Research Article

Expression Pattern of Apoptotic Genes During Different Stages of Corpus Luteum Development in Egyptian Buffaloes

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Abstract | It is fair to know how corpus luteum (CL) is a maestro of regulation of estrus cycle in Egyptian buffaloes. Hence, the study of molecular control of the CL in Egyptian buffaloes let us to know the mechanisms of this regulation. We aimed to (1) figure out the expression profile of apoptotic genes (TNF_{α}- BAX - CASP3 - FASLG - AGTR2 - and NOS2) mRNAs during early, mid, and late stages of CL and (2) clarify the accompanied changes in progesterone (P4), nitric oxide (NO) concentrations and histological evaluation through different stages of CL in buffaloes. Forty five paired samples of ovarian tissue from buffaloes were obtained from slaughterhouse. Ovaries were collected and CLs were classified morphologically into three stages: early, mid and late stages. Moreover, the CLs were frozen at -80 °C for NO estimation in CLs homogenate, RNA isolation and gene expression. Blood samples were obtained for P4 serum levels estimation. Results showed a significant rise of TNF_{α} mRNA during late stage of CL and increase the expression of CASP3, FASLG and AGTR2 mRNAs during mid-stage of CL. Furthermore, NOS2 mRNA was raised during early stage of CL. Surprisingly, NO in CL homogenate was raised through early and late stages of CL. Additionally, P4 increased in mid-stage of CL. Taken together, the fine tuning between apoptotic genes (TNF_{α}- BAX - CASP3 - FASLG - AGTR2 - and NOS2) is required for proper functions of CL in Egyptian buffaloes. Thus, it might be concluded that expression pattern of apoptotic genes is changed according to the stage of CL.

Keywords | Apoptosis, Buffaloes, Corpus luteum, Gene expression, Nitric Oxide.

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INTRODUCTION

Buffaloes (Bubalus bubalis) play an important part in many poor countries. They are used for agricultural economies, producing milk, meat, and draught force (Minervino et al., 2020). One of the greatest constraints in the exploitation of the production potential of buffaloes are its inherently lower reproductive efficiency, manifested by estrus with low behavioral expression and/or not associated with ovulation, postponed sexual maturity, lower conception rates, and longer calving intervals (Baithalua et al., 2013).

The corpus luteum (CL), a dynamic endocrine gland, controls the estrous cycle, embryonic development, implantation, and produce progesterone (P4), which is essential for

pregnancy maintenance (Hojo et al., 2016). Additionally, corpus luteum is one of the ovarian tissue has cyclic periods of development (angiogenesis), steroidogenic activity, and luteolysis (CL regression) (Pate et al., 2012). Moreover, study of corpus luteum give us the mystery of regulation of estrus cycle.

Apoptosis is a physiological process that characterized by tight control, timely process that occurs in all living cells. In addition, apoptosis is a type of pre-programmed cell suicide in which the cell actively destroys itself in order to keep the body functional (Panawala et al., 2017). Furthermore, the delicate balance between cell death and survival factors firmly controls the crucial process of luteal cell apoptosis, which occurs during luteolysis (Skarzynski et al., 2013). When luteolysis occurs, luteal cells exhibit a sort of spontaneous cell death called apoptosis (Sugino and okuda, 2007). It is well known that apoptosis is the major mechanism controlling luteolysis in luteal cells (Jin et al., 2015).

Hence, it is reasonable to assume that apoptotic genes play an interesting rhythm together. Intriguingly, TNF_{α} (tumour necrosis factor) is a cytokine that has many functions in CL (Kapoor et al., 2020). Moreover, activation of caspases (CASPs), which are intracellular cysteine aspartic proteases are remarkable in apoptosis of CL (Hojo et al., 2016). The cellular apoptotic pathway is initiated when the BAX gene (the first pro-apoptotic component), is overexpressed (Yang and Rajamahendran, 2002). The FASL and its receptor have a great role in apoptosis of CL (Skarzynski et al., 2013). The AGTR2 signaling cascade shared in growth inhibition of cells (Nouet et al., 2004). It is well known that, nitric oxide (NO) appears to be essential for luteolysis (Korzekwa et al., 2007). Furthermore, NOS2 (Nitric Oxide Synthase 2) is one of the forms of NOS that had a major control of important ovarian physiological processes (Miyamoto et al., 2009).

Although, many studies (Skarzynski et al., 2013; Hojo et al., 2016) try to give a satisfied knowledge about apoptosis, study molecules changes and mediators controlling apoptosis of CL of buffaloes is still need more explanations. Hence, it is well noted this is the first study have a view of an crucial apoptotic genes: (TNF_{α}, BAX, CASP3, FASLG, AGTR2, NOS2). This knowledge will enhance our understanding of the buffalo reproductive system and potentially increase buffalo population levels. The existing study aimed to (1) investigate the expression of apoptotic genes (TNF_{α} - BAX- CASP3 - FASLG - AGTR2 - and NOS2) mRNAs during different stages of CL (early, mid and late) in Egyptian buffaloes and (2) demonstrate the alterations that happened in progesterone (P4) in serum, nitric oxide (NO) levels in CL homogenate, and histological analysis across the various stages of CL.

MATERIALS AND METHODS

Unless other stated, all chemicals and reagents were got from Qiagen (Hilden, Germany) and Thermo Fisher Scientific (Wilmington, USA).

Collection of ovarian samples and CLs classification

A total of 45 coupled samples of ovarian tissue from buffaloes with unknown reproductive histories with average range (3-7 y) were taken at the El-Warrak abattoir. Animals were rectally palpated to make sure they weren't pregnant and that their genital tracts were clinically normal before being slaughtered.

Ovaries were obtained from January - April 2021 then carried on chilled saline (0.9% NaCl) before being transported on ice. Ovaries were immediately disinfected once in 70% ethanol and then cleaned again with saline a minimum of three times in the laboratory. According to their morphology, one skillful operator split the CLs basically into three phases: the early (days 1–5), developed and mid (days 6–11), and late luteal phase (days 12–16) stages (Rakesh et al., 2013; Horihata et al., 2016).

From early stage through late stage of the estrous cycle, CL was flesh-colored or brilliant red and protruded from the surface of the ovary (Rakesh et al., 2013). During early stage CL was described as bright red in colour, extremely soft to the touch, and barely protruding from the surface of the ovary. The apex of CL was reddish in hue in mid-stage, with a flesh-colored body and a soft consistency. The coloration of CL, on the other hand, was flesh-colored and compact in consistency at late stage, with a well-formed crown and hollow at the apex. Different CL stages were split into two portions; the first portion was snapped frozen at -80 $^{\circ}$C for RNA extraction and later gene quantification as well as measuring of nitric oxide (NO) in CL homogenates. The second one was promptly fixed in 10% neutral buffered formalin for histological evaluation.

Total RNA isolation and first-strand cDNA synthesis

All CL samples (early, mid, and late) had their total RNA extracted using the miRNeasy Mini kit in accordance with the manufacturer's instructions (Qiagen). To get rid of any genomic DNA contamination, the extracted RNA was subjected to on-column DNA digestion using RNase free DNase set on-column (Qiagen). Thermo Fisher Scientific's Nano-drop 2000/c (Wilmington, USA) was used to measure the total RNA concentration and purity (Jyotsna and Medhamurthy, 2009), and 2% agarose gel

Table 1: The primers used in the qRT-PCR analysis.

Gene	Accession No.	Sequences 5′-3′	Annealing temp°C	Product Size (bp)
TNF_{α}	XM_006041930.2	F: CTCTTCTCAAGCCTCAAGTAAC R:AGAGGACCTGTGAGTAGATGAG	57.2	182
BAX	XM_025269476.1	F:GTAACATGGAGCTACAGAGGAT R: ATGATGGTCCTGATCAACTC	54.2	196
CASP3	XM_006075118.1	F:GACTGTGGTATTGAGACAGACA R: CGTACTTTTTCAGCATCTCAC	50	175
FASLG	XM_006063044.2	F: ACAAGGTCTACTCCAGGAACTT R:CTCAAAACTGACCAGAGAGAGT	56	185
AGTR2	XM_006067047.2	F:GTAACATGGAGCTACAGAGGAT R: ATGATGGTCCTGATCAACTC	54.7	194
NOS2	XM_006046821.2	F: GAAGTACATGCAGAATGAGTACC R: CCTCCACCTGGTAGTAGTAGAA	57.2	152
GAPDH	XM_010990867.1	F:GTCTATTACCATCTTCCAGGAG R:AATCTTGAGGGACTTGTCATAC	55	223
β-ACTIN	XM_010997926.1	F:CAGATCATGTTCGAGACCTT R:GTGAGGATCTTCATGAGGTAGT	55	221

electrophoresis was used to confirm the presence of intact RNA.

Using the GScriptfirst-strand synthesis kit (Gene direx, Taiwan), the cDNA for gene expression analysis was created from the isolated total RNA in accordance with the manufacturer's instructions. In a summary, a reaction volume of 20 µL was set up as follows: A PCR strip containing 5 µL of total RNA samples, 4 µL of 5X first strand buffer, 1 µL of Oligo(dT), 1 µL of DTT, 1 µL of dNTP mix, 2 µL of GScript RTase, and 6 µL of RNase-free water was run in a thermocycler (BioRad, USA) set to run at 55°C for 60 min, 70°C Using GAPDH primer in a PCR reaction, the generated cDNA was verified, then kept at -20°C.

Quantitative real-time PCR analysis (qRT-PCT)

Gene-specific primers (Table 1); TNF_{α}, BAX, CASP3, FASLG, AGTR2, NOS2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-ACTIN were configured using Primer3 Program version 4.0 (http://primer3.ut.ee/), according to Rozen and Skaletsky (2006). By sequencing the PCR results, the specificity of each primer amplicon was verified. Quantitative real-time PCR of mRNAs was conducted in a StratageneMx3005P Real-Time PCR System (Agilent Technologies), using SYBR Green/ROX Mix (Thermo Scientific), with the following program: 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The specificity of the amplification was estimated by evaluating the melting curve at the end of the run. The data were analyzed using the comparative threshold cycle (ΔΔCt) approach and normalization was performed by the geometric mean of housekeeping genes (*GAPDH* and *β-ACTIN*). NormFinder was used to select the most stable reference gene for gene expression (Andersen et al., 2004).

2.5. Estimation of Nitric Oxide (NO) in CL homogenate

The CLs were gathered and stored in -80°C freezer. Nitric oxide (Cat. No. NO 25 33; Bio-diagnostic, Egypt) was measured using a colorimetric method to determine the total level of NO. Prior to dissection, the tissues must first be perfused with a PBS solution (phosphate-buffered saline solution, pH 7.4) containing 0.16 mg/ml heparin to remove any red blood cells. Samples were homogenized in 5 - 10 ml cold buffer (100 mM potassium phosphate, pH 7.0, containing 2 mM EDTA) per gram of tissue, and then centrifuged at 4000 rpm for 15 minutes. For testing, the supernatant was removed and frozen at -80°C. The manufacturer's recommendations were followed when determining the NO levels in the supernatant.

Histological examination

The CLs were assembled and preserved in a 10% neutral buffered formalin solution. After fixation, tissues were processed in different grades of alcohols and xylenes and finally embedded in paraffin 5 µm sections. These sections were cut and stained with hematoxylin and eosin (H & E) for light microscopy (Bancroft and Gamble, 2008). Tissue slides were estimated by a BX43 light microscope (Olympus, Japan) equipped with a DP-27 digital camera (Olympus, Japan).

Statistical analysis

Statistical analysis of gene expression data was performed using the Kruskal-Wallis one-way ANOVA test followed by Dunn's multiple comparisons test. The values were graphed and exhibited as mean± SEM, *P*-values <0.05 were considered statistically significant. GraphPad Prism

9.0 is used for data analysis and plotting (Graphpad Software, Inc., SanDiego, CA, USA).

RESULTS

Gene expression of TNF_{α}, BAX, CASP3, FASLG, AGTR2 and NOS2 during early, mid and late stages of CL in Egyptian buffaloes

The TNF_{α} mRNA was increased significantly (P<0.001) in late stage of CL compared to early and mid-stages. There was no significance between expression of TNF_{α} mRNA between early and mid-stages. The BAX gene was decreased in significant manner (P<0.001) in mid and late stages of CL opposed to the expression of BAX mRNA in early stage of CL. There was no significance in expression of BAX mRNA between mid and late stages of CL. Moreover, CASP3 gene expression showed significant up-regulation (P<0.001) in mid stage of CL in comparison to other stages. The expression profile of CASP3 mRNA was down-regulated (P<0.001) in late stage of CL opposed to the mid-stage but more than expression in the early stage (Figure 1).

Additionally, FASLG mRNA was increased with significance (P<0.001) in mid stage of CL compared to early and late stages. There was no significant difference between early and late stages of CL. Furthermore, AGTR2 mRNA was up-regulated with significance (P<0.001) in mid-stage of CL compared to other stages. The AGTR2 gene was declined significantly (P<0.001) in late stage of CL opposed to mid-stage but less than the early stage. The NOS2 expression was dropped in mid and late stages in comparison to early stage of CL (Figure 1).

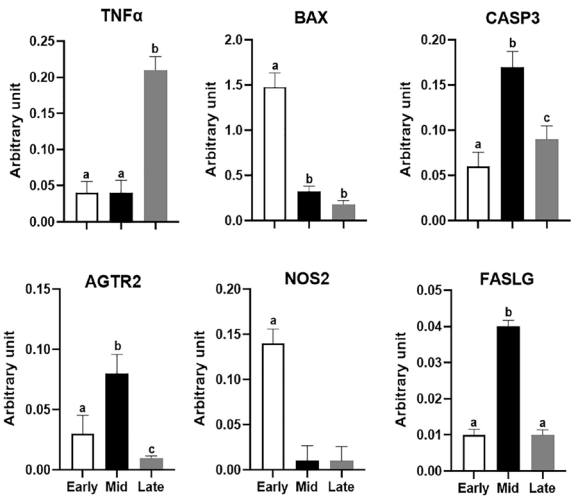

Figure 1: Expression patterns of TNF_{α}, BAX, CASP3, FASLG, AGTR2 and NOS2 mRNAs in different stages of corpus luteum in Egyptian buffaloes. Bars are presented as mean±SEM. [a,b,c]Statistical differences between different stages of CL (statistical significant at P<0.001).

Nitric oxide levels in CL homogenate

The NO levels were increased significantly (P<0.001) in late stage compared to early and mid-stages. Furthermore, NO concentrations were declined (P<0.001) in mid stage when compared to early stage of CL (Figure 2).

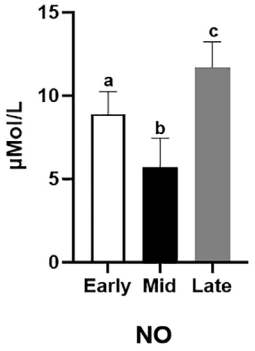

Figure 2: Concentrations of NO in CL homogenate during early, mid and late stages of CL. Bars are presented as mean±SEM. [a,b,c]Statistical differences between different stages of CL (statistical significant at P<0.001).

Histological examination

The early stage of CL showed wide areas of bleeding in the central cavity. Granulosa cells are not yet luteinized, not lipid laden and avascular. Theca cells were vesicular and lipid containing. While, the mid corpus luteum showed complete luteinization of granulosa cells as they become increased in size and filled with lipids. The CL was more vascular and exhibited numerous connective tissue septa. The late stage of corpus luteum is characterized by regression of lutein cells with marked fibrous tissue proliferation (Figure 3).

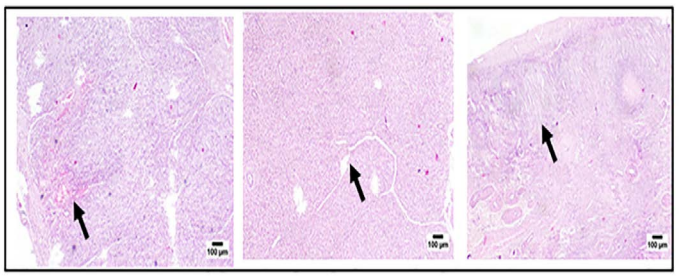

Figure 3: Histological evaluation of different stages of CL (early, mid and late stages). a) Photomicrograph of early stage of corpus hemorrhagicum, showing blood filled cavities (arrow) (H&E). b) Photomicrograph of mid stage of corpus luteum, showing complete luteinization of granulosa cells with existence of connective tissue septa (arrow) (H&E). c) Photomicrograph of late stage of corpus luteum, showing development of fibrotic masses (arrow) (H&E).

Estimation of progesterone levels (P4)

The concentrations of P4 showed significant up-regulation

(P<0.001) in mid-stage compared to early and late stages of CL (Figure 4).

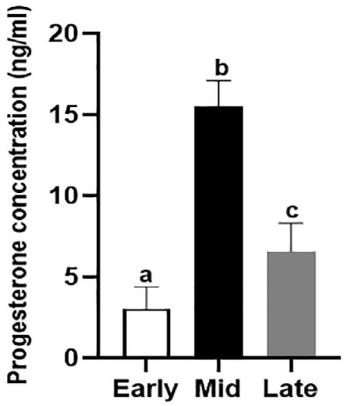

Figure 4: Estimation of progesterone levels during different stages of CL. Bars are presented as mean±SEM. [a,b,c]Statistical differences between different stages of CL (statistical significant at P<0.001).

DISCUSSION

The CL controls the Egyptian buffaloes' estrus cycle. It is logic to know that there is a molecular regulation which mange the functions of CL specially: apoptosis and regression of CL. However, there are many studies have looks at the molecular control of CL apoptosis in cows (Skarzynski et al., 2013; Hojo et al., 2016) and little research in buffaloes (Kapoor et al., 2020; Ibrahim et al., 2022). Interestingly, the study of apoptotic genes controlling the apoptosis and regression of CL in Egyptian buffaloes require a detailed investigation. Hence, we aimed in our research to explore the expression changes of genes regulating apoptosis in Egyptian buffaloes.

Moreover, CL has a crucial role in regulation of estrus cycle and pregnancy maintenance. Once there is no pregnancy, the CL regresses by a mechanism called luteolysis (Hojo et al., 2016). The process of apoptosis is included in structural luteolysis that may be either natural or induced. Apoptosis was regulated by a plenty of molecules, as cytokines (Okuda and Sakumoto, 2003). Tumor necrosis factor-α (TNF_{α}) is a cytokine that exhibits multiple important roles in CL regression (Korzekwa et al., 2008). In our results, the TNF_{α} mRNA was up-regulated significantly in late stage of CL compared to early and mid-stages. In consistent with our results, Kapoor et al. (2020) found the pronouncing distribution of cytokine TNF_{α} during the late luteal phase than other earlier two phases of CL in buffaloes. Pru et al. (2003) showed also that local secretion of TNF_{α} in bovine CL of late stage was prominent than that of the mid-stage, which can pair with our finding.

The BAX gene was declined in significant manner in mid and late stages of CL opposed to the expression of BAX mRNA in the early stage. On the contrary, BAX is a member of Bcl-2 family- is increased during CL regression in cows (Sugino et al., 2007). The difference in the pattern during late stage of CL can be attributed to the study of BAX mRNA not the protein level. Additionally, the species difference may also be one of the causes of the change of BAX mRNA pattern. So, this may need further study for this gene on protein level.

CASP3 mRNA participates in apoptotic signaling during CL regression (Carambula et al., 2002). The current study showed a significant up-regulation of CASP3 expression in mid stage of CL in comparison to early and late stage. The expression of CASP3 mRNA was increased significantly in late stage when compared to the early stage. Our findings are matched with Pelufo et al. (2006); Ibrahim et al. (2022) who stated that the rise of expression of CASP3 mRNA during developmental stage of CL is probably due to its functional role through the early stage of leutolysis. Fas ligand, a member of TNF super family, mainly participates with its receptor (Fas) to induce apoptosis (Okuda and Sakumoto 2003). Our current study showed a significant increase of FASLG gene with significance in mid stage of CL compared to early and late stages. In agreement with our study, Planells-Ferrer et al. (2016) found that, FAS expression combined with apoptosis and expressed in the early stage of CL in cows (Trevisol et al., 2020).

There are two main forms of receptors for Angiotensin II have been classified as angiotensin type 1 and 2 receptor (AGTR1 and AGTR2) (Miyamoto et al., 2009). The AGTR2 mRNA in the present study was up-regulated with significance in mid-stage of CL compared to early stage. In accordance with our study, AGTR2 mRNA increased at the same stage of bovine CL (Berisha et al., 2002).

Additionally, nitric oxide (NO) is an effective mediator of luteolysis in the cow (Korzekwa et al., 2004). Consistent with the present study, Korzekwa et al. (2004) found the prominent rise in the NO level in late stage of CL. It was noted, that NO shared with cytokine TNF_{α} in luteal regression (Korzekwa et al., 2008). Interestingly, the current study showed decline of NOS2 mRNA during mid and late luteal stages of CL. Surprisingly, Yoshioka et al. (2012) is consistent with our idea that NOS2 is inhibited during mid-luteal stage because the progesterone increase (P4) at this stage. Consistent with previous idea, p4 levels were raised during mid-stage of CL.

Manov et al. (2008) is consistent with our findings as

the histological evaluation of CL of buffaloes is that luteotrophs have central nuclei and cytoplasmic lipid droplets, and small luteotrophs with bundle shaped nuclei. The increase of lipid droplets during mid-stage of CL might be attributed to P4 secretion and this was matched with Kapoor et al. (2018).

CONCLUSION

Collectively, this is the first study make a focus on changes occurred in apoptotic genes and their participating in regression of CL. Our findings revealed that TNF_{α} was promoted by expression of BAX at early stage of CL and CASP3 expression at mid-stage of CL. We assumed that NO had a double role during early and late stages of CL. The NO production in CL homogenate was raised by TNF_{α}. In addition, the level of NO had an initial role before apoptosis through activation of FAS, BAX and CASP3 mRNAs. The rise of TNF_{α} and decrease of NOS2 were associated with the drop of P4 at late stage of CL. Moreover, inhibition of AGTR2 mRNA resulted in a decrease of CASP3 gene, which has an essential role in apoptosis.

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CONFLICT OF INTEREST

There are no conflicts of interest declared by the authors.

Data availability statement

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

AUTHORS CONTRIBUTION

Samaa Galal: Conceptualization, Validation, Investigation, Writing. Sally Ibrahim: Conceptualization, Methodology, Validation, Investigation, Writing. Karima Mahmoud: Conceptualization, Formal analysis. Ola Adel and Aya Shokry: Methodology, Validation. Mohamed El-Belely: Conceptualization, Validation. Sayed Ismail: Conceptualization, Investigation, Revision.

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