown that, as a core feature of TCs, the increment of androgen production was driven by LH in a dose-dependent manner (Stewart *et al.* 1995; Campbell *et al.* 1998). Although these findings demonstrated that increased LH secretion may be pivotal for the up-regulation of androgen synthesis, other studies showed that growth factors, plating density, glucose utilization, and co-culture with GCs also affected the competence of androgen production in TCs (Caubo *et al.* 1989; Morley *et al.* 1989; Cara *et al.* 1990; Roberts and

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Skinner 1990a; Allegrucci *et al.* 2003; Magoffin and Weitsman 1993a; Magoffin and Weitsman 1993b; Simone and Mahesh 1993; Stewart *et al.* 1995; Liu *et al.* 2015). There is a complex network of signals on the regulation of androgen synthesis in TCs under different conditions. As far as we know, GC-conditioned medium (GCCM) contains hormones, paracrine factors, and cytokines that are secreted by GCs, which can mimic to a certain extent the local ovarian steroidogenesis by supporting the similar structural integrity of follicles. Thus, further systematic research focusing on the effects of GCCM on androgen production in TCs is warranted.

As one of the leading domestic animals in southern China, buffalo (*Bubalus bubalis*) is widely used for the plow, meat, and milk (Deshun *et al.* 2007; Lu *et al.* 2018; Deng *et al.* 2019; Zhang *et al.* 2020). To our knowledge, the effects of GCCM on androgen synthesis in buffalo TCs have not been reported. Additionally, the mechanism of action underlying the influences of GCCM on steroidogenesis in TCs is likewise unknown. In this study, the effects of GCCM on androgen production competence of buffalo TCs, including the expression levels of androgen synthesis-related genes and the secretion levels of androgen, were studied. This study not only provides a new insight for better understanding of steroidogenesis but also provides a basis for further exploration of ovarian endocrine mechanism.

Materials and Methods

Reagents and Culture Medium All of the chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), except for fetal bovine serum (FBS), which was purchased from Gibco (Carlsbad, CA). The complete cell medium contained high-glucose Dulbecco's modified Eagle's medium (DMEM) and was supplemented with 10% FBS, 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin.

Isolation and Culture of GCs Buffalo ovaries were collected from a local commercial slaughterhouse and transported to the laboratory at 37 °C in physiological saline (0.9% NaCl) within 4 h, then were disinfected with 75% ethanol, and cleaned with physiological saline for 2–3 times. GCs were aspirated from ovarian antral follicles, which ranged from 2 to 6 mm in diameter, with an 18-gauge needle attached to a disposable 10-mL syringe. GCs were then washed three times with phosphate buffered saline (PBS), centrifuged at 300 × *g* for 10 min, and immediately retrieved for cell culture. Primary GCs were inoculated with culture medium (DMEM + 10% FBS) in 12-well plates at a density of 10,000 cells per well at 38.5 °C in a 100% humidified atmosphere of 5% CO_2. The culture medium was replaced on Day 1. When GCs reached 80–90% confluency on Days 3–4, cells were passaged at 12-well plates (Corning Incorporated, Corning, NY) for the following experiments. In order to maintain the consistency of the experimental results, buffalo GCs at passage 1 were used as the cell resources for all experiments in this study.

Collection of GCCM Buffalo GCs at passage 1 were cultured up to 80% confluency in 12-well plates, washed three times with PBS, and incubated with culture medium (DMEM + 10% FBS, with 1 mL medium added per well) at 38.5 °C with 100% humidity and 5% CO_2. GCCM was collected at 24 h, 48 h, and 72 h of culture (1 mL per well) and centrifuged at 300 × *g* for 10 min. The culture medium was not replaced during the process of collecting GCCM. The conditioned medium was then filtered through a 0.2-μm membrane and stored for less than 2 wk at 4 °C until further use.

Isolation and Culture of TCs The antral follicles that were healthy, highly vascularized, and more than 8 mm in diameter were chosen to isolated buffalo TCs for the present research. After collection of follicles, they were cut into two halves by using aseptic scissors. The theca interna layers were peeled off with forceps and washed in PBS by passing them repeatedly through a 1-mL syringe. The layers were then treated with 0.25% trypsin for 10 min to remove any adhering GCs and washed three times with PBS. Cleaned thecal tissues were mechanically disrupted and then enzymatically digested with sterile 0.2% collagenase type II at 37 °C for 45 min with continuous shaking, and digestion was terminated by the addition of complete medium containing FBS. Cells were then filtered through a 40-μm nylon mesh cell strainer (BD Biosciences, San Jose, CA) and separated from pellets by centrifugation. Finally, the cells were resuspended in culture medium (DMEM + 10% FBS) and incubated at 38.5 °C with 100% humidity and 5% CO_2. When buffalo TCs reached 80–90% confluency on Day 5, they were passaged using 0.25% trypsin digestion method or frozen with cryoprotectant of dimethyl sulfoxide (DMSO) for subsequent experiments.

Immunofluorescence Staining Buffalo TCs (primary passage) were seeded in 4-well plates at a density of 5000 cells per well and culture was terminated when cells reached 80% confluency. Immunofluorescence was then performed as follows. (1) Cells were briefly washed three times with PBS, fixed in 4% paraformaldehyde for 30 min, and then washed three times with PBS. (2) Cell membranes were permeabilized with 0.1% TritonX-100 for 15 min and washed three times with PBS. (3) Non-specific antibody binding was blocked by using 5% BSA for 2 h at room temperature followed by washing three times with PBS. (4) Cells were then incubated with rabbit anti-CYP17A1 (1:150; monoclonal; Abcam, Cambridge, MA) and rabbit anti-FSHR (1:150; monoclonal; Abcam) overnight at 4 °C. (5) Cells were washed thrice with PBS the next day and incubated with anti-rabbit secondary

antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature in the dark. The blank control was incubated with secondary antibody only. (6) Nuclei were counterstained with Hoechst 33342 at a final concentration of 5 μM. Finally, immunostaining was assessed using a fluorescence microscope (Nikon, Tokyo, Japan). Purity of buffalo TCs was evaluated as described above, and the primary antibodies were to *CYP17A1* and *FSHR*.

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to product instructions, and cDNA synthesis was performed using PrimeScript TMRT reagent Kit (Osaka, Japan). The cDNA samples were then applied for qRT-PCR using the SYBR Premix Ex Taq^{TM} II (Osaka, Japan). The primer sequences used in this study were displayed in Table 1, and the expression of genes was normalized to that of the internal control gene *GAPDH*. All the experiments were performed in triplicate.

Enzyme-Linked Immunosorbent Assay The enzyme-linked immunosorbent assay (ELISA) was performed to quantify the secretion levels of androgen. The culture media were collected from buffalo TCs, and ELISA was conducted according to the manufacturer's instructions (Jiangsu Jingmei Biological Technology Co., Ltd, Jiangsu, China). The absorbance (A450) was measured using a plate reader (Tecan, Mannedorf, Switzerland), and the readouts were adjusted for the total content of cellular hormone. All of the experiments were performed in triplicate.

Experimental Design Experiment 1: the Effects of GCCM Collection Time on the Expression Levels of Androgen Synthesis-Related Genes and the Secretion Levels of Androgen in Buffalo TCs

The aim of this experiment was to select the optimal collection time for GCCM. Buffalo TCs at passage 3 were cultured in GCCM that was recovered at different collection times (0 h, 24 h, 48 h, and 72 h) for 48 h, and then, the expression levels of androgen synthesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) and the secretion levels of testosterone in TCs were assessed to select the optimum collection time for GCCM.

Experiment 2: the Effects of GCCM Treatment Time on the Expression Levels of Androgen Synthesis-Related Genes and the Secretion Levels of Androgen in Buffalo TCs

The objective of this experiment was to explore the optimal treatment time for GCCM. Buffalo TCs at passage 3 were cultured in GCCM that was collected at 48 h for different treatment times (0 h, 24 h, 48 h, and 72 h), and then, the expression levels of androgen synthesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) and the secretion levels of testosterone in TCs were evaluated to select the optimum treatment time for GCCM.

Statistical Analysis All of the data were presented as the mean ± standard error. Statistical significance was determined using a one-way analysis of variance followed by Student's *t* test. All of the statistical analyses were performed by using the SPSS 22.0 software. P values < 0.05 were deemed to be significant.

Results

Isolation, Culture, and Identification of Buffalo TCs Buffalo TCs were isolated from antral follicles (Fig. 1*a*) using the collagenase type II digestion method. Most of primary cells adhered to plates and exhibited thin and dendritic, indicating their TC origin. Buffalo TCs still remained the original

Table 1. Primers for qRT-PCR

Gene	Primers and probe	Temperature (°C)	Length (bp)
CYP11A1	F: 5′- - 3′GACGTGGCCCATCTCTTCAA	60	144
	R: 5′- - 3′GACCACCCGGTCTTTCTTCC		
CYP17A1	F: 5′- - 3′CTATAGGGGACATCTTCGGGG	60	199
	R: 5′- - 3′AGCACTTCTCTGATGGTCGC		
3β-HSD	F: 5′- - 3′CAGCCAGGTATGGCCGAC	60	89
	R: 5′- - 3′CGGACTACATGTTCCCCCAG		
Star	F: 5′- - 3′AAAGTGATCCCTGACGTGGG	60	175
	R: 5′- - 3′CGTGAGTGATGACCGTGTCT		
LHCGR	F: 5′- - 3′ACCAAAGGCCAGTATTACAACC	60	185
	R: 5′- - 3′GCATGTTTCAGTCGCAGCTT		
GAPDH	F: 5′- - 3′ACAGTCAAGGCAGAGAACGG	60	98
	R: 5′- - 3′CCAGCATCACCCCACTTGAT		

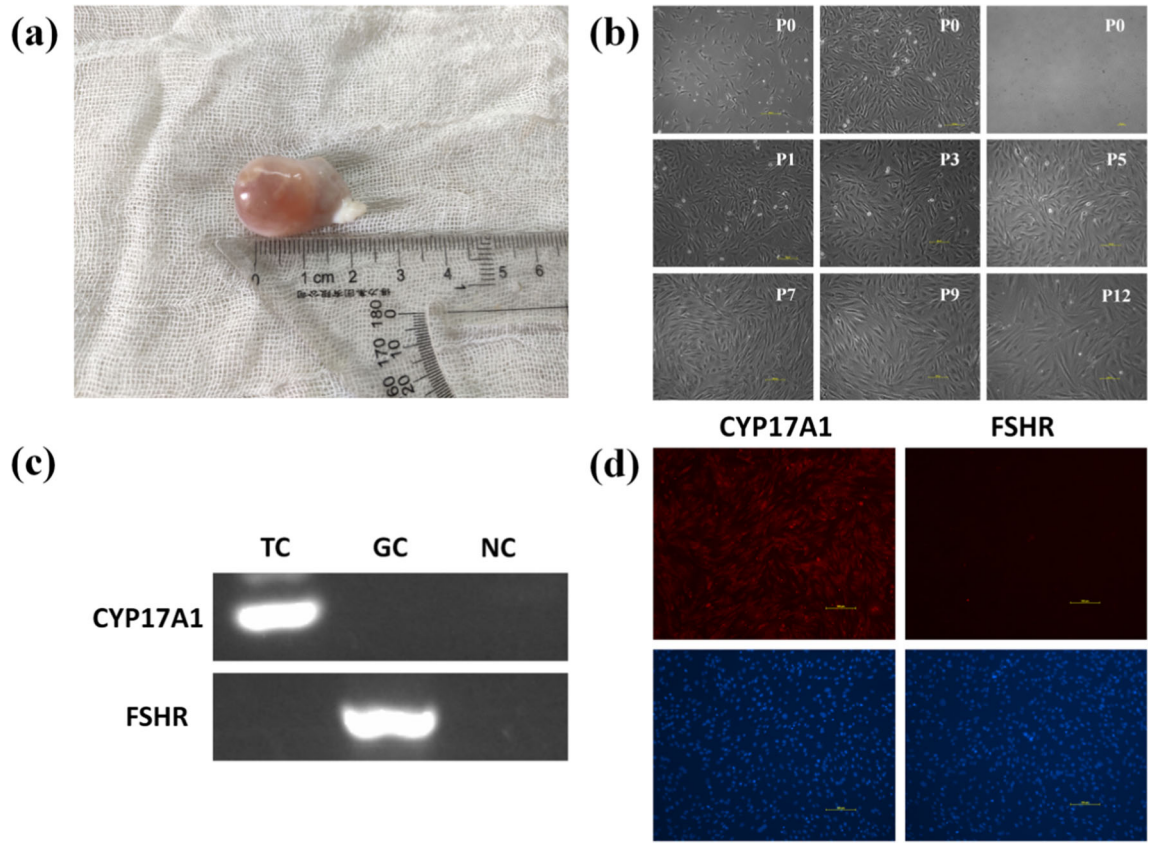

Figure 1. Isolation, culture, and identification of buffalo TCs. (*a*) Ovarian follicles ranging from 8 to 10 mm in diameter were used to peel off the theca interna layers mechanically. (*b*) The morphology of buffalo TCs from primary to passage 12 which were derived from collagenase type II digestion method. (*c*) RT-PCR showed that *CYP17A1*, not *FSHR*, was expressed in buffalo TCs. (*d*) Immunohistochemical analysis indicated that buffalo TCs accounted for 97.81 ± 1.72% of all the cells. NC: the results of no template control; *scale bars* = 100 μm.

morphologic features of TCs when passaged over ten times (Fig. 1*b*).

By identification, buffalo TCs presented typical biologic characteristics of TCs in vitro. RT-PCR results showed that buffalo TCs were positive for TC marker *CYP17A1*, but negative for GC marker *FSHR* (Fig. 1*c*). Besides, immunohistochemical analysis indicated that more than 95% of the cells were stained positive for *CYP17A1*, confirming these cells' TC origin. As *FSHR* is a GC-specific marker, but not TC-specific marker, it was used to confirm that the extracted cells were TCs and contained almost no GCs by reverse identification. The results of control experiments using *FSHR* antibody did not show significant fluorescence signal, indicating that these TC cultures were largely free of GC contamination (Fig. 1*d*). These results demonstrated that the cells that isolated from buffalo antral follicles by collagenase type II digestion were high-purity TCs, which can be used as reliable cell resources for the following research.

The Effects of GCCM Collection Time on the Expression Levels of Androgen Synthesis-Related Genes in Buffalo TCs The effects of GCCM collection time on androgen synthesis in TCs were investigated by testing the expression levels of steroidogenesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) through qRT-PCR. The results showed that the expression levels of genes *CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star* were significantly ($P < 0.05$) up-regulated in TCs with GCCM that was collected at 48 h relative to the control group (Fig. 2). These results suggested that GCCM collected at 48 h improved the expression levels of androgen synthesis-related genes in buffalo TCs.

The Effects of GCCM Collection Time on the Secretion Levels of Androgen in Buffalo TCs The effects of GCCM collection time on the secretion levels of androgen in TCs were evaluated through examining the testosterone secretion levels in the spent medium of GCCM-treated and GCCM-untreated groups by ELISA. The results showed that the testosterone secretion levels of TCs were significantly ($P < 0.05$) higher with GCCM that was collected at 48 h compared with fresh medium (Fig. 3). These results demonstrated that GCCM collected at 48 h promoted the secretion levels of testosterone in buffalo TCs.

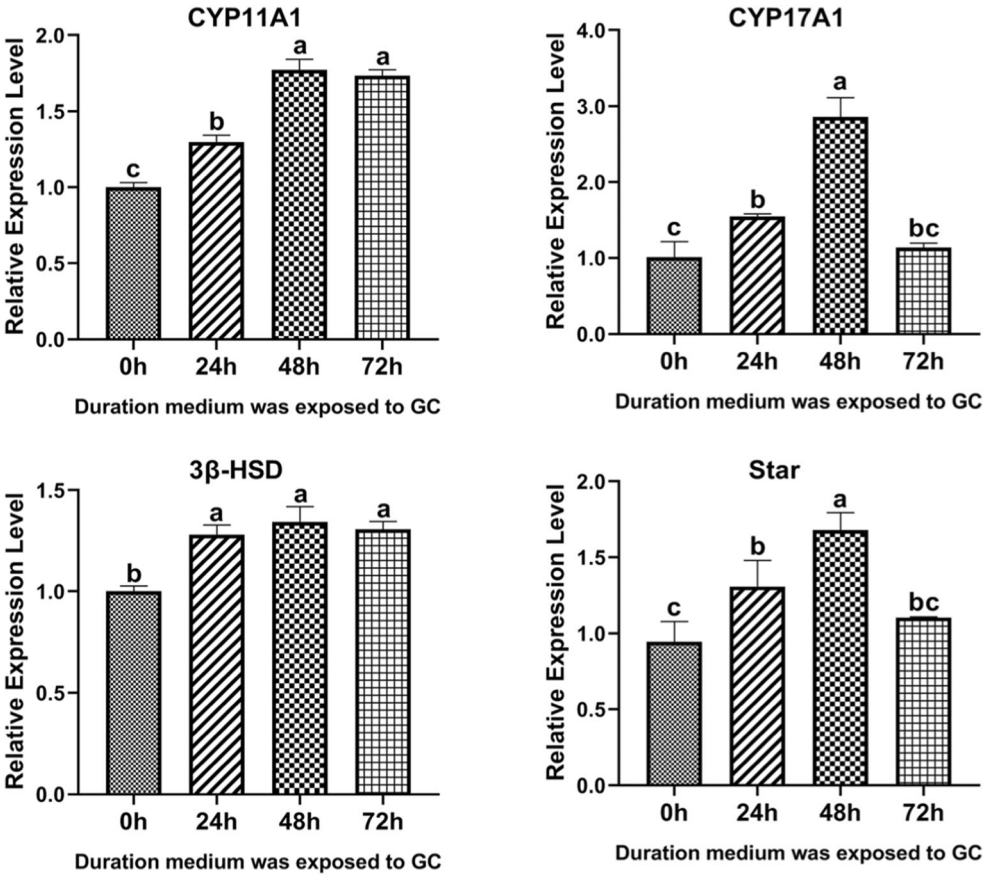

Figure 2. qRT-PCR analyzed the expression levels of androgen synthesis-related genes *CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star* in buffalo TCs. 0 h: buffalo TCs were treated without GCCM; 24 h, 48 h, and 72 h: buffalo TCs were treated with GCCM that was collected at 24 h, 48 h, and 72 h. *GAPDH* expression was used as a standard; marking different *letters* on the *histogram* indicated a significant difference ($P < 0.05$).

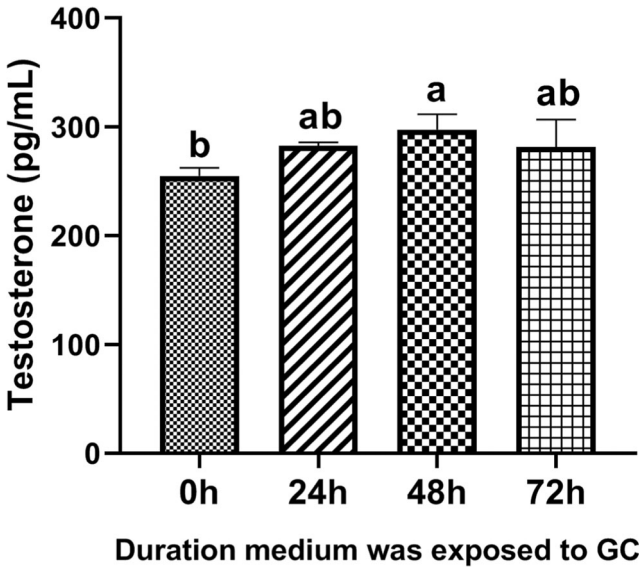

Figure 3. ELISA analyzed the secretion levels of testosterone in buffalo TCs. 0 h: the testosterone levels in GCCM that had been not used to culture buffalo TCs; 24 h, 48 h, and 72 h: the testosterone levels in the spent medium of buffalo TCs treated with GCCM that was collected at 24 h, 48 h, and 72 h. The results were the mean ± SE of three independent experiments. Marking different *letters* on the *histogram* indicated a significant difference ($P < 0.05$).

The Effects of GCCM Treatment Time on the Expression Levels of Androgen Synthesis-Related Genes in Buffalo TCs The effects of GCCM treatment time on androgen synthesis in TCs were investigated by testing the expression levels of steroidogenesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) through qRT-PCR. The results showed that the expression levels of genes *CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star* were significantly ($P < 0.05$) up-regulated in TCs that were treated with GCCM for 48 h relative to the control group (Fig. 4). These results suggested that the treatment time of 48 h with GCCM improved the expression levels of androgen synthesis-related genes in buffalo TCs.

The Effects of GCCM Treatment Time on the Secretion Levels of Androgen in Buffalo TCs The effects of GCCM treatment time on the secretion levels of androgen in TCs were evaluated through examining the testosterone secretion levels in the spent medium of GCCM-treated and GCCM-untreated groups by ELISA. The results showed that the testosterone secretion levels of TCs treated with GCCM for 48 h or 72 h were significantly ($P < 0.05$) higher compared with fresh medium (Fig. 5). These results demonstrated that the treatment time of

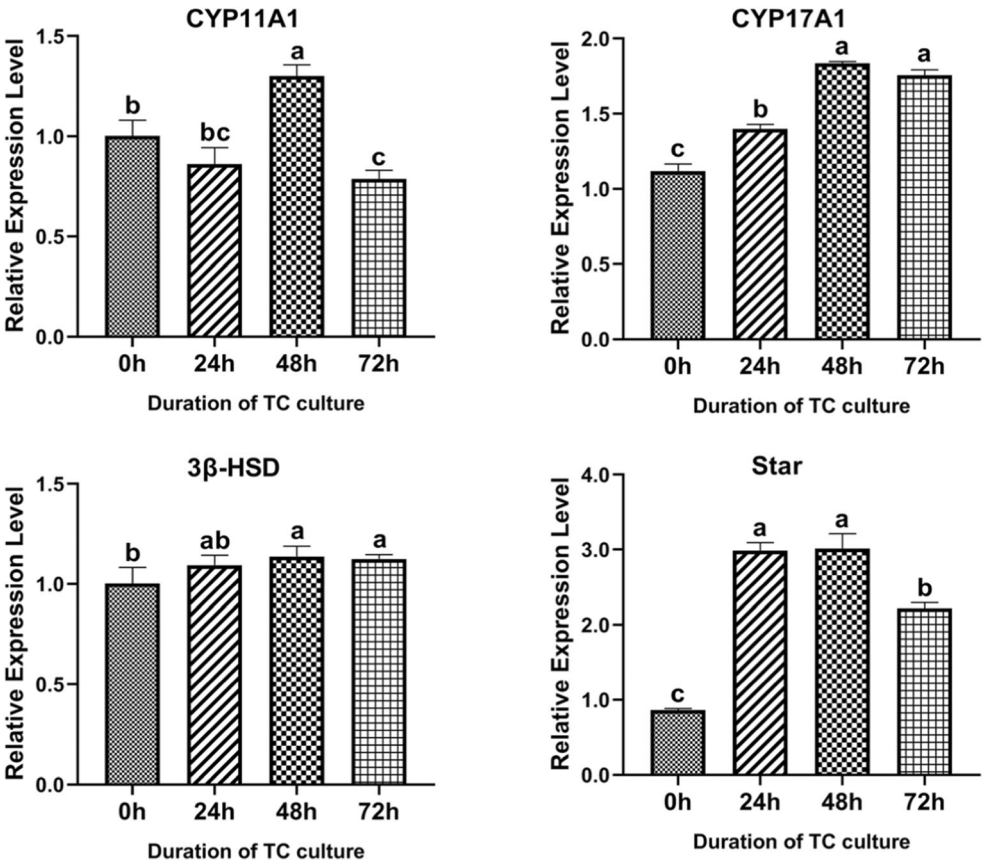

Figure 4. qRT-PCR analyzed the expression levels of androgen synthesis-related genes *CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star* in buffalo TCs. 0 h: buffalo TCs were treated without GCCM; 24 h, 48 h, and 72 h: buffalo TCs were treated with GCCM for 24 h, 48 h, and 72 h. *GAPDH* expression was used as a standard; marking different *letters* on the *histogram* indicated a significant difference ($P < 0.05$).

48 h or 72 h with GCCM promoted the secretion levels of testosterone in buffalo TCs.

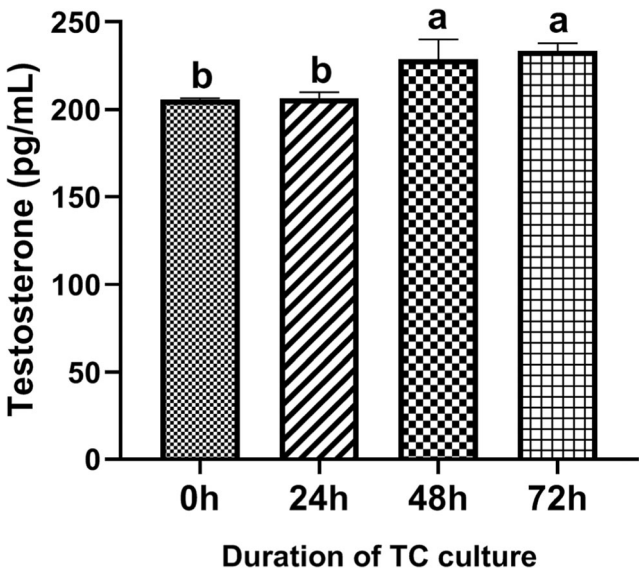

Figure 5. ELISA analyzed the secretion levels of testosterone in buffalo TCs. 0 h: the testosterone levels in GCCM that had not been used to culture buffalo TCs; 24 h, 48 h, and 72 h: the testosterone levels in the spent medium of buffalo TCs treated with GCCM for 24 h, 48 h, and 72 h. The results were the mean ± SE of three independent experiments. Marking different *letters* on the *histogram* indicated a significant difference ($P < 0.05$).

GCCM Enhances the Sensitivity of TCs to LH in Buffalo The effects of GCCM that was collected at 48 h and applied to TCs for 48 h (48 h and 48 h) on the responsiveness of TCs to LH were analyzed by testing the expression levels of gene LH receptor (*LHCGR*) in TCs through qRT-PCR. The results showed that the expression levels of gene *LHCGR* were significantly ($P < 0.05$) up-regulated in TCs that were treated with GCCM (48 h and 48 h) (Fig. 6). These results suggested that GCCM (48 h and 48 h) improved the expression levels of gene *LHCGR* in buffalo TCs.

To further prove that the sensitivity of TCs to LH was changed in GCCM, a dose-response experiment was conducted to compare the ED_{50} value of LH for testosterone production in TCs cultured with and without GCCM. The results showed that the ED_{50} value of LH for TCs with GCCM (ED_{50} value 0.03 μg/L) was significantly ($P < 0.05$) lowered for testosterone production relative to the control group (ED_{50} value 0.1 μg/L) (Fig. 7). These results indicated that GCCM promoted the responsiveness of TCs to LH in buffalo.

Discussion

GCs and TCs are important cell types in follicles and the interactions between GCs and TCs play a crucial role in steroidogenesis, follicular development, and atresia (Orisaka

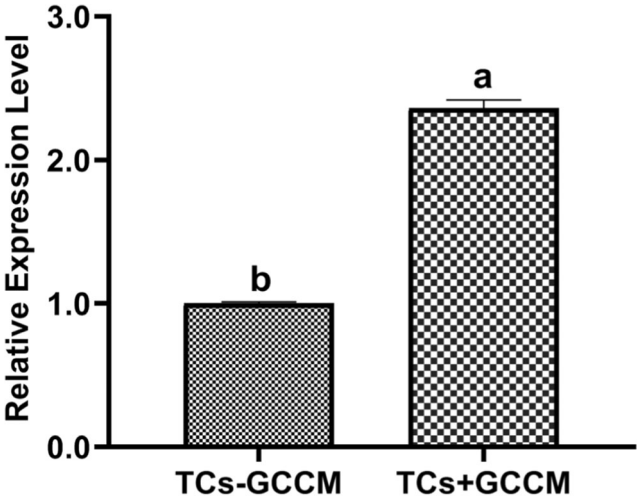

Figure 6. qRT-PCR analyzed the expression levels of gene *LHCGR* in buffalo TCs. TCs − GCCM: buffalo TCs were treated without GCCM; TCs + GCCM: buffalo TCs were treated with GCCM that was collected at 48 h and applied to TCs for 48 h. *GAPDH* expression was used as a standard; marking different letters on the histogram indicated a significant difference ($P < 0.05$).

et al. 2006; Orisaka *et al.* 2009; Young and McNeilly 2010; Richards *et al.* 2018). At present, there are few reports about the interactions between GCs and TCs, especially with respect to the effects of GCs on steroidogenesis of TCs in buffalo. Although previous studies have shown that co-culture with GCs promoted steroidogenic capacity of TCs, the effects of GCCM on androgen synthesis in TCs remained unknown. Thus, further detailed studies are needed. In this study, the effects of GCCM on steroidogenesis in buffalo TCs were investigated. The results showed that GCCM (48 h and 48 h) improved the competence of androgen production in buffalo TCs. These results were in agreement with the findings of other researchers who reported that co-culture with GCs promoted androgen synthesis in TCs of mouse and bovine (Allegrucci *et al.* 2003; Liu *et al.* 2015).

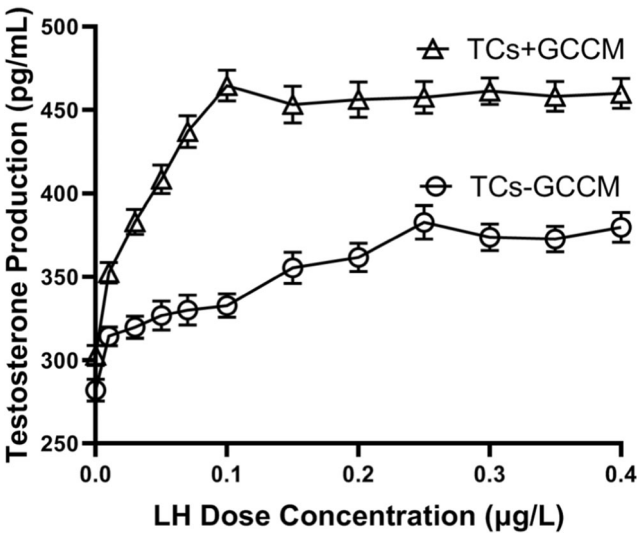

Figure 7. A dose-response experiment compared the ED50 value of LH for testosterone production in TCs with and without GCCM in buffalo. TCs − GCCM: buffalo TCs were treated without GCCM; TCs + GCCM: buffalo TCs were treated with GCCM that was collected at 48 h and applied to TCs for 48 h. Data (mean ± SE) were derived from three independent replicate experiments.

On the basis of the two-cell/two-gonadotropin theory of steroid synthesis, androgens are produced by steroidogenic enzymes in TCs and then subsequently aromatized to estrogens in GCs (Drummond 2006). Under the regulation of LH, TCs express genes that are involved in steroidogenesis including *CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star* which regulate androgen production (Gangloff *et al.* 2002; Magoffin 2005; Lin *et al.* 2006), and of these genes, *CYP17A1* encodes the rate-limiting enzyme of androgen biosynthesis (Richards *et al.* 2018; Słomczyñska and Tabarowski 2001). In the present study, GCCM was used to explore the effects of GCs on androgen production in buffalo TCs. Our results showed that GCCM (48 h and 48 h) improved the expression levels of androgen synthesis-related genes (*CYP11A1*, *CYP17A1*, *3β-HSD*, and *Star*) in buffalo TCs. These results were in accordance with the studies of the effects of GCs on steroidogenesis of TCs in mice (Liu *et al.* 2015).

Previous studies reported that GCs could promote ovarian interstitial cells recruitment to form theca cell layers around the follicles and that inhibin secreted from GCs could induce maturation of dominant follicles and production of androgen (Parrott and Skinner 2000). Besides, it has been confirmed that estradiol secreted by GCs could induce an increased secretion of androstenedione in TCs (Roberts and Skinner 1990b; Wrathall and Knight 1995; Webb *et al.* 1999). These findings suggested that GCs probably provided some essential regulating factors for TCs on steroidogenesis, such as steroids, growth factors, cytokines, and extracellular matrix, and maintained the ability of TCs for androgen synthesis by paracrine regulation. In our study, there was 10% FBS in GCCM, which meant that many nutrients, growth factors, and steroids could be altered, degraded, or decreased in concentration in GCCM. Thus, GCCM can mimic to a certain extent the microenvironment of follicles. In this study, the results showed that TCs with GCCM produced an increased amount of testosterone, which were similar to the findings of other researchers to a certain degree (Fortune 1986; Allegrucci *et al.* 2003).

It is generally accepted that LH sensitivity and responses to LH on androgen synthesis are two established functions of TCs in the ovary (Alviggi *et al.* 2006; Wickenheisser *et al.* 2006; Murayama *et al.* 2012). The capacity of TCs on androgen production and secretion in response to LH appears to be dose-dependent (Stewart *et al.* 1995; Campbell *et al.* 1998). The appropriate dose of LH could regulate the expression of androgen

synthetases and facilitate the transcription of androgen synthesis-related genes, eventually improving the secretion levels of androgens in TCs. To determine the effects of GCCM on androgen production of TCs in response to LH, the acquisition of LH responsiveness in TCs was evaluated. In our study, the results showed that GCCM up-regulated the expression levels of *LHCGR* and reduced the ED_{50} value of LH for testosterone production in TCs, which indicated that GCCM promoted the sensitivity of TCs to LH in buffalo. These results were also consistent with previous studies, which suggested that co-culture with GCs enhanced the responsiveness of TCs to LH in mice (Liu *et al.* 2015). Taken together, these findings indicated that GCCM promoted the steroidogenic competence in TCs mainly through strengthening the sensitivity of TCs to LH in buffalo.

Based on the above results, we speculate the possible explanations for the phenomenon of the effects of GCCM on the responsiveness of TCs to LH in buffalo. The possibility is that growth factors that GCs secrete into GCCM improve the sensitivity of TCs to LH, eventually promoting the capacity of androgen synthesis in buffalo TCs. As we have not conducted the systematic mechanism studies, the action mechanism of GCCM affecting steroidogenesis in buffalo TCs remains unclear. Further studies will focus on the detailed regulation mechanism of GCCM improving androgen production in buffalo TCs.

Conclusion

In conclusion, GCCM (48 h and 48 h) promoted both the expression levels of androgen synthesis-related genes and the secretion levels of testosterone in buffalo TCs. GCCM (48 h and 48 h) enhanced the steroidogenic competence of TCs principally by facilitating the responsiveness of TCs to LH in buffalo. This study provides basic information on the significance of cell interactions for steroidogenesis in the ovary.

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Authors' Contributions Jun Zhang designed the study, performed the experiments, and drafted the manuscript. Fenghua Lu and Deshun Shi designed the study. Yanfei Deng performed the experiments and drafted the manuscript. Jianchun Xu and Xiaofen Yang helped in performing the experiments, collecting and analyzing the data. Haoxin Wang edited the manuscript and analyzed the data.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval All animal procedures used in this study are complied with the guidelines of Institutional Animal Care and Use Committee (IACUC) of Guangxi University.

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