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REGULAR ARTICLES

Dynamic patterns of expressed genes in granulosa cells during follicular and luteal stages in Egyptian buffaloes

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Abstract

A better understanding of the molecular mechanisms in granulosa cells (GC) is warranted, during different follicular and luteal developmental stages in buffalo cows. We aimed to (I) study the expression of selected genes in GC during follicular and luteal phases, (II) evaluate correlations between GC gene expression and steroid concentrations {17-beta estradiol (E2) and progesterone (P4)} in follicular fluid (FF), and (III) study effect of ovarian status on follicular population as well as follicular size frequency. Ovaries were collected in pairs from buffaloes ($n = 178$). Ovaries bearing corpus luteum (CL) were subdivided into hemorrhagic, developing, mature, and albicans. Follicles from luteal groups were classified only into small (< 4 mm) and large (9–20 mm), while follicles from follicular groups were classified into three subgroups: small (< 4 mm), medium (5–8 mm), and large (9–20 mm). The FF and GC were collected for steroid concentrations measurement and gene expression, respectively. In the follicular phase, luteinizing hormone/choriogonadotropin receptor (LHCGR) and cytochrome P450 aromatase (CYP19) in small follicles decreased compared to medium ones. Large follicle showed an increase in LHCGR and CYP19 compared to medium ones. Follicle-stimulating hormone receptor (FSHR) decreased in large compared to medium size follicles. Proliferating cell nuclear antigen (PCNA) increased in small and large follicles. Meanwhile, anti-Mullerian hormone (AMH) and phospholipase A2 group III (PLA2G3) decreased in small and large follicles. The different stages of luteal phase had a profound impact on GC gene expression. There were strong (positive and/or negative) correlations between gene expression and steroid hormones. The different scenarios between expressed genes in GC and steroid concentrations are required for the proper growth and development of follicles and CL.

Keywords Buffaloes · Gene expression · Granulosa cells · Follicular and luteal phases · Follicular fluid

Introduction

Buffalo cows (*Bubalus bubalis*) play a pivotal role in the agricultural economy in many developing countries (Perera 2011). In Egypt, buffaloes are supplying more than 70% of milk and 40% of meat production (FAOSTAT 2018). Despite the buffaloes are well adapt to harsh environment compared to cattle, the reproductive efficiency of buffaloes is mainly hampered by many factors such as late puberty, poor estrous expression, long postpartum anestrus, long calving interval, poor response to multiple ovulation and embryo transfer (MOET), and low conception rates (Fatima et al. 2013; Perera 2011; Salzano et al. 2018).

Ovaries of mature buffalo heifers carry lower number of primordial follicles (10,000–20,000), compared to cattle (100,000). It was reported that the range of total number of surface follicle per ovary was from 5.14 to 6.06 follicles, in collected buffalo ovaries at different reproductive stages (Kumar et al. 1997; Madan et al. 1996). In buffaloes, during estrous cycle the growth of ovarian follicles occurs in a wave-like pattern, whereas Egyptian buffaloes displayed more frequency of three types (53.6%) vs. two types (46.4%) of follicular waves per cycle (Barkawi et al. 2009). These

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waves characterize by presence of different pattern of follicular growth and corpus luteum (CL) at different development stages (Barkawi et al. 2009; Manik et al. 2002). During the follicular waves, groups of small follicles that exist on buffalo's ovary undergo three distinct stages: recruitment, selection, and dominance (Ali et al. 2003; Barkawi et al. 2009; Manik et al. 2002). Furthermore, the antrum cavity is formed and filled with follicular fluid (FF) (Manik et al. 2002). This FF is considered as key player for the regulatory molecules derived from blood or secreted from the follicular cells such as steroids, growth factors, gonadotropins, enzymes, lipoproteins, and proteoglycans (Tripathi et al. 2015). The fate of the developed ovarian follicle either becomes ovulatory follicle or undergoes atresia (Manik et al. 2002). After ovulation, CL is morphologically divided into four different stages: hemorrhagicum, developing, mature, and albicans (Ali et al. 2003).

Many studies showed that the proper follicular and luteal growth as well as development require precise orchestrated cross-talk among FF, oocytes, and their surround somatic cells {cumulus and granulosa cells (GC)} (Munakata et al., 2016; Rao et al. 2011; Uyar et al. 2013). The GC is one of somatic cells that surround oocytes and consider as a key determinant for oocyte quality and subsequently its developmental competence (Kordus and LaVoie 2017; Munakata et al. 2016). These cells also show a unique character like high capabilities for differentiation and proliferation, either in vivo or in vitro (Kulus et al. 2019). In the context, the better understanding of underlying molecular basis of GC is necessary for improving female fertility, using advanced tools of assisted reproduction, as most of the ovarian follicles undergo atresia (Hatzirodos et al. 2014a). Furthermore, some investigations revealed that there were clear alterations in GC gene expression during different follicular phases (growth, plateau, and atretic) in bovine (Douville and Sirard, 2014; Girard et al. 2015). After ovulation, GC stops any further division in order to differentiate into specialized luteal cells (Hatzirodos et al. 2014b). It is well documented that follicle-stimulating hormone receptor (FSHR), luteinizing hormone/choriogonadotropin receptor (LHCGR), and cytochrome P450 aromatase (CYP19A) are responsible for growth, proliferation, and differentiation of GC during follicular development (Hatzirodos et al. 2014b). Furthermore, anti-Mullerian hormone (AMH) revealed a certain dynamic pattern during different stages of follicular growth and development (Alward and Bohlen 2020). The proliferating cell nuclear antigen (PCNA) and apoptosis-related cysteine peptidase (CASP3) control follicular growth, via precise regulation of GC proliferation and/or apoptosis, during different stages of follicular and luteal development (Hatzirodos et al. 2014b; Kfir et al. 2018; Sinderewicz et al. 2017). It was indicated that phospholipase A2 group III (PLA2G3) has a role in regulation of CL, through precise regulation of prostaglandin biosynthesis (PGF_2 alpha and PGE_2), in bovine uterine tissues (Tithof et al. 2007). Thus, FSHR, LHCGR, and CYP19A transcripts were selected as candidate for GC differentiation and proliferation. Furthermore, PCNA and apoptosis-related CASP3 were selected as markers for cell proliferation and apoptosis, respectively. Also, both of AMH and PLA2G3 were investigated as key mediators in steroidogenesis regulation and induced lipid mediator, respectively.

So far, the molecular mechanisms that associate with different stages of follicular and luteal development have been well studied in bovine (Girard et al. 2015), pig (Bonnet et al. 2008), and mouse (McRae et al. 2005). However, there is a lack of studies concerning the molecular regulation of GC during different stages of follicular and luteal development, in buffalo cows. Therefore, we hypothesized that racing the alterations in GC gene expression could be used as noninvasive tools for the proper assessment of different phases of follicular and luteal development in Egyptian buffaloes. Moreover, the better understanding of molecular aspect of GC may be a step forward to improve the response to MOET, in order to maximize exploitation of buffalo cows during their life. Hence, the current study aimed (I) to investigate expression profile of some candidate genes in GC during follicular and luteal phases in Egyptian buffalo cows, (II) to examine correlations between GC gene expression of steroid as well as lipid regulation (CYP19A and PLA2G3) and steroid concentrations (E2 and P4) in FF, and (III) to study the effect of ovarian status on follicular population and follicular size frequency.

Materials and methods

All chemicals and reagents were obtained from Qiagen (Hilden, Germany), unless otherwise stated.

Ethical clearance

As the samples were obtained from the slaughterhouse, the permission of the ethical committee was not required. The study did not involve the handling of live animals.

Ovaries collection as well as ovarian status and follicle classification

At a local abattoir, ovaries ($n = 356$) were collected in pairs from apparently healthy 178 buffalo cows of unknown reproductive history, during breeding season (September–February) directly after slaughtering. Animals (~ 2–4 years old) were rectally palpated to confirm that they were not pregnant before slaughtering and the genital tract was clinically normal. Ovaries were placed in chilled normal saline

(0.9% NaCl) supplemented with 50 µg/L gentamycin, and then transported on ice to the laboratory. In laboratory, ovaries were washed at least three times in warm saline, and then disinfected once in 70% ethanol and washed again with warm saline. According to the ovarian status, ovaries were classified into two main categories (Fig. 1): follicular and luteal. The ovaries bearing CL were subdivided into hemorrhagic (bloody apex over the rupture point), developing (visible vascularization around the periphery of CL), mature (fully developed CL with visible vascularization around its periphery, the apex is red or brown and the rest is grayish), and albicans (the red or brown color disappeared and the entire CL bright red or gray) (Ali et al. 2003). By caliper, the antral follicles from luteal groups were classified only into small (< 4 mm) and large (9–20 mm). It was indicated that during the follicular stage, there are three distinct phases: growing (small size follicles), plateau (medium size follicles), and dominance (large size follicles) (Douville and Sirard 2014; Girard et al. 2015). So, the antral follicles from follicular groups were classified into three subgroups: small (< 4 mm), medium (5–8 mm), and large (9–20 mm) (Kruip and Dieleman 1982).

Collection of follicular fluid and isolation of GC

We gently aspirated FF using 10-mL sterile syringe with an 18-gauge needle, whereas different syringes were used for each category. After aspiration, the FF from each category was placed vertically on ice for 15 min, in order to give the chance for precipitation of cumulus oocyte complexes and decrease the chance of theca cell contamination. The upper layer of FF, which contained GC, was transferred into new tubes for centrifugation at 750 × *g*, for 10 min at 4 °C (Sosa et al. 2020). Afterwards, the clear supernatant was collected for steroid measurement (E2 and P4), while GC pellets were immediately kept at − 80 °C for total RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was extracted from all categories of GC, using miRNeasy mini kit according to the manufacturer's protocol. To remove any possibility of genomic DNA contaminations, the isolated RNA was subjected to on-column DNA digestion, via RNase free DNase kit, and subsequently the isolated RNA was kept at − 80 °C. Total RNA concentration and the level of degradation were checked by Nano-drop 2000/c (Thermo Fisher Scientific, Wilmington, USA) and 2% agarose electrophoresis, respectively. Only samples with high ratio of purity and concentration, as well as showed clear 28 and 18S ribosomal RNA bands, were selected for gene quantification. The cDNA was synthesized, using HiSenScript™ RH(-) cDNA synthesis kit (Intron Biotechnology, Korea), according to the manufacturer's protocol. In brief, 20 µL total reaction volume was prepared; 5 µL of total RNA samples mixed with 10 µL of 2 × reverse-transcription (RT) reaction solution, 1 µL of enzyme mix solution, and dNase/RNase free water up to final volume. Then, tubes were put in a thermocycler machine (BioRad, USA), and program was

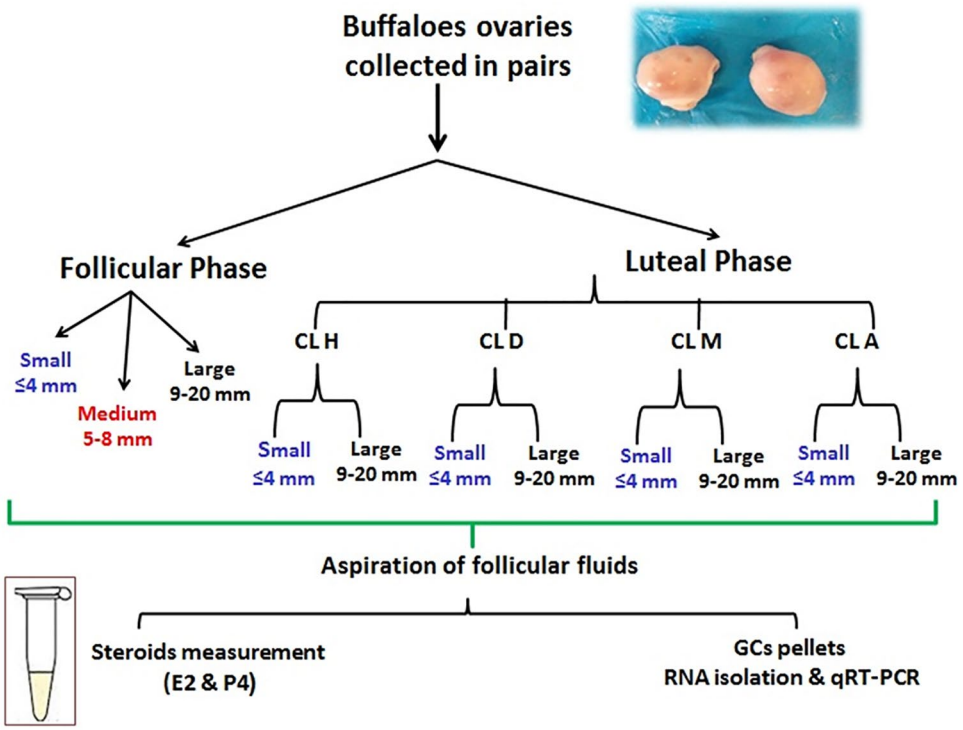

Fig. 1 Schematic diagram shown the whole experimental design. CLH, hemorrhagic corpus luteum; CLD, developing corpus luteum; CLM, mature corpus luteum; CLA, corpus luteum albicans

run, at 42 °C for 60 min, 85 °C for 10 min, and hold at 4 °C. At the end of run, the synthesized cDNA was immediately kept at − 20 °C. In order to confirm the absence of theca cell contamination, a specific marker for theca cells, collagen type I alpha 2 (COL1A2), was used (Hatzirodos et al. 2015), through amplification of cDNA, via conventional PCR (data not shown).

Quantitative real-time PCR analysis

Gene quantifications was performed in Stratagene Mx3000P (Agilent Technologies, USA), using QuantiTect SYBR Green PCR Kit. The primers for selected genes (FSHR, LHCGR, CYP19, PCNA, CASP3, AMH, and PLA2G3), as well as reference genes (18S, GAPDH, and ACTB), were designed using Primer3 program version 4.0 (http://primer3.ut.ee/) (Rozen and Skaletsky 2000). The primers' details are shown in Supplementary Table 1. All samples were amplified using the following conditions: 95 °C for 15 min, 45 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. At the end of run, melting curve was checked to determine the specificity of the amplification. Results were analyzed by the comparative threshold cycle (ΔΔCt) method, and normalization was performed via calculation of data based on geometric mean of three reference genes (18S, GAPDH, and ACTB). NormFinder was used to select the most stable reference gene for gene expression (Andersen et al. 2004).

Determination of steroid concentrations in FF

Both of E2 and P4 concentrations were estimated in the collected FF from all groups, by commercial ELISA kits (Chemux BioScience, South San Francisco, USA), according to the manufacturer's instructions. The optical density (OD) value was measured, using ELISA microplate reader (BioTek ELx800, Vermont, USA), at 450 nm wavelength for both kits. For P4 ELISA kits, sensitivity, intra-assay, and inter-assay coefficients were 0.05 ng/mL, 5.8%, and 9.0%, respectively. For E2 ELISA kits, sensitivity, intra-assay, and inter-assay coefficients were 6.0 pg/mL, 12.1%, and 11.2%, respectively.

Statistical analysis

Mean differences in follicular size were compared between follicular and luteal phases by *t*-test, using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). To compare the means among different follicular size in follicular and luteal stages, ANOVA and LSD test were used (SPSS). The normal distribution was checked via Shapiro–Wilk test and Gaussian distribution, and all data was passed normality test (alpha = 0.05) (GraphPad Software, Inc., San Diego, CA, USA). Statistical analysis of gene expression data and steroid concentrations (E2 and P4) of follicular stage was done, using one-way ANOVA, while the gene expression data and the level of E2 and P4 concentrations in FF concerning luteal stage were performed using two-way ANOVA, followed by Bonferroni's multiple comparisons test (GraphPad Software version 8). The data are shown as the mean ± standard error of the mean (S.E.M) of at least four independent experiments, each done in quadruplicate. The p values ≤ 0.05 were considered statistically significant. In order to evaluate the correlation between steroid concentrations (E2 and P4) and gene expression during follicular and luteal stages, Pearson correlation coefficients were done. GraphPad Prism 8.0 was used for data analysis as well as plotting.

Results

Effect of ovarian status on number of follicular population as well as follicular size frequency in Egyptian buffaloes

We found that the percentage of ovaries in follicular and luteal stages was 21.35% and 78.65%, respectively (Table 1). There were no significant differences between follicular and luteal stages in mean percentage of small, medium, large, and cystic follicles (Table 1). Additionally, we noticed a significant difference ($p < 0.001$) between the small size follicles and cyst in both follicular and luteal stages. However, it was no differences between medium and large size follicles, in both follicular and luteal stages (Table 1). The follicles with size 1.2 and 1.3 mm diameter were higher ($p < 0.05$) in follicular than luteal stage (Table 2).

Table 1 Effect of ovarian status on buffalo follicular population

Ovarian status	No. of ovaries (%)	Total no. of follicles	Follicular category			Cyst No. (%)
			Small No. (%)	Medium No. (%)	Large No. (%)	
Follicular	76 (21.35)	165	90 (57.7 ± 3.4[a])	31 (17.8 ± 1.4[b])	42 (24.2 ± 2.3[b])	2 (0.63 ± 0.6[c])
Luteal	280 (78.65)	644	440 (68.9 ± 4.0[a])	102 (16.3 ± 1.6[b])	101 (14.7 ± 4.9[b])	1 (0.13 ± 0.1[c])
Total	356	809	530 (63.3 ± 3.4)	133 (17.0 ± 1.0)	143 (19.5 ± 3.3)	3 (0.4 ± 0.3)

[a,b,c]Values with different superscripts in the same rows differ ($p < 0.001$), mean ± SEM

Table 2 Effect of ovarian status on buffalo's follicular size frequency

Ovarian status	Total no. of follicles	Follicular size (mm)																		
		0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0
		(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %	(No.) %
Follicular	165	(27) 17.2 ± 1.4	(41) 26.7 ± 1.9	(8) 17.9 ± 1.6	(27) 4.4 ± 0.9	(11) 5.9 ± 0.7	(6) 4.4 ± 0.8	(3) 1.5 ± 0.8	(1) 0.3 ± 0.3	(4) 3.2 ± 0.9	(4) 2.2 ± 1.1	(5) 3.5 ± 0.8*	(8) 5.0 ± 0.2*	(1) 0.3 ± 0.3	(7) 1.9 ± 1.8	(3) 0.9 ± 0.9	(1) 0.3 ± 0.3	(1) 0.3 ± 0.3	(3) 1.8 ± 0.9	(2) 1.5 ± 0.8
Luteal	644	(119) 18.4 ± 0.5	(177) 27.9 ± 2.4	(134) 21.2 ± 2.5	(30) 4.8 ± 0.8	(36) 5.6 ± 0.1	(30) 4.7 ± 0.5	(10) 1.6 ± 0.3	(3) 0.4 ± 0.2	(25) 3.7 ± 1.0	(12) 1.9 ± 0.9	(6) 0.8 ± 0.4	(18) 2.6 ± 0.5	(13) 1.9 ± 0.8	(11) 1.5 ± 1.2	(11) 1.6 ± 1.2	(2) 0.3 ± 0.2	(1) 0.1 ± 0.1	(5) 0.6 ± 0.6	(1) 0.1 ± 0.1

t-test; * $p < 0.05$, mean ± SEM

Ovaries with CL had a clear effect on follicular size population in buffaloes

The effect of CL type on follicular size population was presented in Supplementary Table 2. We noticed that small and medium size follicles were higher ($p < 0.05$) in ovaries that had different stages of CL. However, there was no variation in large size follicles among different stages of CL.

Gene expression patterns of GC in different follicular size during follicular phase

Both of LHCGR and CYP19 mRNAs in small size follicles decreased ($p < 0.001$) compared to medium size follicles, while large size follicle exhibited significant ($p < 0.001$) upregulation in LHCGR and CYP19 genes compared to medium size follicles (Fig. 2). The expression profile of FSHR gene showed a clear downregulation in both small and large size follicles compared to medium size one, whereas the expression of FSHR mRNA was decreased ($p < 0.001$) in large size in comparison with medium size follicles. The relative abundance of PCNA mRNA was increased in GC from small ($p < 0.001$), as well as large ($p < 0.05$) sizes follicles compared to medium size ones. On the other hand, the expression profile of CASP3 mRNA was downregulated ($p < 0.001$) in small size follicle compared to medium size follicles, while in the large size follicles showed an insignificant increase in comparison with medium size follicles (Fig. 2). The expression profiles of AMH and PLA2G3 mRNAs in GC collected from small and large size follicles were decreased ($p < 0.001$) compared to medium size ones (Fig. 2).

The impact of different stages of CL development on GC gene expression

The relative abundance of LHCGR mRNA in the collected GC from large size follicles was increased ($p < 0.001$) in comparison with small size one, during hemorrhagicum and albicans CL. On the other hand, LHCGR mRNA was suppressed ($p < 0.001$) in the collected GC from large size follicles, compared to small size one during mature CL. There was no significant difference in the expression of LHCGR gene, during the developing CL (Fig. 3). We observed that the expression profile of FSHR gene in the collected GC from large size follicles showed downregulation ($p < 0.001$) compared to small size follicles, during the hemorrhagicum and albicans CL. In contrast, the relative abundance of FSHR mRNA in the collected GC from large size follicles exhibited significantly ($p < 0.05$ and $p < 0.001$) upregulation compared to small size one, during the developing and mature CL, respectively (Fig. 3). On the other hand, CYP19 mRNA showed only increased ($p < 0.001$) in collected GC

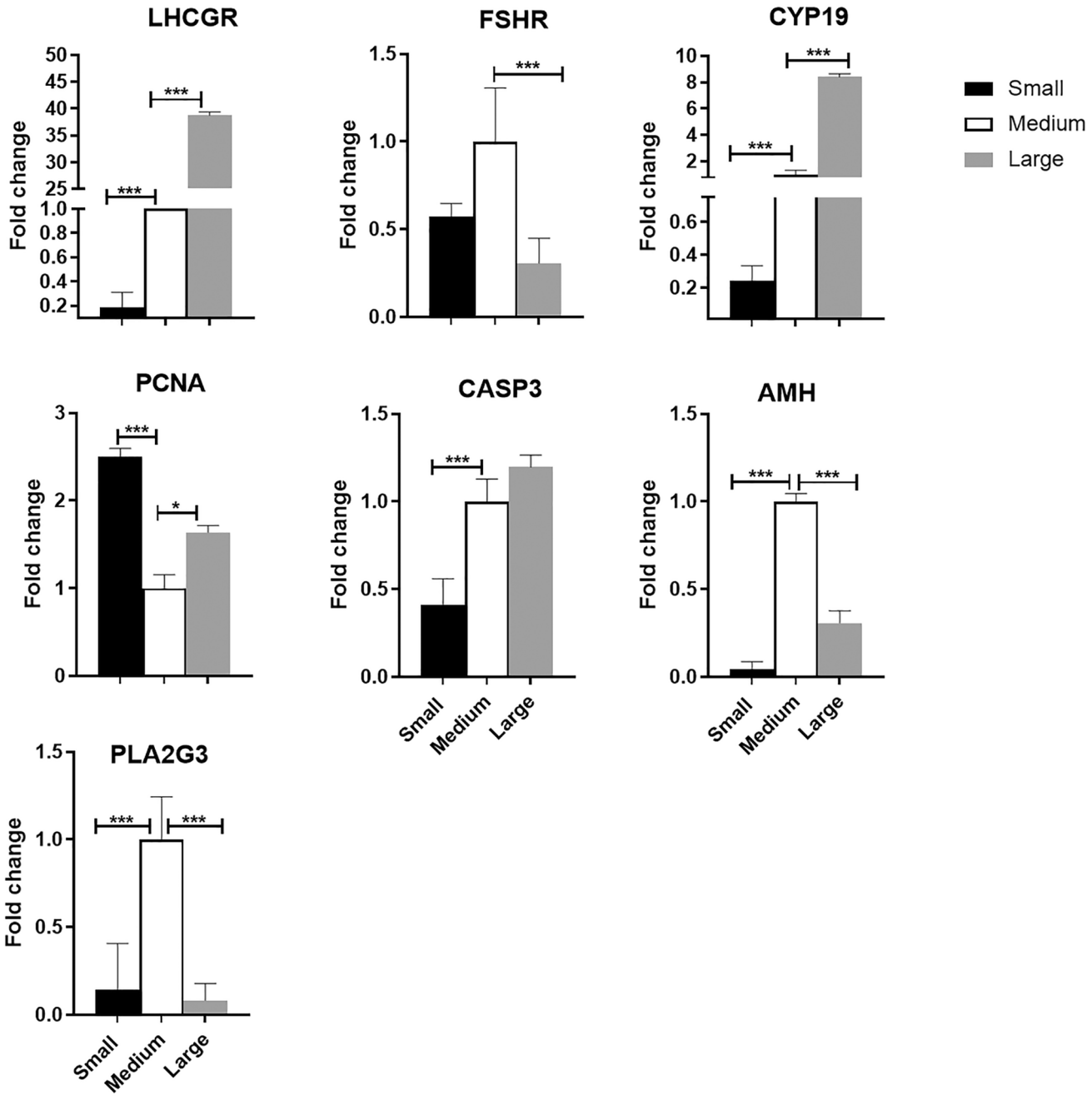

Fig. 2 Expression profile of LHCGR, FSHR, CYP19, PCNA, CASP3, AMH, and PLA2G3 mRNAs in buffalo GC from different follicular sizes (small, medium, and large) during follicular phase. Bars are presented as mean ± SEM. Asterisk(s) represent statistical significance; $^{*}p < 0.05$, $^{***}p < 0.001$

from large size follicles compared to small size one, during the mature luteal stage. However, there were almost no changes in the expression patterns of CYP19 mRNA during hemorrhagicum, developing, and albicans CL (Fig. 3). The PCNA and CASP3 mRNA transcription in the collected GC from large size follicles was upregulated ($p < 0.001$) in comparison with small size one, during hemorrhagicum and albicans CL types (Fig. 3). However, PCNA mRNA transcription was downregulated ($0.001 < p < 0.01$) in the collected GC from large size follicles, compared to small size one during developing as well as mature CL (Fig. 3). Moreover, the relative abundance of CASP3 mRNA in the collected GC from large size follicles was shown a clear ($p < 0.05$) reduction, compared to small size one, during mature CL (Fig. 3). We found that the AMH transcript in the collected GC from large size follicles was significantly

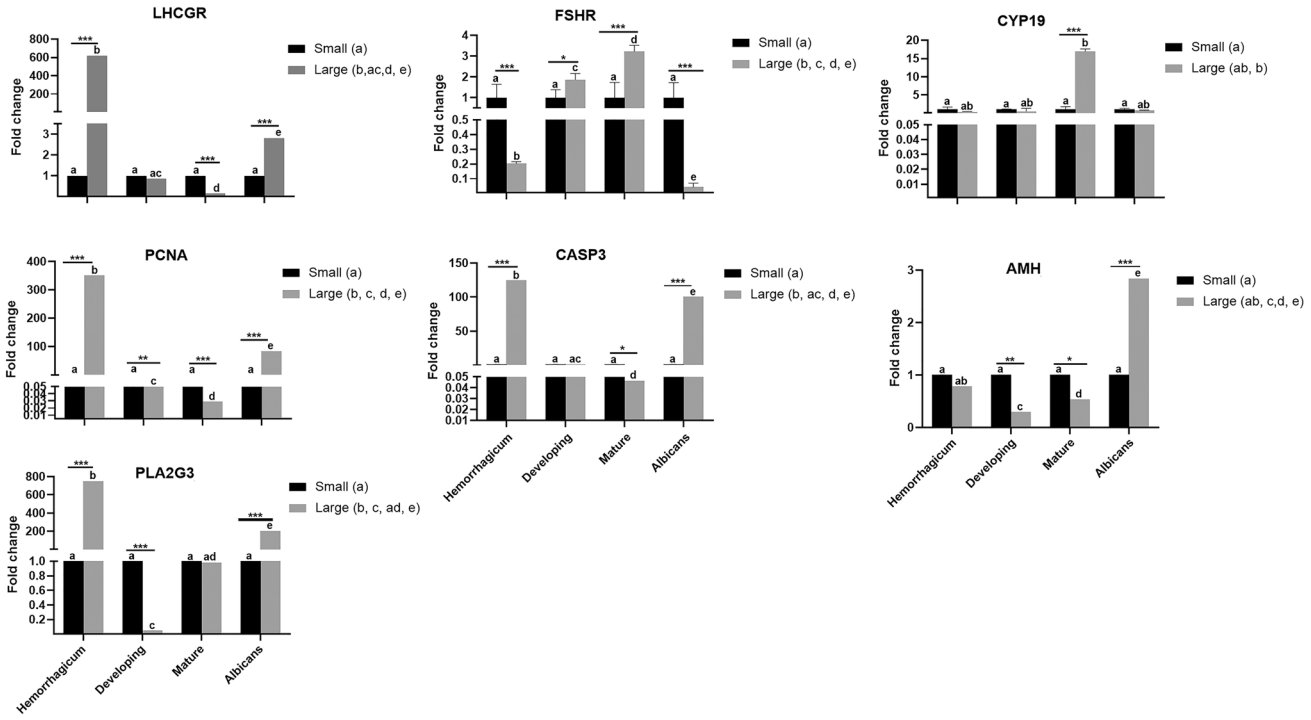

Fig. 3 The relative abundance of LHCGR, FSHR, CYP19, PCNA, CASP3, AMH, and PLA2G3 transcripts in buffalo GCs from small and large size follicles, during different stages of CL (hemorrhagicum, developing, mature, and albicans). Data are presented as mean ± SEM. ^{a,b,c,d,e}Statistical differences among different stages of CL. Asterisk(s) indicate statistical significance between small and large size follicles; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

decreased compared to small size follicles, during developing and mature luteal stages: $p<0.01$ and $p<0.05$, respectively (Fig. 3). On the contrary, the expression of AMH transcript in the collected GC from large size follicles was upregulated ($p<0.001$) compared to small size follicles, during albicans luteal CL (Fig. 3). The relative abundance of PLA2G3 mRNA in the collected GC from large size follicles was over-expressed ($p<0.001$), in comparison with small size follicles, during hemorrhagicum and albicans luteal CL (Fig. 3). On the other hand, the PLA2G3 transcript in the collected GC from large size follicles showed a significant ($p<0.001$) suppression compared to small size one, during developing CL (Fig. 3). Additionally, there was a significant difference ($0.001<p<0.01$) among different types of CL and different follicular sizes.

Dynamic pattern of E2 and P4 levels in FF during follicular and luteal stages

During follicular phase, E2 level in FF was lower ($p<0.05$) in small size follicles, but there was no significant difference from medium and large size follicles (Fig. 4A). The P4 concentration in FF was significantly different ($p<0.05$ and $p<0.001$) among different follicular sizes, whereas the lower P4 level was in FF from large size follicle, and higher P4 level was in small size follicle (Fig. 4A). During luteal phase, we noticed that there was an increased ($p<0.01$) in E2 concentration in FF from large size follicles compared to small ones, during hemorrhagicum, developing, and mature CL, also there was no significant difference in E2 level between small and large size follicles in albicans CL (Fig. 4B). Moreover, there were profound ($p<0.001$) alterations in P4 concentration in FF between small and large size follicles, during hemorrhagicum, developing, and albicans CL (Fig. 4B). Also, there was no significant difference in P4 concentration between small and large size follicles, in mature CL stage (Fig. 4B). Furthermore, it was a clear ($0.001<p<0.01$) effect of different CL types and follicular sizes on E2 and P4 concentrations in FF, during luteal stage (Fig. 4B).

Correlation analysis

During follicular stage (Supplementary Table 3), the correlation between relative abundance of CYP19 mRNAs in GC of small follicles and intrafollicular E2 concentration was negative and strong: $r=-0.8927$ ($p<0.01$). However, there was a positive and strong correlation between PLA2G3 transcripts in GC of small follicles and intrafollicular E2 concentration: $r=0.9661$ ($p<0.001$). The expression pattern

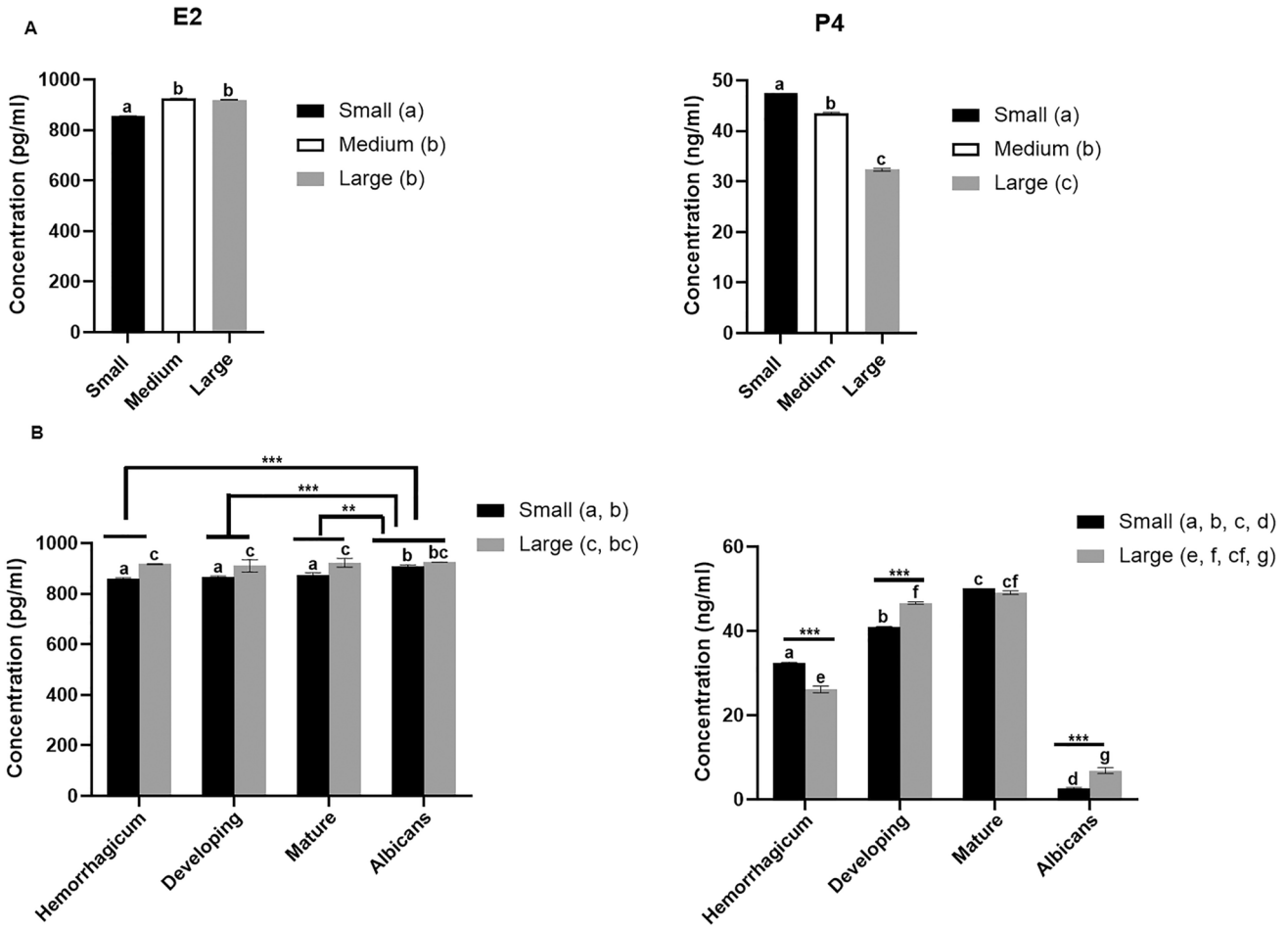

Fig. 4 The steroid levels (E2 and P4) in FF during follicular and luteal stages, in buffalo. **A** Concentrations of E2 and P4 hormones in FF from small, medium, and large size follicles, during follicular phase, ^{a,b,c}statistical differences; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. **B** The E2 and P4 levels in FF from small and large size follicles, during different stages of luteal phase (hemorrhagicum, developing, mature, and albicans). ^{a,b,c,d,e,f}Statistical differences among different stages of CL. Asterisk(s) indicate statistical significance between small and large size follicles; $^{**}p<0.01$, $^{***}p<0.001$

of PLA2G3 mRNAs in GC of medium size follicles was strongly and positively correlated with intrafollicular E2 concentration: $r=0.9937$ ($p<0.001$), but CYP19 transcripts was negatively and strongly correlated with intrafollicular E2 level: $r=-0.8927$ ($p<0.05$). In the large size follicles, intrafollicular E2 was strongly and positively correlated with these GC's transcripts: PLA2G3; $r=0.9764$ ($p<0.05$). However, CYP19 mRNA in GC of large follicles was shown a negative strong correlation ($r=-0.8927$; $p<0.05$), with intrafollicular concentration of E2.

In luteal stage (Supplementary Table 4), the intrafollicular E2 concentration was strongly and positively correlated with PLA2G3 mRNA: $r=0.9352$ and 0.962, respectively ($p<0.05$), in ovaries had developing CL (with large follicles) and mature CL (with small follicles). While in ovaries had developing CL (with small follicles) and albicans CL (with both small and large follicles), the intrafollicular E2 level was strongly and negatively correlated with PLA2G3 transcript: $r=-0.9698, -0.9715$, and -0.9527, respectively ($p<0.01$). The concentration of intrafollicular P4 was strongly and positively correlated with PLA2G3 mRNA: $r=0.9733$ and 0.9612, respectively ($p<0.01$), in ovaries had mature and albicans CL (with large follicles). On the contrary, the P4 level was strongly and negatively correlated with PLA2G3 transcript: $r=-0.9344, -0.9679, -0.971$, and -0.9771, respectively ($p<0.01$), in ovaries that had hemorrhagicum CL (with large follicles), developing CL (with both small and large follicles), and albicans (with small follicles). Correlation data for CYP19 gene did not show any significant differences (data not shown).

Discussion

It is believed that the comprehensive investigations of changes in GC during different phases of follicular and luteal development is needed for a better understanding as well as

a proper assessment of follicular development and oocyte quality.

Herein, the data revealed that both of follicular and luteal stages did not show any significant effect in mean percentage of small, medium, large, and cystic follicular numbers. However, it was a significant difference between the small size follicles and cyst in both follicular and luteal stages. Moreover, we noticed that the follicles with size 1.2 and 1.3 mm diameter were significantly higher in follicular than luteal stage. These findings are in agreement with previous studies in buffalo (Ali et al. 2003; Barkawi et al. 2009; Baruselli et al. 1997). Furthermore, we observed that the small and medium size follicles were significantly higher in ovaries bearing different stages of CL. This may indicate that ovaries bearing CL had a clear effect on follicular size population in buffaloes (Abdoon and Kandil 2001).

This research work was the first to investigate the alterations in GC gene expression in Egyptian buffalo cows, during different follicular and luteal phases. In follicular phase, there are distinct three physiological states: growth (small size follicles), plateau (medium size follicles), and dominance (large size follicles) (Douville and Sirard 2014; Girard et al. 2015; Hatzirodos et al. 2014b). During these stages, GC undergo precise and dynamically changes, in order to support follicular growth and development. For instance, the large size follicles revealed a significant increase in LHCGR and CYP19 mRNAs, in comparison to medium size follicles. In contrast, the small size follicles showed significant decrease in LHCGR and CYP19 transcripts, in comparison to medium size follicles. Furthermore, FSHR gene was significantly decreased only in large size follicles, compared to medium size follicles. Moreover, AMH and PLA2G3 mRNAs were significantly downregulated in small and large size follicles, compared to medium size ones. Additionally, PCNA transcript was significantly upregulated in small as well as large sizes follicles compared to medium size ones. On the other hand, the expression profile of CASP3 gene was significantly decreased only in small size follicle compared to medium size follicles. All these patterns indicated that these transcripts were responsible for regulation of the gene expression in GC during different stages of follicular growth and development (growth, plateau, and dominance), as previously showed in bovine (Douville and Sirard 2014; Girard et al. 2015; Hatzirodos et al. 2014a, 2014b; Luo et al. 2011) and human (Kishi et al. 2018). Furthermore, the expression pattern of AMH provided a strong evidence for its role in regulation of the size of the follicles during follicular development (Alward and Bohlen 2020; Yang et al. 2017). The dynamic pattern of FSHR, PCNA, and CASP3 mRNAs revealed their contribution in follicle growth as well as apoptotic (Feranil et al. 2005; Hatzirodos et al. 2014a; Sinderewicz et al. 2017). It was mentioned that PLA2G3 plays a role in phospholipid digestion as well as a key mediator in lipid metabolism (Diouf et al. 2006). Additionally, PLA2G3 was significantly upregulated in buffaloes primary cultured GC from different follicular sizes (≤ 4, 5–8, 9–15, and 16–20 mm), in comparison with their counterpart uncultured one (Sosa et al. 2020).

It is believed that CL plays vital roles in the estrous cycle regulation as well as the pregnancy maintenance (Stocco et al. 2007). Furthermore, follicular rupture and transformation of follicle into CL associate with a dramatic biochemical as well as morphological alterations (Rao et al. 2011). Here in the current study, we noticed that the different types of CL and different follicular sizes showed clear different dynamic pattern in GC gene expression. For example, the up and/or downregulation of LHCGR, FSHR, CYP19, PCNA, CASP3, AMH, and PLA2G3 mRNAs revealed the proper coordination of GC differentiation during follicular growth as well as different stages of CL formation, maturation, and regression (Cannon et al. 2009; Yoshioka et al. 2013). These findings are in agreement with previous study in dairy cows, which showed that CL had a local effect on follicular development and follicular compositions (Kor 2014a; Romereim et al. 2017). Moreover, these molecular regulations of gene expression in GC may participate in luteinization and maintenance of CL function (Romereim et al. 2017; Stocco et al. 2007). Interestingly, the expression profile of PCNA and CASP3 mRNAs provides strong evidence that both transcripts involved in CL maturation and apoptosis process, respectively during different stages of luteal phase (Carambula et al. 2002; Kfir et al. 2018). It was found a strong relation between AMH concentration and CL numbers after superovulation in high-producing dairy cows (Souza et al. 2015). We did not find any available data about role of PLA2G3 expression during different stages of luteal phase. However, other members of phospholipase A2 enzymes (PLA2G6 and PLA2G4) play a role in regulation of CL, through regulation of prostaglandin biosynthesis ($PGF_{2\alpha}$ and PGE_2) in bovine uterine tissues (Tithof et al. 2007). Collectively, we could say these regulatory patterns in GC gene expression is required for lipid and steroid metabolism/synthesis, as well as cellular proliferation and differentiations, during different follicular/luteal phases, as has been demonstrated in bovine (Romereim et al. 2017).

The accurate detection of estrus and the proper protocol of superovulation are key factors in order to obtain the optimal results (Singh et al. 2015). Previous studies mentioned that buffalo cows still poorly response to superovulation protocols (Fatima et al. 2013; Salzano et al. 2018; Singh et al. 2015). This might be due to lack the information concerning the regulatory mechanisms of GC during different stages of follicular and luteal phase still. Here, we observed that there was a proper orchestrated pattern of the most important genes in regulation of proliferation, differentiation, and viability of GC: LHCGR, FSHR,

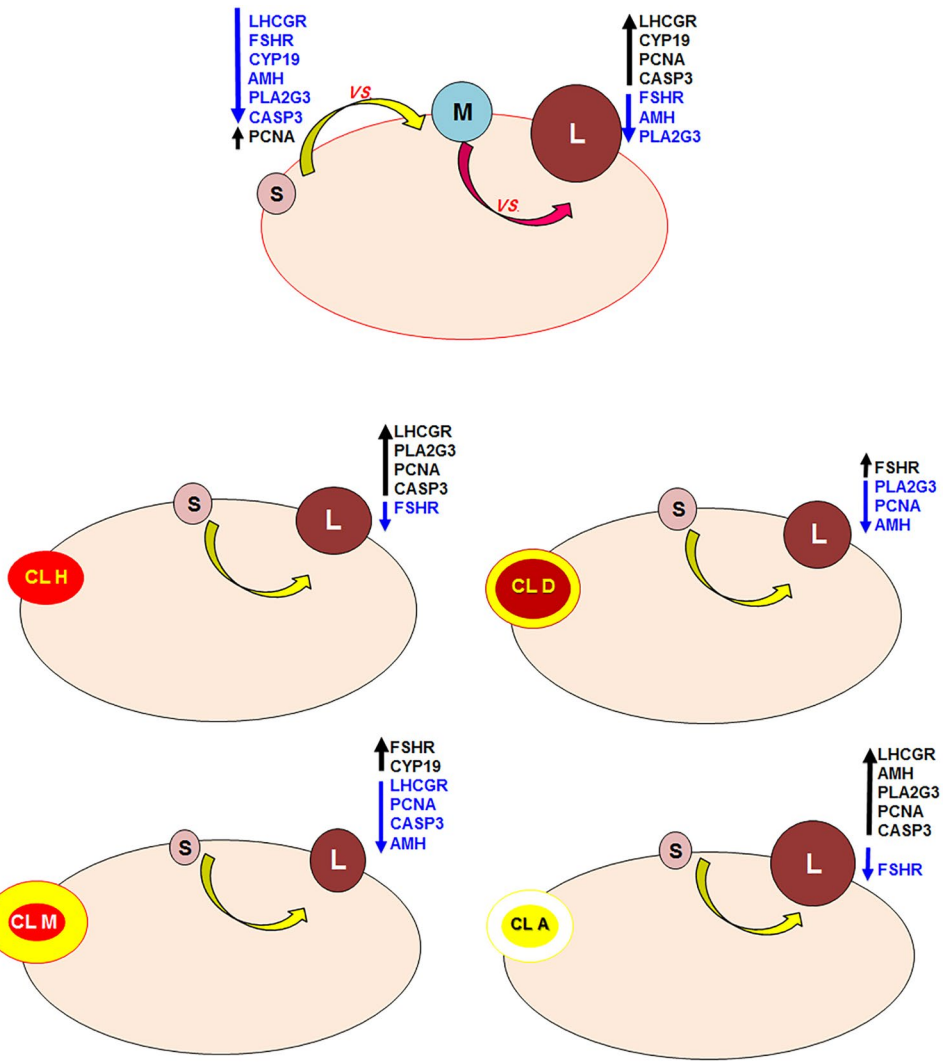

Fig. 5 Schematic model summarized the main findings of follicular and luteal phases. S, small size follicles; M, medium size follicles; L, large size follicles; CLH, hemorrhagic corpus luteum; CLD, developing corpus luteum; CLM, mature corpus luteum; CLA, corpus luteum albicans

CYP19, PCNA, CASP3, and AMH mRNAs, at different stages of luteal phase. These genes revealed an interesting expression patterns mainly at mature stage of CL, represented by upregulation of FSHR and CYP19, as well as downregulation of LHCGR, PCNA, CASP3, and AMH.

The follicular fluid's E2 and P4 is one of the major factors controlling follicular development (Yu et al. 2005). Follicular size and phase of estrous cycle have a profound effect on the concentration of E2 and P4 hormones in FF (Alkalby 2012; Kor 2014b). In the current study, the collected FF from follicles at the follicular phase had higher E2 content than luteal phase with a reverse pattern for P4 content. Our data is in agreement with previous findings in buffaloes (Hozyen et al. 2016) and in bovine (Kor 2014b). Furthermore, we found that P4 concentrations were not related to follicular size. Similarly in buffaloes, it was reported that P4 did not differ among small, medium, and large size follicles.

Regarding the correlation results, both of positive and negative strong correlations between gene expressions of GC: CYP19 and PLA2G3 and steroid hormones (E2 and P4) in FF, during different follicular/luteal phases in buffalo cows, revealed how the complexity of regulatory molecules in GC (up and downregulation of expressed genes) to provide a proper milieu for growth and development of follicles and/or CL, at different reproductive phases (Abedel-Majed et al. 2019; Romereim et al. 2017; Souza A. H., 2015). Moreover, the precise regulation of steroidogenic, proliferative, and apoptotic genes and steroid hormones is essential for optimal GC function, during different follicular and luteal phases in buffalo cows (Romereim et al. 2017; Stocco et al. 2007).

Conclusion

To the best of our knowledge, this is the first study that gives new insights into the regulation of GC during different stages of follicular and luteal development, in buffalo cows. We drew schematic models, in order to illustrate the findings

of the present study, where it was noticed that LHCGR, FSHR, CYP19, PCNA, CASP3, AMH, and PLA2G3 mRNAs revealed different expression pattern, at different follicular and luteal phases (Fig. 5). There were positive and negative strong correlations between the expression profile of selected genes in GC and steroid hormones (E2 and P4) in FF, which gave a solid foundation for precise regulation of GC function in order to maintain the proper growth and development of follicles and CL. Further studies are required for the better understanding the whole transcriptome profile of GC in Egyptian buffalo cows, at different follicular and luteal phases.

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Data Availability The manuscript has data included as electronic supplementary material.

Declarations

Conflict of interest The authors declare no competing interests.

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