Received: 16 June 2020 | Revised: 5 August 2020 | Accepted: 23 August 2020

DOI: 10.1111/rda.13812

ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Hypoxia-inducible factor-1alpha and nitric oxide synthases in bovine follicles close to ovulation and early luteal angiogenesis

Bajram Berisha[1,2] | Dieter Schams[2] | Fred Sinowatz[3] | Daniela Rodler[3] | Michael W. Pfaffl[2]

[1]Animal Biotechnology, Faculty of Agriculture and Veterinary, University of Prishtina, Prishtinë, Kosovo

[2]Animal Physiology and Immunology Weihenstephan, Technical University of Munich, Freising, Germany

[3]Department of Veterinary Sciences, Ludwig Maximilian University of Munich, Munich, Germany

Correspondence
Bajram Berisha, Animal Biotechnology, Faculty of Agriculture and Veterinary, University of Prishtina, Rr. George Bush, n.n., 10000 Prishtinë, Kosovo.
Email: bajram.berisha@uni-pr.edu

Funding information
German Research Foundation (DFG Project number 5270108).

Abstract

The objective of the study was to characterize expression patterns of hypoxia-inducible factor-1alpha (HIF1A), inducible nitric oxide synthase (iNOS) and endothelial (eNOS) isoforms in time-defined follicle classes before and after GnRH application in the cow. Ovaries containing pre-ovulatory follicles or corpora lutea were collected by transvaginal ovariectomy (n = 5 cows/group) as follow: (I) before GnRH administration; (II) 4h after GnRH; (III) 10h after GnRH; (IV) 20h after GnRH; (V) 25h after GnRH; and (VI) 60h after GnRH (early corpus luteum). The mRNA abundance of HIF1A in the follicle group before GnRH was high, followed by a significant down regulation afterwards with a minimum level 25h after GnRH (close to ovulation) and significant increase only after ovulation. The mRNA abundance of iNOS before GnRH was high, decreased significantly during LH surge, with minimum levels afterwards. In contrast, the mRNA of eNOS decreased in the follicle group 20h after GnRH, followed by a rapid and significant upregulation just after ovulation. Immunohistochemically, the granulosa cells of antral follicles and the eosinophils of the theca tissue as well of the early corpus luteum showed a strong staining for HIF1A. The location of the eosinophils could be clearly demonstrated by immunostaining with an eosinophil-specific antibody (EMBP) and transmission electron microscopy. In conclusion, the parallel and acute regulated expression patterns of HIF1A and NOS isoforms, specifically during the interval between the LH surge and ovulation, indicate that these paracrine factors are involved in the local mechanisms, regulating final follicle maturation, ovulation and early luteal angiogenesis.

KEYWORDS
angiogenesis, bovine, corpus luteum, HIF-1alpha, NOS, ovarian follicle

1 | INTRODUCTION

The angiogenesis (development of vascular network) in the ovary is nowadays well established to be of great importance for the development and function of the reproductive tract (Abulafia & Sherer, 2000; Berisha, Schams, Rodler, Sinowatz, & Pfaffl, 2018; Chouhan et al., 2013; Plendl, 2000; Schams, Steinberg, Steffl, Meyer, & Berisha, 2009). The gonadotropin surge in ruminants activates a biochemical cascade that leads to the final follicle growth and ovulation, resulting in corpus luteum (CL) formation and

development (Berisha, 2018; Berisha & Schams, 2005; Berisha, Schams, Rodler, Sinowatz, & Pfaffl, 2016; Tsafriri & Reich, 1999). The folliculo-luteal transition is a complex process that requires the development of angiogenesis in early CL which is closely regulated by a variety of endocrine and paracrine factors (Berisha, Rodler, Schams, Sinowatz, & Pfaffl, 2019; Curry & Smith, 2006; Thompson, Brown, Kind, & Russell, 2015). The key regulators of angiogenesis in ovarian follicles and CL include some molecules like vascular endothelial growth factor (VEGF), angiopoietin (ANPT), fibroblast growth factor (FGF) and insulin-like growth factor (IGF) family members (Abulafia & Sherer, 2000; Berisha, Schams, Rodler, & Pfaffl, 2016; Fraser & Lunn, 2000; Hayashi, Berisha, Matsui, Schams, & Miyamoto, 2004).

Some recent studies, however, suggested multiple roles of tissue hypoxia and its interaction with different paracrine factors in ovarian angiogenesis (Meidan, Klipper, Zalman, & Yalu, 2013; Thompson et al., 2015). In this context, the locally produced pro-angiogenic molecules, hypoxia-inducible factor-1alpha (HIF1A) and nitric oxide (NO) synthases namely their inducible (iNOS) and endothelial (eNOS) isoforms seem to be highly critical regulatory mediators for the regulation of the oestrous cycle (Anteby et al., 1996; Guo et al., 2019; Jablonka-Shariff & Olson, 1997). Hypoxia-inducible factor-1alpha expression in mature follicles changes dramatically during the periovulatory period, suggesting them to have an essential role during ovulation and CL formation (Van den Berisha, Schams, Rodler, Sinowatz, & Pfaffl, 2017; Faes et al., 2009; Rico et al., 2014; Driesche, Myers, Gay, Thong, & Duncan, 2008). The ovarian eNOS and iNOS showed distinct cell-specific expression patterns and are differentially regulated during follicular and luteal development (Anteby et al., 1996; Nath & Maitra, 2018; Olson, Jones-Burton, & Jablonka-Shariff, 1996). The role of eNOS and iNOS in the regulation of folliculogenesis is confirmed by its involvement in the control of ovulation rate, apoptotic cell death and granulosa-luteal cell steroidogenic activity (Faes et al., 2009; Guo et al., 2019; Jablonka-Shariff & Olson, 1997).

The HIF1A and members of NOS family have been demonstrated to have critical roles in angiogenesis via regulation of different angiogenic factors (Abe et al., 2013; Krock, Skuli, & Simon, 2011; Nishimura & Okuda, 2010). In addition, the presence of eosinophilic cells in the ovary of different species at the time of oestrus seems also to contribute to the follicle growth, ovulation and development of the early CL (Aust et al., 2000; Cavender & Murdoch, 1988; Reibiger & Spanel-Borowski, 2000). Our previous experiment (Kliem et al., 2013) showed that eosinophilic cells accumulate in the capillaries of the bovine Graafian follicle and in the developing CL after ovulation, playing an important role in ovarian angiogenesis.

The HIF1A and NOS isoforms were shown to have effects in the regulation of ovarian function in ruminants. However, the examination of these factors under specific physiological phases, especially in follicle just before ovulation and during CL formation, has not been thoroughly elucidated to date. Thus, we tested the hypothesis that the pre-ovulatory LH surge may affect HIF1A and examined NOS family members, which may have further effects on the folliculo-luteal transition and CL formation. Therefore, the objective of the study was to provide a closer look at changes in ovarian HIF1A and NOS expression patterns in bovine follicles, specifically during the interval between the ovulatory gonadotropin surge and ovulation in time-defined follicle classes before and after GnRH application (LH surge) and after ovulation (early CL).

2 | MATERIAL AND METHODS

2.1 | Animals, procedure of superovulation and collection of ovaries

The animal experiment was approved by the animal ethics committee located at the government of Upper Bavaria (reference number 211-2531.3-33/96). The study was performed on 30 German Fleckvieh cows with a normal oestrous cycle, and the superovulation procedure was conducted as described by Berisha, Steffl, Amselgruber, and Schams (2006), Berisha et al. (2019). For confirmation of LH surge, blood samples were collected at −24h, −12h, −1h and 0h before and 3h and 12h after GnRH application (Berisha et al., 2008). The bovine ovaries (containing pre-ovulatory follicles or early CL) were collected by transvaginal ovariectomy and

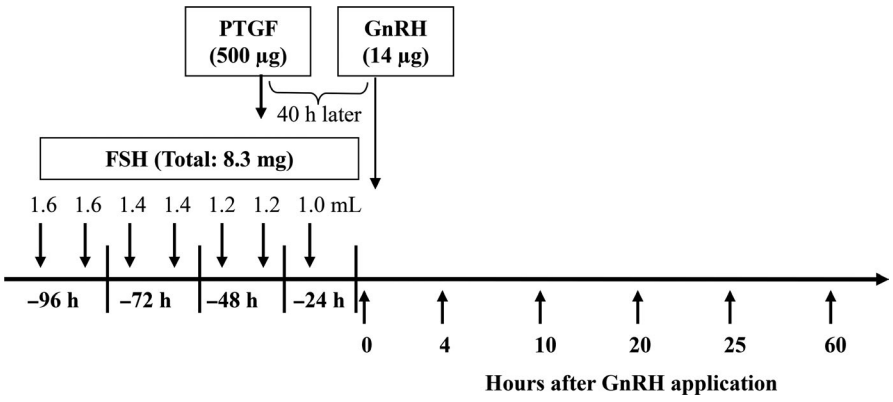

FIGURE 1 Time schedule of the treatment for multiple ovulation and ovary collection in cows. Ovaries containing pre-ovulatory follicles or new CL were collected at (I) 0h, (II) 4h, (III) 10h, (IV) 20h, (V) 25h (follicles) and (VI) 60h (early CL, Day 2–3) relative to injection of gonadotropin-releasing hormone (GnRH) to induce an luteinizing hormone (LH) surge ($n = 5$ cows/group). FSH (Follicle-stimulating hormone), PTGF (Prostaglandin F2-alpha)

classified as follow: (I) 0h, (II) 4h, (III) 10h, (IV) 20h, (V) 25h (follicles) and (VI) 60h (early CL, 2–3 days after ovulation) relative to injection of GnRH (Figure 1).

2.2 | Collection, classification and preparation of periovulatory follicles and early CL

Only follicles that appeared healthy (i.e. well vascularized with transparent follicular wall and fluid) and whose diameter were > 10 mm were collected. The follicular fluid (FF) was aspirated from follicles first, and after that, the follicle tissue aliquots were stored at −80C until extraction of RNA. The follicular fluid (FF) was stored at −20°C until the determination of oestradiol, progesterone, prostaglandin F2alpha and prostaglandin E2 (Berisha et al., 2019). The follicles and CL tissue were fixed in Bouin´s solution for immunohistochemical analysis of HIF1A and eosinophil major basic protein (EMBP). Regarding the experimental protocol, in our previous studies, we have shown that the superovulated follicles after GnRH application were comparable to natural ovulation in the cow (Berisha et al., 2019; Schams, Schallenberger, Hoffmann, & Karg, 1977).

2.3 | Characterization of follicle classes

For a better characterization of follicle classes close to ovulation, oestradiol, progesterone prostaglandin F2alpha and prostaglandin E2 were measured in FF. The patterns of oestradiol, progesterone, prostaglandin F2alpha and prostaglandin E2 (FF concentration) in this superovulation model agreed with our experimental data and confirm the validity of our method of follicle classification during final follicle growth and maturation close to ovulation (Berisha et al., 2019). The follicle samples corresponded to whole follicle tissue (without separation of GC from TI) to compare the expression patterns of examined factors between follicles close to ovulation and CL after ovulation.

2.4 | Total RNA extraction and quality determination

Total RNA from the tissues, either follicles or CL, was extracted with peqGOLD TriFAst (PeqLab, Erlangen, Germany) according to the manufacturer´s instructions as described previously (Berisha, Steffl, et al., 2006). After RNA extraction, DNA digestion was performed using the DNA-free kit (Ambion, Austin, USA). Isolated total RNA was dissolved in RNase-free water and spectroscopically quantified at 260 nm and 280 nm. RNA integrity was evaluated by RIN value (RNA Integrity Number) by using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) in conjunction with the RNA 6000 Nano Assay according to the manufacturer´s instructions. The automatically calculated RIN allows classification of total RNA integrity and quality based on a numbering system from 1 to 10 with 1 being the most degraded profile and 10 being the most intact RNA (Mueller et al.,).

2.5 | RNA reverse-transcription (RT) and real-time PCR

Amounts of 1 μg of total RNA per biological sample were reverse-transcribed to cDNA using the following master mix: 26 μl RNAase-free water, 12 μl 5xBuffer (Promega, Mannheim, Germany), 3 μl Random Primers (50μM) (Invitrogen, Carlsbad, Germany), 3 μl dNTPs (10mM) (Fermentas, St. Leon-Rot, Germany) and 200 U of M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). A master mix of the reaction components was prepared as reported earlier (Berisha et al., 2018), and real-time PCR amplification protocol was employed for all investigated factors. In brief: 1) denaturation and polymerase activation for 10 min at 95°C; 2) 40 cycles of a three-segmented amplification and quantification programme 2a) denaturation for 10 s at 95°C, 2b) annealing for 10 s at 60°C, 2c) elongation for 15 s at 72°C; 3) a melting curve step by slow heating from 60°C to 99°C with a rate of 0.5°C/sec and continuous fluorescence measurement; 4) and a final cooling step down to 40°C. Gene expression data were analysed using Rotor-Gene 3000 software (Qiagen, version 5.03).

The relative expressions of each target gene were calculated using the 'comparative quantification' algorithm and by using the 'take off' points method. The changes in mRNA abundance of examined target gene expression were assessed by normalization to a stable expressed internal control gene ubiquitin (UBQ). In order to obtain the gene expression differences, the ΔΔCT (cycle threshold) method was used (Livak & Schmittgen, 2001; Pfaffl, Buschmann, Mussack, Kirchner, & Berisha, 2019). To visualize the expression data ΔCT between experimental groups, the ΔCP value was subtracted from the constant CT value 40, so that a high '40 minus ΔCT' value indicated a high-gene expression level and vice versa (Berisha et al., 2018).

2.6 | Confirmation of primer specificity and sequence analysis

The mRNA abundance in our study was measured by the reverse-transcription quantitative polymerase chain reaction (RT-qPCR), as described in our previous study (Berisha, Meyer, & Schams, 2010). For confirmation, the PCR products were separated on agarose gel electrophoresis and commercially sequenced (TopLab, Munich, Germany). The amplified RT-qPCR products showed 100% homology to the known bovine gene sequence published in NCBI GenBank. The primer sequences and expected PCR product length are shown in Table 1.

2.7 | Gene expression

The housekeeping genes ubiquitin (UBQ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined in all samples in order to evaluate equal quantity and quality of the preceding

TABLE 1 Primer sequences of ubiquitin (UBQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HIF-1alpha (HIF1A) and nitric oxide (NO) synthases namely the inducible (iNOS) and endothelial (eNOS) isoforms

Target	Sequence of nucleotide fragment	size (bp)	Reference
UBQ	For 5'-AGATCCAGGATAAGGAAGGCAT-3'	198	Berisha et al. (2009)
	Rev 5'-GCTCCACCTCCAGGGTGATT-3'		
GAPDH	For 5'-GTCTTCACTACCATGGAGAAGG-3'	197	Berisha, Schams, and Miyamoto (2002)
	Rev 5'-TCATGGATGACCTTGGCCAG-3'		
HIF1A	For 5'-GAGCCTGATGCTTTAACTTTGC-3'	199	Berisha et al. (2017)
	Rev 5'-GAGTTTCAGAGGCAGGTAATGG-3'		
eNOS	For 5'-AGGAGTGGAAGTGGTTCCG-3'	126	Ulbrich et al. (2006)
	Rev 5'-GCCCCGGTACTACTCTGTCA-3'		
iNOS	For 5'-ACCTACCAGCTGACGGGAGAT-3'	316	Ulbrich et al. (2006)
	Rev 5'-TGGCAGGGTCCCCTGTGATG-3'		

Abbreviations: For, forwards; Rev, reverse.

RT reaction in each follicle and CL sample. While both genes were constantly expressed in all samples, we choose UBQ as normalizer. The mRNA abundance data of examined factors (Figures 2 and 3) were presented as changes (40-ΔCT ± *SEM* from 5 follicles or CL per group) in the target gene expression, normalized to UBQ as described previously by Berisha et al. (2018).

2.8 | Immunohistochemistry of HIF1A and EMBP

Bovine ovarian follicles and corpora lutea were obtained within 10 min after slaughter. Small tissue pieces (1 × 1 × 0.5 mm) were then fixed in Bouin´s solution. After fixation, tissues were immersed for 3 days in 70% ethanol. The samples were then dehydrated in a series of solutions of increasing ethanol content, isopropanol and xylene and then embedded in paraffin wax at 61oC. Then, appropriate tissue samples of antral follicles and early corpora lutea (day 1 and day 2 after ovulation) were cut using a rotary microtome (Leitz Microtome HM, 340, Wetzlar, Germany) to obtain 5 μm serial sections, which were mounted on amino-propyltriethoxysilane coated slides (SupraFrost Ultra Plus; Menzel-Gläser, Braunschweig, Germany). The dewaxed sections were washed three times (3 × 5 min) with PBS (pH 7.4). Endogenous peroxidase activity was blocked with 7.5% H_2O_2 at room temperature for 10 min. Non-specific antibody binding was blocked with Dako protein block serum-free (Dako Deutschland GmbH; Hamburg, Germany) for 10 min.

To reach an optimal signal-to-background ratio, systematic titration of the primary antibodies (1:50; 1:100; 1:200; 1:300; 1:400) was performed. Then, the sections were incubated with polyclonal primary antibodies against HIF1A (diluted 1:100, host goat, secondary antibody rabbit-anti-goat IgG(*F*(ab′)2), diluted 1:300; Santa Cruz Biotechnology Inc., Heidelberg, Germany) and EMBP (primary antibody: mouse anti-human eosinophil major basic protein, diluted 1:200 in PBS, AbDSerotec, Düsseldorf, Germany, secondary antibody: rabbit-anti-mouse polyclonal antibody, Sigma-Aldrich Hamburg, diluted 1:200 in PBS). Signal generation of the appropriate biotinylated secondary antibodies was achieved with treatment with streptavidin-ABComplex–HRP (Dako, Deutschland GmbH) and was performed for 30 min at room temperature. Treatment with 1 mg/ml 3,3-diaminobenzidine tetrahydrochloride (DAB tablets (10 mg), BIOTREND Chemikalien GmbH, Köln, Germany) was performed for 5 min. All the incubations were performed in a humidified chamber at 20°C. Negative controls were performed by either omission of the incubation step with the first-step antibody to exclude antigen-independent signal generation, by replacement of the first antibody by buffer or non-immune serum or by incubating with the 3,3-diaminobenzidine reagent alone to exclude the possibility of detecting non-suppressed endogenous peroxidase activity. A lack of detectable staining in the negative controls demonstrated that the reactions were specific. The images were captured with a Leica Labor-Lux microscope equipped with a Zeiss Axiocam camera (Zeiss, Munich, Germany). As positive controls, ovarian tissues from quails

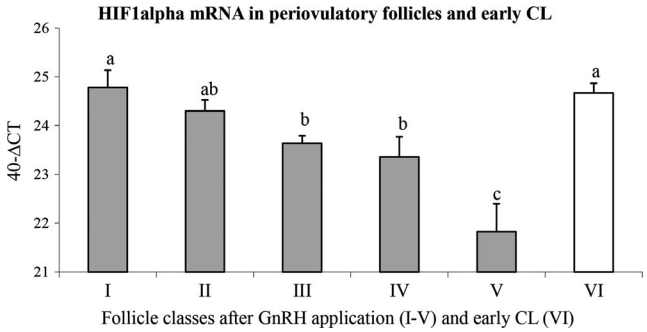

FIGURE 2 Changes of mRNA for hypoxia inducible factor-1alpha (HIF1A) in periovulatory follicles collected at (I) 0h, (II) 4h, (III) 10h, (IV) 20h, (V) 25h (follicles) and (VI) 60h (early CL, 2–3 days) after injection of GnRH to induce an LH surge. The changes in mRNA abundance of examined factors were assayed by normalization to the UBQ internal control. In order to obtain the CT (cycle threshold) difference, the data were analysed using the ΔΔCT method described previously by Livak and Schmittgen (2001). Results are shown as 40–ΔCT ± *SEM* ($n = 5$ follicle/class), so that a high 40–ΔCT value indicates a high-gene expression level and vice versa. Different superscripts denote statistically different values ($p < .05$)

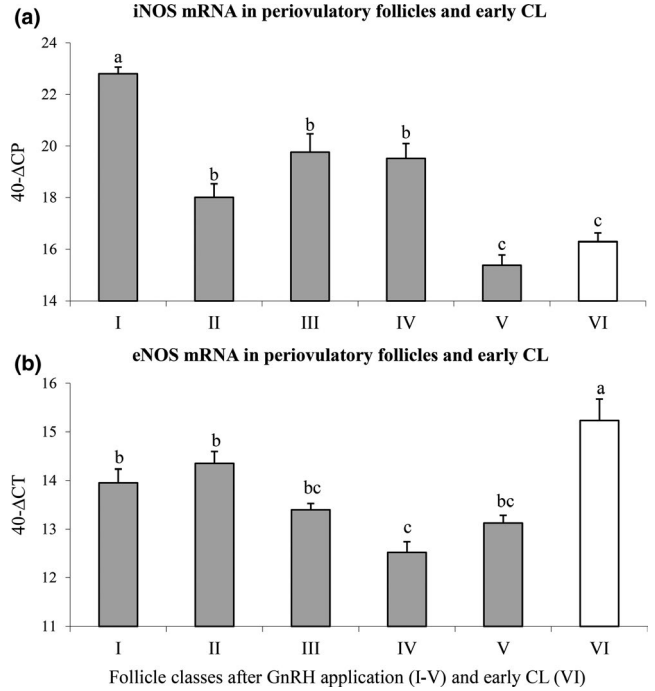

FIGURE 3 Expression of mRNA for (A) inducible nitric oxide synthases (iNOS) and (B) endothelial nitric oxide synthases (eNOS) in periovulatory follicles collected at (I) 0h, (II) 4h, (III) 10h, (IV) 20h, (V) 25h (follicles) and (VI) 60h (early CL, 2–3 days) after injection of GnRH to induce an LH surge. The changes in mRNA abundance of examined factors were assayed by normalization to the UBQ internal control. In order to obtain the CT (cycle threshold) difference, the data were analysed using the ΔΔCT method described previously by Livak and Schmittgen (2001). Results are shown as 40−ΔCT ± *SEM* ($n = 5$ follicle/class). Different superscripts denote statistically different values ($p < .05$)

(Coturnix japonica) and cats (Felis silvestris) of proven immunoreactivity were used. The signal intensity was evaluated independently by at least two observers into the following categories: - (no staining), + (weak), ++ (moderate), +++ (strong).

2.9 | Transmission electron microscopy (TEM)

Small pieces (side length of 1 mm) of bovine antral follicles and corpora lutea were immediately fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 hr. Afterwards, the specimens were brought into 1% osmium tetroxide (Plano, Wetzlar, Germany) containing 1.5% potassium ferrocyanide (Sigma-Aldrich, Steinheim, Germany), dehydrated in a graded series of ethanols (50, 70 and 90% for 30 min each) and embedded in Polyembed 812 BDMA (Polysciences, Eppelheim, Germany). Semi-thin sections (1 μm thick) were cut and stained with Richardson's solution. Ultra-thin sections were obtained using a diamond knife (Reichert-Labtec, Wolfratshausen, Germany), placed on 150-mesh coppergrids (Polysciences) and stained with uranyl acetate (Scientific LTD. Stansted, UK) and lead citrate (Agar Aids, Stansted, UK). They were then examined with a transmission electron microscope (EM 902, Zeiss, Oberkochen, Germany). EM photographs were obtained with a digital camera.

2.10 | Statistical analysis

To assess the statistical significance in gene expression results, a one-way ANOVA followed by the Holm Sidak as a multiple comparison test was applied (Sigma Stat 3.0). The mRNA expression data which are not normally distributed or failing a testing of equal variance test were evaluated by one-way ANOVA on ranks followed by the Kruskal–Wallis test (Sigma Stat 3.0). All expression data are shown as means ± *SEM*, and differences were considered significant if $p < .05$. The follicle and CL samples ($n = 5$) were obtained from 5 cows/group.

3 | RESULTS

3.1 | Transcript abundance for HIF1A, iNOS and eNOS in periovulatory follicles

The HIF1A, iNOS and eNOS transcript abundance level in follicle tissue before and after GnRH and after ovulation (early CL) are shown in Figures 2 and 3. The HIF1A mRNA abundance (Figure 2) in the follicle group before GnRH application (control group) was high, followed by a continuous and significant down regulation ($p < .05$) afterwards with a minimum level 25h after GnRH (close to ovulation) and significant increase ($p < .05$) only after ovulation (early CL). The highest HIF1A mRNA abundance was detected in early CL (period of angiogenesis).

The mRNA abundance of iNOS (Figure 3a) before GnRH application (control group) was high, decreased significantly ($p < .05$) during LH surge (4h after GnRH), with a minimum level close to ovulation (25h after GnRH) and after ovulation (in early CL). In contrast, the mRNA abundance of eNOS (Figure 3b) decreased in the follicle group 20h after GnRH, followed by a rapid and significant upregulation ($p < .05$) just after ovulation (early CL, luteal angiogenesis).

3.2 | Immunohistochemical localization of HIF1A and EMBP

The luminal layers of Graafian follicles (pre-ovulatory) were distinctly immunostained for HIF1A (Figure 4a). In the surrounding theca layers and the ovarian stroma, HIF1A-positive cells, probably eosinophils, could regularly be observed. After ovulation, the collapsed Graafian follicle consists of granulosa cells with morphological signs of beginning luteinization. Compared to the Graafian follicle, HIF1A immunostaining of granulosa cells in the early CL appeared distinctly reduced (Figure 4b). In contrast, eosinophils and macrophages were strongly positive and were located near

or within blood vessels and seem to migrate through granulosa cells and accumulate in the stroma of tissue folds (Figure 4b). Eosinophils were identified by strong positive immunostaining for EMBP (Figure 4d,e). The stroma of the tissue folds contained numerous eosinophils (Figure 4b) and macrophages, which started to migrate between the granulosa cells. They could be then located near or even within the blood vessels (Figure 5) of the early CL. Eosinophils, which could be precisely located in the early CL with antibodies against EMBP (Figure 4d,e) on the other hand, were still distinctly positive for HIF1A (Figure 4b).

3.3 | Characterization of eosinophilic cells in the CL using transmission electron microscopy

Ultrastructural studies showed that a usually reniform or bi-lobulated nucleus characterizes the eosinophilic cells of the corpus luteum (Figures 5 and 6). Their cytoplasm contains numerous round granules of different size and electron density. Many of the eosinophilic cells also showed considerable amounts of glycogen in their cytoplasm (Figure 6). Although they appeared somewhat different from eosinophils from bovine blood (Kliem et al., 2013).

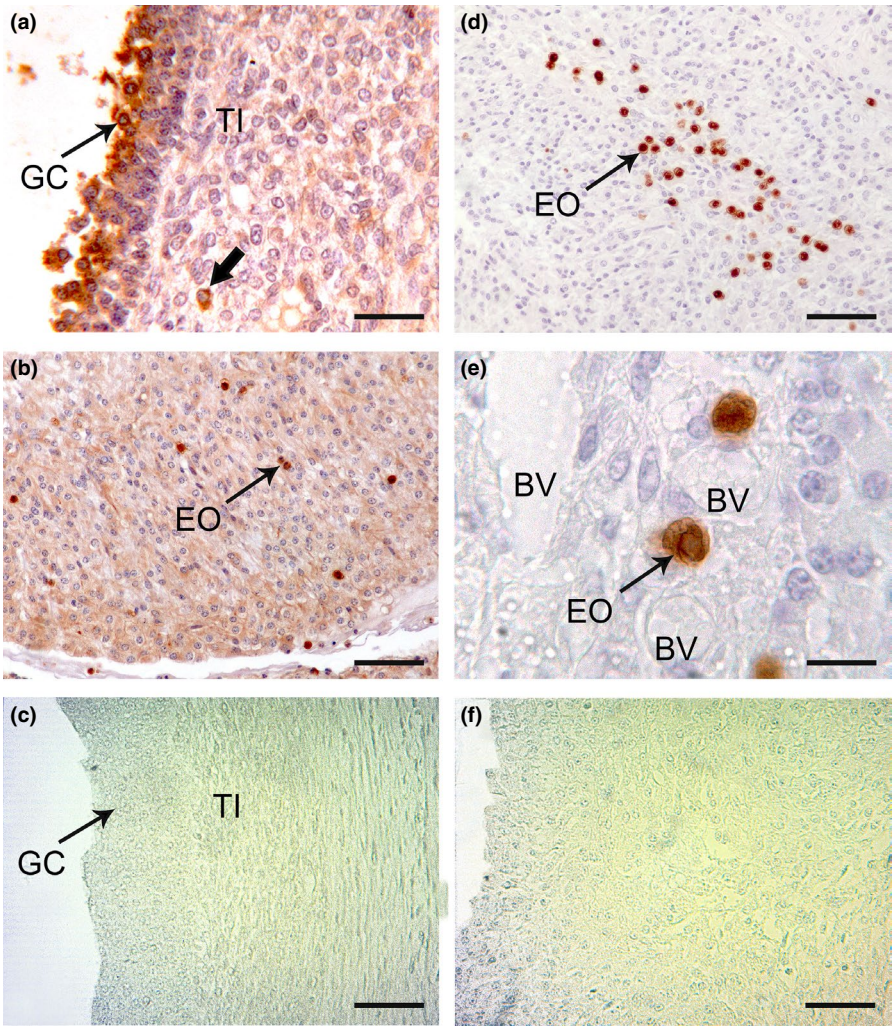

FIGURE 4 Immunohistochemical demonstration of HIF-1alpha (HIF1A) and EMBP. (a) Immunohistochemical localization of HIF1A in a pre-ovulatory follicle. The luminal layers of the granulosa cells (GC) show distinct reaction with HIF1A antibodies, whereas the basal cell layers remain negative. The arrow points to a distinctly positive eosinophil in the theca interna (TI). SB = 100 μm. (b) Immunohistochemical localization of HIF1A in the early corpus luteum (d1-2). Numerous eosinophils (EO) in the stroma of the corpus luteum show strong immunostaining with HIF1A. The granulosa-luteal cells show only a weak reaction with the antibody. SB = 200 μm. (c) Negative control for HIF1A in a pre-ovulatory follicle. No immunostaining for HIF1A was observed. SB = 200 μm. (d) Immunohistochemical localization of EMBP in the early corpus luteum (d1-2). Using the marker antibody EMBP, the eosinophils (EO) can clearly be identified in the early corpus luteum. SB = 200 μm. (e) Immunohistochemical localization of EMBP in eosinophils, higher magnification. Many strongly EMBP positive eosinophils (EO) can be localized near to blood vessels (BV). SB = 20 μm. (f) Negative control for EMBP in the early corpus luteum (d1-2). No immunostaining for EMBP was observed. SB = 200 μm

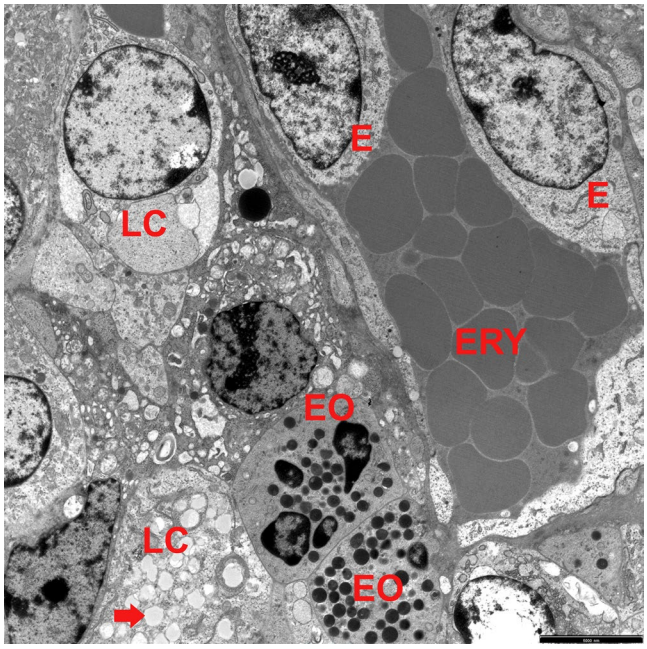

FIGURE 5 Transmission electron microscopy of eosinophils and vessel in the early corpus luteum (d1). Two eosinophils (EO) with their characteristic round granules can be detected near a vessel with erythrocytes (ERY) and endothelial cells (E). They are surrounded by luteinizing granulosa cells (LC), which, as typical steroid hormone producing cells, show many lipid droplets in their cytoplasm (arrow). SB = 5,000 nm

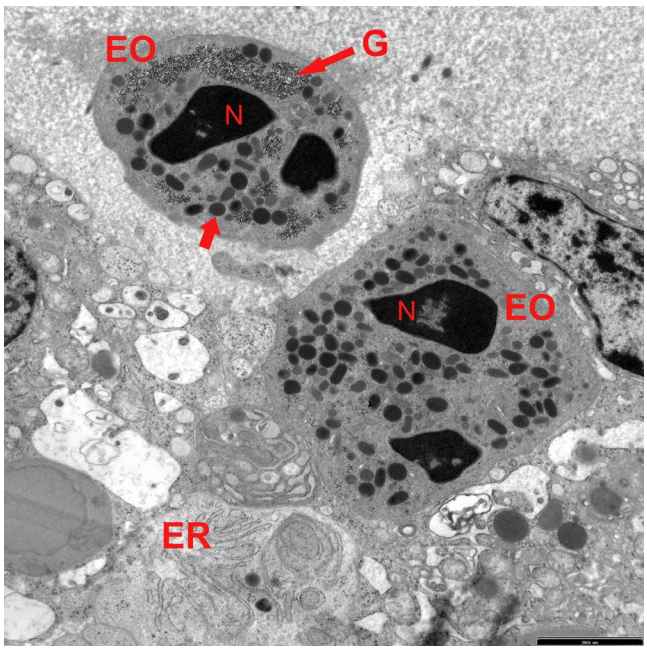

FIGURE 6 Transmission electron microscopy of eosinophils in the early corpus luteum (d1). These two eosinophils (EO) show rounded granula (small arrow) of different sizes and density. Their nuclei (N) are bilobulated. In the cytoplasm of the upper eosinophil, a large area of glycogen (G) can be detected. The luteinizing cells contain a high amount of smooth and rough endoplasmic reticulum (ER). SB = 2,500 nm

4 | DISCUSSION

Our results demonstrate the transcript abundance and pattern of protein localization of HIF1A and NOS isoforms (iNOS and eNOS) in different timely defined follicle classes after GnRH application as well as after ovulation (in early CL tissue) in the cow. Regarding the experimental protocol, in our previous studies (Berisha et al., 2019; Berisha, Steffl, et al., 2006), we have shown that the superovulated follicles after GnRH application were comparable to those naturally developed in the cow (Schams et al., 1977). To compare the expression patterns of examined factors between whole follicles (GC and TI) and CL tissue during folliculo-luteal transition in the present study, we did not separate follicle cells (without GC and TI separation).

Numerous studies have demonstrated that LH surge in ruminants initiates a series of biochemical events in the ovary, such as regulation of many endocrine and locally produced growth factors to complement gonadotropins action during ovulation and CL formation (Berisha et al., 2008, 2019; Curry & Osteen, 2003; Schams et al., 2009; Schilffarth, Antoni, Schams, Meyer, & Berisha, 2009; Shirasuna et al., 2008; Skarzynski et al., 2013). During this folliculo-luteal transition, angiogenesis seems to be a critical regulatory event for ovarian function. It is well known that the leading regulators of angiogenesis in ovarian follicles and CL include molecules like VEGF, ANPT, FGF and IGF family members (Berisha & Schams, 2005; Berisha, Schams, Rodler, & Pfaffl, 2016; Ribatti, Vacca, & Presta, 2000; Woad & Robinson, 2016). Some recent studies, however, suggested that the pro-angiogenic molecules HIF1A and NOS isoforms seem to be highly important regulatory mediators for the regulation of final follicle development, ovulation and CL formation (Anteby et al., 1996; Berisha et al., 2017; Faes et al., 2009; Guo et al., 2019; Jablonka-Shariff & Olson, 1997; Li et al., 2020).

In the context of the locally produced pro-angiogenic molecules, HIF1A seems to be a highly crucial regulatory mediator for the follicle growth, development and ovulation in mice (Li et al., 2020). The present study demonstrates a specific pattern of HIF1A expression, which depends on the developmental stage of the follicles before and after the LH surge and after ovulation (early CL). The mRNA abundance of HIF1A (Figure 2) in the follicle group after LH surge decreased continuously during periovulation with the rapid and significant upregulation only after ovulation (early CL). The mRNA abundance profile in our follicle classes correlates well with a positive staining for HIF1A, mainly in GC after ovulation (Figure 4). It is assumed that a low oxygen, but not a hypoxic environment, exists in the mature follicle, because granulosa and cumulus cells tolerate the lower oxygen environment without the need to activate a hypoxic stress response (Thompson et al., 2015).

In contrast to the follicles before ovulation, the HIF1A activity is considerably upregulated following the ovulatory signal and is significantly involved in post-ovulatory cellular differentiation (Duncan, van den Driesche, & Fraser, 2008; Van den Driesche et al., 2008; Thompson et al., 2015; Rico et al., 2014). It seems that

the ruptured follicle just after ovulation is under extreme hypoxic condition (Amselgruber, Schäfer, & Sinowatz, 1999; Nishimura & Okuda, 2010). It is well known that cellular responses to hypoxia are mediated by HIF1A that binds to the hypoxia responsible elements and is considered as a master regulator of angiogenesis in early CL (Krock et al., 2011; Meidan et al., 2013). As shown in Figure 4a, GC layers close to the basal membrane stained negative for HIF1A. A steady increase of HIF1A immunostaining in the GC was observed towards the lumen of the follicle (Berisha et al., 2017). The interaction signalling pathways regulating the most important angiogenic factor VEGFA via HIF1A is suggested to be the key mechanisms during ovulation and CL formation (Li et al., 2020; Zhang, Yin, & Wang, 2011). The critical role of HIF1A for angiogenesis has been reported in different mammalian species and cattle (Boonyaprakob, Gadsby, Hedgpeth, Routh, & Almond, 2005; Duncan et al., 2008; Meidan et al., 2013; Nishimura & Okuda, 2010). In addition, the results of some recent studies (Kim, Bagchi, & Bagchi, 2009; Li et al., 2020) indicated that the inhibition of HIF activity impairs ovulation by preventing the rupture (ovulation) of the pre-ovulatory follicles.

Follicular angiogenesis is proposed to be initiated by an interaction between LH surge, several extracellular matrix (ECM) degrading proteases, VEGFA and other various angiogenic factors (Curry & Smith, 2006; Van den Driesche et al., 2008; Berisha et al., 2008; Thompson et al., 2015; Trau, Brännström, Curry, & Duffy, 2016). We have previously demonstrated the interaction of LH surge to ECM proteases, VEGFA and other pro-angiogenic factors in the bovine ovary (Acosta et al., 2005; Berisha et al., 2009; Berisha, Steffl, et al., 2006; Berisha, Welter, et al., 2006; Shimizu, Berisha, Schams, & Miyamoto, 2007a, 2007b). The specific regulation of ECM degrading proteinases, namely matrix metalloproteinase (MMP) and plasminogen activator (PA) systems close to ovulation suggesting them to be key mediators of ovulation and early CL formation. The rapid increase of MMP and PA proteases in response to the LH surge, and the consistently high levels of them throughout the superovulation experiment (Berisha et al., 2008; Markosyan & Duffy, 2009), may be linked to the role of these proteases in the degradation of the basal membrane that occurs in the GC and TI tissue around ovulation and CL formation (Kliem et al., 2007). The ECM activity in pre-ovulatory follicles supported the important role of these proteases in remodelling and transition of the follicle into the luteal cell (Curry & Smith, 2006).

The high HIF1A mRNA abundance during early luteal development (period of angiogenesis), in our present study (Figure 2), agrees with previous results in pig (Boonyaprakob et al., 2005) and cow (Nishimuara & Okuda, 2010). Our results suggest that the hypoxic situation in the early CL regulates the expression of HIF1A, followed by VEGFA and FGF2 upregulation (Berisha, Schams, Kosmann, Amselgruber, & Einspanier, 2000a, 2000b). Blocking the key angiogenic factors, for example VEGFA and FGF2 by specific antibodies, causes significant decrease in CL function (Yamashita et al., 2008). The protein localization HIF1A was weak in granulosa-lutein cells, but several positive eosinophilic granulocytes were observed during early CL development (Figure 4b). In our previous study, we assumed that migration of the eosinophils into the early CL is an essential stimulus for angiogenesis during early luteal development (Kliem et al., 2013). An increased number of eosinophils were observed in the periovulatory follicle shortly before ovulation in human, porcine, ovine and bovine (Aust et al., 2000; Cavender & Murdoch, 1988; Reibiger & Spanel-Borowski, 2000). In addition, VEGFA content and CL vascularization in early CL tissue were significantly decreased by suppression of the eosinophilic migration (Kliem et al., 2013; Murdoch & Van Kirk, 2000). An increased number of eosinophils invade the theca externa and later the theca interna of the pre-ovulatory follicles in several species (Puxeddu et al., 2005), including the bovine (Kliem et al., 2013).

It has been already clearly shown by Zietzschmann and Krölling (1955) that the overwhelming numbers of immune cells in the early bovine CL are eosinophil granulocytes. Only rarely neutrophil granulocytes and macrophages were found. This is in contrast to the findings of Jiemtaweeboon et al. (2011), who described that a significant number of polymorphonuclear neutrophils infiltrate the developing bovine CL during the first day after ovulation, and to our own results, where we could find numerous eosinophils but few other immune cells using histological, immunohistological and ultrastructural techniques. As shown in our results, after ovulation, the eosinophils can precisely be located within the early bovine CL using antibodies against EMBP and transmission electron microscopy (Figure 5). Different to the eosinophils in the circulating blood stream, our transmission electron microscopy (TEM) studies showed that eosinophils within the early CL contain moderate to large amounts of glycogen (Figure 6). We assume that the eosinophils of the blood show a change in their phenotype due to changes in their metabolism when they leave the capillaries and invade the forming CL.

It is well known that NO produced by various eNOS and iNOS is crucial for vascular remodelling and angiogenesis (Boopathy et al., 2017; Rudic et al., 1998). The specifically modulated transcript abundance of eNOS and iNOS in our study (Figure 3) provides evidence for an involvement of NO in the regulation of folliculo-luteal transition and early luteal development in the cow. Various NOS isoforms are known to act as paracrine factors during different processes associated with female reproduction (Ulbrich et al., 2006). A regulatory role for iNOS and eNOS in follicle development and ovulation process is assumed in numerous species. The role of eNOS and iNOS isoform in the regulation of folliculogenesis is demonstrated by its involvement in the control of ovulation rate and steroidogenic activity of granulosa-luteal cells (Faes et al., 2009; Guo et al., 2019; Jablonka-Shariff & Olson, 1997). Moreover, a positive correlation between follicular size and follicular NOS concentration has been reported (Anteby et al., 1996). The eNOS and iNOS isoforms show distinct cell-specific expression patterns and are differentially regulated during follicular and luteal development (Jablonka-Shariff & Olson, 1997; Nath & Maitra, 2018; Olson et al., 1996). These findings agree well with our iNOS and eNOS transcript abundance data in follicle classes before ovulation (Figure 3). The mRNA abundance of

iNOS (Figure 3a) before GnRH application (control group) was high, decreased significantly during LH surge (4h after GnRH), achieving a minimum level close to ovulation and after ovulation in early CL.

The recent studies, however, clearly demonstrated that especially eNOS plays a leading role in VEGF-induced angiogenesis and vascular permeability (Amano et al., 2003; Fukumura et al., 2001; Kimura & Esumi, 2003). This correlates with a rapid and significant upregulation of eNOS mRNA abundance (Figure 3b) just after ovulation (early CL, luteal angiogenesis) in our experiment. In addition, the upregulation of eNOS agrees very well with mRNA and protein expression profiles for VEGFA as the most important factor for the early luteal angiogenesis (Berisha, Schams, Kosmann, Amselgruber, & Einspanier, 2000a). There are clear evidences that VEGFA promotes NO production and induces eNOS and iNOS expression in vascular endothelial cells in vitro (Hood, Meininger, Ziche, & Granger, 1998; Kroll & Waltenberger, 1998). The positive interaction between NO and VEGFA may have implications for endothelial function and angiogenesis (Dulak et al., 2000). Furthermore, it is demonstrated that VEGFA expression and angiogenesis can be blocked by inhibition of eNOS-mediated NO production (Murohara et al., 1998; Papapetropoulos, García-Cardena, Madri, & Sessa, 1997). In addition, the data of Abe et al. (2013) indicate that NO induces VEGFA expression in vivo and in vitro in the rat placenta, suggesting that peaked NO production was maintained by a reciprocal relationship between NO and VEGFA via HIF1A. Furthermore, it is assumed the reciprocal regulation between HIF1A, NOS and VEGFA during angiogenesis (Kimura & Esumi, 2003).

The significant upregulation of HIF1A and eNOS just after ovulation (early CL) in our study (Figures 2 and 3) demonstrates the important role of these pro-angiogenic factors which play roles in ovulation process, luteinization and CL formation. Moreover, in the early CL, hypoxia modulates the expression of different angiogenic factors during early luteal phase (Berisha & Schams, 2005; Berisha et al., 2000a; Boonyaprakob et al., 2005; Duncan et al., 2008; Pugh & Ratcliffe, 2003). High expression levels of angiogenic factors in early CL suggest them to be essential for angiogenesis and optimal function during the early luteal phase (Berisha & Schams, 2005; Duncan et al., 2008; Yamashita et al., 2008). The rapid upregulation and co-expression of HIF1A and eNOS soon after ovulation in early CL correlate well with the mRNA expression, protein concentration and tissue localization of the key angiogenic factors like VEGF, ANTP, FGF and IGF system members (Berisha, Schams, Rodler, & Pfaffl, 2016).

In conclusion, the parallel and acute regulated expression of HIF1A and NOS family members in our study, specifically during the interval between the ovulatory gonadotropin surge and ovulation, indicates that these paracrine factors are involved in the local mechanisms, regulating final follicle maturation, ovulation and early luteal angiogenesis in the cow.

ACKNOWLEDGEMENT

This work was supported by the German Research Foundation (DFG Project number 5270108).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Berisha B. and Schams D designed and executed all the experiments. Berisha B analysed data and wrote the manuscript. Pfaffl W. M involved in qRT-PCR and data analysis. Sinowatz F. and Rodler D involved in Immunohistochemistry and Transmission electron microscopy and data analysis. All the authors edited the manuscript.

DATA AVAILABILITY

The data used to support the findings of this study are available on request to the corresponding author.

ORCID

Bajram Berisha https://orcid.org/0000-0001-9112-054X

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How to cite this article: Berisha B, Schams D, Sinowatz F, Rodler D, Pfaffl MW. Hypoxia-inducible factor-1alpha and nitric oxide synthases in bovine follicles close to ovulation and early luteal angiogenesis. *Reprod Dom Anim*. 2020;55:1573–1584.https://doi.org/10.1111/rda.13812