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Cellular and molecular mechanisms of the preovulatory follicle differenciation and ovulation: what do we know in the mare relative to other species

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Abstract:

Terminal follicular differentiation and ovulation are essential steps of reproduction. They are induced by the increase in circulating LH, and lead to the expulsion from the ovary of oocytes ready to be fertilized. This review summarizes our current understanding of cellular and molecular pathways that control ovulation using a broad mammalian literature, with a specific focus to the mare, which is unique in some aspects of ovarian function in some cases. Essential steps and key factors are approached. The first part of this review concerns LH, receptors and signaling, addressing the description of the equine gonadotropin and cloning, signaling pathways that are activated following the binding of LH to its receptors, and implication of transcription factors which better known are CCAAT-enhancer-binding proteins (CEBP) and cAMP response element-binding protein (CREB). The second and major part is devoted to the cellular and molecular actors within follicular cells during preovulatory maturation. We relate to 1) molecules involved in vascular permeability and vasoconstriction, 2) involvement of neuropeptides, such as kisspeptin, neurotrophins and neuronal growth factor, neuropeptide Y (NPY), 3) the modification of steroidogenesis, steroids intrafollicular levels and enzymes activity, 4) the local inflammation, with the increase in prostaglandins synthesis, and implication of leukotrienes, cytokines and glucocorticoids, 5) extracellular matrix remodeling with involvement of proteases, antiproteases and inhibitors, as well as relaxin, and finaly 6) the implication of oxytocine, osteopontin, growth factors and reactive oxygen species. The third part describes our current knowledge on molecular aspect of in vivo cumulus-oocyte-complexe maturation, with a specific focus on signaling pathways, paracrine factors, and intracellular regulations that occur in cumulus cells during expansion, and in the oocyte during nuclear and cytoplasmic meiosis resumption. Our aim was to give an overall and comprehensive map of the regulatory mechanisms that intervene within the preovulatory follicle during differentiation and ovulation

Introduction:

In mammals, while the basal concentrations of FSH and LH are sufficient for the proliferation of follicle cells, preovulatory maturation occurs under the influence of an increase in circulating LH levels. LH and FSH levels are highly dependent on GnRH secretion from hypothalamic neurons. The hypothalamic-pituitary-gonadal axis has been recently revisited by using knockouts animals, leading to hypothesis related to mammalian evolution [1]. It is well known that kisspeptin stimulates GnRH neurons leading to GnRH release in both *in vitro* and *in vivo* studies [2]. Two other neuropeptides (neurokinin B (NKB) and dynorphin (DYN)) interact with kisspeptin as key hypothalamic regulators of reproductive function, and are thought to be co-secreted with kisspeptin to regulate GnRH secretion [3-5]. The kisspeptin neural system play therefore a major role in the control of reproduction, and may be highly valuable for the development of novel strategies for the management of fertility in mammals [6]. Nevertheless kisspeptin seems not to be an universal ovulation-inducing agent since it failed to have an effect in the mare [7, 8].

The increase in circulating LH level causes a series of morphological and functional changes in the follicle destined to ovulate that results in: 1/ the differentiation of follicle cells, granulosa and thecal, in preparation for follicular rupture, referred to as ovulation, 2/ the differentiation of follicle cells, granulosa and thecal, for the formation of a functional corpus luteum, referred to as luteinisation, 3/ the expansion of cumulus cells surrounding the oocyte and, lastly, 4/ oocyte maturation. All these events must be coordinated in order to result in the production of mature and fertilisable oocytes and of corpus luteum capable of supporting the beginning of pregnancy. The delay between LH peak and ovulation vary according to species (Table 1), being the longest in sows and mares.

The conditions under which preovulatory maturation occurs are unusual in the equine species compared to other mammals. The circulating LH level does not exhibit a short-term high-amplitude peak before ovulation but rather a gradual increase over several days reaching a maximum 1-3 days after ovulation [9, 10]. In addition, the concomitant increase in FSH observed before ovulation in cows and in women does not take place in the mare [11].

Under breeding conditions, preovulatory maturation and ovulation can be induced by hormone injection. Human Chorionic Gonadotropin (hCG), has been used to induce ovulation in mare since 1960's. Chorulon® (lyophilised hCG) is largely used in horse breeding. However, repeated administration of hCG results in immunological reaction and antibody formation in most mares [12, 13]. In order to avoid this deleterious effect, equine pituitary gland extracts can also be used [13]. These extracts typically induce ovulation in 34 hours when administrated to mares in estrus with a follicle larger than 35 mm. Nevertheless, equine pituitary gland extracts are not commercially available, and were only experimentally used. The efficiency of GnRH agonists in hastening ovulation over consecutive cycles has also been reported. Deslorelin acetate (Ovuplant®) is the most widely used in Australia, North America and in Europe. Repeated injections of buserelin (Receptal®), another GnRH agonist proved as effective as hCG [14, 15]. Few years ago, the 14kDa "ovulation inducing factor" from the seminal plasma of camelids has been identified as nerve growth factor beta (NGF) [16, 17]. To date, it is able to induce ovulation in alpacas and llamas but no data are available concerning the capability of neurotrophins and/or neuronal growth factors to induce ovulation in the mare. Last year, the absence of NGF in equine ejaculates has been demonstrated [18]. Nevertheless, the use of recombinant equine gonadotropins proved efficiency (see below).

1 - LH, receptor and signal transmission

LH is a glycoprotein made up of two subunits, A and B, bound together non-covalently. In each species, subunit A is responsible for binding the hormone to its receptor, and is common to all glycoprotein hormones (LH, FSH, TSH and CG), whereas subunit B confers biological specificity [19]. In horse, subunit A is made up of 96 amino acids and subunit B is made up of 149 amino acids. Glycosidic bonds are joined to the asparagine residues of subunit A and to serine residues of subunit B. The protein structure of eLH is identical to that of eCG, since these two hormones come from the same gene, expressed in the pituitary gland and placenta respectively. However, eLH and eCG differ at least in terms of their glycosidic bonds. In human, LH and CG show differences in their expression pattern, biopotency and regulation [20]. It is interesting to note also that eLH (and therefore eCG) exhibits dual heterologous LH/FSH activity [21, 22]. The cDNAs coding for the A and B subunits of eLH/CG have been cloned [23, 24] and used for co-transfecting COS-7 cells [25]. Since then, several works described the development and efficiency of genetically cloned recombinant equine gonadotropins to induce ovulation in the mare [26-29]. Three years ago, molecular characterization, modelling and in silico analysis of eLH/CG A has been realized [30]. This enable the use of recombinant eLH to induce ovulation in mare.

For preovulatory follicle maturation occurence, LH receptors (LHR) must be expressed at the follicular cell surface and the signal generated must be transmitted. As a general rule, LHR are present on thecal cells in growing follicles and are also present on granulosa cells in the preovulatory follicle. The expression of LHR by granulosa cells depends on FSH, or even on prolactin [31, 32]. In mare, LHR are present in the granulosa cells once follicles have reached 5mm in diameter [33]. Similarly, we have shown during this work that, at the end of the follicle phase, the number of LHR in the mural granulosa cells increases in relation to the follicle diameter. This study confirms that conducted by Fay & Douglas [34] in the mare, and is consistent with several studies carried out in other species.

LHR are members of the family of G protein-coupled receptors that interact with adenylate cyclase via the Gs subunit. They catalyse the formation of cAMP which activates protein kinase A (PKA). LH binding to its receptor also increases the intracellular levels of inositol phosphate [35] and activates the pathway of PKC [36]. It also acts via tyrosine kinases [36, 37]. The activation pathway taken by PKA is nonetheless the best known. It has been shown that in the rat, the differentiation of granulosa cells at the preovulatory stage does not occurs together with an increase in the intracellular concentration of the catalytic subunit of PKA, but rather with a change in its cellular location [38]. On the other hand, the expression of its regulatory RIIB subunit is induced in the preovulatory follicle cells, which would make it possible to specifically modulate the activity of PKA and therefore the effects of LH [39, 40]. In mare, it has been demonstrated few year ago that LHR expression on the ovarian epithelium surface is linked with expression of metalloproteinases (MMP), implicated in tissue remodelling [41]. The same year, a study reported the up-regulation and regulatory control of Regulator of G-protein Signaling protein-2 (RGS2) in equine preovulatory follicles [42], a protein known to control signalling through G-protein coupled receptors.

The involvement of the two transcription factors of the family of CCAAT-enhancer-binding proteins CEBPA and CEBPB, in preovulatory follicle maturation was reported [43]. In rats, the intrafollicular levels of CEBPA mRNA and protein increase during follicle growth, suggesting that they are dependent on oestrogen and/or FSH expression. Their decrease after ovulation have been induced using hCG [44]. A few years later, the same authors clearly showed the key role played by CEBPA in the capacity of the dominant follicle to respond to preovulatory stimulation [45]. On the other hand, the expression of the CEBPB gene (also referred to as LAP) is induced in the granulosa cells of the dominant follicle following stimulation by LH or hCG. In other systems (3T3 cell line, adipocytes), CEBPA and CEBPB are believed to contribute to cell growth and cell differentiation and to intervene in regulating the expression of numerous metabolic enzymes; their expression is regulated mainly by the cytokines IL-6 and TNF [46, 47]. By analogy, it is convenient to imagine that these factors are involved in regulating certain ovarian functions. CREB, a transcription factor of the bZIP family, is regulated by gonadotropic hormones. In the ovary, CREB regulates the transcription of the *Cyp19* gene, coding for P450 aromatase, via the modulation of cAMP levels [40, 48, 49] and also regulates the gene coding for the A sub-unit of inhibin *InhA* [50]. CREB also regulates *Hspa1a* gene (Computational analysis) [51]. It has been shown that the CREB transcript and CREB protein levels do not vary during the rat oestrous cycle, but that activity concerning the regulation of the transcription of this factor, which depends on its phosphorylation state, increases under the influence of gonadotropic hormones [52]. In the mouse, CREB activity (i.e. phorphorylation) is required for LH-induced expression of EGF-like factors in granulosa cells [53]. The miRNA miR-132, known to be regulated by CREB, has been shown to decrease in equine preovulatory follicle [54].

Other transcription factors such as SF1, SP1, PBX1, NGFIB, MYC [55], as well as histone acetylation- or methylation-related genes [56] are also involved in the hormonal regulation of the expression of certain genes in the ovary. To our knowledge, the transcription factors involved during preovulatory differentiation have not been studied yet in domestic mammals such as the bovine, ovine, caprine and equine species.

All these studies clearly show the complexity of the mechanisms involved in regulating preovulatory follicle maturation.

2 – Cellular aspects of the follicle maturation

Ovulation, luteinisation and maturation of the oocyte-cumulus complex depend on the presence of numerous factors within the preovulatory follicle. Most of these factors are produced by the follicle cells in response to LH stimulation, which induces cell differentiation towards the luteal phenotype and the resumption of oocyte meiosis. In the rat, it has been shown that follicle cells are entirely reprogrammed approximately 7 h after LH injection [57, 58]. Major steps are summarized in Figure 1.

Increase in vascular permeability: Shortly after the endogenous gonadotropin surge or injection of LH/hCG, observations of the follicle destined to ovulate indicate that considerable changes occur in the capillaries of the internal theca which becomes oedematous. The blood flow increases, associated with hyperaemia of the preovulatory follicle [59-61]. Gonadotropins modulate capillary permeability of the ovarian blood-follicle barrier, mainly through increased numbers of large pores, similar to a classical inflammatory response [62].

The local production of bradykinin and histamine is associated with these modifications in the vascularisation of the preovulatory follicle [63, 64]. Moreover, it has been shown that the intrafollicular concentration of certain vasoactive molecules such as platelet-activating factor (PAF) increases after the LH peak [65, 66]. On the other hand, work by Pellicer [67], and then by Daud et al [68] and Naftolin et al. [69, 70], have shown that molecules of the Renin-Angiotensin System, known to affect blood pressure and vasoconstriction, are involved in ovulation and luteinisation. The precise mechanism of action of these various molecules remains to be determined but has been discussed in cattle [71, 72]. Their ovarian expression in mares is unknown, with the exception of histamine, the concentration of which remains stable after ovulation has been induced [73].

Cell proliferation arrest: At preovulatory maturation, the size of the follicle destined to ovulate increases only little or not at all due to the arrest of cell multiplication. In the rat, hCG via the cAMP/PKA pathway, is known to rapidly induces (about 4 h) the arrest of the transcription of cyclin D2 (CCND2) which, when associated with CDK4 or CDK6, enables the granulosa cells to progress to phase 1 [74, 75]. The inhibition of cyclin E (CCNE) -which when associated with CDK2 enables the cells to advance onto phase S- occurs less rapidly, and is visible 24 h after the injection of hCG [74]. At the same time, the induction of the transcription of P27KIP1, a protein that inhibits the activity of CDK2, 4, 6 and CDC2, is initiated. This blocks the phosphorylation cascades at various points, and confirms the hypothesis that granulosa cells withdraw from the proliferation process. However, numerous studies also suggest that P27KIP1 is involved in luteinisation and the formation of the functional corpus luteum [76-78]. More recent data [79] suggest that cumulus granulosa cells continue to proliferate for up to 10 h after an ovulatory stimulus, possibly via CCNE/CDK2. A very recent study performed also in rodent demonstrated that at least two factors known to regulate differentiation and cell division (ERRFI1 and IFRD1) may be essential for follicular cell differentiation and cumulus expansion [80].

Neuropeptide expression:

Kisspeptin: Kisspeptin is a family of neuropeptides well known to be released from the neurons of hypothalamus and to stimulate GnRH release via the GPR54 receptor [81]. Moreover, GPR54 is present on granulosa and cumulus cells [82-84]. Immunohistochemical studies have shown that kisspeptin is distributed in theca, granulosa, luteal, and interstitial cells of human, marmoset, hamster, and rat ovaries [83, 85, 86]. It has been shown in rodents that kisspeptin mRNA synthesis, that increases in the ovary during proestrous, is stimulated by hCG [86], and that estrogens regulate kisspeptin expression in granulosa cells [87]. More recent data confirm and extend this study and demonstrated the relevance of kisspeptin to the differentiation of granulosa cells into luteal cells [88], suggesting an important role in ovulation.

NGF, ADCYAP and VIP

A recent review describes the importance of neurotrophins and neuronal growth factors in the ovary [89]. The neurotrophin family consists of NGF, the neurotrophins 3 and 4 (NTF3 and NTF4), and brain-derived neurotrophic factor (BDNF), and the neuronal growth factors are glial derived neuroptrophic factor (GDNF) and vasointestinal peptide (VIP). The two neuropeptides, VIP and ADCYAP (previously named PACAP for Pituitary Adenylate Cyclase Activating Polypeptide), have been identified in the rat ovary [90], in granulosa and cumulus cells of mouse [91] and in women [92]. The work of Julio-Pieper et al. [93] suggests a role of neurotrophins in the maintenance of follicular and luteal vasculature. The addition of VIP to secondary mouse follicles cultured *in vitro* induced follicular growth and oestradiol production [94]. NGF that was identified in antral follicles of rats, goats, sheep and humans [95-98], promotes ovarian secretion of steroid hormones and prostaglandins [99-101]. The work by Salas et al. [101] shows that NGF also induces an increase in FSH receptor expression and exhibits an increase in FSH sensitivity in human granulosa cells [101]. The high affinity NGF receptor NTRK1 (formely named Trka), has been identified in granulosa cells and oocytes of mouse and rat [95, 102]. Moreover, NTRK1 has been implicated in ovulation [103]. ADCYAP and VIP have two major groups of receptors sites. ADCYAP and its type 1 receptor ADCYP1R1 are expressed by granulosa cells after the LH stimulation of the preovulatory follicle [104], under the regulation of other genes [105]. It requires in particular the prior synthesis of the progesterone receptor [106]. ADCYAP and VIP are activators of cAMP production and thus stimulate the preovulatory production of progesterone [107, 108]. Moreover, ADCYAP has a role in oocyte maturation [109], and displays an apoptosis-inhibiting role [105] . The localisation and functional activity of ADCYAP, VIP and their receptors has been described in mouse ovary [110].

Neuropeptide Y

Few studies have been carried out on the presence and role of neuropeptide Y (NPY) in the ovary. The studies conducted *in vitro* by Barreca et al. [111], show that NPY is involved in regulating the production of oestradiol at the beginning of luteinisation. Otherwise, oestradiol modifies the number of NPY immunoreactive neurones and regulates NPY release in the hippocampal dentate gyrus in the female rat [112]. Moreover, it has been suggested that NPY directly inhibits ovarian cell proliferation and promotes apoptosis via transcription factor p53 [113].

Nevertheless, the expression of neurotransmitters in the preovulatory follicle in the equine species remains unknown.

Modification of steroidogenesis*:* Ovarian steroidogenesis is entirely modified after LH surge since, in many domestic mammals, the production of androgens and oestrogens decreases, whereas the production of progesterone increases [114]. This change in steroidogenesis occurs together with modulations in the expression and activity of the enzymes involved. In mare, steroid evolution during final dominant follicle maturation is similar, with a decrease in oestradiol level and an increase in progesterone level in the follicular fluid [33, 115-118], whereas a transient increase in oestradiol disrupts periovulatory LH surge in mare [119]. The decrease in oestradiol level is related to the decrease in the intracellular amount of aromatase [33, 117]. In the rat adrenomedullin2 has been proposed, to regulate estradiol synthesis and to support ovulation [120]. Moreover, it has been shown that the granulosa cell concentration of mRNA for the StAR protein (Steroidogenic Acute Regulatory protein), involved in transporting cholesterol from the external membrane to the internal membrane of mitochondria, increases after the LH surge in mares [121]. Our study on the expression of the protein itself in the lysates of equine granulosa cells did not confirm this observation [117]. Slough and collaborators [122] showed that StAR mRNA decreases after day 12 in the mare. This decline is time-related to a decrease in circulating progesterone level. These results correspond to data from other species, indicating that StAR plays a rate-limiting role in steroidogenesis [123], but are not in agreement with the results obtained in the mare [121].

Progesterone is well known for its endocrine action. It also exhibits local action since receptors are present in the follicle. It has been shown in the rat that the expression of progesterone receptors (PGR) by granulosa cells of the preovulatory follicle after LH stimulation occurs rapidly but transiently [124-126]; it is amplified by progesterone. Mutant mice deficient for PGR do not ovulate even after the injection of an ovulating dose of hCG [127]. PGR are transcription factors and so they intervene in inducing the expression of several genes involved in preovulatory maturation. PGR activity requires interactions with many transcriptional coregulators, which can be coactivators or corepressors, as SRC, FRG2, NCOA3 and MSANTD1 (formely named SRC1, SRC2, SRC3 and NCO1, respectively) which interact with the ligand binding domain of PGR. The absence of NCOA3 in mice decreases the ovulation rate [128]. Nowadays, steroid receptors modulators could be considered as therapeutic targets in case of ovulation deficiency [129].

It appears that progesterone, in many species, is crucial to ovulation and luteinisation [40, 130]. In fact, it was shown several years ago that LH-induced ovulation can be blocked by HSD3B-inhibitors (rat: [131]; primate: [132]), by anti-progesterone antibodies (rat: [133]) and by RU486 (mouse: [134, 135]).

Local inflammation: The ovarian synthesis of prostaglandins (PGF2, PGE2, PGI2) is increased after LH stimulation, particularly in the preovulatory follicle [136-140]. At the basal level, they are synthetized via the transformation of arachidonic acid by PTGS1 that displays cyclooxygenase and peroxidase functions and is expressed in the theca. Following LH stimulation, the PTGS2 isoform is induced in granulosa cells and therefore increases prostaglandin production in the ovary (rat: [141-143]; cow: [144, 145]). The transcription of the PTGS2 gene is induced by the activation of the PKA, PKC and tyrosine kinase pathways [36] and involves the transcription factor CEBPB [146]. Moreover, transcription factors from the RUNX family would be involved in the LH-induced PTGS2 gene expression [147]. The involvement of prostaglandins in follicular rupture has been shown by the use of anti PGF2alpha antibodies [148] or indomethacin, a nonsteroidal anti-inflammatory and PTGS-inhibitor [149-152]. The specific PTGS2 inhibitor NS398 also decreases prostaglandin production and ovulation rate in rats, in a dose-dependent manner [153]. PTGS2 deficient mice are infertile, due to the absence of ovulation [154]. However, the luteinisation phenomenon (functional corpus luteum) and oocyte nuclear maturation are not disrupted by the inhibition of PTGS [155]. As recently reviewed, PTGS2 inhibitor may be suitable for use as an emergency (but not monthly) contraceptive [156]. In mare, intrafollicular PGF concentration has been measured firstly by Watson & Sertich [115], who observed no variation after injection of an ovulating dose of hCG. Then, Watson & Sertich [73] and Sirois & Dore [157] observed a late increase (>30h) in PGE2 and PGF in the preovulatory follicular fluid in mare after injection of an ovulating dose of hCG. This increase is due to the induced expression of PTGS2 by equine granulosa cells of the preovulatory follicle [121, 157]. The intrafollicular injection of indomethacin does not inhibit ovulation in mare, but increases the induction-ovulation interval [73]. Again in the mare, the intrafollicular injection of PGE2 and PGF restores ovulation of luteinized unruptured follicles [158] and induction of luteolysis with PGF2alpha alters the level of follicular fluid factors (IGF1 and PGE2) [159].

Leukotrienes, other derivatives of arachidonic acid, also appear to be involved in final follicle maturation [160-163]. In mares the intrafollicular levels of leukotrienes B4 and C4 have been measured and, as opposed to observations in rats [164, 165], they do not increase after induction of ovulation [73]. This same result was observed in goats [166]. The current hypothesis is that eicosanoids (prostaglandins and leukotrienes) stimulate the synthesis and activity of collagenases involved in matrix degradation at follicular rupture (rat: [167]). This has not been confirmed for all the species studied so far.

On the other hand, the presence of several inflammatory cytokines has been shown in the ovary during the preovulatory period and seems involved in ovulation. In fact, ovulation in the broad sense of the term is often compared to an inflammatory-type reaction [130, 168, 169]. Inflammation that is induced by gonadotropin stimulation has a physiologic role, creating a weakening in the follicle wall and eventual rupture [130, 168]. Several studies show that these cytokines potentiate the LH effect [170]. The mechanism of action of cytokines during preovulatory maturation is probably similar to that observed during inflammation; in particular, TNF and IL-1 activate proteolytic enzyme activity and the production of prostaglandins and nitric oxide [171]. Moreover, they modulate steroidogenesis [171] and stimulate the maturation of the oocyte-cumulus complex (rabbit : [172]). A recent study showed that specific inflammatory mediators are altered in PRKO mice ovaries, leading to the hypothesis that progesterone regulates features of inflammation at ovulation [173].

The interleukin-1 system, composed of IL-1A, IL-1B, the natural antagonist IL1RA and IL1 receptors, is the best known. IL1B, IL1RN and the type 1 receptor IL1R1 are expressed in the human ovary [170]. IL1A and IL1B come from two different genes, but act on the same receptors. They must undergo proteolytic cleavage to become biologically active. The antagonist IL1RA has a local role as a regulator of IL1 activity. Biologically active IL1 has been measured in the follicular fluid of women [174, 175] and sows [176]. In the rat, IL1B is expressed by thecal cells, mainly after the injection of a dose of hCG [177], as well as by the oocyte [178], whereas in women granulosa cells are the source of ovarian IL1 [179-182]. We demonstrated the presence of IL1B in equine follicular fluid and the presence of IL1B and IL1RA mRNA in equine granulosa cells [183, 184]. Importantly, the expression of IL1B gene in granulosa cells, as well as IL1B follicular fluid content seem to be influenced by equine gonadotropin in mare [183]. Moreover, it has been demonstrated that IL1B promotes the ovulation process in the rat [185], the rabbit [172] and the mare [186]. In mare, intrafollicular injection of IL1B induces synchronized ovulations [187]. These results are in agreement with those obtained by Martoriati et al. showing that the intrafollicular injection of IL1RA reduces the ovulation rate or delays the ovulation time [186]. The effects of IL1B on the oocyte could be mediated by cumulus cells. IL1B increases *in vitro* the germinal vesicle breakdown of oocytes in the rabbit model [172], as well as *in vivo* in the mare [186], demonstrating its beneficial role in oocyte nuclear maturation. For review see Gerard and collaborators [188].

Only the type 1 receptor IL1R1 appears to be expressed in human ovary [180], whereas in rats, IL1R2 has also been identified [106]. Other cytokines such IL6, the granulocyte macrophage colony stimulating factor (CSF), and TNF have also been measured in the follicular fluid of the preovulatory follicle (rat: [189]; human: [170, 190, 191]). IL6 may intervene in the IL1 role in rat ovary [192]. In human, exogenous IL8 induces a similar increase in follicular growth to that produced by the LH surge, suggesting that IL8 is an important actor of the ovulatory process [193]. However, no study has been conducted in the mare on the role of IL8 in the ovary. The potential role of other interleukins (IL2, IL4, IL7, IL11, IL12, IL13, IL15, IL18, IL23) on folliculogenesis, oocyte maturation and ovulation is treated in details in the revue by Smolikova et al. [194]. A very recent study performed in the rat preovulatory follicle showed that IL11 is stimulated during ovulation, and increases progesterone production [195]. Moreover, it has been shown that chemokine receptor 4 (CXCR4) was induced by hCG in both granulosa and theca equine cells [196].

A preovulatory increase in cortisol, anti-inflammatory glucocorticoid, has also been reported in several studies (human: [197, 198]). According to the studies of S. Hillier, conducted mainly in humans, this increase is due to the synthesis of the isoform 1 of the hydroxysteroid dehydrogenase 11B (HSD11B1) by granulosa cells stimulated by LH. This enzyme reduces cortisone to cortisol [199]. HSD11B2 is expressed by follicles at the earlier stages.

In the human ovarian follicle, glucocorticoid metabolism is a scale between HSD11B2 dehydrogenase activity (with high cortisol-binding affinity) and HSD11B1 dehydrogenase and reductase activities (with low cortisol-binding affinity) [200-203]. Responsiveness of granulosa cell HSD11B to gonadotropins favors metabolism of cortisol to cortisone in immature follicles, and the converse is true in periovulatory follicles [202, 204], with increased cortisol production by luteinized granulosa cells positively associated with oocyte maturation [205]. Its level decreases after LH stimulation [199], explaining why the cortisol/cortisone ratio increases in follicular fluid at ovulation [197]. The role of the preovulatory increase in cortisol is not clearly understood. Cortisol may intervene in cell differentiation in relation to ovulation and/or the formation of the corpus luteum; a local role of glucocorticoids in oocyte maturation has also been suggested [205, 206]. In the mare, involvement of glucocorticoids in follicular and oocyte maturation has recently been shown [207].

Degradation of the extracellular matrix (ECM): In order the tissue to be reorganised, which takes place during follicular rupture and the formation of the corpus luteum, the extracellular matrix has to be remodelled. The involvement of proteases in ovulation was initially suggested in 1916 by Schochet [208]. In the ovulatory follicle after LH stimulation, the degradation of the basal lamina is initiated following an increase in the local production of plasminogen activator (PLA) (mainly tissue type PLAT), plasmin activator and metalloproteinase/collagenase activator (see reviews [64, 209, 210]). PLA transforms plasminogen into plasmin, which in turn activates certain collagenases [211]. At the same time, the inhibiting activity of SERPINE1 (formely PAI1) increases at the intrafollicular level after ovulation has been induced (human: [212]; rat: [213]; pig: [214]). The preovulatory increase in collagenase activity within a follicle destined to ovulate was demonstrated in 1985 by Reich et al. [215]. Then, the zymography technique showed that this collagenase activity is mainly due to MMP1 and MMP-2 [216]. On the other hand, collagenase inhibitors TIMP1 and TIMP2 are present in the preovulatory follicle after ovulation has been induced (rat: [167, 217, 218]; ewe: [219, 220]; cow: [221] women: [222]). The expression of MMP and TIMP is regulated by steroids (increase in progesterone and/or decrease in oestradiol) and by prostaglandins [167, 223, 224]. In the equine ovary, changes in MMP2 and MMP9 activities occur in follicular cells, and may be essential to the tissue organization necessary for ovulation [225, 226]. Alpha 2 macroglobulin which exhibits collagenase inhibiting activity, is also present in the follicular fluid in the murine species [227], in humans [228, 229], and in the mare [230]. Its expression by granulosa cells increases following injection of hCG [227]. The concomitant expression of proteases and antiproteases in the preovulatory follicle probably makes it possible to modulate both the site and degree of degradation of the follicle wall at rupture. Collagenase alpha 2 has recently been described in the equine preovulatory follicle from young and old animals [231].

Relaxin participates to extracellular matrix remodelling by regulating proteolytic enzymes activity in the ovary. Relaxin is a member of the insulin family; it has been studied in particular for its role in parturition and lactation. In the ovary, relaxin has been found in the follicular fluid (women [232]), and its level significantly increases with follicle size in the sow

[233]. Its production by granulosa cells is stimulated *in vitro* by LH [234, 235]. Relaxin appears to be a paracrine factor of preovulatory follicle maturation [236], probably by modulating the activity of certain collagenases. In sows, it appears that exogenous relaxin influences its own receptors expression, improves oocyte nuclear maturation [237]. Equine relaxin was purified and characterised by Stewart & Papkoff in 1986 [238], and its expression in the mare ovary has been studied [239]. These authors confirmed that relaxin is present in equine follicular fluid, but its concentration does not increase during follicle growth or maturation. Moreover, it has not been possible to detect any mRNA transcript in the follicle cells. Indirect evidence indicates that, similarly to the rat, relaxin might contribute to the ovulatory process in the mare. Actually, Song et al. [240] studied the role of relaxin in stromal tissue remodeling by evaluating its regulating activity of gelatinase and TIMP, PLA and SERPINE1 produced by ovarian equine stromal cells in culture. MMP and PLA systems are coordinated during ECM proteolysis. Equine relaxin modulates both the MMP and PLA systems in culture, which suggests that it potentially participates in ECM degradation in the ovary by simultaneously regulating proteolytic enzymes and their inhibitors, providing additional evidence that this hormone plays significant roles in tissue remodeling associated with follicle growth and ovulation in equine ovaries [240], for recent review see Klein [241].

Other molecules:

Oxytocin like progesterone is a luteinisation marker. In fact in the ovary, oxytocin is produced mainly by the corpus luteum. However, it has been shown in monkey and cow that the granulosa cells of antral follicles already produce oxytocin [242, 243] and that this production is stimulated by the preovulatory increase in LH. In women, the presence of oxytocin and its receptor have been demonstrated in cumulus cells at the preovulatory stage [244]. However, the studies conducted in mare did not confirm these observations [245, 246].

As in other species, the release of equine endometrial PGF2 is stimulated by oxytocin. During late dioestrus, initial oxytocin secretion comes from the pituitary. In contrast to other species, no significant luteal oxytocin synthesis exists in the mare. Of note is the fact that equine is the only domestic species in which oxytocin has been localized in the endometrium where specific secretory cells containing oxytocin have been described [247]. These data suggest that, in this species, oxytocin is not involved in regulating the preovulatory maturation or the luteal differentiation.

Osteopontin (SPP1) is a 41.5kDa secreted glycoprotein whose mRNA was described for the first time in mice ovary in 1991 [248] and in human ovary in 1992 [249]. Expression of this protein, implicated in angiogenesis and tissue remodelling, was then studied in bovine ovarian follicles and in corpus luteum. SPP1 mRNA was detected in bovine granulosa, theca and luteal cells, but the authors showed no effect of gonadotrophic hormones mRNA expression of ovarian cells culture [250]. More recently, upregulation of SPP1 has been shown in mouse granulosa cells in response to a gonadotropin surge through epidermal growth factor receptor (EGFR) signaling [251], which seems the main mediators of LH surge in the ovulatory follicle [252]. Thus, SPP1 expression is involved in corpus luteum formation and function during the early-luteal phase, and enhances progesterone synthesis but also promotes the expression of VEGF, which encodes a prominent factor in the early luteinizing period [251]. To date no data is available to our knowledge concerning SPP1 and follicle maturation or ovulation in the mare.

Growth factors are important actors of the preovulatory maturation and ovulation. Intense expression of VEGF, receptors and angiopoietins have been detected in periovulatory equine ovaries [253]. A contradictory work described a decrease in preovulatory intrafollicular VEGF [254].The concentration of few other growth factors has been shown to change in the preovulatory follicle, without any direct effect on ovulation. In the mare, modulation of intrafollicular levels of insulin-like growth factor 1 (IGF1), IGFBPs, *inhA*, and activin A have been observed [116, 254]. Moreover, epiregulin and amphiregulin (members of the EGF family) mRNA significantly increase in follicular cells from equine preovulatory follicles [255, 256]. Interestingly, a recent study performed in the mare, indicated that TGF/BMP signaling pathway in granulosa cells is regulated by exosomes present in the preovulatory follicular fluid, that thus may intervene in follicle maturation [257]. Very recently, a protein array analysis performed in mouse granulosa cells highly suggest that signaling parthways of IGF1R, FGFR2 and EPHB1, are activated by the preovulatory LH surge [258].

Reactive oxygen species (ROS) and antioxidant are highly important in overall health, and have significant roles in the ovary to ensure ovulation of a well-matured oocyte [259]. Ovarian levels of superoxide dismutase 1-2 and catalase vary in relation to steroids, and are regulated by gonadotropins. Few years ago, the observation of stimulation of sulfiredoxin and hyperoxidization of peroxiredoxin during the periovulatory period led to suggest the presence of an antioxidant system within the ovary at the time of ovulation [260].

Only few other **global analysis** i.e. proteomic, transcriptomic and metabolomics studies describe follicular factors which synthesis is regulated by the LH surge in the mare, and may intervene in the terminal differentiation of the follicle before ovulation in this species [116, 261-264]. These approaches are of great interest to point out key actors of this process, but are highly dependent to availability and performance of biochemical and bioinformatics tools and strategies.

3 - Maturation of the oocyte-cumulus complex

In several mammalian species, including horses [265], oocytes that have finished growing are able to mature when isolated from their follicles and are cultured *in vitro*. This observation suggests that follicle inhibition exists. Maturation of the oocyte-cumulus complex takes place *in vivo* in the ovulatory follicle following the LH surge. It consists of expansion of the cumulus oophorus (population of granulosa cells surrounding the oocyte), of nuclear maturation of the oocyte with resumption of meiosis and cytoplasmic maturation, i.e. organelle reorganization and molecular changes. Cumulus cells play an important role during oocyte maturation, acting by gap juntional communications and soluble factors [266, 267]. Recent studies focused on the second messenger cyclic adenosine monophosphate (cAMP) and oocyte secreted factors (OSFs) which possibly play a key role in the regulation of oocyte maturation [268]. cAMP is produced by cumulus cells and transported through gap junctions into the oocyte. In the mare, phosphodiesterase 4D increases in cumulus cells after LH induction of ovulation, as well as activation of both oocyte specific G-protein-coupled receptor 3 and phosphodiesterase 3A [256]. Oocyte regulates cumulus cell differentiation via the secretion of soluble paracrine growth factors, known as the OSFs regulation of folliculogenesis [269]. OSFs regulate cumulus cells, which in turn modulate oocyte development. This microenvironment is crucial for oocyte development and maturation [270]. The role of cumulus cell’s macrophage colony-stimulating factor (M-CSF) in the process of LH-induced resumption of meiosis has recently been demonstrated in the mouse to act via the vital inhibition of natriuretic peptide receptor2 (NRP2) [271]. Growth differentiation factor 9 (GDF9) [272] and bone morphogenetic protein 15 (BMP15) [273], from the transforming growth factor beta superfamily (TGFB), are the most important OSFs in this context. The preovulatory LH surge upregulates BMP15 secreted by the oocyte, and may be used to interact with somatic follicular cells [256, 274]. Moreover, it has been recently hypothesized that alteration of oocyte-specific galactosyl- and acetylglucosaminyltransferases may affect

BMP15 activity and hyaluronan production, a vital matrix constituent of the cumulus-oocyte complexe [275]. Recently in the mare, immunization against BMP15 and GDF9 demonstrated a lower ovulation rate, and has been proposed as a contraceptive [276]. In addition to gap junctional communications and paracrine signalling between the oocyte and surrounding cumulus cells, is a less well characterized way of signalling including exosomal transfer which allow the transfer of various molecules, as very recently reviewed [277]. All these communication pathways regulate signalling events around ovulation that lead to oocyte maturation and competence for fertilization and embryo development.

The morphological studies conducted on the equine *cumulus oophorus* [278, 279], show it similar to that in other domestic species: the 3 or 4 regular layers of cells close to the oocyte make up the corona radiata. It is itself surrounded by a large number of less organised cells making up the *cumulus* [280]. However, the equine cumulus is more strongly attached to the follicle wall than the bovine cumulus [281]. As studied in mouse and pig, the cumulus expansion is characterized by the synthesis of hyaluronan which associates to proteins and proteoglycans (mainly the inter alpha trypsin inhibitor family) to constitute an expanded hyaluronan-rich extracellular matrix [282]. In parallel to the cumulus expansion, meiotic oocyte resumption and organelle and cytosqueletal reorganization are orchestrated for successful oocyte maturation. These are under the control of molecular changes, such as proteins degradation or de novo synthesis. Thus, maturing oocytes depend on post-transcriptional regulation of stored transcripts. Reyes & Ross recently published a review that encompass the key importance of cytoplasmic polyadenylation mechanism and associated factors during oocyte maturation in mammals, based on studies performed mainly in Xenopus [283]. These authors describe the expression of polyadenylation and translational regulator associated factors in human, mouse and cattle metaphase 2 oocytes, and address the complexity of the molecular mechanisms associated to oocyte maturation. Factors affecting meiotic and developmental competence of the equine oocyte have been reviewed [284]. At least, the oocyte of the dominant follicle resumes meiosis when it has reached 80% of its definitive size, i.e. just a few hours before ovulation [285]. In mare, no clear relationship has been established between the size of an oocyte and its capacity to resume meiosis [286]. As is the case for other domestic mammals, the oocyte nuclear maturation stages in the equine species include nuclear envelope breakdown, completion of the first meiotic division, with polar body extrusion and arrest in metaphase II [287]. Equine oocytes are ovulated at the metaphase II stage [288, 289] [290], as in most mammals. Meiosis is completed at fertilisation. A chronology of *in vivo* oocyte maturation in the equine species after induced ovulation showed that the metaphase I and metaphase II stages were reached respectively 24 and 35 h after the injection of exogenous gonadotropins [291].Two important phenomena occur during nuclear maturation: chromatin condensation, and the disappearance of the nuclear membrane. It has been shown that the M phase Promoting Factor (MPF) is involved in both these phenomena. The expression of mitogen-activated protein kinases (MAPK) and of MPF components CDK1 (formely CDC2) and cyclin B in mare oocytes during both *in vivo* and *in vitro* maturation was studied in our team several years ago [292, 293]. It has been shown that MAPK activity in equine oocyte is regulated by calcium homeostasis [294]. Nuclear configuration, spindle morphology, histone acetylation and microtubules/microfilaments content were recently described during meiosis resumption in equine [295-297]. At cytoplasmic scale, few studies describe the ultrastructure of the equine oocyte and the organisation of cytoplasmic organelles. It was reported the absence of granular endoplasmic reticulum and Golgi apparatus in mare oocytes [298], as well as a particular abundance of lipid droplets. During *in vivo* maturation of the equine oocyte, Bézard et al. [290] and Gröndahl et al. [287] in 1995 observed the disruption of the junctions between cumulus cells and the oocyte, the migration of cortical granules towards the periphery of the cytoplasm and the migration of mitochondria towards the centre of the cytoplasm. Since few years, research has focused on mitochondrial DNA in oocytes. In the mare, the reduced fertility observed with maternal aging is related to mitochondrial damage and loss [256, 299, 300]. If several cytoplasmic changes were reported during maturation of equine oocytes [286, 287, 301], migration of cortical granules has been assessed as an indicator of cytoplasmic maturation, the final sign being the ability of the oocyte to undergo fertilization [302, 303].

Concerning intra-oocyte lipids, mainly stored in lipid droplets, Prates et al. [304] recently reviewed their implication during oocyte-cumulus complex maturation and oocyte competence for fertilization. Lipid droplets distribution is related to cumulus expension and oocyte maturation in the mare [305]. Moreover, ovarian leptin has been studied in the horse [306], leading these authors to proposed that oocyte leptin and leptin receptors contents, its maturation competence, and adiposity are related. Metabolic changes and in particular the increase in the intra-oocyte level of glutathione, seem to have considerable consequences for fertilisation. Some glutathion comes from the cumulus cells, which would explain the involvement of these cells in the quality of maturation [307]. Few year later, we studied the glutathione content and glutathione peroxidase expression in equine oocytes and cumulus cells before and after maturation [308]. The growth hormone (GH) receptor was detected in the oocyte and in cumulus and granulosa cells, and *in vitro* addition of eGH to maturation medium increased the rate of cytoplasmic maturation of equine oocytes [309-311]. Recently, hemoglobin has been described in mouse oocytes, and has been hypothesized to intervene during cumulus oocyte complex maturation [277]. It may be transferred from the cumulus cells to the oocyte and may act as O2/NO gas binding protein. Nevertheless, strong evidences about the regulation, function and mechanism of action of hemoglobin in the cumulus oocyte complex during the ovulatory period, as well its role during oocyte maturation have yet to be established.

Conclusion:

As described here, a large number of regulatory factors have been shown within ovarian follicles and are potentially involved in controlling the development and maturation of the follicle and/or of the oocyte that it contains. Some of them originate from local synthesis and others from the passage from the circulatory compartment to the follicular fluid, and sometimes the two simultaneously. However, few have been conducted on mares in comparison to other species.

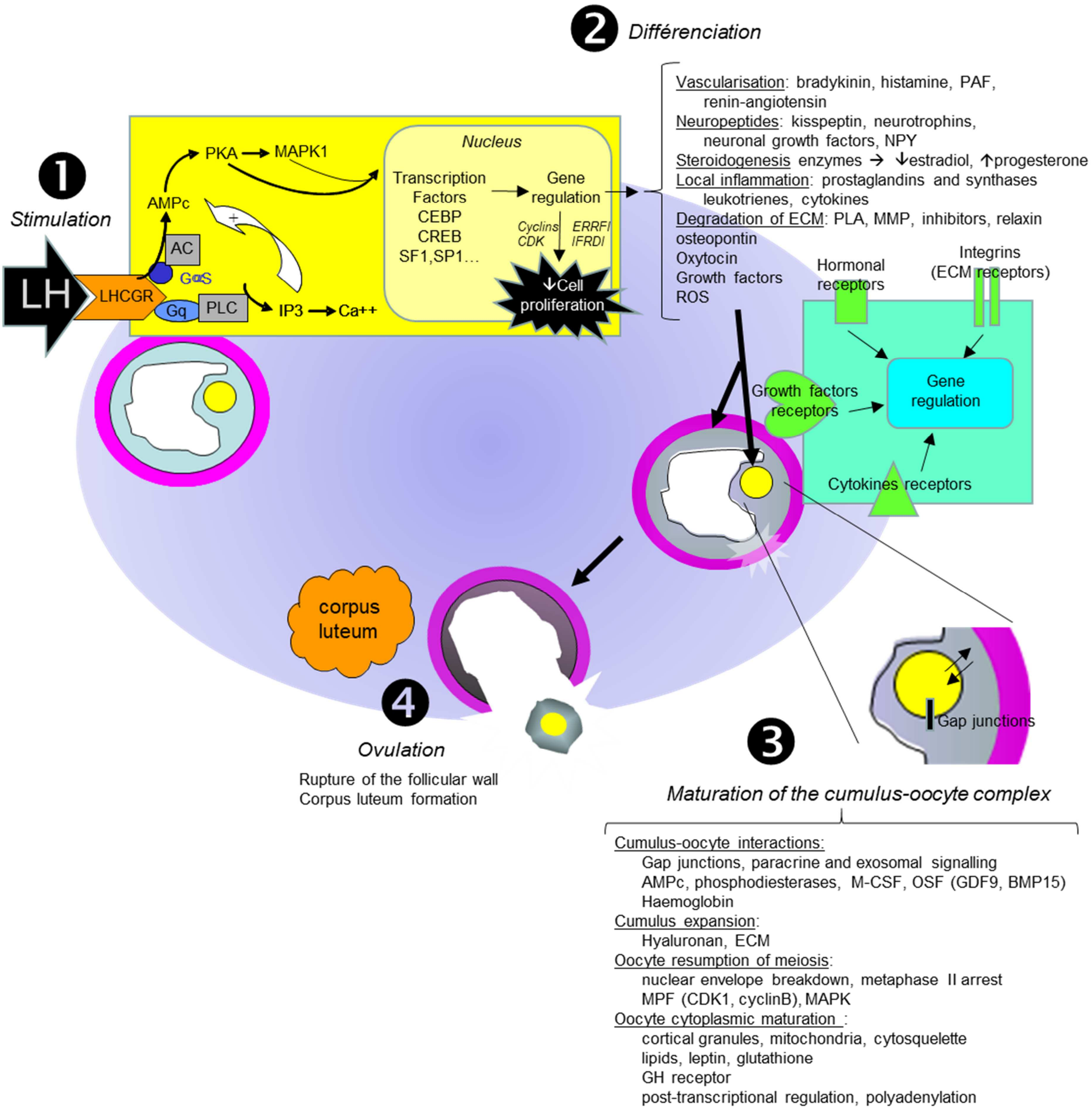
However in the equine species, requirements in the field of reproduction often resemble those in human medicine; it is often preferable to obtain descendants from individuals capable of conceiving *in vivo* or of carrying a pregnancy to term. On the other hand, compared to other mammals, the horse exhibits certain particularities that make it an interesting model for studying follicle development and maturation. Some of these particularities are quoted in this report, such as ovary morphology, the length of the oestrus phase, and the absence of any real preovulatory LH peak. Moreover, the follicle and oocyte maturation mechanisms appear to be unusual. In fact, the *in vitro* maturation rate of oocytes is low and the success of IVF and ICSI still limited. The equine species is also characterised by a relatively high rate of anovulatory cycles.

Consequently, although the physiological mechanisms that regulate folliculogenesis and oogenesis do not appear to be fundamentally different from the mechanisms observed in the other domestic mammal species, these observations taken together suggest that some unique types of regulation may be involved in the mare. A better understanding of the mechanisms and factors involved in final maturation in this species is therefore a prerequisite for obtaining quality oocytes.

Figure legends:

Figure 1 : Schématic representation of molecular events triggered by the LH surge that may intervene during preovulatory differenciation and ovulation in the mare. Based on data colllected in the mare as well as other mammalian literature. Differenciation of the preovulatory follicle is a consequence of LH binding to receptors localized in the plasma membrane of follicular cells (❶), activation of signaling pathways induces regulation of gene expression within follicular cells (❷), as well as maturation of the cumulus-oocyte complexe (❸), that finaly leads to the follicular rupture (❹), that liberates a fertilizable oocyte and forms a corpus luteum that both allow pregnancy.

Figure1 :



❶
Stimulation
LH
LHCGR
GαS
AC
AMPc
PKA
MAPK1
+
Gq
PLC
IP3
Ca++
Nucleus
Transcription Factors
CEBP
CREB
SF1,SP1…
Gene regulation
Cyclins
CDK
ERRFI
IFRDI
↓Cell proliferation
❷ Différenciation
Vascularisation: bradykinin, histamine, PAF, renin-angiotensin
Neuropeptides: kisspeptin, neurotrophins, neuronal growth factors, NPY
Steroidogenesis enzymes → ↓estradiol, ↑progesterone
Local inflammation: prostaglandins and synthases leukotrienes, cytokines
Degradation of ECM: PLA, MMP, inhibitors, relaxin
osteopontin
Oxytocin
Growth factors
ROS
Hormonal receptors
Integrins (ECM receptors)
Growth factors receptors
Gene regulation
Cytokines receptors
Gap junctions
❸
Maturation of the cumulus-oocyte complex
Cumulus-oocyte interactions:
Gap junctions, paracrine and exosomal signalling
AMPc, phosphodiesterases, M-CSF, OSF (GDF9, BMP15)
Haemoglobin
Cumulus expansion:
Hyaluronan, ECM
Oocyte resumption of meiosis:
nuclear envelope breakdown, metaphase II arrest
MPF (CDK1, cyclinB), MAPK
Oocyte cytoplasmic maturation :
cortical granules, mitochondria, cytosquelette
lipids, leptin, glutathione
GH receptor
post-transcriptional regulation, polyadenylation
corpus luteum
❹
Ovulation
Rupture of the follicular wall
Corpus luteum formation

Tables:

Table 1: Interval between LH preovulatory peak and ovulation in different mammals. Of note is the fact that there is not LH peak in the mare but a gradual LH increase starting before ovulation to reach a maximum level 24 to 48 hours after ovulation.

Species	**Period of time between hCG injection or endogenous LH peak and ovulation (hours)**
Mouse	12
Rat	14
Ewe	25
Cow	28
Sow	40
Mare	35-40
woman	36

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