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RESEARCH ARTICLE

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Role of intraluteal and intrauterine prostaglandin signaling in LH-induced luteolysis in pregnant rats

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Abstract

Luteal dysfunctions lead to fertility disorders and pregnancy complications. Normal luteal function is regulated by many factors, including luteinizing hormone (LH). The luteotropic roles of LH have been widely investigated but its role in the process of luteolysis has received little attention. LH has been shown to have luteolytic effects during pregnancy in rats and the role of intraluteal prostaglandins (PGs) in LH-mediated luteolysis has been demonstrated by others. However, the status of PG signaling in the uterus during LH-mediated luteolysis remains unexplored. In this study, we utilized the repeated LH administration (4×LH) model for luteolysis induction. We have examined the effect of LH-mediated luteolysis on the expression of genes involved in luteal/uterine PG synthesis, luteal $PGF_{2\alpha}$ signaling, and uterine activation during different stages (mid and late) of pregnancy. Further, we analyzed the effect of overall PG synthesis machinery blockage on LH-mediated luteolysis during late pregnancy. Unlike the midstage of pregnancy, the expression of genes involved in PG synthesis, $PGF_{2\alpha}$ signaling, and uterine activation in late-stage pregnant rats' luteal and uterine tissue increase 4×LH. Since the cAMP/PKA pathway mediates LH-mediated luteolysis, we analyzed the effect of inhibition of endogenous PG synthesis on the cAMP/PKA/CREB pathway, followed by the analysis of the expression of markers of luteolysis. Inhibition of endogenous PG synthesis did not affect the cAMP/PKA/CREB pathway. However, in the absence of endogenous PGs, luteolysis could not be activated to the full extent. Our results suggest that endogenous PGs may contribute to LH-mediated luteolysis, but this dependency on endogenous PGs is pregnancy-stage dependent. These findings advance our understanding of the molecular pathways that regulate luteolysis.

KEYWORDS
cAMP/PKA/CREB pathway, Cox1, LH, luteolysis, prostaglandin

1 | INTRODUCTION

In female mammals, the process of luteolysis is complex and varies from species to species. Luteinizing hormone (LH) induces luteolysis in rats during the mid and late-stage of pregnancy (Vashistha et al., 2021). Stocco and colleagues emphasized the role of intraluteal prostaglandin (PG) $F_{2\alpha}$ in LH-induced luteolysis (C. Stocco & Deis, 1998). However, they did not analyze the role of uterine $PGF_{2\alpha}$ signaling in LH-mediated luteolysis. Our study explains the effect of 4×LH on luteal and uterine $PGF_{2\alpha}$ biosynthesis machinery, uterine activation, and the effect of blockage of overall PG synthesis machinery on LH-mediated luteolysis.

$PGF_{2\alpha}$ is a well-established luteolysis and plays a crucial role during luteolysis in many species, including bovines (Metcalf et al., 1992),

primates (Stouffer et al., 2013), and rodents (Bjurulf et al., 1998). In rodents, $PGF_{2\alpha}$ of both the luteal and uterine origin are involved in luteolysis (McCracken et al., 1999). The uterus remains in a quiescent state throughout pregnancy until the beginning of parturition (Renthal et al., 2010). Uterine activation in rats is associated with increased expression of a set of genes encoding PG synthesis machinery and contraction-associated proteins (CAPs) (JRG et al., 2000; Mitchell et al., 2005). PG synthesis machinery comprises of four main enzymes including cyclooxygenase (COX) 1, COX 2, PGF synthase (PGFS), and carbonyl reductase 1 (CBR1). PGs are synthesized through the hydrolysis of membrane phospholipid into arachidonic acid by the action of phospholipases (Ricciotti & FitzGerald, 2011). Arachidonic acid is further converted to PGH_2 by the enzymes COX1 and COX2, this step is the rate-limiting step in the production of PGs (Ricciotti & FitzGerald, 2011). PGH_2 is quite unstable in nature and is further converted into different PGs namely PGE_2, PGD_2, PGF_2, and PGI_2 by PGE2 synthase, PGD2 synthase, PGFS, and PGI2 synthase, respectively (Ricciotti & FitzGerald, 2011). COX1 and COX2 are bifunctional enzymes that contain both cyclooxygenase and peroxidase activity. The two cyclooxygenase isoforms, COX1 and COX2, are targets of nonsteroidal anti-inflammatory drugs (NSAIDs) (Allaj et al., 2013). NSAIDs binds and inactivate the COX, which results in a decrease in the peripheral and central PG production (Allaj et al., 2013). Many NSAID have been used for this purpose including, indomethacin, naproxen, diclofenac sodium (DIC), ketoprofen, aspirin, and many others (Allaj et al., 2013). DIC [2-(2,5-dichlorophenyl) amino] benzene acetic acid, is one of the most widely used NSAID for treatment of pain and inflammation (van Walsem et al., 2015). Its specificity for both the isoforms of cyclooxygenase is almost the same and is metabolized in the liver (Tomic et al., 2008). It has been used for reproductive studies to analyze the effects of suppression of PGs on oxytocin sensitivity and parturition (Chan, 1983).

Some of the CAPs known to be upregulated at the time of uterine activation include oxytocin (Lefebvre et al., 1992), fibronectin (Tuo & Bazer, 1996), interleukin-1 (IL-1), osteopontin (Waterhouse et al., 1992), P-glycoprotein (Axiotis et al., 1991), and insulin-like growth factor binding protein 2 (IGFBP-2) (Rutanen, 2000). Cytokines also play an important role in uterine contractility, cervical ripening, and birth (Orsi & Tribe, 2008). Reports suggest an association of nuclear factor *K*-light-chain-enhancer of activated B cells and IL-1β with the contraction of the uterus (Orsi & Tribe, 2008). Other cytokines like transforming growth factor-β1, IL-1α, interferon-γ (IFN-γ), and tumor necrosis factor-α have also been implicated in uterine activation (Orsi & Tribe, 2008).

For sufficient activation of the uterus along with activation of CAP, repression of the relaxation system is required (Challis et al., 2000). Inducers of myometrial relaxation include progesterone (P_4), nitric oxide, parathyroid hormone-related peptide, and others (Challis et al., 2000). A study in 2003 (Girotti & Zingg, 2003) utilized microarray technique to identify different factors that may regulate the process of uterine relaxation. Among the expression of other genes identified in the analysis, a gene named *Ugn*, which codes uroguanylin (UGN) protein, decreased its expression up to 20-fold immediately before parturition (Jaleel et al., 2002). UGN ligand binds to its transmembrane enzyme-linked receptor guanylate cyclase C (GC-C) present on the uterus (Jaleel et al., 2002). Upon ligand binding, GC-C catalyses the conversion of GTP to cGMP, and cGMP relaxes smooth muscle cells by inhibiting Ca^{2+} entry (Carvajal et al., 2000). P_4 has been shown to upregulate the expression of PGE2 receptor 2 subtype (EP2) to maintain uterine quiescence, however, expression of EP2 and EP4 becomes down-regulated at the time of parturition in rodents to potentiate the process of contraction (Breyer et al., 2001).

We conducted experiments to determine the involvement of $PGF_{2\alpha}$ signaling during LH-mediated luteolysis. Our specific objectives were to analyze: (1) the effect of 4xLH on PG synthesis and its signaling in the corpus luteum (CL) and uterus during mid and late pregnancy in rats and (2) the effect of inhibition of endogenous PG synthesis on LH-mediated luteolysis and $PGF_{2\alpha}$ signaling in the CL and uterus during late pregnancy.

2 | RESULTS

2.1 | Effect of 4xLH treatment on the expression of $PGF_{2\alpha}$ biosynthesis machinery during pregnancy

We examined the participation of endogenous $PGF_{2\alpha}$ signaling in LH-induced luteolysis. The expression of genes involved in $PGF_{2\alpha}$ synthesis (*Cox1*, *Cox2*, *Pgfs*, and *Cbr1)* was analyzed at the transcript level in the CL and uterine tissue. During late pregnancy, 4xLH treatment decreased the luteal expression of *Cox1* ($p < 0.01$) and *Cox2* (nonsignificant) (responsible for the synthesis of precursor PG, PGH2) (Figure 1a,b). However, the expression of genes *Pgfs* ($p < 0.01$) and *Cbr1* ($p < 0.05$) increased in the CL, the enzymes coded by the genes are responsible for the conversion of PGH2 and PGE2 to $PGF_{2\alpha}$, respectively (Figure 1c,d). Transcript levels of $PGF_{2\alpha}$ biosynthesis genes increased (*Cox1* [nonsignificant], *Cox2* [$p < 0.01$], *Pgfs* [$p < 0.01$], and *Cbr1* [nonsignificant]) in the intact uterus post 4xLH treatment during late pregnancy (Figure 1e–h). No change occurred in the levels of $PGF_{2\alpha}$ biosynthesis genes in the CL (Figure 1i–l) and uterine tissue collected from midpregnant rats (data not shown).

2.2 | Effect of 4xLH treatment on genes associated with activated $PGF_{2\alpha}$ signaling and uterine activation during pregnancy

During late pregnancy, 4xLH significantly decreased luteal expression of 11β-hydroxysteroid dehydrogenase (*11β-hsd2*) ($p < 0.001$) and tissue inhibitors of metalloproteinases 3 (*Timp3*) ($p < 0.05$) compared with Veh treated rats (Figure 2a,c). However, expression of sterol carrier protein 2 (*Scp2*) decreased nonsignificantly upon 4XLH treatment (Figure 2b). We analyzed the transcript levels of CAPs and genes involved in uterine relaxation to assess uterine activation. During late pregnancy, expression of *Il-1β* (nonsignificant), *Infr-γ* ($p < 0.01$), and *Igfbp2* ($p < 0.01$) increased in the uterine tissue of rats receiving 4xLH treatment (Figure 2d–f). Expression of *Ugn* ($p < 0.01$), *Ep2* (nonsignificant), and *Igfbp5* ($p < 0.05$) decreased in the uterine

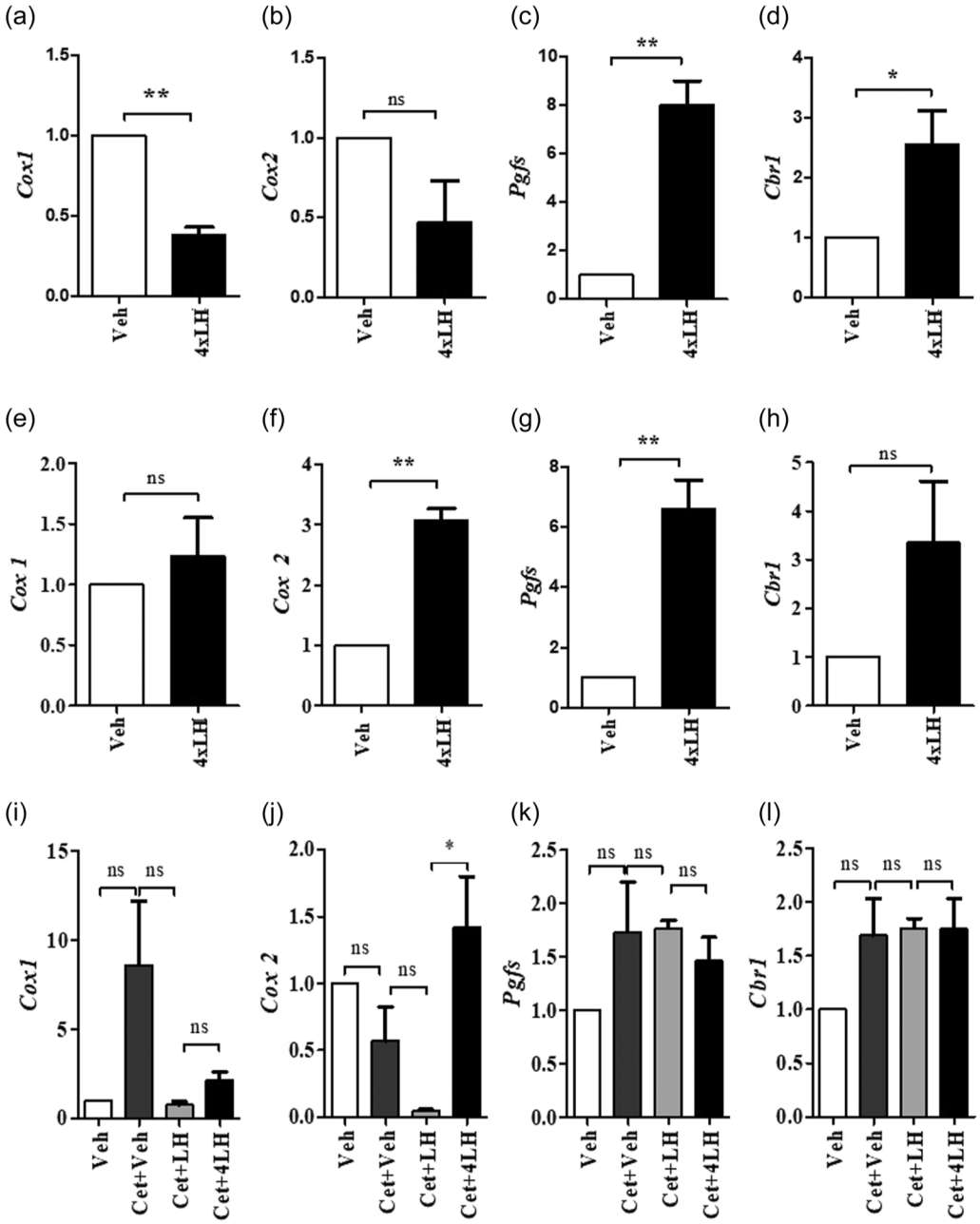

FIGURE 1 Effects of 4xLH treatment on prostaglandin synthesis machinery in late and midpregnant rats. *Cox1*, *Cox2*, *Pgfs*, *Cbr1* mRNA expression during late (a–d) and midpregnancy (i–l) in the luteal tissue. *Cox1*, *Cox2*, *Pgfs*, *Cbr1* mRNA expression during late pregnancy (e–h) in the uterine tissue. L19 mRNA expression levels were used as internal control for qPCR analyses. The data represent mean ± SEM of three rats/group. LH, luteinizing hormone; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction. *<0.05, **<0.01, and ***<0.001.

tissue post 4xLH treatment during late pregnancy (Figure 2g–i). No change occurred in the levels of $PGF_{2\alpha}$ signaling genes in the CL and uterine tissue collected from midpregnant rats (data not shown). Expression analysis of $PGF_{2\alpha}$ biosynthesis and its downstream signaling genes suggested that $PGF_{2\alpha}$ signaling is activated in LH-induced luteolysis during the late stage and not during the midstage of pregnancy. Therefore, to further examine the role of $PGF_{2\alpha}$ signaling in LH-induced luteolysis, we administered DIC (an inhibitor of PG synthesis) to block the endogenous synthesis of PG during the late stage of pregnancy in rats.

2.3 | Effect of 4XLH treatment on expression of $PGF_{2\alpha}$ biosynthesis genes and uterine activation in DIC pretreated late-pregnant rats

We utilized DIC to inhibit the expression of genes involved in $PGF_{2\alpha}$ biosynthesis. A significant reduction in *Cox1*, *Cox2*, *Pgfs*, *and Cbr1* expression was observed in DIC treated group compared with Veh group in both the luteal and uterine tissue (data not shown). After confirming inhibition of the genes associated with $PGF_{2\alpha}$ biosynthesis by DIC treatment, 4xLH treatment was given to DIC-treated rats to

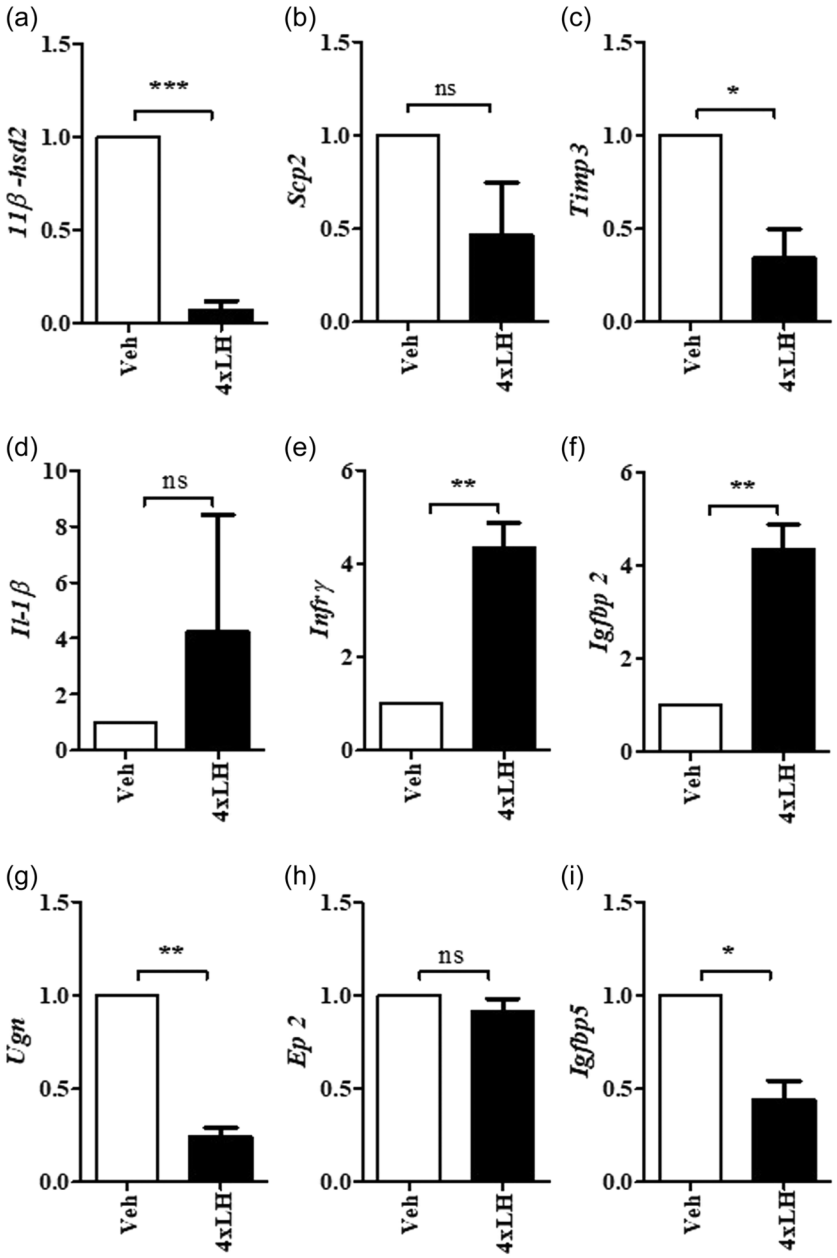

FIGURE 2 Effects of 4xLH treatment on genes associated with $PGF_{2\alpha}$ signaling during late pregnancy. *11β-hsd2* (a), *Scp2* (b), *Timp3* (c) mRNA expression in the luteal tissue. *Il-1β* (d), *Infr-γ* (e) and *Igfbp2* (f) *Ugn* (g), *Ep2* (h), *Igfbp5* (i) mRNA expression in the uterine tissue. L19 mRNA expression levels were used as internal control for qPCR analyses. The data represent mean ± SEM of three rats/group. LH, luteinizing hormone; mRNA, messenger RNA. *<0.05, **<0.01, and ***<0.001.

examine if LH could still mediate its luteolytic effects in the absence of endogenous PG signaling. The experimental regimen followed is described in Section 4. For simplicity, group of rats that received only DIC treatment will be referred to as DIC group and the group that received a combination of DIC plus 4xLH treatment will be referred to as DIC + 4LH group henceforth. Luteal expression of *Cox2* ($p < 0.01$), *Pgfs* ($p < 0.05$), and Cbr*1* (nonsignificant) increased in the DIC + 4LH compared with DIC group (Figure 3b–d). However, the expression of *Cox1*, the constitutively expressed isoform, decreased in the DIC + 4LH group compared with DIC group (Figure 3a). The expression of *Cox1* (nonsignificant), *Cox2* (nonsignificant), *Pgfs* ($p < 0.01$), and *Cbr1* (nonsignificant) increased in the uterine tissue collected from DIC + 4LH group (Figure 3e–h). The expression of *CAPs* (*Il1-β* [$p < 0.01$], *Infr-γ* [$p < 0.05$], and *Igfbp2* [nonsignificant]) increased in the DIC + 4LH group compared with DIC group (Figure 4a–c). The expression of genes associated with uterine relaxation (*Ugn* [$p < 0.001$], *Ep2* [$p < 0.05$], and *Igfbp5* [nonsignificant]) decreased in the uterine tissue collected from the DIC + 4LH group compared with DIC group (Figure 4d–f).

2.4 | Effect of 4xLH treatment on cAMP/PKA/CREB pathway in CL of rats treated with DIC

The desensitization of cAMP/PKA/CREB pathway post 4xLH treatment plays a crucial role in LH-mediated luteolysis (Vashistha et al., 2021). We next analyzed the effect of blockage of endogenous PGs on LH-induced downregulation of

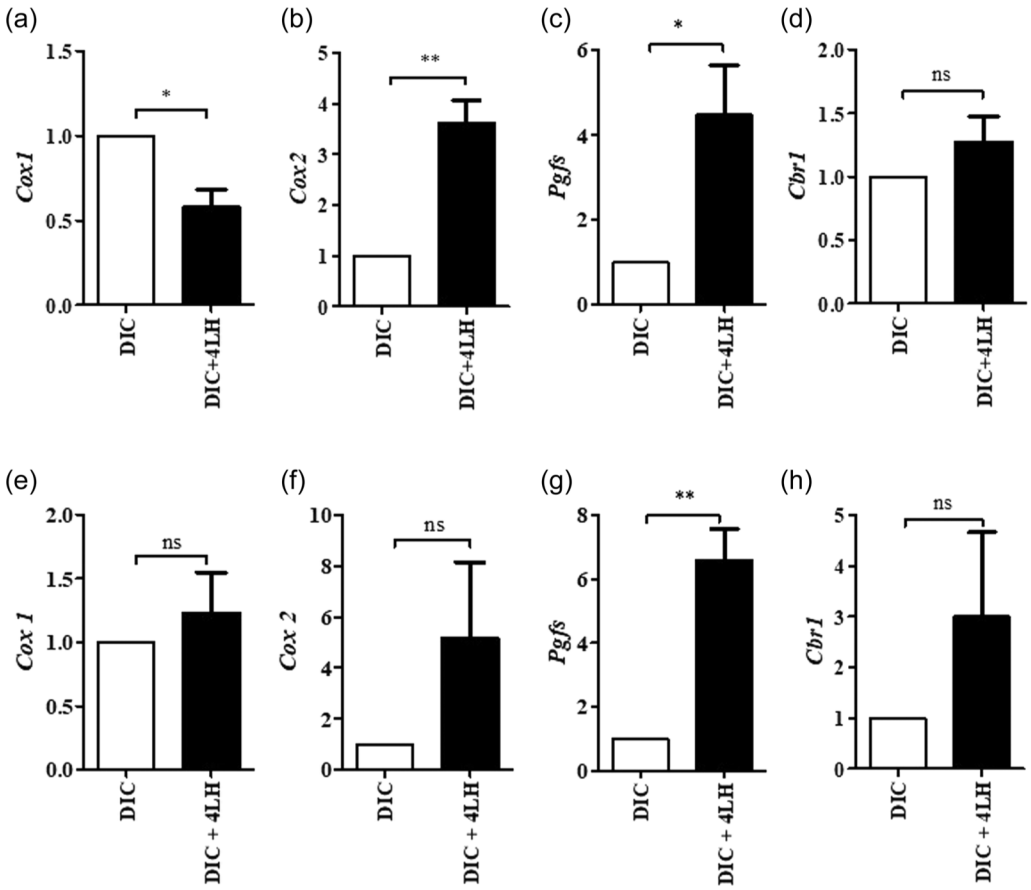

FIGURE 3 Effects of 4xLH treatment on prostaglandin synthesis machinery during late pregnancy. The rats were also treated with DIC to inhibit endogenous prostaglandin secretion before administration of single or multiple injections of LH. *Cox1* (a), *Cox2* (b), *Pgfs* (c), *Cbr11* (d) mRNA expression in the luteal tissue. *Cox1* (e), *Cox2* (f), *Pgfs* (g), *Cbr1* (h) mRNA expression in the uterine tissue. L19 mRNA expression levels were used as internal control for qPCR analyses. The data represent mean ± SEM of three rats/group. LH, luteinizing hormone; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction. *<0.05, **<0.01, and ***<0.001.

cAMP/PKA/CREB pathway. As can be seen in Figure 5a–c, 4xLH treatment decreased the luteal messenger RNA (mRNA) expression of luteinizing hormone receptor (*Lh/cgr*) ($p < 0.01$), cAMP content ($p < 0.01$), and pCREB protein levels ($p < 0.01$) compared with Veh-treated late-pregnant rats. Inhibition of endogenous PG biosynthesis (DIC group) did not affect the cAMP/PKA/CREB pathway as revealed by no significant change in *Lh/cghr* transcript levels, luteal cAMP levels, and phosphorylation status of CREB in comparison with Veh treated animals (Figure 5a–c). We observed a reduction in *Lh/cghr* ($p < 0.001$) expression, cAMP levels ($p < 0.05$), and pCREB (nonsignificant) in the luteal tissue of DIC + 4LH treated rats compared with DIC treated group (Figure 5a–c). However, rats that received either LH or a combination of DIC and 4xLH (DIC + 4LH group) had no significant difference in the mRNA levels of *Lh/cgr*, luteal cAMP content, and phosphorylated CREB (Figure 5a–c). The results indicate that 4xLH treatment following inhibition of endogenous $PGF_{2\alpha}$ synthesis can still cause desensitization of the cAMP/PKA/CREB pathway. This suggests that endogenous $PGF_{2\alpha}$ does not play a role in LH-induced downregulation of the cAMP/PKA/CREB pathway.

2.5 | Effect of 4xLH treatment on functional and structural luteolysis in rats treated with DIC

Analysis of the effect of inhibition of endogenous PG biosynthesis on LH-mediated functional luteolysis revealed that the expression of the luteolytic marker *20α-hsd* increased ($p < 0.001$) upon 4xLH treatment compared with Veh treated group (Figure 5d). Inhibition of endogenous PGs did not significantly alter its expression compared with the Veh-treated group (Figure 5d). An increase ($p < 0.05$) in its expression was observed in the DIC + 4LH group compared with the DIC group (Figure 5d). However, the increase in the expression of the gene *20α-hydroxysteroid dehydrogenase* (*20α-hsd*) in the DIC + 4LH group was significantly less than the group in which endogenous PGs were not depleted (LH injections group) (Figure 5d). Nonetheless,

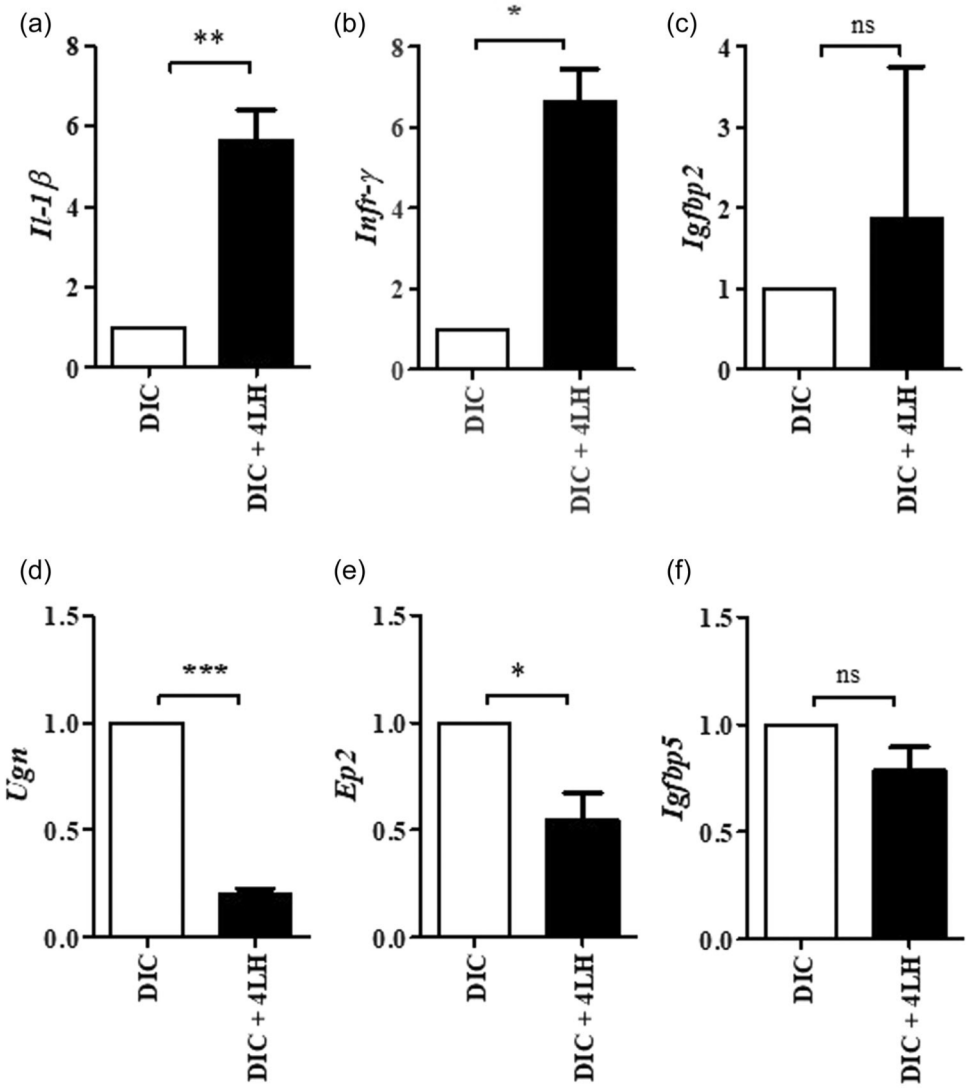

FIGURE 4 Effects of 4xLH treatment on genes associated with $PGF_{2\alpha}$ signaling during late pregnancy. The rats were also treated with DIC to inhibit endogenous prostaglandin secretion before administration of single or 4xLH treatment. *Il-1β* (a), *Infr-γ* (b) and *Igfbp2* (c) *Ugn* (d), *Ep2* (e), *Igfbp5* (f), mRNA expression in the uterine tissue. L19 mRNA expression levels were used as internal control for qPCR analyses. The data represent mean ± SEM of three rats/group. LH, luteinizing hormone; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction. *<0.05, **<0.01, and ***<0.001.

the findings indicate that endogenous PG plays some role in functional luteolysis initiated by 4xLH treatment during the late stage of pregnancy. However, we did not observe any change in the serum P_4 levels between the DIC and DIC + 4LH groups (Figure 5e). Further analysis of the effect of depletion of endogenous $PGF_{2\alpha}$ on LH-mediated structural luteolysis was carried out by analyzing the protein levels of apoptosis (Cleaved caspase 3) and autophagy (LC3I/II, Beclin1) markers. Protein levels LC3I/II, Beclin1, and Cleaved caspase 3 did not change in the luteal tissue collected from the DIC + 4LH group compared with DIC group (Figure 5f–h). The findings suggest that 4xLH treatment protocol in the absence of endogenous $PGF_{2\alpha}$ signaling activates only functional luteolysis.

3 | DISCUSSION

4xLH treatment initiates the process of luteolysis during different stages of pregnancy in rats (Vashistha et al., 2021). In this study, we have analyzed the effect 4xLH treatment on $PGF_{2\alpha}$ biosynthesis machinery, uterine activation, and the effect of blockage of overall PG synthesis machinery on LH-mediated luteolysis.

LH is known to have stage-dependent effects on pregnancy (Vashistha et al., 2021). During midpregnancy, we did not observe any significant change in the expression of $PGF_{2\alpha}$ synthesis and $PGF_{2\alpha}$ responsive genes in the luteal and uterine tissue post 4xLH treatment. The results suggest that endogenous $PGF_{2\alpha}$ signaling may not play a

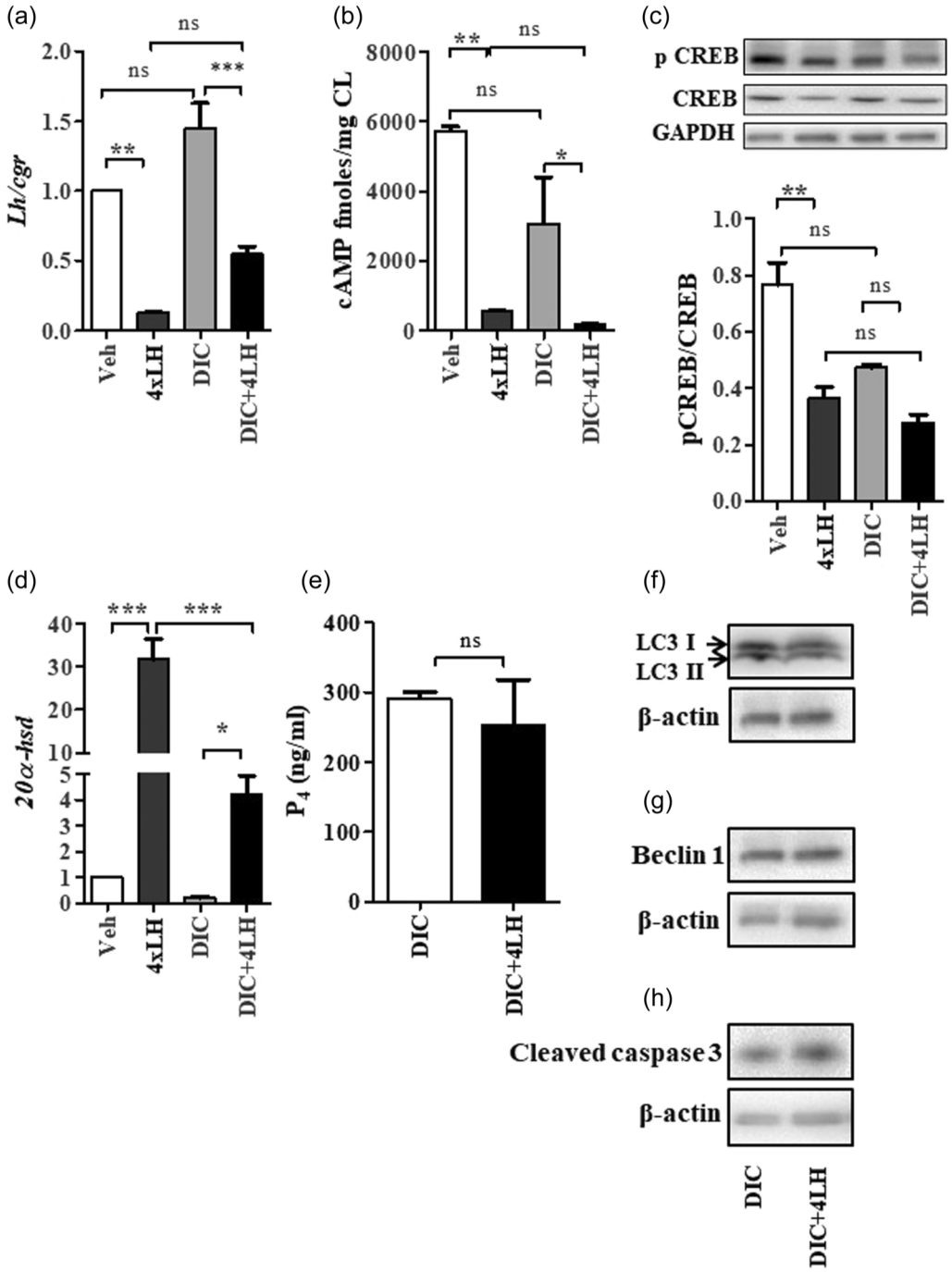

FIGURE 5 Effects of 4xLH treatment on various components of cAMP signal transduction pathway and markers of luteolysis in late-pregnant rats. The rats were also treated with DIC to inhibit endogenous prostaglandin secretion before administration of single or 4xLH treatment. mRNA expression of *Lh/cgr* (a), cAMP levels (b), representative immunoblot analysis image and quantitation data for pCREB and CREB (c), and mRNA expression of luteal *20α-hsd* (d) in the luteal tissue. Serum P_4 levels (e), representative immunoblot analysis image and quantitation data for LC3 II (f), Beclin 1 (g), and Cleaved Cas 3 (h) in the luteal tissue. L19 mRNA expression was used as an internal control for qPCR. GAPDH and β-actin protein levels were used as internal controls for qPCR and immunoblot analyses. Data represented mean ± SEM of three–five rats/group. LH, luteinizing hormone; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction. *<0.05, **<0.01, and ***<0.001.

significant role in LH-induced luteolysis during midpregnancy (Farina et al., 2004). It was demonstrated in the lab previously that the serum levels of P_4 do not change upon 4xLH treatment during the midstage of pregnancy (Vashistha et al., 2021). The limited role of $PGF_{2\alpha}$ signaling during midpregnancy can be explained as the inhibitory effects of P_4 are still intact and prevent the activation of $PGF_{2\alpha}$ signaling during 4xLH treatment. P_4 has been demonstrated to play a crucial role in maintaining uterine quiescence by various groups (Farina et al., 2004). In several

mammalian species, excepting humans, a drop in P_4 is a prerequisite for the onset of labor (Kota et al., 2013). Various studies corroborate that a drop in P_4 levels is required to initiate the luteolytic events and uterine activation (Kota et al., 2013; C. O. Stocco & Deis, 1996). P_4 has been shown to mediate its effect of maintaining uterine quiescence through a number of mechanisms which include increase in the expression of calcium and potassium channels (Mendelson & Condon, 2005; Soloff et al., 2011), downregulation of expression of uterotonic agents and members of their signaling cascades (Mendelson & Condon, 2005; Soloff et al., 2011) and decrease in the cross-linking of the actin with the myosin filament (Soloff et al., 2011).

In the present study, 4xLH treatment decreased the expression of *Cox1* and *Cox2* in the late-stage pregnant rat luteal tissue suggesting a downregulation in overall PG production. An increase in the expression of *Pgfs* and *Cbr1* suggests that 4XLH treatment specifically shifts the PG synthesis machinery toward $PGF_{2\alpha}$ production. However, whether the increase in Pgfs/Cbr1 expression leads to increased PG synthesis requires further investigation. Other effectors of $PGF_{2\alpha}$ signaling in the CL include *11β-hsd2*, *Scp2*, and *Timp3* (Berridge & Irvine, 1984). *11β-hsd2* is an enzyme that converts corticosterone to 11-dehydrocorticosterone (an inactive product). Corticosterone binds to the GR through which P4 is believed to mediate its actions (Lei et al., 2012). PGF2α has been reported to inhibit *11β-hsd2* expression (Hardy et al., 1999). Another important gene on which PGF2α act includes *Timp3*, it is involved in tissue remodeling and is known to get downregulated in response to PGF2α treatment (C. Stocco et al., 2001). siRNA-mediated reduction of TIMP3 has been associated with decreased progesterone synthesis. *Scp2* is involved in the delivery of cholesterol to the outer mitochondrial membrane and plays an important role in the process of steroidogenesis (McLean et al., 1995). PGF2α treatment has been reported to down-regulate *Scp2* mRNA expression. We observed a decrease in their expression upon 4xLH treatment. These genes get downregulated on $PGF_{2\alpha}$ treatment (Berridge & Irvine, 1984; Hardy et al., 1999) and hence serve as the markers of increased $PGF_{2\alpha}$ signaling. Unlike the midstage, the results obtained during the late stage suggest that the 4xLH treatment stimulates $PGF_{2\alpha}$ signaling in the CL. Similar observations were made by Stocco et. al., where they demonstrated an increase in luteal PG content which further led to an increase in 20α-HSD activity in LH-induced luteolysis during the late-stage of pregnancy in rats (C. Stocco & Deis, 1998).

In rats, luteolysis precedes the process of parturition. For the initiation of parturition, the uterus transforms from a quiescent state to a highly active state. This transformation involves increased expression of genes encoding PG synthesis machinery, CAPs, and repression of uterine relaxant systems (JRG et al., 2000; Mitchell et al., 2005). During the late stage of pregnancy, an increase in the mRNA expression of uterine PG synthesis at machinery was observed post 4xLH treatment. Similar observations have been made by others as well in Leydig cells (Chen et al., 2007). In bovines, LH stimulates the expression of *Cox2*, *Pgfs*, and *Cbr1* (Chen et al., 2007).

In the present study, the expression of CAPs increased, and the expression of genes involved in uterine relaxation decreased post 4xLH treatment during the late stage of pregnancy. The results suggest that LH-mediated luteolysis activates $PGF_{2\alpha}$ signaling in the uterine tissue. An increase in the PG signaling during luteolysis and parturitions has been observed by others as well. Among the CAPs, *Ugn* is known to undergo rapid reduction just before parturition (Girotti & Zingg, 2003). EP receptor subtypes EP1, EP2, EP3, and EP4 receptors have different effects on the uterus: EP1 and EP3 are involved in uterine contraction while EP2 and EP4 are responsible for uterine relaxation (Astle et al., 2005). We observed a decrease in the expression of EP2 receptor subtype suggesting activation of the uterus. Brodt-Eppley and colleagues (Astle et al., 2005) analyzed the expression of EP2 receptor throughout gestation and observed that the mRNA levels of EP2 were highest on Day 16 of pregnancy and declined significantly until parturition. We observed an increase in the mRNA levels of cytokines post 4xLH treatment. An increase in the levels of cytokines during $PGF_{2\alpha}$ induced luteolysis in bovine CL has been reported by others as well (Chen et al., 2007).

4xLH treatment has been shown to activate luteolytic events both in-vivo and in-vitro via desensitization of the cAMP/PKA/CREB pathway (Vashistha et al., 2021). $PGF_{2\alpha}$ is a well-established luteolysin in rodents and is known to interact with the cAMP/PKA/CREB pathway (Astle et al., 2005). Hence, we analyzed the effect of inhibition of endogenous PGs on the cAMP/PKA/CREB pathway. Inhibition of endogenous PG synthesis did not have any effect on cAMP/PKA/CREB pathway. Similar effects on cAMP/PKA/CREB pathway upon stimulation with $PGF_{2\alpha}$ have also been reported in bovine luteal cell culture (Astle et al., 2005). The enzyme AC generally acts as the site for interaction between the PG signaling and cAMP/PKA/CREB pathway (Astle et al., 2005). Out of the nine ACs, AC 1, AC 3, and AC 8 are known to be stimulated by Ca^{2+} released on FP receptor activation. AC 5 and AC 6 are inhibited whereas AC 2, AC 4, AC 7, and AC 9 are unaffected by Ca^{2+} (Astle et al., 2005). This suggests that the type of interaction between the FP receptor and adenylate cyclase pathway might depend on the AC isoform expressed.

$PGF_{2\alpha}$ positively regulates the expression of the luteolytic marker *20α-hsd* (C. O. Stocco et al., 2000). In rats, $PGF_{2\alpha}$ administration induces premature expression of 20α-HSD in the luteal tissue (C. O. Stocco et al., 2000). The increased expression of 20α-HSD gives the signal for parturition in rodents. The primary function of 20α-HSD is to increase the catabolism of P_4 into an inactive metabolite 20α-dihydroprogesterone (C. O. Stocco et al., 2000). Our results indicate that even in the absence of endogenous $PGF_{2\alpha}$ signaling, 4xLH treatment could initiate functional luteolysis but not structural luteolysis. Although the data suggest that desensitization of the cAMP pathway and an active $PGF_{2\alpha}$ signaling are required for the initiation of luteolysis to the full extent. Although the data give some insights in the luteolytic process more studies utilizing relevant signaling markers are required for proper understanding of the process.

In summary, our results suggest that endogenous $PGF_{2\alpha}$ may contribute to LH-mediated luteolysis, but this dependency on endogenous $PGF_{2\alpha}$ is pregnancy-stage dependent. Unlike the mid-stage of pregnancy, endogenous $PGF_{2\alpha}$ plays some role during the

late stage. Even during the late stage of pregnancy, the participation of $PGF_{2\alpha}$ signaling appears to be subtle compared with the cAMP/PKA/CREB pathway in the initiation of LH-mediated luteolysis.

4 | MATERIALS AND METHODS

4.1 | Reagents

Ovine LH and Cetrorelix (Cet) were kind gifts from Professor M. R. Sairam (University of Montreal, Quebec, Canada) and Asta Medica, respectively. Trizol (93289) and Diclofenac (D6899) were purchased from Sigma Aldrich Co. P_4 RIA kit (IM1188) was procured from Beckman Coulter. Antibody against cyclic adenosine monophosphate (cAMP), CV-27 pool was obtained from Dr. A. F. Parlow, NHPP (CA, USA). PKA assay kit (ADI-EKS-390A) was purchased from Enzo Life Sciences. Table 1 contains details of the antibodies employed. All the other reagents were purchased from Sigma Aldrich Co. or sourced from local distributors.

4.2 | Animals

The Institutional Animal Ethics Committee, Indian Institute of Science (Bangalore, India), approved procedures for animal handling and experimentation. *Rattus norvegicus* (Harlan-Wistar strain) were housed in a controlled environment and kept under 12 h light and 12 h dark cycles with ad libitum access to food and water. To obtain pregnant rats, 2–3 month-old female virgin rats were cohabitated with male rats. Vaginal smears were screened daily for the presence of sperm. We designated the day of the appearance of sperm as Day of pregnancy (DOP 1). After establishing pregnancy, female rats were housed individually for further experimentation. The gestation period in our colony of rats is 23 days. We collected blood and CL at suitable intervals depending on the experiment.

4.3 | LH-mediated induction of luteolysis in pregnant rats

We carried out studies in the late (DOP 19) and midstage (DOP 8–10) pregnant rats. Previously published protocol was followed (Vashistha et al., 2021). Briefly, late-stage pregnant rats were assigned into two groups with three animals/group. On DOP 19, each group received either four i.p. injections of Veh (100 µL of 1× phosphate-buffered saline [PBS]) or LH (10 µg dissolved in 100 µL of 1× PBS) at 08:00, 09:00, 10:00, and 11:00 h. On DOP 20, rats from both groups received one i.p. injection of LH (10 µg). Circulating LH levels are high during midpregnancy, and CL functions are dependent on LH during this period (Loewit et al., 1969). To rule out the participation of endogenous LH during exogenous administration of LH, Cet, a GnRH receptor antagonist, was administered to inhibit endogenous levels of LH. The tested dose and duration of Cet treatment were reported previously (John et al., 2016). Administration of 150 µg/Kg BW of Cet every 12 h on DOP 8–9 of pregnancy resulted in luteolysis and pregnancy loss (John et al., 2016). We assigned rats on DOP 8 to four different groups with three rats/group. Rats in the first and second groups received four s.c. injections of either Veh (100 µL of 1× PBS) or Cet (150 µg/Kg BW) at 12 h intervals for 48 h beginning on DOP 8, respectively. Post Cet treatment, we administered one i.p. injection of Veh on DOP 10. Rats in the third and fourth groups received Cet treatment as per the second group. Furthermore, on DOP 10, rats from the third and fourth groups received one or four injections of LH (10 µg: at hourly intervals), respectively. The first, second, third, and fourth groups are referred to as Veh, Cet+Veh, Cet+LH, and Cet+4LH groups, respectively. Blood and CL were collected from rats during both stages, 40 min after the last Veh/LH injection.

4.4 | Effect of PG synthesis blockade on LH-induced luteolysis

Endogenous PGs were inhibited by employing DIC during late pregnancy to confirm the role of PG signaling during LH-induced luteolysis. Previously published protocol with few modifications (Chan, 1983) was followed. Briefly, we assigned 18-day-old pregnant rats (late-pregnant stage) into two groups (three animals/group). Rats in both groups received DIC by oral gavage three times daily (6 a.m. [1 mg], 12 p.m. [0.5 mg], and 6 p.m. [0.5 mg]) on Days 18–19 of pregnancy. On Day 20 of pregnancy, both the groups received one injection of DIC (6 a.m.) and a single i.p. injection of LH (10 µg: dissolved in 100 µL of 1X PBS)

TABLE 1 List of antibodies used for immunoblotting.

Si. No.	Antibody	Company	Catalog number	Dilution	Secondary antibody
1.	GAPDH D16H11	CST	#5174	1:1000	Anti-rabbit
2.	Beclin 1	CST	#3738	1:1000	Anti-rabbit
3.	LC3I/II D3U4C0	CST	#12741	1:1000	Anti-rabbit
4.	Cleaved caspase 3	CST	#9661	1:1000	Anti-rabbit
5.	pCREB Ser133	CST	#9198	1:1000	Anti-rabbit
6.	CREB 48H2	CST	#9197	1:1000	Anti-rabbit

at 11 a.m. Additionally, rats in the second group received four injections of LH (10 µg: dissolved in 100 µL of 1X PBS) at hourly intervals on Day 19 of pregnancy as well. Blood, CL, and uterine tissue were collected from rats during both stages, 40 min after the last Veh/LH injection.

4.5 | Tissue and blood processing

Fat was cleared from the CL and uterine tissue upon collection. The tissues were then flash-frozen in liquid nitrogen and stored at −70°C for RNA and protein analysis. Blood was further processed into plasma and serum and stored at −20°C until analysis.

4.6 | quantitative polymerase chain reaction (qPCR)

Total RNA from CL tissues was isolated using TRI reagent as per the manufacturer's recommendations. The qPCR analysis was performed as described previously (Vashistha et al., 2021). Table 2 contains the list of primers used in the qPCR analysis.

TABLE 2 List of primers used for real-time PCR.

Si. No.	Gene name	Primer sequence (5′ to 3′)	Annealing temp (°C)
1.	*rpL19*	**F-** CGTCCTCCGCTGTGGTAAA	62
		R- AGTACCCTTCCTCTTCCCTATGC	
2.	*20α-hsd*	**F-** CTGTAAACCAGGTCGAATGTCAC	64
		R- GGGTAGTTCGGGTTCACCC	
3.	*Cox1*	**F-** GGTACTCACGGTGCGGT	62
		R- GAATGAACTCCCTTCTCAGC	
4.	*Cox 2*	**F-** CATGGGTGTGAAAGGAAATAAG	62
		R- CACCGTAAACATGATTTAAGTCC	
5.	*Pgfs*	**F-**GGGAGGCCATGGAGAAGTGTAAG	62
		R-GGCAACCAGAACGATGTCAT	
6.	*Cbr1*	**F-** ACAACCCGCAGAGCATCCG	62
		R- TTGATACATTCACCACTCTGCCTTGG	
7.	*Timp3*	**F-** GCTGACAGGGCGCGTGTATG	59.4
		R- TAGCCAGGGTACCCGAAATTGG	
8.	*Scp2*	**F-** TGTACGGTGAATCCACCTGCG	59.4
		R- GTACCAAGGGATCCCTTTTCCATC	
9.	*Ugn*	**F-**AGAAACCCAGAGGTGTGAGC	59.4
		R-CGGACATCTGCTTCTCTTCC	
10.	*Ep2*	**F-** CTGGTAACGGAACTGGTGCT	**68**
		R- CGTGGCCAGACTAAAGAAGG	
11.	*Lh/cgr*	**F-**TCCAAGGGATGAATAACGAGTCTG	62
		R-TGGAAGGCTCCACTGTGCAT	
12.	*Igfbp5*	**F-**GGAATCTGACCAAGGCCCC	**63**
		R-GAGAAGGCTTGCACTGCTTTCTC	
13.	*Il-1β*	**F-** GAACATAAGCCAACAAGTGGTA	**60**
		R- TTGGGATCCACACTCTCC	
14.	*Infr--γ*	**F-** GGACGGTAACACGAAAATACTTG	**60**
		R- TCACCTCGAACTTGGCGAT	
15.	*Igfbp2*	**F-** GCCTGTACAACCTCAAACAG	**62**
		R- GAAGAGTGACACTCGGGGT	

Abbreviation: PCR, polymerase chain reaction.

4.7 | Immunoblotting

Immunoblot analysis of the total protein lysates from CL tissue was carried out as per the procedures reported previously (Kunal et al., 2012). Table 1 contains the list of antibodies used in the immunoblotting analysis.

4.8 | RIA of cAMP

cAMP lysate preparation and the assay protocol followed have been described previously (Kunal et al., 2012). The antibody (CV-27) at a dilution of 1:30,000 (~ 40% binding) was used for the assay of samples.

4.9 | P_4 assay

Serum P_4 levels were determined by employing a commercially available RIA kit as per the manufacturer's instructions. The inter-and intra-assay coefficients of variation were found to be ≤9%.

4.10 | Statistical analysis

All data are expressed as mean ± SEM. The graphs were plotted and analyzed using GraphPad Prism® 5 software (GraphPad Software, Inc.). A two-tail paired "*t*" test with 95% confidence intervals was used for statistical analysis of the results between two groups. One-way analysis of variance and Bonferroni posttests with 95% confidence intervals were used to compare three or more groups. A $p < 0.05$ was considered to be statistically significant.

AUTHOR CONTRIBUTIONS

Akshi Vashistha conceived and conceptualized the research program, designed experimental strategies, and supervised all experiments. Akshi Vashistha and Habibur Rahaman Khan collected samples. Akshi Vashistha performed data acquisition, data analysis and wrote the draft of the manuscript. All authors approve submission of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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