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Development of equine preovulatory follicles: Morphological studies and regulation of steroidogenic acute regulatory protein (StAR)

par

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présenté par

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SOMMAIRE

Chez la jument, l'augmentation préovulatoire de l'hormone lutéinisante provoque un remodelage rapide de la structure et des caractéristiques fonctionnelles du follicule préovulatoire. On assiste ainsi à des changements cellulaires et vasculaires marqués dans le follicule et à un réaménagement de la production d'hormones stéroïdiennes des oestrogènes vers la progestérone. Deux expériences ont été menées pour caractériser deux aspects des dernières étapes du développement des follicules préovulatoires équins. La première avait comme objectif de caractériser la morphologie histologique des follicules préovulatoires équins pendant la différenciation finale. La deuxième devait permettre de caractériser la structure primaire de la protéine de régulation rapide de la stéroïdogenèse (StAR), et la régulation de son ARN messager (ARNm) dans le follicule préovulatoire à la suite d'une ovulation induite à l'hCG.

Afin de caractériser ces changements cellulaires et vasculaires, 5 à 6 follicules préovulatoires ont été isolés pendant l'oestrus 0, 12, 24, 30, 33, 36 et 39 h après l'administration d'une dose ovulatoire d'hCG (0h = moment de l'administration de l'hCG). Des échantillons de la paroi folliculaire ont été fixés à la formaline, inclus dans la paraffine puis évalués au microscope optique. Nous avons pu observer des changements très marqués de la morphologie histologique des follicules équins dans les heures précédant l'ovulation. L'épaisseur de la couche cellulaire granuleuse (GCL) a doublé entre 0 h et 39 h (77,8 ± 4,8 contre 158,8 ± 4,8 μm; $p<0,01$), un phénomène attribué à l'augmentation significative du nombre de cellules de la granulosa. Des substances mucoïdes ont tout d'abord été détectées dans la GCL (coloration au bleu Alcian) à 12 h et elles ont atteint leur concentration maximale entre 36 h et 39 h. Nous avons été

frappés par l'apparition soudaine d'éosinophiles dans la thèque interne et la thèque externe respectivement à 33 h et 36 h, à 39 h, nous avons pu ainsi observer respectivement 14,0 ± 0,8 et 5,6 ± 0,3 éosinophiles par champ (40X) dans la thèque interne et la thèque externe. Nous avons également noté un œdème marqué, de l'hypérémie et des hémorragies de même qu'une augmentation significative du nombre de vaisseaux sanguins dans la thèque interne et la thèque externe entre 36 h et 39 h.

Afin de caractériser la StAR équine, une génothèque d'ADN complémentaire (ADNc) a été préparée à partir de follicules préovulatoires isolés à 36 h puis criblée à l'aide d'une sonde d'ADNc de StAR. Les résultats du séquençage des clones positifs ont permis d'identifier deux formes d'ADNc de StAR équine, qui diffèrent seulement par leur région 3' non traduite (3'-UTR), dans un cas, il s'agit d'une forme longue de 2 918 paires de bases (pb) et dans l'autre, d'une forme courte de 1 599 pb. La forme longue renferme une région 5'-UTR de 117 pb, un cadre ouvert de lecture (ORF) de 855 pb et une région 3'-UTR de 1946 pb. L'ORF encode une protéine de 285 acides aminés qui s'est avérée respectivement identique à 90%, 89%, 88%, 87%, 85%, 87% et 88% à la protéine de StAR d'humain, de porc, de vache, de souris, de hamster, de rat et de mouton. L'analyse d'extension d'amorces a démontré que le clone d'ADNc ne possédait pas les premières 10 pb du transcrit primaire, sa région 5'-UTR de StAR renfermait donc un total de 127 pb.

Afin de caractériser la régulation de l'ARNm de StAR dans les follicules préovulatoires, ces derniers ont été isolés pendant l'oestrus à 0, 12, 24, 30, 33, 36 et 39 h (n=4-5 follicules par moment étudié) après une dose ovulatoire de hCG. En plus, trois corps jaunes (CL) ont été recueillis au huitième jour du cycle. Les résultats des

transferts de Northern n'ont permis de mettre en évidence aucun changement significatif dans les niveaux d'ARN de StAR à la suite du traitement à l'hCG lorsque les analyses étaient effectuées sur une paroi folliculaire intacte (thèque interne à laquelle sont fixées des cellules granuleuses). Cependant les transferts de Northern effectués avec des cellules de follicules isolées ont permis de mettre au jour un mécanisme de régulation imprévue de l'ARNm de StAR. Dans les cellules de la granulosa, les transcrits de StAR étaient indétectables à 0 h, mais augmentaient de manière significative à 30 h. Cette induction était associée à une augmentation des concentrations de progestérone dans le liquide folliculaire ($p<0,05$). Par contre, les niveaux d'ARNm de StAR étaient élevés dans la thèque interne à 0 h, demeuraient inchangés jusqu'à 33 h et chutaient par la suite ($p<0,05$).

Dans l'ensemble, ces études ont permis de caractériser pour la première fois les changements cellulaires et vasculaires marqués qui surviennent dans les follicules préovulatoires équins lors d'une ovulation induite à l'hCG. En outre, elles ont permis de décrire la structure primaire de la StAR équine et d'identifier une nouvelle régulation inverse des transcrits de StAR dans la thèque interne et les cellules de la granulosa des follicules équins avant l'ovulation.

SUMMARY

A rapid remodeling of the structure and function of the preovulatory follicle is induced by the preovulatory rise of luteinizing hormone in mares. The gonadotropin rise leads to marked cellular and vascular changes in the follicle, and a switch in steroid hormone production from estrogen to progesterone. Two experiments were performed to characterize two aspects of the final development of equine preovulatory follicles. The first experiment characterized the morphology of the developing equine preovulatory follicle during its final differentiation. The objectives of the second experiment were to 1) determine the primary nucleotide structure of the equine steroidogenic acute regulatory protein (StAR), a key protein involved in the rapid regulation of steroid hormone production, and 2) examine the regulation of its messenger RNA (mRNA) in preovulatory follicles after hCG-induced ovulation.

To characterize the cellular and vascular changes associated with ovulation in mares, preovulatory follicles were isolated during estrus at 0, 12, 24, 30, 33, 36 and 39 h after an ovulatory dose of hCG. Five to six follicles per time-point were isolated. Pieces of follicle wall were formalin-fixed, paraffin-embedded and evaluated by light microscopy. Results showed a dramatic change in the morphology of equine follicles in the hours prior to ovulation. The thickness of the granulosa cell layer (GCL) doubled between 0 and 39 h post-hCG (77.8 ± 4.8 versus 158.8 ± 4.8 μm, respectively, $P<0.01$), and was associated with a significant increase in the number of granulosa cells. Acid mucosubstances in the GCL (Alcian blue staining) were first detected 12 h post-hCG, and reached maximal levels 36-39 h post-hCG. One striking histological feature was the sudden appearance of eosinophils in theca interna and theca externa layers 33 h and 36 h

post-hCG, respectively. At 39 h post-hCG, there were 14.0 ± 0.8 and 5.6 ± 0.3 eosinophils per field (40X) in theca interna and theca externa, respectively. Severe edema, hyperemia and hemorrhages, and significant increases in numbers of blood vessels in theca interna and externa were observed 36-39 h post-hCG.

To characterize the equine StAR, a complementary DNA (cDNA) library, prepared from a preovulatory follicle isolated 36 h post hCG, was screened with a mouse StAR cDNA probe. Sequencing on positive clones identified two forms of equine StAR cDNA that differ only in their 3'-untranslated region (3'-UTR), a long form of 2918 base pairs (bp) and a short form of 1599 bp. The StAR-long form cDNA contains a 5'-UTR of 117 bp, an open reading frame (ORF) of 855 bp, and a 3'-UTR of 1946 bp. The ORF encodes a 285 amino acid protein that is 90%, 89%, 88%, 87%, 85%, 87%, and 88% identical to the human, pig, cow, mouse, hamster, rat, and sheep StAR protein, respectively. Primer extension analysis showed that the cDNA clone lacked the first 10 bp of the primary transcript, giving a total of 127 bp for the complete StAR 5'-UTR.

To characterize the regulation of StAR mRNA in preovulatory follicles prior to ovulation, preovulatory follicles were isolated during estrus at 0, 12, 24, 30, 33, 36, and 39 h (n=4-5 follicles/time point) after an ovulatory dose of hCG. In addition, three corpora lutea (CL) were collected on day 8 of cycle. Results from Northern blots showed no significant changes in StAR mRNA levels after hCG treatment when analyses were performed on intact follicle wall (theca interna with attached granulosa cells). However, Northern blots performed on isolated follicle cells revealed an unexpected regulation of StAR mRNA. In granulosa cells, StAR transcripts were undetectable at 0 h but significantly increased at 30 h post-hCG, and this induction was associated with a

rise in follicular fluid concentrations of progesterone ($P<0.05$). In contrast, StAR mRNA levels were high in theca interna at 0 h, remained unchanged until 33 h post-hCG, and dropped dramatically thereafter ($P<0.05$).

Collectively, these studies characterize, for the first time, the dramatic cellular and vascular changes in equine preovulatory follicles during hCG-induced ovulation. Also, they describe the primary structure of equine StAR and identify a novel inverse regulation of StAR transcript in the theca interna and granulosa cells of equine follicles prior to ovulation.

AVANT-PROPOS (PREFACE)

Le mémoire comprend une introduction générale, une revue de la littérature, deux articles scientifiques, une discussion générale, une conclusion générale, et une liste de références.

This thesis comprises a general introduction, a general literature review, two scientific articles, a general discussion, general conclusion, and a list of references.

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ABBREVIATIONS

µm:	micrometer
8-bromo-cAMP:	8 bromoadenosine 3', 5'-cyclic monophosphate
ACTH:	adenocorticotropic hormone
Ad4BP:	adrenal 4 binding protein
bp:	base pair
cDNA:	complementary DNA
CL:	corpus luteum
CRRA:	Centre de recherche en reproduction animale
DHEA:	dehydroepiandrosterone
eCG:	equine chorionic gonadotropin
EFTu:	elongation factor Tu
FSH:	follicle stimulating hormone
IGF:	insulin-like growth factor I
GCL:	granulosa cell layer
GnRH:	gonadotropin releasing hormone
h :	hour
hCG:	human chorionic gonadotropin

HDL:	high-density lipoprotein
kDa:	kiloDalton
LDL:	low-density lipoprotein
LH:	luteinizing hormone
mRNA:	messenger ribonucleic acid
min:	minute
ORF:	open reading frame
PGE:	prostaglandins of the E series
PGF2 alpha:	prostaglandin F2 alpha
PGH2:	prostaglandin H2
PGHS:	prostaglandin G/H synthase
PGI2:	prostacycline
PKA:	protein kinase A
PMSG:	pregnant mare serum gonadotropin
SEM:	standard error of the mean
SF1:	steroidogenic factor 1
StAR:	Steroidogenic acute regulatory protein
UTR:	untranslated region

1. INTRODUCTION

The ovarian follicle is the structural and functional unit of the mammalian ovary, providing the microenvironment necessary for the growth and maturation of the oocyte and production of steroid hormones. It consists of a cumulus oophorus-enclosed oocyte that is surrounded by granulosa cells in the inner layers and by theca cells in the outer layers. Each follicle develops through primordial, primary, and secondary stages before acquiring an antral cavity. Further growth and differentiation leads to large Graafian or preovulatory follicles, which, under proper conditions, ovulate and transform into corpora lutea (Adashi, 1994; Richards, 1994). The ovulatory process, defined as the time between the luteinizing hormone (LH) surge and follicular rupture, is a period of rapid tissue remodeling in the follicle. The duration of ovulatory process varies among different species. In the mare, the ovulatory process lasts approximately 36-48 h, which is relatively long compared to other species (Duchamp et al., 1987; Sirois, 1994; Sirois and Doré, 1997).

The process of follicular rupture involves a complex series of biochemical and biophysical changes in the developing preovulatory follicle. To release the oocyte from the follicle, several tissue layers (the surface epithelium, tunica albuginea, theca externa, theca interna, and granulosa layer) must rupture (Espey, 1994; Espey and Lipner, 1994). Follicular remodeling involves degradation of extracellular matrix components, and is associated with prominent changes in the vascular architecture and with leukocyte infiltration, all features common to an acute inflammation (Espey, 1980; Espey, 1994). The preovulatory gonadotropin rise is the trigger for ovulation. It increases blood flow, causes vasodilatation (Damber et al., 1987; Kranzfelder et al., 1992) and increases

vascular permeability (Abisogun et al., 1988) which lead to hyperemia, oedema and extravasation of erythrocytes within the theca interna (Parr, 1974; Cavender and Murdoch, 1988; Espey, 1994; Espey and Lipner, 1994). As ovulation approaches, different types of leukocytes infiltrate the theca layers of different species, including neutrophils and eosinophils in sheep (Cavender and Murdoch, 1988), mast cells in rats (Gaytan et al., 1991) and cows (Nakamura et al., 1987), basophils in rabbits (Zachariae et al., 1958), eosinophils in pigs (Standaert et al., 1991), and neutrophils in humans (Brannstrom et al., 1994).

The preovulatory rise in gonadotropins also induces marked steroidogenic changes in the ovarian follicle, as both theca interna and granulosa cells start to luteinize, and the follicle switches from an oestradiol-secreting to a progesterone-secreting structure. The first rate-limiting step in steroid hormone production is the rapid mobilization of the substrate cholesterol from the outer to inner mitochondrial membrane where it is converted into pregnenolone by the cytochrome P450scc enzyme (Miller, 1988). This fast movement of cholesterol depends on a rapidly synthesized and highly labile protein that appears in response to tropic hormone (Stocco and Clark, 1996; Stocco, 1998). This key protein that is involved in the acute regulation of steroid hormone production was purified, its cDNA was cloned in MA-10 mouse Leydig tumor cells (Clark et al., 1994), and it was named steroidogenic acute regulatory protein (StAR).

StAR is synthesized as a 37-kDa preprotein in the cytoplasm of the cell and is imported into the mitochondria, where cleavage of mitochondria targeting sequences occurs, yielding 30-kDa StAR protein (Epstein, 1991; Stocco and Sodeman, 1991).

StAR has also been cloned in human (Sugawara et al., 1995), cattle (Hartung et al., 1995; Pilon et al., 1997), rat (Sandhoff and McLean, 1996; Selvaraj et al., 1996; Lee et al., 1997; Mizutani et al., 1997), hamster (Fleury et al., 1996), and in the pig (Pilon et al., 1997). Only a partial sequence has been isolated in sheep (Juengel et al., 1995). Comparisons of deduced StAR amino acid sequences across species revealed that they are more than 85% identical and more than 90% similar (Stocco and Clark, 1996; Stocco and Clark, 1997; Stocco, 1998). It is now known that StAR is essentially confined to steroidogenic tissues, both during development and in the adult (Clark et al., 1995; Sugawara et al., 1995). The genetic studies of patients with lipoid congenital adrenal hyperplasia, in which the synthesis of all gonadal and adrenal steroid hormone production is severely impaired, reveal mutations in the StAR gene (Lin et al., 1995; Nakae et al., 1997; Saenger, 1997; Miller et al., 1998).

In contrast to other animal species, the morpholgical histology of equine preovulatory follicles and the regulation of the steroidogenic acute regulatory protein have not been characterized in mares. Thus, the general objective of this study was to characterize these two aspects of the final development of equine follicles prior to ovulation.

The specific objectives were to:

1. Characterize the cellular and vascular changes present during the final differentiation of equine preovulatory follicles following the administration of hCG.
2. Clone and determine the primary structure of equine StAR.
3. Characterize the regulation of StAR mRNA in granulosa and theca interna cells of equine preovulatory follicles after the administration of an ovulatory dose of hCG.

2. REVIEW OF LITERATURE

2.1. The equine estrous cycle

2.1.1. Seasonality and length of the cycle

The mare is a seasonally polyestrous breeder. The reproductive season normally extends from April to October with the greatest degree of functional activity occurring in June, July and August (Hughes et al., 1972; Palmer, 1978; Ginther, 1992; Noakes, 1996). The length of the estrous cycle and the duration of the follicular phase are influenced by season, individual variation, breed and follicular states at the onset of estrus (Palmer and Jousset, 1975; Hughes et al., 1980; Alexander and Irvine, 1991; Ginther, 1992).

The length of the equine estrous cycle is defined as the period between two ovulations that coincide with estrus (Hughes et al., 1975; Hughes et al., 1980). The average length of the estrous cycle is about 20-21 days, including a follicular phase (estrus) of 5-6 days during which the mare is sexually receptive to the stallion and ovulation occurs, and a luteal phase of 14-15 days during which the ruptured ovulatory follicle develops into a corpus luteum (CL) that secretes progesterone (Ginther, 1992; Daels and Hughes, 1993; Noakes, 1996). However, the equine estrous cycle in mares is different from that of other large animal species. It has a long period of estrus (5-6 days) and the ovulatory follicle reaches a relatively large size (40-45 mm) (Hughes et al., 1980; McDonald, 1989; Ginther, 1992; Johnson and Becker, 1993). Ovulation occurs at a specific region of the ovary, the ovulation fossa (Whitmore et al., 1973; Geschwind et al., 1975; Noden et al., 1975; McDonald, 1989), and follicular rupture is not triggered by a typical LH surge but by a gradual increase in LH over five to six days (Ginther, 1992; Daels and Hughes, 1993; Noakes, 1996).

2.1.2. Endocrinology of the normal estrous cycle.

Gonadotropin releasing hormone (GnRH) is the master switch of reproductive function in the mare. GnRH is responsible for the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Johnson, 1987; Irvine and Alexander, 1988; Price, 1991; Turner and Irvine, 1991; Irvine and Alexander, 1997). Changes in patterns of the GnRH release have an important role in seasonality and ovulatory cycles in mares (Alexander and Irvine, 1987; Irvine and Alexander, 1988; Alexander and Irvine, 1991).

Early in the breeding season, the mean FSH concentration increases twice during the cycle. The first peak is observed about two days after ovulation, while the second peak occurs in the middle of cycle (Evans and Irvine, 1975; Ginther, 1992; Noakes, 1996). This second increase in FSH secretion is responsible for priming the development of a new group of follicles, of which one will ovulate during following cycle, and for stimulating the follicular estrogen secretion (Evans and Irvine, 1975). Bergfelt and Ginther (1985) proposed that low concentrations of FSH during estrus (Figure 1) are caused by the secretion of inhibin-like proteins by the Graafian follicle.

LH concentrations increase slowly but progressively during estrus, reaching maximal levels two days after ovulation. Then, they decline progressively over the next four to five days and remain low between days 5 and 16 of the cycle (Pattison et al., 1974; Evans and Irvine, 1975; Geschwind et al., 1975; Noden et al., 1975; Oxender et al., 1977; Noakes, 1996) (Figure 1). The physiological process of follicular luteinization in mares is initiated before ovulation (Sirois et al., 1990; Tucker et al., 1991).

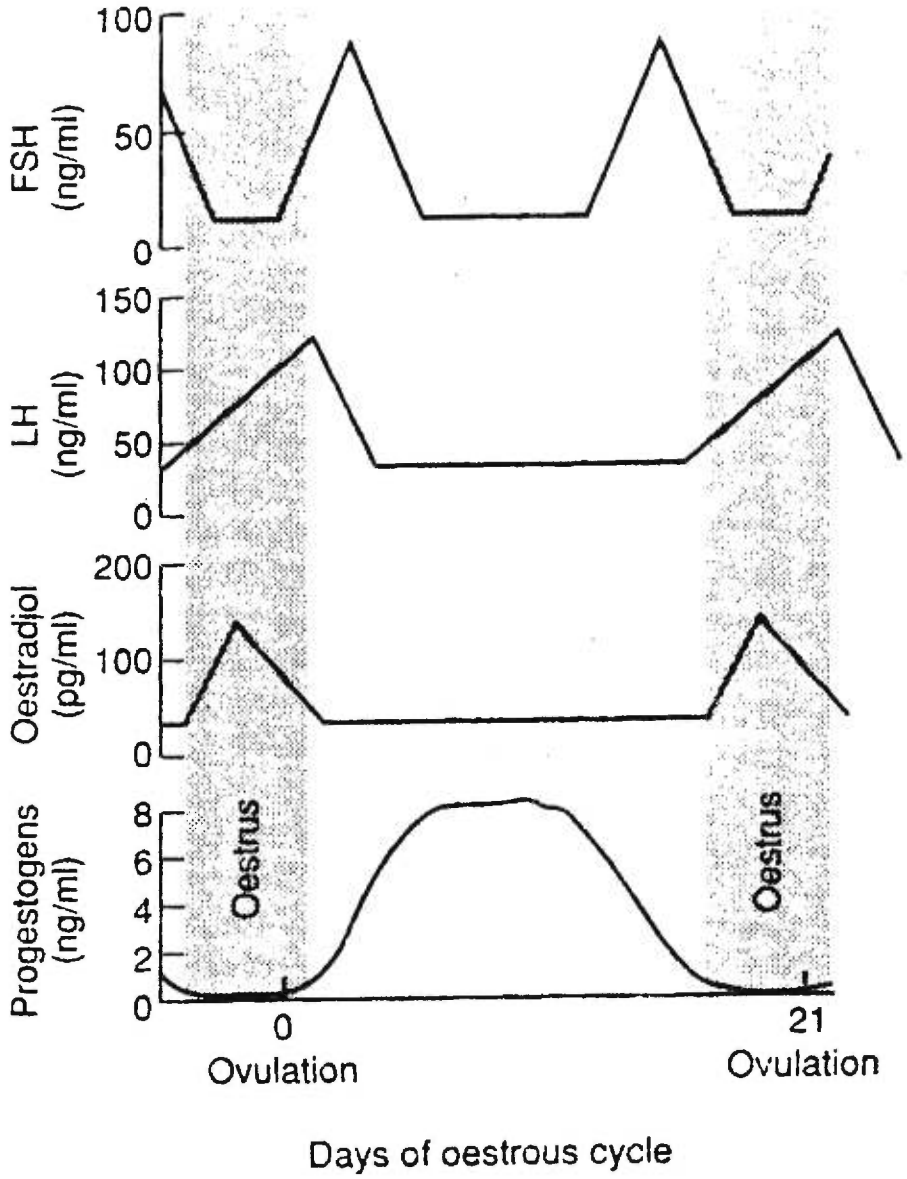

Figure 1. Trends in hormonal concentrations in peripheral circulation of the mare during the estrous cycle. From Arther (1996).

Estradiol secretion begins to rise about six days prior to ovulation, close to the beginning of estrus, and it peaks approximately one to two days before ovulation. Then, estradiol decreases to low diestrus levels within two days (Pattison et al., 1974; Hillman and Loy, 1975; Palmer and Jousset, 1975; Palmer and Terqui, 1977; Daels et al., 1991; Noakes, 1996) (Figure 1).

Circulating levels of progesterone are below 1 ng/ml during the follicular phase. However, they increase rapidly after ovulation, reaching maximal levels within six days. Progesterone remains high throughout the luteal phase before declining rapidly during luteolysis (Stabenfeldt et al., 1972; Plotka et al., 1975; Hughes et al., 1980; Daels and Hughes, 1993; Noakes, 1996) (Figure 1).

2.2. Folliculogenesis

The entry of a primordial follicle into the growth and differentiation processes of folliculogenesis represents an irreversible commitment to one of two fates: atresia or ovulation (Greenwald and Roy, 1994).

2.2.1. Control of follicular development

Follicular growth during the estrous cycle of the mare is a dynamic process, and gonadotropins are the primary regulators of follicular growth (Pierson, 1993). During late diestrus, the growth of large follicles is attributed to the presence of a high concentration of circulating FSH (Irvine, 1981; Ginther, 1992). Alexander and Irvine (1987) showed that pusatile secretion of GnRH results in the concomitant pulsatile release of FSH and LH; 91% of the GnRH pulses produced gonadotropin pulses.

During early follicular development in mammals, preantral follicles acquire LH receptors in theca interna cells and FSH receptors in granulosa cells (Hansel and Convey,

1983; Pierson, 1993; Gore-Langton and Armstrong, 1994). Theca cells produce androgens under the influence of LH, and androgens then diffuse across the basal lamina into granulosa cells where they are aromatized into estrogens under the influence of FSH. Estrogens are responsible for inducing LH receptors in granulosa cells, and prepare the follicle to respond to preovulatory increases in gonadotropin secretion that cause the final phase of follicular maturation (Pierson, 1993; Richards, 1994).

Presumptive ovulatory follicles obtained from mares during the first or the fourth day of estrus were found to be larger in size, to contain more proteins in the granulosa cell component, higher follicular fluid estradiol concentration, and higher granulosa cell LH/ hCG receptor content than follicles obtained during the luteal phase on day 14 of cycle (Fay and Douglas, 1987).

2.2.2. Equine follicular development

Mares have one or two major follicular waves per cycle. Each follicular wave consists of three consecutive phases. The first is a recruitment phase, during which a group of follicles (cohort) enters a period of growth; the second is a selection phase during which only one (in some cases two) follicle is selected to continue its growth; and the third is a dominance phase during which the selected follicle is morphologically and functionally dominant (Sirois et al., 1989; Ginther, 1992; Fortune, 1994). A major follicular wave forming during early diestrus and giving rise to a dominant anovulatory follicle or a dominant ovulatory follicle (diestrus or secondary ovulation) is defined as a secondary wave. A major wave beginning during the mid-diestrus and giving origin to the ovulatory associated with estrus (primary ovulation) is defined as a primary wave (Ginther, 1992) (Figure 2). The diameter of the preovulatory follicle is significantly

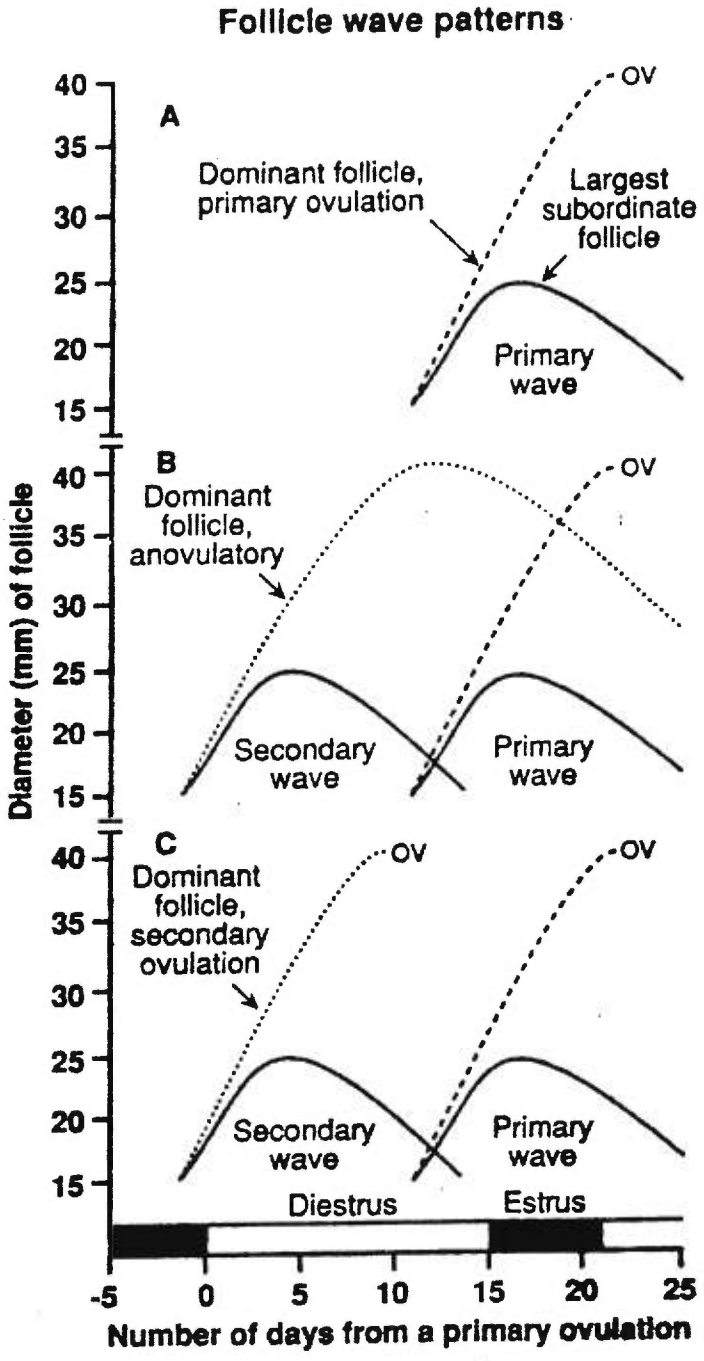

Figure 2. Patterns of follicular waves during the equine estrous cycle. From Ginther (1992).

larger in the adult mare than in the young mare (4.1 mm vs. 36.8 mm respectively), and it is larger in summer than in spring (Hohenhaus and Bostedt, 1992).

2.2.3. Selection and ovulation of the dominant follicle

The recruitment of the preovulatory follicle occurs 10 to 12 days before ovulation (Driancourt and Palmer, 1984). A follicle of about 25 mm is usually present at the time of regression of the corpus luteum, and this follicle is usually the dominant follicle that will ovulate within six to seven days later (Pierson and Ginther, 1987).

Based on the increase in the number of LH receptors in granulosa cells and in estradiol concentrations in the follicular fluid, the presumptive preovulatory follicle has already been selected by day 14 of the cycle (Fay and Douglas, 1987). Richards (1994) suggested that increases in FSH and LH are required to select mammalian follicles to grow beyond the small antral stage and to enter the preovulatory stage. Pierson and Ginther (1990) reported that the selection of the equine ovulatory follicle occurred when the diameter of the largest follicle reached 25 - 30 mm. Studies using transrectal palpation and ultrasonographic examinations of equine ovaries have indicated that the selection becomes an identifiable phenomenon six to seven days before ovulation, approximately when the dominant follicle is identifiable by its largest size, and when subordinate follicles cease to grow (Sirois et al., 1989; Ginther, 1992; Fortune, 1994).

Ovulation marks the culmination of a series of events initiated by the surge of luteinizing hormone (LH) and characterized by resumption of meiosis, initiation of luteinization of the granulosa cells, and rupture of the follicle to release a fertilizable ovum (Espey and Lipner, 1994). Ovulation of the equine follicle is a rapid process, with the majority of the follicular fluid disappearing in less than 2 min (Carnevales et al.,

1988; Townson and Ginther, 1989). Ovulation generally occurs within 24h to 48h before the end of estrus (Daels et al., 1991; Ginther, 1992; Daels and Hughes, 1993; Noakes, 1996). In one study, Hughes et al. (1972) found that 78% of mares ovulated within 24h before the end of estrus, 12% ovulated more than 48h before the end of estrus, and 10% ovulated after the end of estrus.

2.3.1. Morphological changes during the ovulatory process

2.3.1.1. General morphology of the preovulatory follicle

The outer wall of the preovulatory follicle is composed of five layers. The most superficial layer is the surface germinal epithelium, which is formed by a single layer of cuboidal epithelial cells. These cells are characterized by large polymorphic nuclei and by the presence of a number of dense granules in their cytoplasm (Murdoch and Cavender, 1987; Espey, 1994; Espey and Lipner, 1994).

The second layer is the tunica albuginea. This layer consists of fibroblasts and collagen that form a tenacious sheath around the entire ovary, and usually consists of about five to seven cell layers. The extracellular collagen fibers, which these cells produce, are distributed as a matrix around the entire ovary (Murdoch and Cavender, 1987; Espey, 1994; Espey and Lipner, 1994).

The third layer is the theca externa. This layer is composed of several layers (5-7 cells) of fusiform cells and an extensive capillary network, and is the outermost coat of the preovulatory follicle. The fusiform cells are of two types, the first is designated as stromal or fibroblast cells, while the second is ultrastructurally similar to smooth muscle (Murdoch and Cavender, 1987; Espey, 1994; Espey and Lipner, 1994).

The fourth layer, the theca interna, is located just inside the theca externa. This is a thin layer of steroid-secreting cells characterized by a cytoplasm containing numerous mitochondria and lipid droplets. This metabolically active layer receives most of its capillary supply of the follicle (Murdoch and Cavender, 1987; Espey, 1994; Espey and Lipner, 1994).

The fifth layer, the stratum granulosa, is avascular and contains 5-7 cell layers except for the cumulus oophorus which supports the oocyte. The outermost ring of granulosa cells is separated from theca interna and its vascular supply by the basal lamina (Murdoch and Cavender, 1987; Espey, 1994; Espey and Lipner, 1994). Gap junctional complexes provide a means by which granulosa cells can communicate with each other and with the oocyte (Gilula et al., 1978).

2.3.1.2. Changes in the follicle associated with ovulation

2.3.1.2.1. Morphological changes

Between the preovulatory gonadotropin surge and ovulation, mammalian follicular cells begin to luteinize (Espey, 1967; Parr, 1974; Cavender and Murdoch, 1988; Espey, 1994; Espey and Lipner, 1994). The fibroblast of the tunica albuginea and the theca externa change from a quiescent, resting stage to an active proliferating state. These cells become more elongated, and cytoplasmic processes extend far from the central mass. Also, fibroblasts begin to dissociate from one another; the tunica albuginea and theca externa at the apex of the follicle become loose and less tenacious. The theca interna remains relatively unchanged, most of the large capillaries in this layer continue to be patent and flowing. The granulosa cells begin to exhibit lipid droplets in their cytoplasm during the final hours before rupture (Espey, 1994; Espey and Lipner, 1994).

There is an increase in follicular diameter, the follicle wall becomes slightly folded (Priedkalns et al., 1968; McClellan et al., 1975). There is a decrease in number of gap junctions among granulosa cells, and the cumulus oocyte complex becomes free-floating (Murdoch and Cavender, 1987; LeMaire, 1989). During the final minutes before actual rupture, there are marked changes at the apex of the follicle wall (Espey, 1967; Espey, 1994; Espey and Lipner, 1994). The fibroblasts become much more dissociated, and the apex balloons out to form the stigma, the site where the rupture normally occurs (Espey, 1994; Espey and Lipner, 1994).

The histological changes associated with the ovulation process have been studied in many mammalian species, including in rabbits (Espey, 1967; Bjersing and Cajander, 1974; Cherney et al., 1975; Motta and Van Blerkom, 1975), sheep (Cavender and Murdoch, 1988), rats (Parr, 1974; McClellan et al., 1975), mice (Byskov, 1969; Talbot et al., 1987), pigs (Espey, 1967), cows (Priedkalns and Weber, 1968; Priedkalns et al., 1968), bat (Mori and Uchida, 1981), hamsters (Pendergrass and Reber, 1980; Martin and Talbot, 1987; Talbot et al., 1987) and humans (Okamura et al., 1980). In contrast to all these species, the histological changes associated with the ovulatory process have not been characterized in mares (Ginther, 1992), although the histological morphology of the corpus luteum has been well-studied (Harrison, 1946; Van Niekerk et al., 1975; Levine et al., 1979). In general, a few days before ovulation in the mare, the fibroblast cells in the theca interna proliferate and enlarge into oval or round cells with light-staining nuclei. The granulosa cells then cease dividing (Van Niekerk et al., 1975; Ginther, 1992). Just before ovulation, the theca cells are in various stages of degeneration. The granulosa cells change from a compact mass to a lacelike layer of spindle-shaped cells. These cells

secret a mucoid substance that lines the antrum (Van Niekerk et al., 1975; Ginther, 1992).

2.3.1.2.1. Vascular changes

Important vascular changes occur during the ovulation process, including increases in blood flow, vasodilation and increases in vascular permeability (Damber et al., 1987; Abisogun et al., 1988; Kranzfelder et al., 1992). As a result of increase in the permeability of ovarian blood vessels, hyperemia, edema and extravasation of erythrocytes are observed within the theca interna (Espey, 1967; Espey, 1967; Parr, 1974; Cavender and Murdoch, 1988; Espey, 1994; Espey and Lipner, 1994).

In the ewe, the blood supply to the follicular wall was found to increase after the LH surge, but to decrease during the last 10 to 12 h before ovulation (Murdoch and Myers, 1983; Murdoch et al., 1983). Also, cross-sectional areas of vascular lumens increased after the preovulatory LH surge, decreased before ovulation, and then increased again as corpora lutea formed. The follicular stigma that develops near the time of ovulation was completely devoid of blood vessels (Cavender and Murdoch, 1988).

In the rat, the blood flow to the follicle in gonadotropin-primed immature animals did not increase during the ovulatory process (Damber et al., 1987). However, an increase in the capillary area of preovulatory follicles was found after hCG stimulation (Kranzfelder et al., 1992).

In the rabbit preovulatory follicle, the ovarian blood flow increased rapidly within 1 h following hCG, remained high between 2 to 5 h post-hCG, and then decreased gradually from 5 to 8 h after hCG (Makinoda et al., 1988). Using carbon particles as a tracer to examine perifollicular capillaries, reveals capillary engorgement between 9 and

12 h after hCG, and edema in the pericapillary space between 10 and 12 h post-hCG (Okuda et al., 1980). Also, slight carbon leakage was observed in the inner vascular ring of the theca interna of antral follicles, but blood vessels in the other ovarian compartments were unstained. Between 4 and 10.5 h after hCG treatment or mating, vascular leakage was marked in the blood vessels of the interstitial gland and in the theca interna of antral follicles. Just before ovulation, carbon particles were observed between granulosa cells and some carbon had seeped into the follicular fluid of preruptured follicles (Gerdes et al., 1992).

During the ovulatory process in humans, there are prominent changes in the regional follicular blood flow with a marked increase at the base of the follicle, and a concomitant decrease at the apex. These changes may be essential for the release of a mature oocyte (Brannstrom et al., 1998).

Mammalian follicular hyperemia is likely to be initiated by histamine (Hunter and Leathem, 1968; Lipner, 1971; Piacsek et al., 1971; Morikawa et al., 1981), and propagated by prostaglandin E2 (Espey, 1980; Murdoch and Cavender, 1987; Espey, 1994). Each of these vasoactive factors causes contraction of the endothelium and relaxation of smooth muscle cells resulting in vasodilatation and increased capillary permeability (Espey, 1980; Kaley et al., 1985). LH stimulates cyclooxygenase activity, which lead to synthesis of prostaglandins from arachidonate (Espey, 1980; Murdoch and Cavender, 1987; LeMaire, 1989). Follicular prostaglandin synthesis is dramatically increased in the hours proceeding ovulation in several species, and prostaglandin synthesis inhibitors have been shown to block ovulation (Armstrong, 1981).

Because of increased blood flow and vascular permeability, fluid crosses the endothelium, enters intercellular spaces, which leads to edema in the theca interna and theca externa. Although follicular edema does not have any direct role in the ovulation process, it may help the luteinization process by facilitating the access of LH to unvascularized granulosa cells (Espey, 1994). Also, treatment with antihistamine does not inhibit ovulation, but prevents follicular hyperemia and edema (Halterman and Murdoch, 1986).

2.3.1.2.3. Cellular changes

Mammalian ovulation shares several similarities with a local inflammatory reaction, as it involves the participation of leukocytes and inflammatory mediators. In response to a preovulatory LH surge, there is an influx of leukocytes into the preovulatory follicle, and an uncharacterized chemotactic attraction for these cells has previously been reported to be present in follicular fluid of several species (Herriot et al., 1986; Seow et al., 1988; Watson et al., 1991; Brannstrom and Norman, 1993; Arici et al., 1996; Runesson et al., 1996). In sheep, neutrophils and eosinophils migrate from the vascular compartment before ovulation. Around the time of ovulation, there are masses of extravasated blood in thecal tissues, and numerous platelets adhere to the damaged vascular endothelium. Extravasated monocytes/macrophages are evident after ovulation. The number of extravascular lymphocytes remain relatively constant, but the lymphocytes are often localised along the endothelium in ovulatory and postovulatory follicles. Basophilic cells accumulate in association with the development of new capillaries during luteinization (Cavender and Murdoch, 1988).

In the rat, neutrophils and macrophages are the major leukocyte populations present in the follicular wall of the preovulatory follicles. There is a selective increase in the numbers of macrophages and neutrophilic granulocytes in the medullary region and in the thecal layer as the ovulatory period progresses (Brannstrom et al., 1993). Mast cells are also were found in rat follicles, but are limited to the ovarian hilum and are not observed in follicles, corpora lutea and interstitium (Jones et al., 1980); this contrasts with larger species such as humans, cows and monkeys where mast cells are observed throughout the ovary. (Krishna et al., 1989).

In humans, macrophages and neutrophilic granulocytes are present in high numbers in the follicle wall and are further increased in the theca at ovulation. Macrophages and neutrophils are also abundant in the CL, and an increase in the macrophage density during CL regression could imply a role for these cells in luteolysis (Brannstrom et al., 1994).

The pig follicle has been investigated by Standaert et al., (1991). Eosinophils are the most prominent blood cell type present in theca layers of preovulatory follicles and in the regressing CL. In the CL at other stages, few eosinophils are observed. Neutrophil numbers remain moderate and unchanged throughout most of the estrous cycle except in the regressing CL, where the number of neutrophils is slightly increased. Low numbers of plasma cells are observed in all structures. The number of macrophages increases in freshly luteinized follicles, decreases in the developing and mature CL, and then increases again in the regressing CL. However, the relative proportion of macrophages as a percent of total blood leukocytes present does not change throughout the cycle.

In the cow, mast cells are observed in the theca externa of follicles, the external capsule of the corpus luteum, and in the stromal and hilar regions of the ovary (Nakamura et al., 1987). The same authors show that the stromal and hilar regions exhibit the highest number of mast cells compared with the follicular and luteal compartments. A significant number of mast cells is observed in the theca externa of the dominant follicle. After ovulation, the number of mast cells surrounding the corpus luteum is significantly decreased.

In rabbits, an increased number of polymorphonuclear leukocytes migrating into the surrounding interstitial tissue is observed at about 6 h post-hCG (Gerdes et al., 1992). The number of leukocytes seen in the follicular wall and ovarian medulla increases markedly towards ovulation.

The cause and purpose of this dramatic alteration in ovarian leukocyte populations remain unclear and are largely unexplored. Recruitment of leukocytes elsewhere in the body usually occurs under the influence of chemotactic factors from local tissues (Norman and Brannstrom, 1994). Several substances with chemotactic properties have been described in follicular fluid from cattle, sheep and human (Herriot et al., 1986; Seow et al., 1988; Murdoch and McCormick, 1989; Castilla et al., 1990; Murdoch and Steadman, 1991; Sirotkin and Luck, 1995; Runesson et al., 1996). In mares, chemotactic activity was measured in fluid aspirated from follicles of estrous mares 0, 12, 24, and 36 hours after hCG treatment (Watson et al., 1991). In that study equine follicular fluid acted as a chemoattractant for neutrophils, but there was no significant change in chemotactic activity at different times after administration of hCG.

2.3.1.3. Proteolytic degradation of follicular wall during ovulation

Proteolytic enzymes are among the factors involved in the rupture of the mature follicle. Mature ovarian follicles produce proteolytic enzymes which reach maximal levels near the time of ovulation (Reich et al., 1985; Reich et al., 1986; Liu and Hsueh, 1987; Espey, 1994; Espey and Lipner, 1994). The proteolytic enzymes are capable of physically weakening the follicle wall (Beers, 1975).

2.3.1.3.1. Plasminogen activator and plasmin

Plasminogen activator may be involved in the ovulatory process, considering that its level increases in rat preovulatory follicles, and it is able to decrease the tensile strength of graafian follicle wall (Beers, 1975 ; Beers and Strickland, 1978). It was suggested that plasmin, the product of plasminogen activator is action on plasminogen, activates latent collagenase and thereby initiates the proteolytic processes culminating in follicular rupture (Beers and Strickland, 1978).

Two plasminogen activators have been identified in mammalian tissues, the urokinase type and the tissue type. These enzymes are products of different genes (Ny et al., 1984; Suenson et al., 1984). Both plasminogen activators are present in granulosa and theca compartments of the follicle (Reich et al., 1986; Reinthaller et al., 1990). The granulosa cells contribute 80% to 90% of the total follicle plasminogen activator activity. After gonadotropin stimulation, a significant increase in tissue plasminogen activator is observed in both rat (Reich et al., 1986) and human granulosa cells (Reinthaller et al., 1990). However, urokinase plasminogen activator, rather than tissue plasminogen activator, seems to be the gonadotropin stimulated in mice (Canipari et al., 1987; Sappino et al., 1989). Inhibitors of serine proteases, a class which includes plasminogen

activator, prevent ovulation in vivo (Akazawa et al., 1983; Reich et al., 1985) and in vitro (Ichikawa et al., 1983).

2.3.1.3.2. Collagenase

Morphological changes like thinning of the apical wall of the preovulatory follicle and dissociation and fragmentation of thecal collagen fibrils suggest that collagen degrading enzymes play a role in follicular rupture (Woessner et al., 1989; Espey, 1994). Collagenolytic activity has been demonstrated at the time of ovulation in rats (Morales et al., 1983) and rabbits (Kawamura et al., 1981 and Kawamura et al., 1984). Collagenase, synthesized in granulosa cells of preovulatory follicles, is consumed at the follicular apex which results in collagen degradation and disruption of the follicular wall (Fukumoto et al., 1981). Collagenase activity in the ovaries of immature rats primed with PMSG/hCG peaks at 8 h after-hCG and remains high at 12 h, which corresponds to the time of the release of the ova (Curry et al., 1985).

2.3.1.4. Other chemical changes during ovulation

2.3.1.4.1. Steroid hormones

Early during the ovulatory process, relative estrogen synthesis is high and progesterone synthesis is low. As the gonadotropin hormone rises, estrogen synthesis drops dramatically while progesterone synthesis increases several fold (Espey, 1994). A relation between this steroid hormone switch and ovulation has been established by using of antiprogesterone antiserum and antitestesterone antiserum in rats (Mori et al., 1977a, b), humans and sheep (Baird, 1983). Ovulation was blocked in gonadotropin-primed immature rats with antiserum against either progesterone or testosterone, and the effect of the antiprogesterone antiserum was reversed by hCG treatment (Mori et al.,

1977a, b). Also, antiprogesterone antiserum was inefective when administered more than 6 h after hCG treatment (Kohda et al, 1980).

Blockage of 3β-hydroxysteroid dehydrogenase in gonadotropin-primed immature rats by the inhibitor epostane was found to inhibit ovulation in a dose-related fashion. The drug also decreased plasma progesterone levels in these animals. The inhibitory effect of epostane on gonadotropin-stimulated ovulation was reversed by injections of progesterone (Snyder et al., 1984).

2.3.1.4.2. Prostaglandins

Prostaglandin endoperoxide synthase (PGHS) is an enzyme that converts arachidonic acid into prostaglandin H2. Prostaglandin H2 is then further metabolized into biologically active prostaglandins (PGE, and PGF2 alpha) by specific synthases. Two isoforms of PGHS, refer to as PGHS-1 and PGHS-2, have been identified (Herschman, 1996; Williams and DuBois, 1996). The two forms differ in their pattern of expression. PGHS-1 is present in a wide variety of tissues and is referred to as the constitutive form. PGHS-2 is generally undetectable in most tissues, but can be induced by a variety of agonists and is referred to as the inducible form (Herschman, 1996; Williams and DuBois, 1996).

The preovulatory rise in gonadotropin levels stimulates prostaglandin synthesis in the preovulatory follicles of different species (LeMaire et al., 1973; Ainsworth et al., 1975; Armstrong and Zamecnik, 1975; Bauminger et al., 1975; Ainsworth et al., 1984). The marked increase in follicular prostaglandin before ovulation is associated with gonadotropin dependent induction of PGHS enzymes in rat granulosa cells (Hedin et al., 1987; Huslig et al., 1987). High levels of gonadotropins were shown to selectively

induce PGHS-2, but not PGHS-1 in rat preovulatory follicles in vivo and in vitro (Wong and Richards, 1991; Sirois and Richards, 1992; Sirois et al., 1992; Morris and Richards, 1995). The induction in rats is very rapid, within 2-4 h after hCG in vivo (Sirois et al., 1992). In cows, the induction of PGHS was observed at 18 h after hCG (Sirois, 1994; Liu et al., 1997). In mares, PGHS was induced in granulosa cells at 30 h post-hCG (Sirois and Doré, 1997; Boerboom and Sirois, 1998). In all species studied so far, the interval from PGHS induction to ovulation was found to be about 10 h (Sirois et al., 1992; Sirois, 1994; Sirois and Doré, 1997; Boerboom and Sirois, 1998).

The role of prostaglandins in ovulation was confirmed by the blockage of ovulation with indomethacin (Armstrong and Grinwich, 1972; Orczyk and Behrman, 1972; Tsafriri et al., 1972; Wallach et al., 1975) or the intrafollicular injection of antiserum to PGF2 alpha in rabbits (Armstrong et al., 1974). The inhibitory action of indomethacin on ovulation was overcome by the administration of PGE2 in rats (Tsafriri et al., 1972). Also, the obligatory role of PGHS-2 during the ovulation process was highlighted by recent gene targeting studies in mice (Lim et al., 1997).

2.4. Steroidogenesis

The biosynthesis of steroid hormones involves several enzyme systems. Steroid hormones have a common 17-carbon cyclopentanoperhydrophenanthrene structure with four rings A, B, C, and D (Miller, 1988; Granner, 1990; Ginther, 1992; Gore-Langton and Armstrong, 1994; Noakes, 1996) (Figure 3). Steroid biosynthesis involves the progressive loss of carbon atoms from the precursor cholesterol, which is metabolized in a series of hydroxylation reactions catalyzed by three members of a superfamily of genes known collectively as cytochrome P450: P450scc, P45017α, and P450arom (Nebert et

al., 1991). P450 enzymes are important in the metabolism of numerous physiological substrates such as steroids, fatty acids, and prostaglandins (Graham-Lorence and Peterson, 1996). All these enzymes consist of about 500 amino acids and contain a single heme group. Their designation is based on a characteristic absorption from 420 to 450 nm upon reduction with carbon monoxide (Miller, 1988; Graham-Lorence and Peterson, 1996)

The biological action of sex steroid hormones can usually be predicted by their structure. A 21-carbon steroid has progestin activity and is produced by the cleavage of the side chain of cholesterol between C-20 and C-22. A 19-carbon steroid has androgen activity when an additional cleavage occurs between C-17 and C-20. A 18-carbon steroid has estrogen activity when the angular methyl group at C-10 is removed and aromatization of the A ring occurs (Miller, 1988; Granner, 1990; Ginther, 1992; Gore-Langton and Armstrong, 1994; Noakes, 1996) (Figure 4).

2.4.1. Site of follicular steroidogenesis

Granulosa cells and theca interna cells are the two steroidogenic cell types of the follicle. However, interactions between these two cell types are required for estrogen biosynthesis in the preovulatory follicle (Short, 1962; Younglai and Short, 1970; Hay et al., 1975 and Ginther, 1992). Mahajan and Samuels (1974) reported that the production of estrogen in vitro is more in the theca / granulosa cell combination than in each cell alone. Meanwhile, Seamans and Sharp (1982) found that equine granulosa cells secreted more estrogen than theca cells when cultured in the presence of aromatizable androgens. In equine preovulatory follicles, the granulosa cell layer is the only site for estrogen

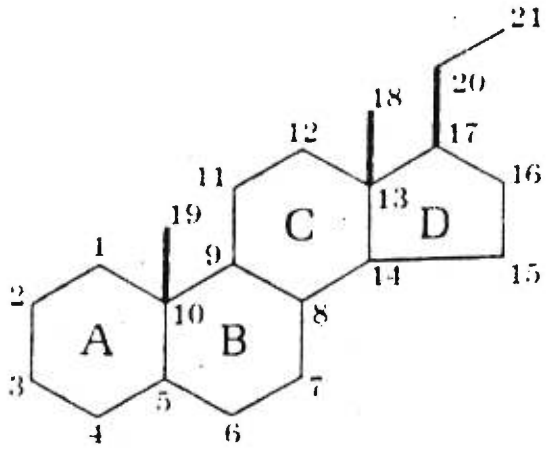

Figure 3. The conventional representation of the steroid ring system, showing the lettering system used to designate the rings and the numbering system used to identify the carbon atoms. From Granner (1990).

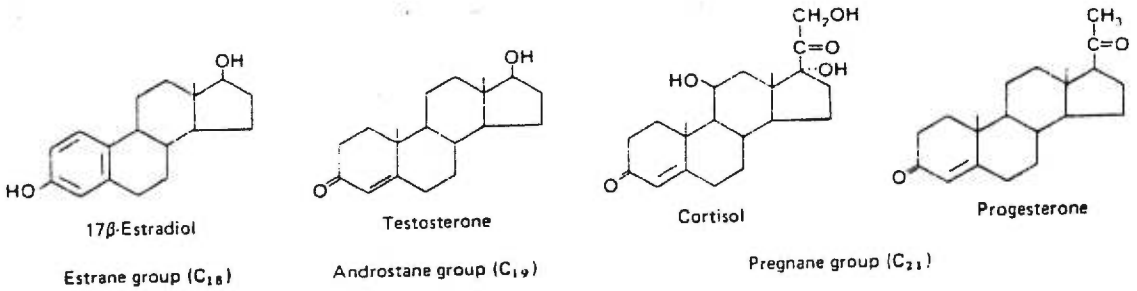

Figure. 4. Basic steroid hormone structures.. From Granner (1990).

production (Sirois et al., 1991; Tucker et al., 1991). The pattern of hormone secretion of equine granulosa cells changes as follicular development proceeds, and these cells becomes primary progesterone secreting cells in late estrus (Sirois et al., 1991; Tucker et al., 1991).

2.4.2. Biosynthesis of steroid hormones

Gonadotropin hormones trigger a rapid increase in gonadal steroid production in the testis and the ovary. This acute response occurs within minutes of hormonal stimulation and involves the mobilization and delivery of cholesterol to the inner mitochondrial membrane (Ferguson, 1963; Carren et al., 1965; Davis and Garren, 1968; Simpson et al., 1972; Privalle et al., 1983; Jefcoate et al., 1986; Jefcoate et al., 1987). Chronic stimulation, on the other hand, requires several hours and results in an increase in the transcription of genes encoding steroidogenic enzymes (Simpson and Waterman, 1988). Both the acute and chronic responses are hormonally controlled via activation of adenylate cyclase, resulting in an increase in intracellular levels of cAMP and activation of the protein kinase A (PKA) pathway (Simpson and Waterman, 1988; Hajnes et al., 1959, Sala et al., 1979).

Steroid hormones are derived from cholesterol that can be obtained from three sources: (1) cholesterol synthesized de novo from two carbon units (acetyl coenzyme A); (2) cholesterol liberated from cholesterol esters stored within lipid droplets; and (3) preformed cholesterol circulating in blood in the form of high-density lipoproteins (HDL) and low-density lipoproteins (LDL). LDL cholesterol esters are taken up by receptor-mediated endocytosis, while HDL cholesterol is taken up by a distinct mechanism (Miller, 1988; Murphy and Silavin, 1989).

Although there are two potential pathways for steroid hormone biosynthesis in the equine follicle, the Δ^4 and the Δ^5 pathway, the Δ^4 pathway is considered to be quantitatively the most important (Short, 1962 and Younglai and Short, 1970; Pierson, 1993) (Figure 5). Cholesterol is first transported from the outer to the inner mitochondrial membrane by the action of protein named the steroidogenic acute regulatory protein (StAR) (Clark et al., 1994; Stocco and Clark, 1996; Stocco, 1998) (Figure 6).

The conversion of cholesterol to pregnenolone is the first rate-limiting and hormonally regulated step in steroidogenesis (Miller, 1988; Gore-Langton and Armstrong, 1994; Richards, 1994) (Figure 6). The conversion of cholesterol to pregnenolone occurs in the mitochondria and involves three distinct chemical reactions, cholesterol 22- hydroxlase, cholesterol 20α-hydroxylase, and cholesterol side-chain cleavage at the bond between C-20 and C-22 (C-20 / C-22 lyase) (Lieberman et al., 1984; Miller, 1988; Gore-Langton and Armstrong, 1994).

Pregnenolone diffuses to the smooth endoplasmic reticulum, where it is dehydrogenated to form progesterone in a reaction catalyzed by 3β-hydroxysteroid dehydrogenase / Δ^5 - Δ^4 isomerase (3βHSD) (Niswender et al., 1974; Miller, 1988; Ginther, 1992; Gore-Langton and Armstrong, 1994; Noakes, 1996) (Figures 5 and 6).

Both pregnenolone and progesterone may undergo a 17α-hydroxylation to form 17α-hydroxypregnenolone and 17 α -hydroxyprogesterone, respectively. These latter 17 hydroxylated steroids may then undergo scission between C-17 and C-20 to yield dehydroepiandrosterone (DHEA) and androstenedione, respectively (Figure 5 and 6).

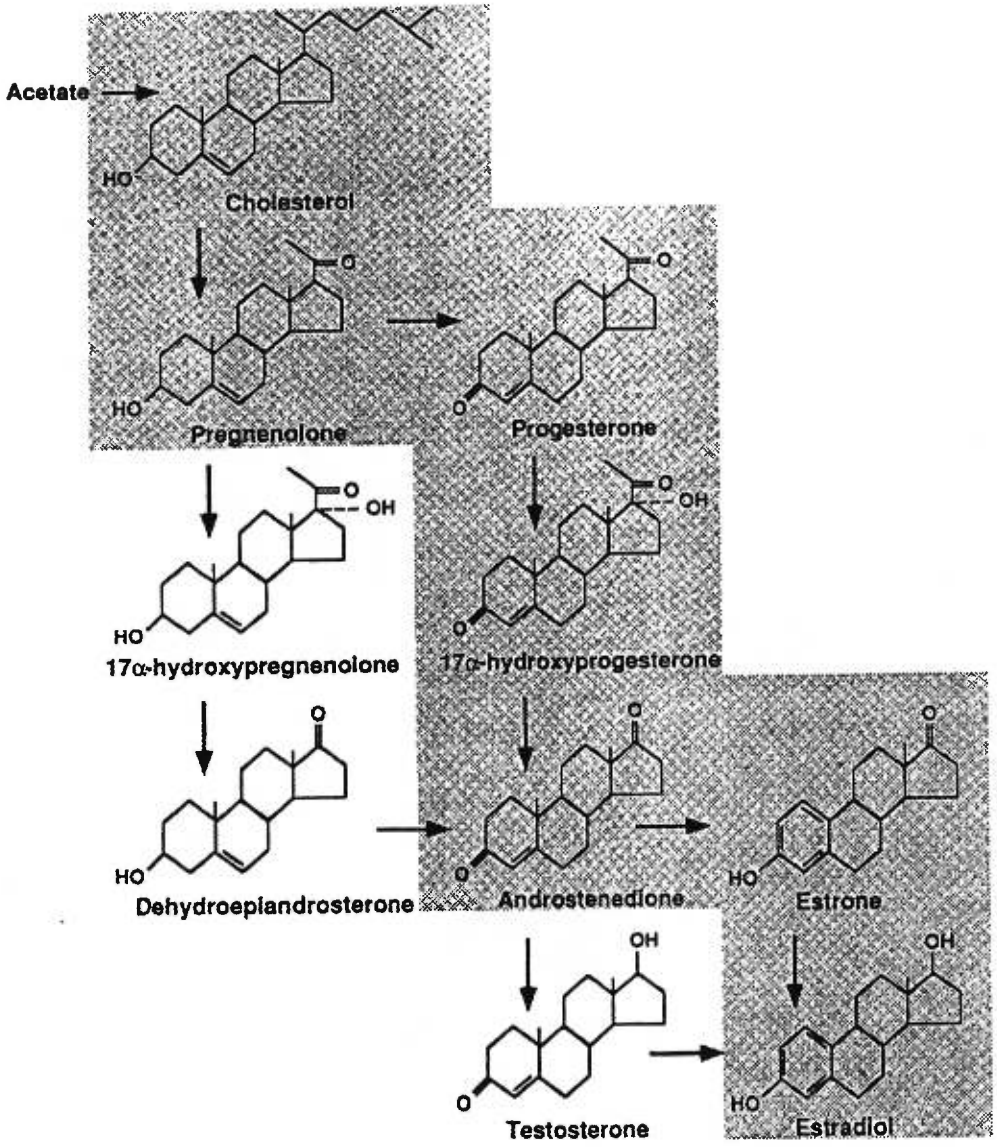

Figure. 5. Pathways for the biosynthesis of estrogens in equids. Both the Δ^4 and Δ^5 pathways are depicted and may be used for synthesis of estrogens. The preferred pathway for biosynthesis of estrogens in the testis and in the follicle is the Δ^4 pathway (shaded). From Pierson (1993)

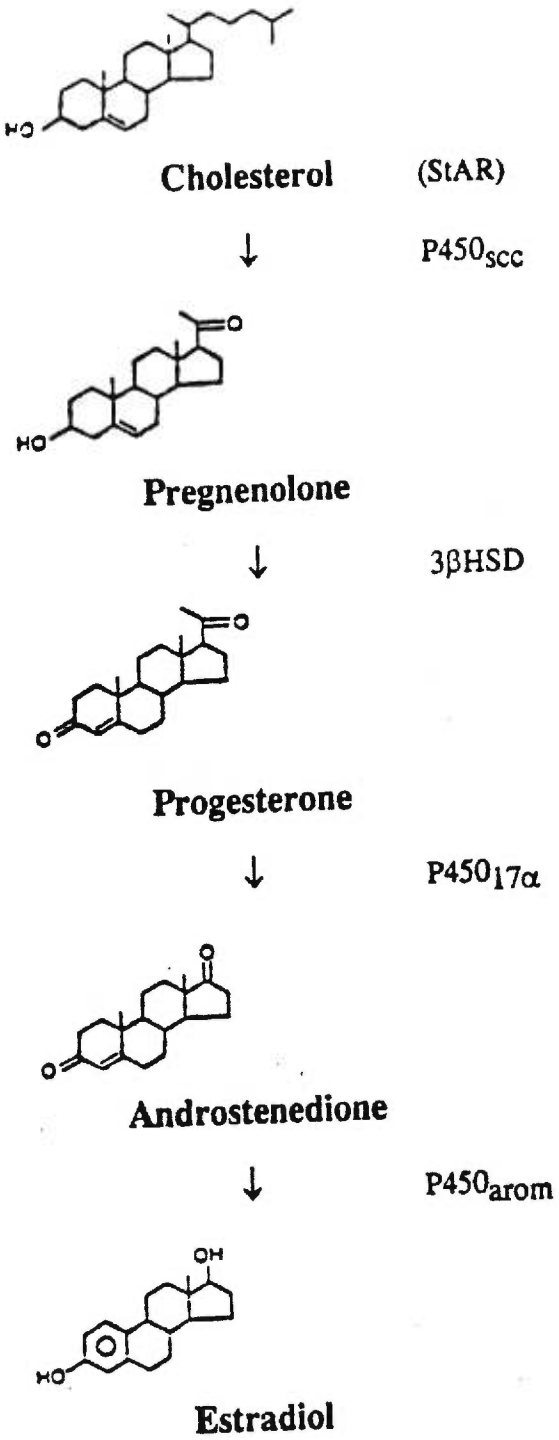

Figure 6. Simplified version of the biosynthesis of steroid hormones

It is well demonstrated that all four of these reactions are mediated by a single enzyme, P45017 α-hydroxylase (Kominami et al., 1980; Miller, 1988).

Estrogen hormones are formed from androgens by elimination of the C-19 methyl group and aromatization of the A ring (Miller, 1988; Ginther, 1992; Gore-Langton and Armstrong, 1994; Simpson et al., 1994; Noakes, 1996). The C-19 androgen hormones are converted to C-18 estrogens is done by the aromatase cytochrome P450 enzyme (P450 arom) (Figure 5 and 6). This enzyme complex is located in the membrane of the agranular endoplasmic reticulium of several cell types (Lieberman et al., 1984; Miller, 1988; Gore-Langton and Armstrong, 1994; Richards, 1994; Simpson et al., 1994). The P450 arom enzyme catalyzes a multistep reaction. The initial steps in the aromatization reaction involve two sequential hydroxylations at C-19, yielding 19-hydroxyandrostenedione and 19-dihydroxyandrostenedione. The mechanism of the third oxidation is still undefined (Miller, 1988; Korzekwa et al., 1993; Gore-Langton and Armstrong, 1994).

2.4.3. Steroidogenic acute regulatory protein (StAR)

The rapid increase in steroid hormone production by steroidogenic cells in response to hormone stimulation is well documented (Carren et al., 1965; Cooke et al., 1975; Medelson et al., 1975). In order to initiate and sustain steroidogenesis, it is necessary to provide constant supply of cholesterol to the P450 scc enzyme. Although the rate-limiting enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone by P450 scc (Stone and Hechter, 1959; Karaboyas and Koritz, 1965; and Carren et al., 1971), the true rate-limiting step in this process is the delivery of the substrate to the inner mitochondrial membrane (Crivello and Jefcoate, 1980; Privalle et

al., 1983; Jefcoate et al., 1987). The major barrier to overcome in the delivery of cholesterol to the P450scc is the aqueous space between the outer and inner mitochondrial membranes. Since the aqueous diffusion of cholesterol is extremely slow (Phillips et al., 1987), and can not provide sufficient substrate to account for the rapid and large increase in steroid production observed in steroidogenic cells, it follows that the successful stimulation of steroidogenesis would require the activation of a mechanism that rapidly transports cholesterol across this barrier (Stocco and Clark, 1996) (Figure 7). In a number of early studies, it was observed that the inhibition of protein synthesis blocked hormonally induced steroid production (Ferguson, 1962; Ferguson, 1963; Carren et al., 1965; Davis and Garren, 1968; Privalle et al., 1983). The precise location of this inhibition was pinpointed to the transport of cholesterol from the outer to the inner mitochondrial membrane (Ohno et al., 1983; Privalle et al., 1983). Thus, a hormonally regulated, newly synthesized protein was proposed to function in the transfer of cholesterol from the outer to the inner mitochondrial membrane (Stocco and Clark, 1996; Arakane et al., 1997; Stocco, 1997; Sugawara, 1997; Stocco, 1998).

A 30-kDa phosphoprotein was firstly described in hormone treated rats and in mouse adrenocortical cells (Krueger and Orme-Johnson, 1983; Pon et al., 1986; Pon and Orme-Johnson, 1988). Also, a series of mitochondrial proteins of 37, 32, and 30 kDa have been described in mouse MA-10 Leydig tumor cells. They are synthesized in response to LH, hCG, and to stimulation with the cAMP analog dibutyryl-cAMP (Stocco and Kilgore, 1988; Stocco and Chen, 1991; Stocco and Sodeman, 1991; Stocco, 1992; Stocco and Ascoli, 1993). These proteins are derived from a single protein named Steroidogenic Acute Regulatory Protein (StAR) (Clark et al., 1994).

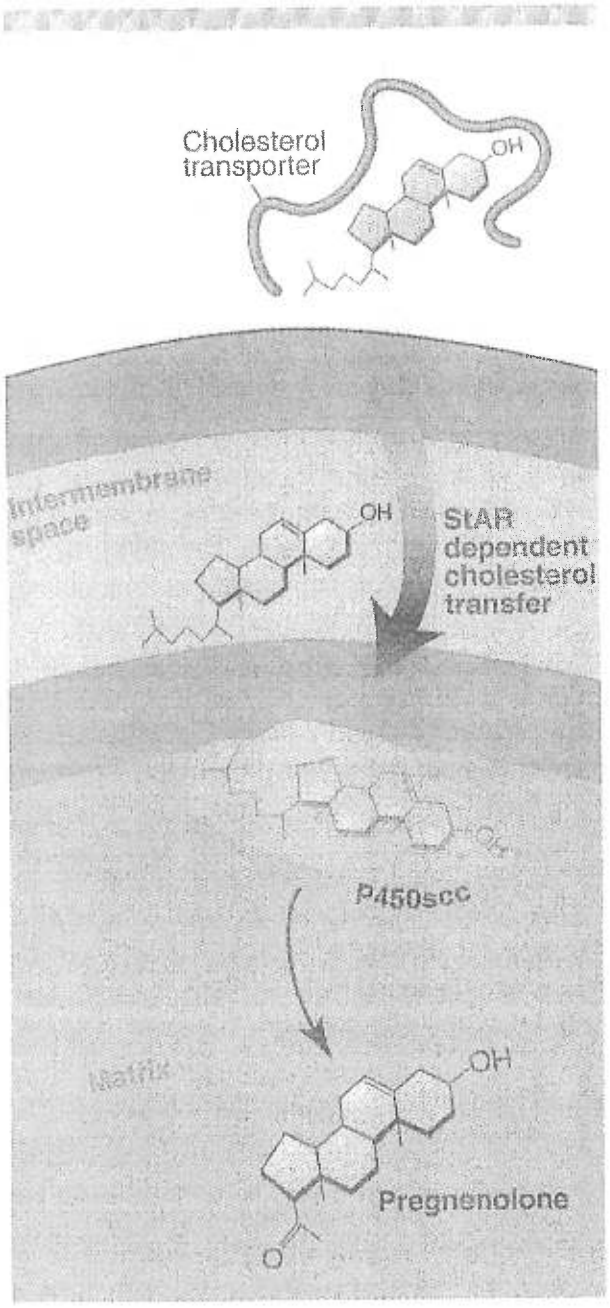

Figure 7. Putative site of StAR action. From Waterman (1995).

The mouse Leydig tumor cell line MA10 proved useful for purification and microsequencing of peptides derived from these proteins. This information in turn allowed the design of degenerate nucleotide primers and the PCR amplification of partial cDNA sequences for the mouse StAR gene (Clark et al., 1994). These sequences were used to screen a mouse Leydig tumor cell cDNA library, and a full-length StAR cDNA sequence of 1456 bp was isolated. It was composed of an open reading frame (ORF) of 852 nucleotides that encoded a protein of 284 amino acids, with a calculated molecular mass of 31.6 KDa. In the human, a full-length StAR cDNA has been cloned as an orphan sequence identified via chimeric association with human P220 translation initiation factor (Sugawara et al., 1995). In cattle, the full-length cDNA was cloned as an orphan sequence identified via differential cDNA expression studies of corpus luteum cells taken early and late during the bovine estrous cycle (Hartung et al., 1995). Also, full-length StAR cDNAs were cloned in the rat (Sandhoff and McLean, 1996; Selvaraj et al., 1996; Lee et al., 1997; Mizutani et al., 1997) and the pig (Pilon et al., 1997). In addition, a partial cDNA has been isolated in sheep (Juengel et al., 1995). Deduced amino acid sequences of all cloned cDNAs are highly similar, with 85-88% identity and greater than 90% similarity (Stocco and Clark, 1996; Stocco, 1997; Stocco, 1998).

2.4.3.1. StAR expression

In all species studied so far, StAR was found to be expressed in steroidogenic tissues exhibiting very high steroid production (ovaries, adrenal gland, and testis) (Stocco and Clark, 1996; Stocco, 1997; Stocco, 1998). However, species differences have been observed in StAR expression. StAR mRNA has been detected in the placenta of cows (Pescador et al., 1996; Pilon et al., 1997) and pigs (Pilon et al., 1997), but not in mice

(Clark and Stocco, 1995), rats (Sandhoff and McLean, 1996a) and humans (Sugawara et al., 1995). Also, StAR transcripts have been observed in kidney of human (Sugawara et al., 1995), but not in all other species studied so far. Elevated levels of StAR transcripts have been reported in theca interna of large follicles in rats (Ronen-Fuhrmann et al., 1998; Sandhoff and McLean, 1996a), cows (Soumano and Price, 1997) and humans (Pollack et al., 1997, Kiriakidou et al., 1996). No StAR mRNA was observed in granulosa cells before gonadotropin treatment in rats (Ronen-Fuhrmann et al., 1998; Sandhoff and McLean, 1996a), cows (Soumano and Price, 1997) and humans (Kiriakidou et al., 1996). However, detection of the transcript was observed after gonadotropin treatment in vivo (Ronen-Fuhrmann et al., 1998; Sandhoff and McLean, 1996a; Soumano and Price, 1997) and after stimulation with agonists of the PKA pathway in vitro (Kiriakidou et al., 1996; Selvaraj et al., 1996, Pescador et al., 1996, Ronen-Fuhrmann et al., 1998, Pescador et al., 1997, Balasubramanian et al., 1997, Thompson et al., 1997).

Differences in the number and size of StAR transcripts have been observed among species. Four StAR mRNAs of 0.65, 1.7, 3.1 and 5.25 kb have been observed in hamsters (Fleury et al., 1996). Three sizes of StAR mRNAs have been detected in the mouse, including two major bands of 3.4 and 1.6 kb, and a minor and inconsistent band of 2.7 kb (Clark and Stocco, 1995). In humans, one major transcript has 1.6 kb and two minor transcripts have 4.4 and 7.5 kb (Gradi et al., 1995; Sugawara et al., 1995). In rats, two major bands at 3.4 and 1.6 kb and a less abundant transcript of 1.2 kb have been reported (Sandhoff and McLean, 1996a). Two StAR mRNAs are reported in cows, a strong band of 3.0 kb and weaker one of 1.8 kb (Hartung et al., 1995; Pescador et al., 1996). Several StAR mRNAs have been recorded in pigs, a major hybridizing band of

1.8 kb and a fainter band of about 1.1 kb in one study (Pilon et al., 1997), and a major band of 2.8 kb and two weaker bands of 1.6 and 0.8 kb in another study (Pescador et al., 1997). However, only one band of 2.8 kb was observed in sheep (Juengel et al., 1995).

Several studies have supported the putative role of StAR in steroidogenesis. In sheep, the removal of the pituitary caused a decrease in StAR mRNA and in progesterone secretion from the corpus luteum (Juengel et al., 1995). Treatment of these hypophysectomized animals with LH or growth hormone lead to increase of StAR mRNA to levels similar to those of sheep with an intact pituitary (Juengel et al., 1995). Injection of PGF2 alpha lead to a decrease in peripheral progesterone concentrations and StAR mRNA within 4 h post-treatment (Juengel et al., 1995). Also, concentrations of progesterone and levels StAR mRNA were found to be higher in pregnant than non-pregnant sheep 15 days post-estrus (Juengel et al., 1995).

In humans, StAR mRNA is induced in granulosa cells by cAMP, and the effect of cAMP are antagonized by activators of protein kinase C (Kiriakidou et al., 1996). Moreover, in vitro studies, hCG increased StAR transcripts and progesterone production by human luteal cells. Whereas, PGF2 alpha treatment significantly decreased both StAR mRNA and media progesterone levels (Chung et al., 1998).

In cattle, the synthesis of StAR mRNA and protein are tightly coupled in the corpus luteum. Levels of StAR mRNA were low during the early luteal phase, increased 9 to 15 fold during the mid to late luteal phase, and disappeared in regressed CL. Corpora lutea from intact animals treated with PGF2 alpha displayed a 50% decline in StAR mRNA over 12h, and no StAR mRNA was detected after 24h post-treatment (Pescador et al., 1996). Soumano and Price (1997) compared follicular levels of StAR mRNA in animals superovulated with two different gonadotropin treatments. The

study concluded that stimulation with a gonadotropin preparation rich in LH activity (eCG) significantly increases the accumulation of StAR mRNA in large and medium follicles, in comparison to stimulation with a gonadotropin preparation low in LH activity (FSH).

In rats, the hormonal regulation of ovarian StAR mRNA was examined in animals treated with eCG to induce follicular development, and hCG to induce ovulation. Result showed that eCG increased StAR mRNA two fold, and serum levels of progesterone were increased by 84%. StAR mRNA was further increased eight to nine fold after 3 h post-hCG (Sandhoff and McLean, 1996a). Time course experiments following PGF2 alpha administration showed a significant decrease in StAR expression as early as 30 min following injection (Sandhoff and McClean, 1996b). In another study, treatment of immature rats with eCG and hCG caused a biphasic regulation of StAR in the follicle. The first peak of StAR was generated by eCG and lasted 24 h. The second peak was observed in response to hCG stimulation (Ronen-Fuhrmann et al., 1998). Also, StAR mRNA was found to be expressed in rat immortalized granulosa cell lines, and was upregulated by FSH, hCG, isoproterenol and foskolin (Selvaraj et al., 1996).

In rabbits, StAR mRNA levels were stable and levels of progesterone were high in rabbits treated continuously with exogenous estradiol during the first 13 days of pseudopregnancy. In contrast, StAR mRNA and progesterone concentrations dropped when animals were deprived of estradiol, and replacement of estradiol deprivation stimulated the reappearance of StAR and progesterone secretion (Townson et al., 1996).

Studies in hamsters showed that the administration of ACTH hormone provoked an increase in the level of three of the StAR mRNAs one hour after stimulation.

However, levels of the 0.65 kb mRNA were not changed after ACTH treatment, suggesting that the regulation of StAR in hamster adrenals could be under the control of different genes, promoters, or polyadenylation signals (Fleury et al., 1996).

In pigs, treatment of serum-free cultures of granulosa cells with FSH or insulin-like growth factor I (IGF-I) alone had a small but consistent stimulatory effect on StAR mRNA and protein accumulation. However, the combined effect of FSH and IGF-I was strongly synergistic (Balasubramanian et al., 1997). In that study, the protein kinase A agonist, 8-bromo-cAMP alone elicited a 3.5-fold increase in StAR message by 48 h of culture, whereas the combination of IGF-I and FSH or 8-bromo-cAMP evoked a 26- to 40-fold synergistic rise in StAR message accumulation. In luteinized porcine granulosa cells, FSH elevated StAR message in a dose-dependent manner, and actinomycin D eliminated constitutive StAR message (Pescador et al., 1997). These authors showed that pretreatment with cycloheximide prevented FSH induction of StAR, and dibutyryl cAMP caused time-dependent increases in StAR, indicating the importance of the protein kinase A pathway in StAR gene expression. However, activation of the protein kinase C pathway by a phorbol ester eliminated FSH induction of StAR mRNA.

2.4.3.3. Mechanism of StAR action

Stocco and Clark (1996) proposed that as a result of trophic hormone stimulation, the StAR 37-kDa precursor protein was rapidly synthesized in the cytosol and was quickly targeted to the mitochondria via its signal sequence. The nature of this targeting is not known. However, as the precursor protein begins entering into the mitochondrial inner compartment, contact sites between the inner and outer membranes are formed and the signal sequence is removed by a matrix processing protease and this

leads to the formation of the 32-kDa StAR protein. Further processing of the protein by the mitochondrial intermediate processing peptide removes the targeting sequence, and the mature 30-kDa protein remains associated with either the inner mitochondrial memberane or the inter membrane compartment (King et al., 1995). It is further proposed that during the formation of contact sites, cholesterol is transferred from the outer to the inner mitochondrial membranes (Epstein and Orme-Johnson, 1991; Stocco and Sodeman, 1991). Following processing of the 30-kDa protein, the membranes again separate and no further cholesterol transfer can occur. Therefore, the continued synthesis and processing of additional StAR precursor protein is necessary to allow for the continued transport of cholesterol to the inner membrane. Since the half-life of the 37-kDa and 32-kDa precursors of the 30-kDa mitochondrial protein are very short (Epstein and Orme-Johnson, 1991), this would explain the observation that steroidogenesis decays very quickly in the absence of new protein synthesis (Figure 8).

This hypothesis may not be completely correct in the light of more recent findings (Arakane et al., 1996). Deletion of 62 amino acids from the StAR amino-terminus did not diminish its stimulatory activity on pregnenolone synthesis. Western blots analysis confirmed the N-62 truncated protein was not imported or processed by the mitochondria (Arakane et al., 1996). Despite the lack of a mitochondrial targeting sequence, the N-62 protein was associated with the mitochondrial outer membrane as determined by the immunogold labeling of transfected cells (Arakane et al., 1996). According to these findings, it was proposed that StAR importation is the "off" switch instead of being the "on" switch for cholesterol transfer (Arakane et al., 1996; Arakane et al., 1997) (Figure 9).

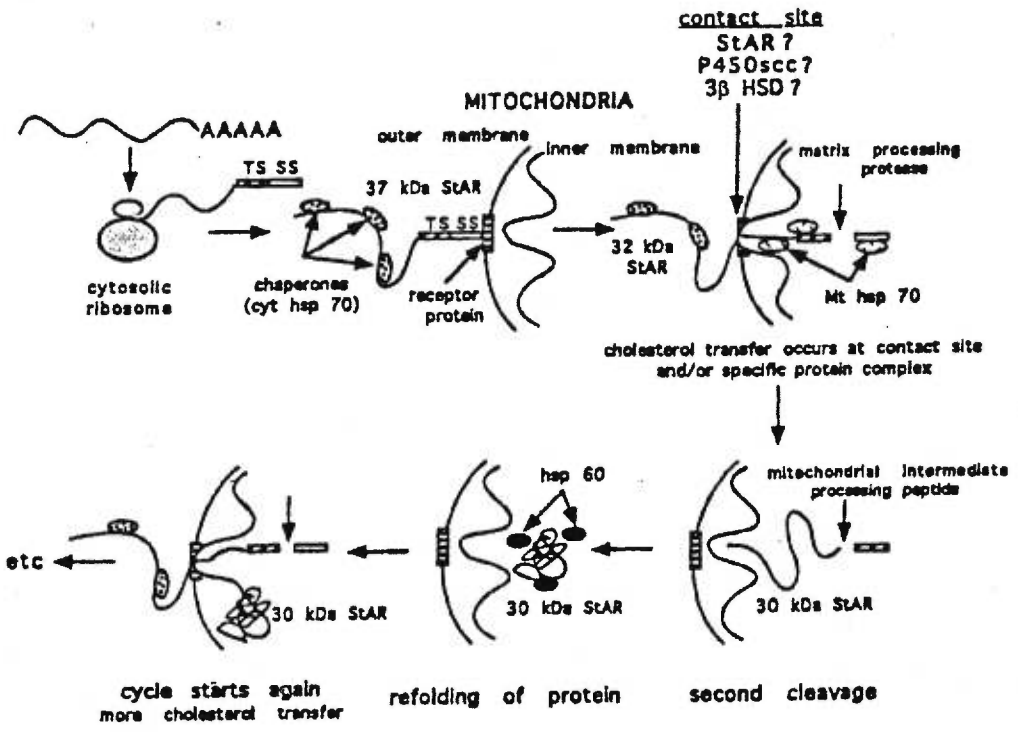

Figure 8. Proposed model for the acute regulation of steroidogenesis by the StAR protein. Upon stimulation of steroidogenic cells with trophic hormones, the 37-KDa StAR protein precursor is rapidly synthesized in the cytoplasm. The 37-kDa protein is transported to the mitochondria where the insertion process begins, and "contact sites" between the outer and inner mitochondria membrane are formed concomitant with the first cleavage event. The cleavage of the first signal precursor by the matrix processing protease results in the formation of the 32-kDa intermediate form of the StAR protein. It is during this time that cholesterol is able to transfer from the outer to the inner mitochondrial membrane. Lastly, the inner and outer membranes separate and the 30-kDa product that is no longer able to function. From Clark and Stocco (1996).

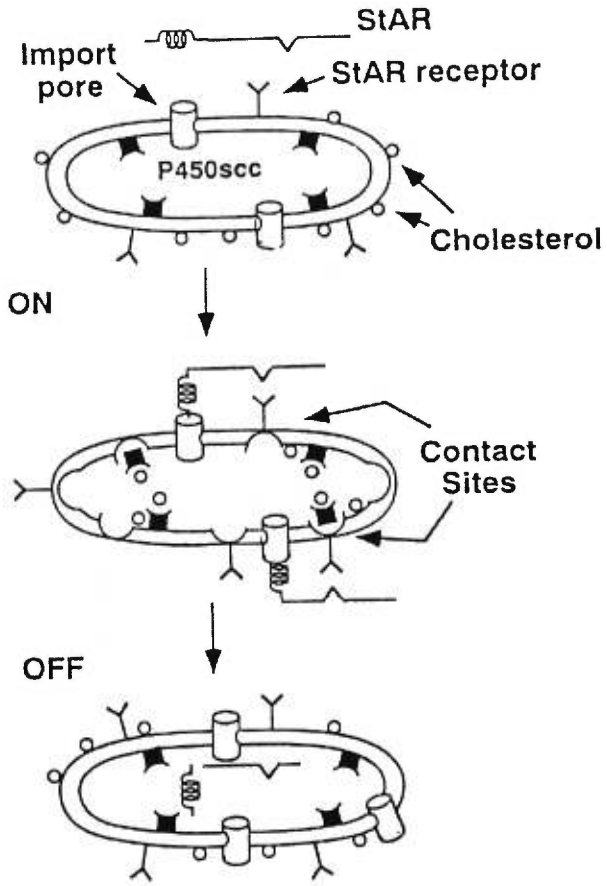

Figure 9. A model for StAR action. StAR is thought to bind to a receptor on the outer mitochondria membrane. This interaction triggers the formation of contact sites between the outer and inner membrane that allows cholesterol to flow down a chemical gradient to reach the P450scc enzyme. When StAR is imported into the mitochondria and processed, it is removed from its receptor and the membrane contact sites are dismantled, terminating the facilitated flux of substrates to P450scc enzyme. From Arakane et al. (1997).

The latest model of StAR function proposes that the C-terminal is critical for the specific interaction of StAR with the mitochondrial outer membrane, and that this interaction facilitates or signals, somehow, the translocation of cholesterol (Clark and Stocco, 1997). This proposal was based on the experimental study of Arakane et al., (1996) in which he found that deletion of 10 amino acids from carboxy-terminal region decreased pregnenolone production by 50% and decreased mitochondrial import efficiency of StAR. Furthermore, when the last 28 amino acids were deleted, the protein was inactive and was not imported into mitochondria, indicating that the C-terminal region of StAR may contribute to the efficiency of StAR import into mitochondria (Arakane et al., 1996).

3.0 ARTICLE ONE

Characterization of cellular and vascular changes in equine preovulatory follicles during hCG-induced ovulation

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Short Title: Histological morphology of equine preovulatory follicles

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3.1. SUMMARY

In contrast to other species, the histological morphology of the equine follicle during the ovulatory process has not been described. To characterize the cellular and vascular changes associated with ovulation in mares, preovulatory follicles were isolated during oestrus 0, 12, 24, 30, 33, 36 and 39 h (n = 5-6 follicles per time point) after an ovulatory dose of hCG. Pieces of follicle wall were formalin-fixed and processed for light microscopy to evaluate the general follicular morphology and quantify selected parameters. Results showed dramatic changes in the histology of equine follicles in the hours prior to ovulation. The thickness of the granulosa cell layer doubled between 0 and 39 h post-hCG (77.8 ± 4.8 versus 158.8 ± 4.8 m, respectively, $P < 0.01$). This expansion was caused primarily by a pronounced accumulation of acid mucosubstances between granulosa cells, first detected 12 h post-hCG and reached maximal levels at 36-39 h. In contrast, a significant thinning of the theca interna layer was observed following hCG treatment. Fewer cell layers were present, theca interna cells appeared smaller than before hCG, and the presence of occasional pyknotic cells was noted at 36 and 39 h post-hCG. Another dramatic finding was the invasion of theca layers by numerous eosinophils. No eosinophils were observed in preovulatory follicles isolated between 0-24 h post-hCG, but their numbers increased to 14.0 ± 0.8 and 5.6 ± 0.3 eosinophils per field (X400) in theca interna and theca externa, respectively, 39 h after hCG treatment ($P < 0.01$). Severe oedema, hyperemia and hemorrhages, and significant increases in numbers of blood vessels in theca interna and externa were observed 36 and 39 h post-hCG. Thus, this study provides the first in-depth characterization of the sequential cellular and vascular changes that occur in equine follicles prior to ovulation.

3.2. INTRODUCTION

The process of ovarian follicular development and ovulation in mares has some unique characteristics when compared to other large animal species. The mare is a seasonal polyoestrous animal with a cycle length of about 20-21 days that is characterized by a relatively long period of oestrus (follicular phase of 5-7 days) (Palmer, 1978; Ginther, 1992; Daels and Hughes, 1993). In most cases, a single cohort or wave of follicles larger than 15 mm develops during the equine oestrous cycle, with the preovulatory follicle being recruited 13-14 days before ovulation (Driancourt and Palmer, 1984; Pierson and Ginther, 1987; Sirois *et al.* 1989). The maximal size reached by the preovulatory follicle (40-45 mm in diameter) is considerably larger than in other species. Ovulation in mares is not triggered by a typical LH surge. Instead, there is a progressive rise in LH during oestrus that generally peaks one day after ovulation (Whitmore *et al.*, 1973; Stabenfeldt *et al.*, 1975; Alexander and Irvine 1982), suggesting that the ovulatory cascade in horses is initiated when a threshold level of gonadotropin is reached. However, the hormonal and cellular control of estradiol biosynthesis in the developing equine preovulatory follicle appears to follow the two-cell (theca interna and granulosa cells), two-gonadotropin (LH and FSH) model common to other species (Sirois *et al.*, 1991; Almadhidi *et al.*, 1995).

Ovulation in mares can be induced during the follicular phase by the administration of hCG, and the length of the ovulatory process, defined as the interval from hCG injection to follicular rupture, is approximately 36-48 h (Duchamp *et al.*, 1987; Watson *et al.*, 1991). In contrast to other species where follicular rupture occurs over the majority of the ovarian surface, the equine ovulatory process is restricted to a

specific region of the ovary, the ovulatory fossa (Stabenfeldt *et al.*, 1975). Some of the biophysical characteristics of equine ovulation have been described using transrectal palpation and ultrasonography. As in other species, rupture does not appear to result from a buildup of pressure within the follicle (Ginther, 1992). The shape and consistency of the equine follicle change as ovulation approaches, going from a relatively firm spherical structure to a soft non-spherical (pear-shaped or conical) structure (Pierson and Ginther, 1985). Two patterns of follicular fluid evacuation have been observed following rupture, an one abrupt, where 90% of the fluid is evacuated during the first 60 seconds, and the second more gradual where complete evacuation requires 6-7 minutes (Townson and Ginther, 1987; 1989).

Unravelling the complex cascade of cellular and biochemical events leading to follicular rupture has been the focus of much attention, and the subject of excellent reviews (Murdoch and Cavender, 1987; Thibault and Levasseur, 1988; Goetz *et al.*, 1991; Tsafriri *et al.*, 1993; Espey and Lipner, 1994). Descriptive histological studies on the ovulatory process have been performed in numerous species, including rabbits (Espey, 1967; Bjersing and Cajander, 1974), various rodents (Byskov, 1969; Parr, 1974; Martin and Talbot, 1987), sheep (Cavender and Murdoch, 1988), cattle (Priedkalns *et al.*, 1968), and humans (Okamura *et al.*, 1980). Whereas differences were noted amongst species, the collective findings revealed a clear parallel between the gonadotropin-dependent process and a classic inflammatory reaction. As observed in acute inflammation, hyperemia, congestion, increased vascular permeability, and the recruitment of white blood cells are present during ovulation. Surprisingly, the sequential histological morphology of the developing ovulatory process has not been

characterized in mares, although the microscopic anatomy of the corpus luteum has been described (Harrison, 1946; Van Niekerk *et al.*, 1975; Levine *et al.*, 1979). To better understand the precise sequence of events leading to follicular rupture in horses, the objective of this study was to characterize the cellular and vascular changes occurring during the final differentiation of equine preovulatory follicles following a gonadotropin-induced ovulation.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Lutalyse was purchased from UpJohn (Kalamazoo, MI), whereas hCG was obtained from The Buttler Company (Columbus, OH). Rompun was purchased from Haver (Bayvet Division, Shawnee, KS). Torbugesic was obtained from Fort Dodge Laboratories Inc (Fort Dodge, IA). Dormosedan was purchased from SmithKline Beecham, Animal Health (West Chester, PA). Culture media was obtained from Gibco Bethesda Research Laboratories Life Technologies Inc (Gaithersburg, MD).

3.3.2. Animals

Adult Standardbred and Thoroughbred mares, weighing approximately 375-450 kg, were kept outdoors and fed daily with hay and concentrates. They were teased daily with a pony stallion for detection of oestrus, and ovarian follicular development was monitored daily by transrectal real-time ultrasonography (Sirois *et al.*, 1989). When the preovulatory follicle reached 35 mm in diameter during oestrus, the animal was injected intravenously with 2500 IU hCG to induce the ovulatory process.

3.3.3. Ovariectomy and isolation of preovulatory follicles

Mares were ovariectomized via colpotomy 0, 12, 24, 30, 33, 36 and 39 h post-hCG with a chain ecraseur (n = 5-6 follicles per time point) (Vaughan, 1988; Sirois *et al.*, 1989). During the procedure, neuroleptanalgesia was induced with a combination of xylazine (Rompun; 0.65 mg/kg, iv), butorphanol (Torbugesic; 0.005 mg/kg, iv) and detomidine (Dormosedan; 0.02 mg/kg, iv), as previously described (Sirois and Dor_, 1997). The recovered ovary was kept in ice-cold Eagles's Minimal Essential Medium (MEM) supplemented with penicillin (50 U/ml)-streptomycin (50 µg/ml, Gibco), L-glutamine (2.0 mM; Gibco) and nonessential amino acids (0.1 mM; Gibco). The preovulatory follicle was dissected from the surrounding ovarian tissue with a scalpel, and pieces of the follicle wall were fixed in 10% neutral buffered formalin. All animal procedures were approved by the institutional animal use and care committee.

3.3.4. Histology

Formalin-fixed tissues were paraffin-embedded, and 3-µm-thick sections were prepared and stained with hematoxylin-eosin-safran (HES). Alcian blue (pH 2.5) staining was also performed on selected sections. The morphology of the follicle wall and its cellular constituents were characterized under light microscopy. More specifically, the following parameters were quantified in follicle wall: 1) the number of cells and thickness of the granulosa layer; 2) the number of cells and thickness of the theca interna layer; 3) the number of blood vessels (capillaries, venules and small veins) in theca interna and theca externa; and 4) the number of eosinophils in theca interna and theca externa. Five random representative high-power fields (X400) were selected on each section to determine these parameters. Additionally, the following parameters were

evaluated in a semi-quantitative way in follicle wall: 1) degree of hyperemia (0 = absent, 1 = little, 2 = moderate, and 3 = marked); 2) degree of oedema (0 = absent, 1 = light, 2 = moderate, and 3 = marked); 3) presence of hemorrhage (0 = absent, 1 = few, 2 = numerous); and 4) presence of acid mucosubstances as detected with Alcian blue staining (pH 2.5) (0 = absent, 1 = little, 2 = moderate, 3 = high; 4 = very high). Five representative fields (X100) were selected on each section to determine these parameters.

3.3.5. Statistical analyses

The effect of time after hCG treatment on each parameter was tested by one-way analysis of variance (ANOVA). When ANOVAs indicated significant differences ($P < 0.05$), multiple comparisons of means were made using the Dunnett's test to compare different time points post-hCG with O h. Analyses were performed with JMP software (Statistical Analysis System Institutes, Cary. NC). Data are presented as mean ± SEM (n = 5-6 follicles per time point).

3.4. RESULTS

3.4.1. Macroscopic characteristics of equine preovulatory follicles

Thirty-six equine preovulatory follicles were isolated during oestrus between 0 and 39 h post-hCG, including five follicles at 0, 12, 24, 30, 33 and 39 h, and six follicles at 36 h after hCG treatment. The overall mean follicle diameter was 39.4 ± 0.5 mm, and no significant difference was observed across time points post-hCG ($P > 0.05$). All preovulatory follicles recovered between 0 and 36 h were intact, whereas two of the five follicles obtained 39 h post-hCG accidentally ruptured during the procedure. Follicles

isolated at this latter time (39 h post-hCG) appeared easily deformable at the ultrasound examination prior to the surgery and were very soft upon palpation, suggesting imminent ovulation. Interestingly, after follicles were dissected and opened with scissors, a clear circular hemorrhagic area of approximately 1-1.5 cm in diameter located towards the ovulatory fossa was observed in the wall of four of the five follicles obtained 39 h post-hCG. Only one follicle isolated at 36 h contained a similar hemorrhagic region, whereas none was observed in follicles at earlier time points. Another important macroscopic finding was the presence of a mucoid substance coating the luminal side of the follicle following hCG treatment. This mucoid substance became more apparent macroscopically at 30 h post-hCG, and made the granulosa cell layer sticky.

3.4.2. Microscopic gonadotropin-induced changes in the granulosa cell layer

The administration of hCG induced striking microscopic changes in the granulosa cell layer (Fig. 1). Prior to hCG treatment (0 h), the granulosa cell layer was composed of four to nine compact layers of small elongated cells. These cells contained a small dark-staining nucleus that was often located in a basal position. Mitotic figures were occasionally observed (0-2 mitoses per field; X400). Gonadotropin treatment caused a marked loosening of the granulosa cell layer that resulted in a significant increase in the layer's thickness, rising from 77.8 ± 4.8 to 158.8 ± 4.8 μm between 0 and 39 h post-hCG ($P < 0.01$, Figs. 1 and 3). The marked loosening of the granulosa cell layer resulted from the accumulation of a mucoid material between the cells. Alcian blue staining revealed that this material was composed of acid mucosubstances (Fig. 2a and b). The accumulation was progressive and reached highest levels at 36 and 39 h post-hCG ($P < 0.01$, Fig. 3b). No mitotic figures were observed in the granulosa cell layer after hCG

treatment. The mean number of layers of granulosa cells increased between 0 and 24 h post-hCG (5.5 ± 0.2 and 6.4 ± 0.2 cells, respectively, $P < 0.01$), but did not rise thereafter.

3.4.3. Microscopic gonadotropin-induced changes in theca layers

The theca interna layer of equine preovulatory follicles isolated prior to hCG treatment (O h) was relatively thick (85.5 ± 4.5 μm; Fig. 5), and composed of plump polyhedral cells containing a light-staining nucleus (Figs. 1 and 2). The layer was well-vascularized, containing numerous vessels (6.6 ± 0.5 vessels per field; X400; Fig. 6). The theca externa was less vascularized (3.5 ± 0.4 vessels per field; X400; Fig. 6), and composed of small fibroblasts. The boundary between theca interna and theca externa was easily detectable at this time point (0 h post-hCG), because of marked differences in cell morphology.

The administration of hCG caused profound changes in the morphology of theca layers (Fig. 1). A significant thinning of the theca interna layer was observed after hCG treatment (Fig. 5). Fewer cell layers were present, theca interna cells appeared smaller than before hCG, and the presence of occasional pyknotic cells was noted at 36 and 39 h post-hCG. Another dramatic finding was a marked infiltration of theca layers by eosinophils. Equine eosinophilic leukocytes were easily recognizable by their very large and tightly packed cytoplasmic granules giving the cells a raspberry-like appearance (Jain, 1986). No eosinophils were observed in theca interna and theca externa of preovulatory follicles isolated between 0-24 h post-hCG, but a rapid and significant increase in their number occurred thereafter (Figs. 2c, 2d and 4). The number of eosinophils per field (X400) in theca interna increased from 0.65 ± 0.06 to 14.0 ± 1.5

between 30 h and 39 h post-hCG, respectively ($P < 0.05$). Numerous eosinophils were observed along the margins of the endothelium of small vessels, and emigrating in the extravascular compartment (Fig. 2c and d).

Dramatic vascular changes were observed in theca layers after hCG treatment (Fig. 1). They included significant increases in degrees of oedema, hemorrhages and hyperemia, with maximal levels observed at 36 and 39 h post-hCG (Fig. 7). The number of blood vessels (per field; X400) in theca interna and theca externa were significantly increased at 33 and 30 h post-hCG, respectively ($P < 0.01$; Fig. 6).

3.5. DISCUSSION

This study is the first to provide an in-depth characterization of the sequential cellular and vascular changes that occur in equine follicles prior to ovulation. In contrast to detailed reports in other species, histological investigations of ovarian structures have been very limited in the mare, and have focused primarily on the corpus luteum (Harrison, 1946; Van Niekerk *et al.*, 1975; Levine *et al.*, 1979). In this study, the changing microscopic anatomy of the equine follicle was characterized using a group of thirty-six presumptive preovulatory follicles identified by ultrasonography and isolated during oestrus at specific times after the administration of an ovulatory dose of hCG. The induction of the ovulatory process was associated with a profound remodeling of the granulosa cell layer. The marked expansion of the layer was, to some extent, reminiscent of a phenomenon usually associated with the cumulus oocyte complex (Salustri *et al.*, 1996). Whereas a loosening of granulosa cells just prior to ovulation has been reported in other species (Priedkalns *et al.*, 1968; Parr, 1974; Cherney et *al.*, 1978),

the degree of expansion observed in the mare appears unique. The precise nature of the mucosubstances secreted during cellular expansion remains to be determined, but it will likely include members of the glycosaminoglycan family (e.g. hyaluronan, heparan sulfate or dermatan sulfate) as shown in other species (Jackson *et al.*, 1991; Chen *et al.*, 1993; Tirone *et al.*, 1997). The phenomenon is not a function restricted to hCG treatment since it has also been observed in equine preovulatory follicles isolated during late oestrus where the native goadotropin rise was present (J. Sirois and J.E. Fortune, unpublished data). A direct consequence of the large amount of mucosubstance produced is a progressive entrapment of granulosa cells, making the isolation of equine cells for *in vitro* studies particularly tedious during the latter stages of the ovulatory process (need for repetitive centrifugations and washes).

In a study focusing primarily on the histology of the developing equine corpus luteum, Van Niekerk *et al.* (1975) reported that theca interna cells degenerate at the time of ovulation in the mare and therefore, in contrast to other species, do not contribute to the luteal tissue. Results from the present report are consistent with this earlier finding, as marked thinning of the theca interna layer and occasional cell pyknosis were observed after hCG treatment. These changes were observed primarily at the end of the ovulatory process, which likely relates to the need to provide an adequate thecal androgen production during the follicular phase to maintain follicular estradiol biosynthesis. Interestingly, a potential biochemical consequence of thecal degeneration was recently suggested by the marked drop in transcripts coding for the steroidogenic acute regulatory protein (StAR) in these cells at 36 and 39 h post-hCG (Kerban *et al.*, 1997). However, the putative degeneration of the theca interna in mares remains puzzling, since it has not

been reported in other species. Future investigations should concentrate on elucidating the nature of the process involved (apoptosis *versus* necrosis), as well as the cellular and molecular mechanisms responsible for cell demise.

This is the first study to report the massive emigration of eosinophilic leukocytes into the equine follicle wall during the ovulatory process. The attraction of white blood cells from the circulation into theca layers of preovulatory follicles has been reported in other species (Cavender and Murdoch, 1988; Standaert *et al.*, 1991; Gesdes *et al.*, 1992; Brannstrom *et al.*, 1993), supporting their role in the inflammatory-like response in the ovary at the time of ovulation. Interestingly, there seem to be species-related differences in the leukocyte subtype attracted into the preovulatory follicle, with eosinophils being predominant in equine (this study) and porcine follicles (Standaert *et al.*, 1991), and neutrophils being the principal leukocyte detected in rat (Brannstrom *et al.*, 1993) and rabbit follicles (Gerdes *et al.*, 1992). Eosinophils were first detected 33 h post-hCG in equine follicles, which coincides precisely with the gonadotropin-dependent induction of prostaglandin synthesis in granulosa cells prior to ovulation (Watson and Sertich, 1991; Sirois and Dor_, 1997). However, a causal relationship between prostaglandin synthesis and leukocyte migration was not supported by studies in other species (Espey *et al.*, 1981; Chun *et al.*, 1993). Leukocyte chemoattractant activities have been detected in fluid or cellular extracts of bovine (Sirotkin and Luck, 1995), equine (Watson *et al.*, 1991), human (Seow *et al.*, 1988) and ovine follicles (Murdoch and McCormick, 1989). The fact that Watson *et al.* (1991) reported no significant difference in the neutrophil chemotactic activity of fluid aspirated from equine preovulatory follicles between 0 and 36 h post-hCG is not inconsistent with results of the present study since eosinophils,

and not neutrophils, appeared to be predominantly attracted in the equine follicle. Although the precise nature of the follicular chemotactic signal has not been identified, studies in sheep (Murdoch and McCormick, 1989; 1993) and cattle (Sirotkin and Luck, 1995) suggest that potential candidates include collagen-like peptides, collagenase, ascorbic acid and calcium ions. Also, a potential regulation of ovarian endothelial adhesion molecules during the ovulatory process could provide an additional mechanism for leukocyte recruitment in follicles. The initial step in leukocyte extravasation involves a series of interactions with endothelial cells that are mediated by adhesion molecules, including P-selectin (Smith, 1993). Interestingly, recent studies have shown that eosinophils use P-selectin to tether and roll on vascular endothelial cells (Kamala et al., 1997; Kitayama *et al.*, 1997). The precise function(s) of the massive follicular eosinophilia in mares during the ovulatory process remains uncertain. Depletion of circulatory eosinophils in sheep using prednisolone prevented the characteristic eosinophilia in preovulatory follicles but did not affect ovulation (Murdoch and Steadman, 1991). Likewise, severe depletion of circulatory leukocytes in rats did not prevent ovulation, which prompted Chun *et al.* (1993) to suggest that influx of leukocytes may be a consequence of vascular changes during the ovulatory process and not an obligatory requirement for follicular rupture.

As the time of ovulation approached, the equine follicle was the site of marked vascular changes, including an apparent increase in blood supply, hyperemia, vasodilation, oedema and hemorrhages. These changes have been described in other species and helped establish a clear parallel between the ovulatory process and a classic inflammatory reaction (Espey, 1980). However, different attempts to identify the

follicular mediators responsible for the gonadotropin-induced vascular response have not provided a consistent picture (Goetz *et al.*, 1991; Tsafriri *et al.*, 1993; Espey, 1994). Numerous potent vasoactive agents playing an important role in inflammation, such as histamine, bradykinin and products of the cyclooxygenase and lipoxygenase pathways, have been identified in preovulatory follicles and have been proposed to participate in the ovulatory process (Piacsek *et al.*, 1971; Guraya and Dhanju, 1992; Murdoch *et al.*, 1993; Priddy and Killick, 1993; Tsafriri *et al.*, 1993). It appears that follicular hyperemia and increased vascular permeability would be initiated by histamine, and be propagated by prostaglandins (Murdoch and Cavender, 1987). Prostaglandins are thought to play a key role during the ovulatory process (Murdoch *et al.*, 1993; Priddy and Killick, 1993). This concept was recently reinforced by the finding that female mice deficient for PGHS-2, the first rate limiting enzyme in the biosynthetic pathway of prostaglandins from arachidonic acid (Herschman, 1996), have an impaired ovulatory process (Dinchuk *et al.*, 1995; Lim H *et al.*, 1997). In equine preovulatory follicles, a selective induction of PGHS-2 in granulosa cells beginning 30 h after hCG treatment is responsible for the dramatic increase in follicular prostaglandin synthesis prior to ovulation (Watson and Sertich, 1991; Sirois and Dor_, 1997; Boerboom and Sirois, 1998). Elevated levels of prostaglandins are likely involved in the vascular response observed, as well as in the proteolytic and collagenolytic activities needed for follicular rupture (Tsafriri *et al.*, 1993).

In summary, this study provides a first characterization of cellular and vascular changes in equine follicles during the ovulatory process. Considering the variable length of the follicular phase in mares and the absence of a discrete LH preovulatory surge

(Whitmore *et al.*, 1973; Stabenfeldt *et al.*, 1975; Alexander and Irvine 1982), exogenous gonadotropin (hCG) was used to induce ovulation and better study the chronology of the follicular response. Some of the more interesting findings about the equine ovulatory process include a marked expansion of the granulosa cell layer, a putative degeneration of theca interna cells, and a pronounced follicular eosinophilia. Future studies are needed to unravel the cellular and molecular basis for these gonadotropin-induced changes in equine follicles prior to ovulation.

3.6. ACKNOWLEDGMENTS

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Fig. 1. Histological morphology of equine preovulatory follicles during hCG-induced ovulation. Sections of follicle walls were prepared from equine preovulatory follicles isolated during estrus 0 (a), 12 (b), 24 (c), 30 (d), 36 (e), and 39 h (f) after hCG treatment. Note the loosening of the granulosa cell layer (c, d, e, f), thinning of the theca interna (d, e, f), developing oedema (d, e, f) and progressive vascular changes (e, f). Magnification, X200.

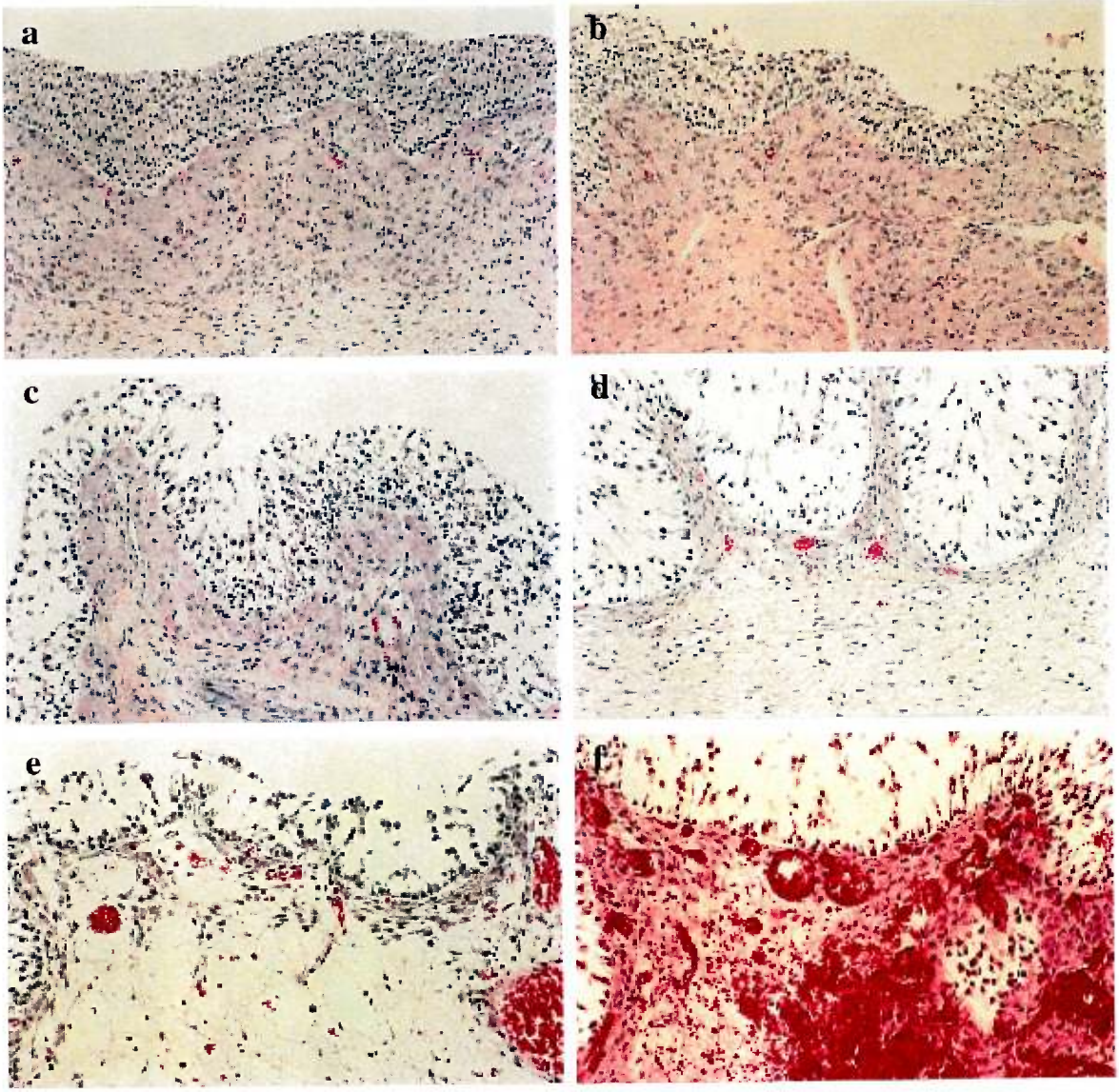

a
b
c
d
e
f

Fig. 2. Effect of hCG on the accumulation of acid mucosubstances in the granulosa cell layer (a, b) and the infiltration of eosinophils in theca layers (c, d). Sections of follicle walls were prepared from equine preovulatory follicles isolated 0 (a), 30 (c) and 39 h (b, d) post-hCG, and stained with hematoxylin-eosin-saffran (a, b) or alcian blue, pH 2.5 (c, d). Arrowheads (c, d) indicate eosinophils. Magnifications, X200 (a, b); X400 (c, d).

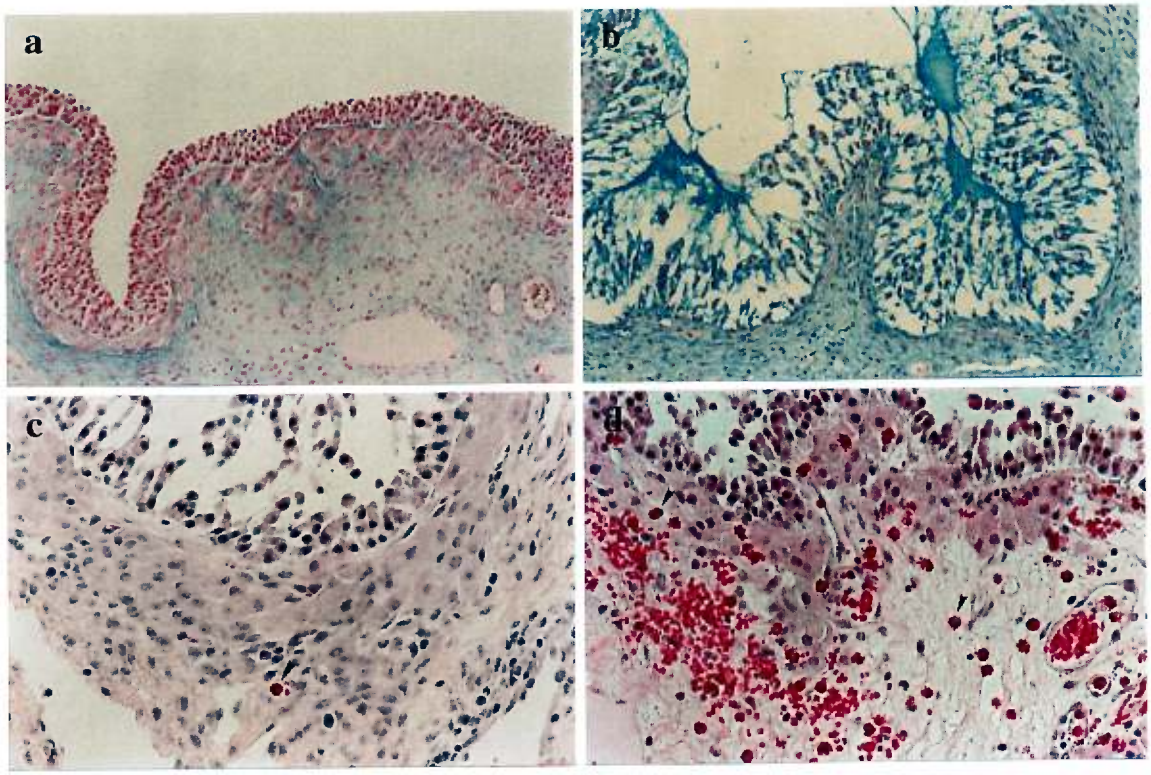

a
b
c
d

Fig. 3. Changes in the granulosa cell layer of equine preovulatory follicles after hCG treatment. (a) Effect of hCG on the thickness of the granulosa cell layer. Sections of follicle walls of equine preovulatory follicles were isolated between 0-39 h post-hCG, and the thickness of the granulosa cell layer was measured. (b) Time- and gonadotropin-dependent accumulation of acid mucosubstances. The relative amount of acid mucosubstances substances in the granulosa cell layer was estimated using an arbitrary scale, as described in Materials and Methods. Results are presented as mean ± SEM (n = 5-6 follicles/time-point). Columns with an asterisk are significantly different from time 0 h (ANOVA, Dunnett's test, $P<0.01$).

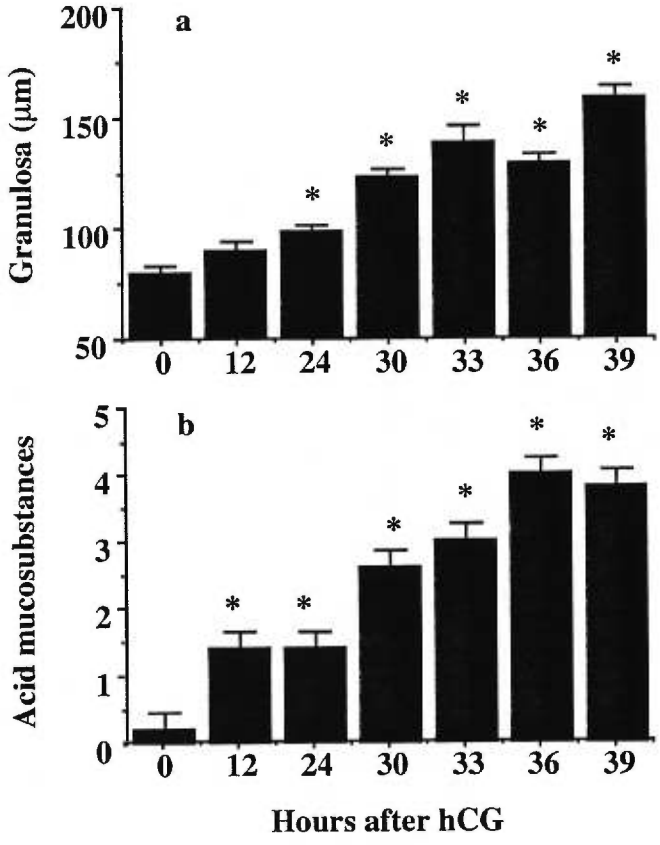

a
b
Granulosa (μm)
Acid mucosubstances
200
150
100
50
5
4
3
2
1
0
0
12
24
30
33
36
39
Hours after hCG

Fig. 4. Time-course of induction of eosinophil infiltration in theca interna and theca externa after hCG treatment. Sections of follicle walls of equine preovulatory follicles were isolated between 0-39 h post-hCG, and the number of eosinophils per field (X400) was determined in theca interna (filled bars) and theca externa (open bars). Results are presented as mean ± SEM (n = 5-6 follicles/time point). Columns with an asterisk are significantly different from time 0 h (ANOVA, Dunnett's test, $P < 0.01$).

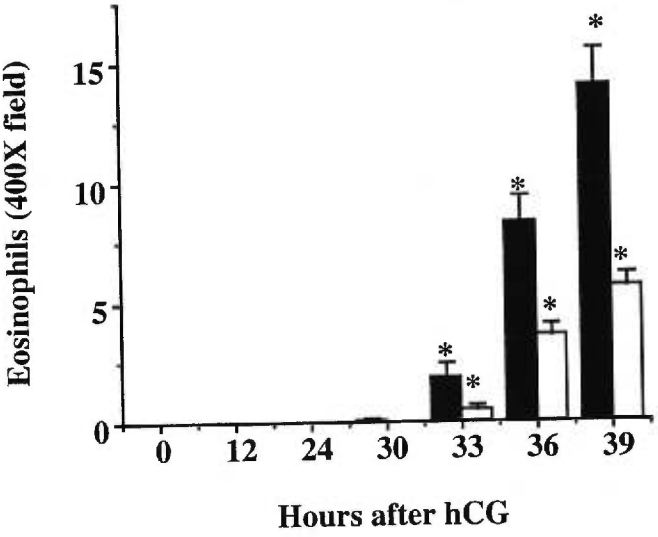

Eosinophils (400X field)
15
10
5
0
0
12
24
30
33
36
39
Hours after hCG
*

Fig. 5. Changes in the theca interna layer of equine preovulatory follicles after hCG treatment. Effects of hCG on the number of cells (a) and thickness (b) of the theca interna layer were determined on sections of follicle walls of preovulatory follicles isolated between 0-39 h post-hCG. Results are presented as mean ± SEM (n = 5-6 follicles/time-point). Columns with an asterisk are significantly different from time 0 h (ANOVA, Dunnett's test, $P<0.01$).

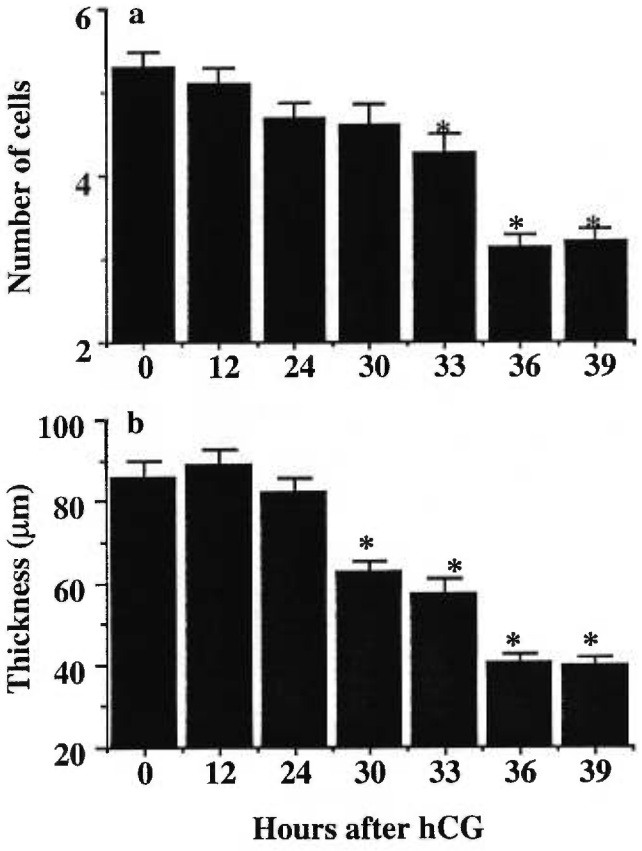

a
6
4
2
Number of cells
0
12
24
30
33
36
39
*
b
100
80
60
40
20
Thickness (μm)
Hours after hCG

Fig. 6. Number of thecal blood vessels in equine follicles prior to ovulation. Sections of follicle walls of equine preovulatory follicles were isolated between 0-39 h post-hCG, and numbers of blood vessels per field (X400; n=5 fields/follicle) were determined in theca interna (filled bars) and theca externa (open bars). Results are presented as mean ± SEM (n = 5-6 follicles/time point). Columns with an asterisk are significantly different from time 0 h (ANOVA, Dunnett's test, $P < 0.01$).

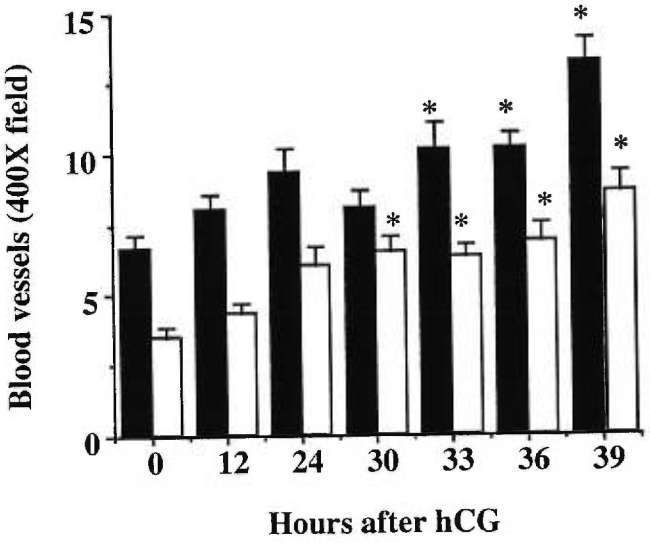
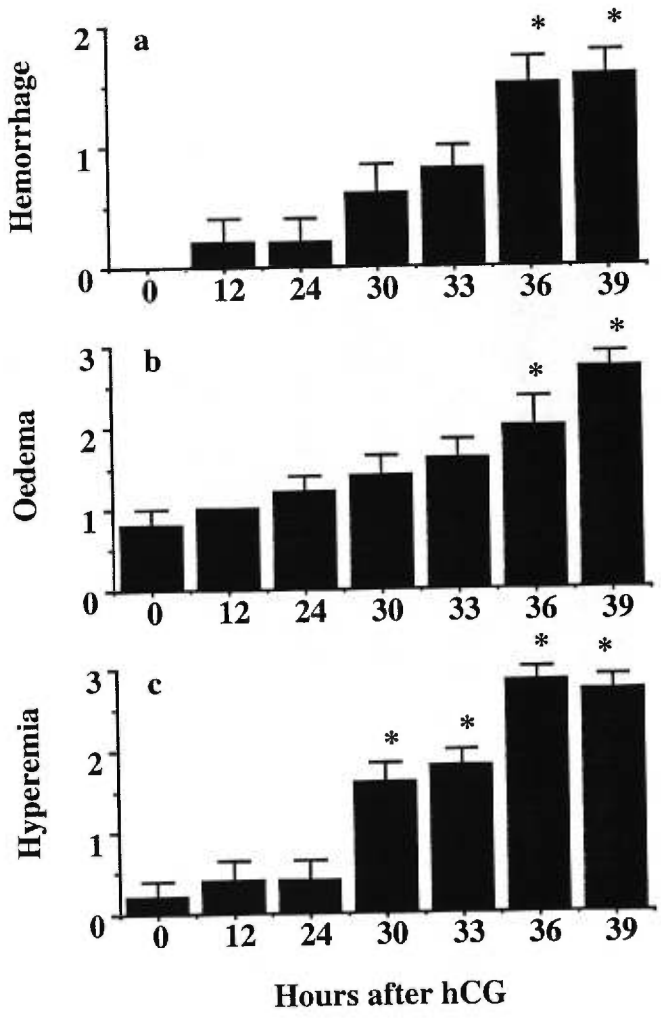

Blood vessels (400X field)
15
10
5
0
0
12
24
30
33
36
39
Hours after hCG
*
*
*
*
*
*
*

Fig. 7. Degree of hemorrhage (a), oedema (b) and hyperemia (c) in equine follicles after hCG treatment. Sections of follicle walls of equine preovulatory follicles were isolated between 0-39 h post-hCG, the degrees of hemorrhage, oedema and hyperemia were determined using an arbitrary scale, as described in Materials and Methods. Results are presented as mean ± SEM (n = 5-6 follicles/time point). Columns with an asterisk are significantly different from time 0 h (ANOVA, Dunnett's test, $P < 0.01$).


Hemorrhage
a
2
1
0
0
12
24
30
33
36
39
*
*
Oedema
b
3
2
1
0
0
12
24
30
33
36
39
*
*
Hyperemia
c
3
2
1
0
0
12
24
30
33
36
39
*
*
*
*
Hours after hCG

4.0. ARTICLE TWO

Human Chorionic Gonadotropin Induces an Inverse Regulation of Steroidogenic Acute Regulatory Protein Messenger Ribonucleic Acid in Theca Interna and Granulosa Cells of Equine Preovulatory Follicles*

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Key Words: Steroidogenic acute regulatory protein, StAR, cDNA, ovary, follicle, granulosa cells, theca interna, horse

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4.1. ABSTRACT

The time- and gonadotropin-dependent regulation of steroidogenic acute regulatory protein (StAR) has not been characterized *in vivo* in preovulatory follicles of large monoovulatory species and of sexually mature animals. The objectives of this study were to clone equine StAR and describe the regulation of its mRNA in equine follicles after the administration of an ovulatory dose of hCG. The screening of an equine follicle complementary DNA (cDNA) library with a mouse StAR cDNA probe revealed two forms of equine StAR that differ only in the length of their 3'-untranslated region (3'-UTR); a long form of 2918 base pairs (bp) and a short form of 1599 bp. The StAR-long form cDNA contains a 5'-UTR of 117 bp, an open reading frame (ORF) of 855 bp and a 3'-UTR of 1946 bp. Primer extension analysis showed that the cDNA clone lacked the first 10 bp of the primary transcript, giving a total of 127 bp for the complete StAR 5'-UTR. The ORF encodes a 285 amino acid protein that is 86%-90% identical to StAR of other species characterized thus far. The regulation of StAR mRNA *in vivo* was studied in equine preovulatory follicles isolated during estrus at 0, 12, 24, 30, 33, 36, and 39 h (n = 4-5 follicles/time point) after an ovulatory dose of hCG. Results from Northern blots showed no significant changes in StAR mRNA levels after hCG treatment when analyses were performed on intact follicle wall (theca interna with attached granulosa cells). However, Northern blots performed on isolated follicle cells revealed an unexpected regulation of StAR mRNA. In granulosa cells, StAR transcripts were undetectable at 0 h but significantly increased at 30 h post-hCG, and this induction was associated with a rise in follicular fluid concentrations of progesterone ($P<0.05$). In contrast, StAR mRNA levels were high in theca interna at 0 h, remained unchanged until

33 h post-hCG, and dropped dramatically thereafter ($P<0.05$). Thus, this study describes the primary structure of equine StAR, documents for the first time the regulation of StAR mRNA *in vivo* in preovulatory follicles of a large monoovulatory species, and identifies a novel inverse regulation of StAR transcripts in theca interna and granulosa cells of equine follicles prior to ovulation.

4.2. INTRODUCTION

The biosynthesis of all steroid hormones begins in mitochondria with the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side chain cleavage enzyme complex (P450scc; 1, 2). Adequate delivery of hydrophobic cholesterol to the inner mitochondrial membrane, where resides P450scc, is a key rate-limiting step in the acute regulation of steroidogenesis (3-5). While the mechanism of intracellular transport of cholesterol to the mitochondrion remains unresolved, its translocation from the outer to the inner mitochondrial membrane appears to involve a protein originally described by Orme-Johnson and collaborators (6-7), and recently purified, cloned and named steroidogenic acute regulatory protein (StAR) by Clark *et al.* (8). StAR is a phosphoprotein synthesized in the cytosol as a short-lived 37-kDa precursor that is processed into more stable 30-kDa proteins after mitochondrial import (9-11). Interestingly, the 37-kDa precursor protein is believed to represent the active form of StAR involved in moving cholesterol across mitochondrial membranes, while the role, if any, of the 30-kDa proteins remains unknown (12, 13). The deduced amino acid sequence of the StAR protein has been characterized in mouse (8), human (14), cow (15), rat (16-19), sheep (20), pig (21) and hamster (22).

The critical role of StAR in steroid hormone synthesis has been clearly demonstrated using various models, including a biochemically defined *in vitro* system (11), cultures of intact cells (14, 23), and a targeted gene disruption approach to generate StAR knockout mice (24). Moreover, the finding that mutations within the StAR gene are responsible in humans for congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which the synthesis of all adrenal and gonadal steroid is severely impaired, further underscores the importance of the protein (25, 26).

Results from recent studies have documented the pattern of expression and regulation of StAR in ovarian cells during various physiological processes. High levels of StAR mRNA and protein were observed in corpora luteum of sheep (20), cows (15, 21, 27), rats (18, 28), humans (29, 30) and pigs (31). Luteal StAR transcripts were increased by LH and GH in hypophysectomized sheep (20), and by estradiol-17ß in rabbits (32). In contrast, regression of the corpus luteum is accompanied by a marked decrease in StAR expression (20, 27, 28, 30). StAR is also regulated in a gonadotropin-dependent and stage-specific manner during follicular development (17, 29, 31, 33). Experiments *in vitro* showed that gonadotropins and activators of the protein kinase A pathway up-regulate StAR expression in granulosa cells (16, 27, 29, 33-36), whereas prostaglandin F2α and phorbol 12-myristate 13-acetate appeared to be negative regulators of StAR expression *in vivo* and *in vitro* (20, 28, 29, 33). The equine CG/human CG-treated immature rat model was used to study the control of StAR expression *in vivo* in preovulatory follicles (17, 33). However, the regulation of follicular StAR in a more physiological system using sexually mature animals has not been characterized, and in-depth studies in large monoovulatory species are lacking.

Therefore, the general objective of this study was to use the equine preovulatory follicle as a model to study the cell-specific and time-dependent regulation of StAR by gonadotropins *in vivo*. The specific objectives were to clone and characterize equine StAR, describe the regulation of its mRNA in preovulatory follicles after the administration of an ovulatory dose of gonadotropins, and determine the contribution of theca interna and granulosa cells to follicular StAR expression.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Lutalyse was purchased from UpJohn (Kalamazoo, MI); hCG was obtained from The Buttler Company (Columbus, OH); Torbugesic was purchased from Fort Dodge Laboratories Inc (Fort Dodge, IA); Rompun was obtained from Haver (Bayvet Division, Shawnee, KS); Dormosedan was purchased from SmithKline Beecham, Animal Health (West Chester, PA); RNAsin, Prime-a-Gene labeling system, DNA 5'-End Labeling System and AMV reverse transcriptase were obtained from Promega (Madison, WI); Biotrans nylon membranes (0.2 m) was purchased from ICN Pharmaceuticals (Montreal, Quebec); [-^{32}P]dCTP, [-^{32}P]ATP and [^{35}S]dATP were obtained from Mandel Scientific NEN Life Science Products (Mississauga, Ontario); TRIzol total RNA isolation reagent, RNA ladder (0.24-9.5 kilobases), synthetic oligonucleotides and culture media were purchased from Gibco Bethesda Research Laboratories Life Technologies Inc (Gaithersburg, MD); QuikHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA); T4 polynucleotide kinase and all sequencing reagents

were purchased from Pharmacia Biotech Inc (Baie D'Urfé, Québec); Kodak film X-OMAT AR was obtained from Eastman Kodak Company (Rochester, NY); Electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA).

4.3.2. Cloning and sequencing of equine StAR

To clone the equine StAR cDNA, an expression library prepared with equine follicle mRNA (37) was screened with a mouse StAR cDNA (8). The probe was labeled with [-^{32}P]deoxy-CTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1 x 10^8 cpm/ g DNA. Approximately 100,000 phage plaques were screened, and hybridization was performed at 55 C with QuikHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screening, and pBluescript phagemids containing the cloned DNA insert were excised *in vivo* with the Ex-Assist/SOLR system (Stratagene). DNA sequencing was performed by the Sanger dideoxy nucleotide chain termination method (38) using the T7 Sequencing Kit (Pharmacia), vector based primers (T3 and T7) and specific primers synthesized as internal StAR sequences were obtained. Nucleotide and amino acid analyses were performed using the FASTA program of Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI) and the MacDNASIS software version 2.0 (Hitachi, Hialeah, FL).

4.3.3. Primer extension analysis

Primer extension analysis was performed in aqueous buffer as previously described (37, 39). The reaction used total RNA extracted with TRIzol (Gibco BRL) from a corpus luteum isolated on day 8 of the estrous cycle and from spleen (negative

control), and a 30-mer antisense oligonucleotide 5'-GGCTCCGAGGCAGTGCTGGA GGAG-3' corresponding to base pairs 46 to 75 of the longest StAR cDNA clone (Fig. 1). The extension product was analyzed by electrophoresis on a 6% polyacrylamide/7M urea gel, and its size was determined by comparison with the products of an unrelated sequencing reaction run in adjacent lanes.

4.3.4. Isolation and dissection of equine preovulatory follicles

Standardbred and Thoroughbred mares were teased daily with a pony stallion for detection of estrus, and ovarian follicular development was monitored daily by transrectal real-time ultrasonography (40). Ovulation was induced during estrus with hCG (2500 IU, iv) when the preovulatory follicle reached 35 mm in diameter. Ovariectomy was performed via colpotomy 0, 12, 24, 30, 33, 36 and 39 hours post-hCG with a chain ecraseur (n=4-6 follicles per time-point)(42). During the procedure, neuroleptanalgesia was induced with a combination of xylazine (Rompun; 0.65 mg/kg, iv), butorphanol (Torbugesic; 0.005 mg/kg, iv) and detomidine (Dormosedan; 0.02 mg/kg, iv), as described (41). The recovered ovary was kept in ice-cold Eagles's Minimal Essential Medium (MEM) supplemented with penicillin (50 U/ml)-streptomycin (50 μg/ml, Gibco), L-glutamine (2.0 mM; Gibco) and nonessential amino acids (0.1 mM; Gibco). Each preovulatory follicle was dissected into three cellular preparations, as previously described (42, 43). They included pieces of follicle wall (theca interna with attached granulosa cells) and isolated preparations of theca interna and granulosa cells. All samples were stored at -70 C until RNA extraction. Animal procedures were approved by the Comité de déontologie animale of the Université de Montréal.

4.3.5. RNA extraction and Northern blot analysis

Total RNA was extracted with TRIzol (Gibco BRL) using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific) from equine tissues. For Northern analysis, RNA samples (10 g) were denatured at 55 C for 15 min in 50% deionized formamide-6% formaldehyde, electrophoresed in a 1% formaldehyde-agarose gel and transferred onto a nylon membrane as described (36, 43). A ladder of RNA standards was run with each gel and ethidium bromide (10 μg) was added to each sample prior to electrophoresis to compare RNA loading and determine migration of standards. The membrane was first hybridized to the ^{32}P-labeled equine StAR cDNA probe using QuikHyb solution (Stratagene). After stripping the radioactivity with 0.1% SSC-0.1% SDS for 30 min at 100 C, the same blot was subsequently hybridized with a rat elongation factor Tu cDNA as a control gene for RNA loading and transfer (45).

4.3.6. Progesterone RIA

Nonextracted aliquots of follicular fluid were assayed for progesterone by a specific RIA (46). The sensitivity of the assay was 7.29 pg/assay tube, and the intra- and inter-assay coefficients of variations were 11.4% and 18.6%, respectively.

4.3.7 Statistical analysis

One-way ANOVA was used to test the effect of time after hCG on relative StAR mRNA levels and concentrations of progesterone in follicular fluids. When ANOVAs indicated significant differences ($P<0.05$), the Dunnett's test was used for multiple comparisons with the control (0 h post-hCG). Data were transformed to logarithms before analysis when heterogeneity of variance was observed with the Hartley test.

Statistical analyses were performed using the Software (SAS Institute Inc, Cary, NC). Relative levels of StAR mRNA were quantified by determining the optical density of the StAR band on autoradiograms with a computer-assisted image analysis system (Collage Macintosh program, Fotodyne Inc., New Berlin, WI). The EFTu signal was also quantified and used to normalize results. For each cellular preparation, data were expressed as ratios of StAR mRNA to EFTu, and are presented as means ± SEM (n=4 follicles/time point).

4.4. RESULTS

4.4.1. Characterization of the equine StAR cDNA

Twelve positive clones were isolated from an equine follicle cDNA library after an initial screen of approximately 100,000 phage plaques. DNA sequencing analyses revealed that the clones represent two forms of equine StAR, a short form composed of 1599 bp (clone 10-1) and a long form of 2918 bp (clone 1-2). The short and long clones had 5'-untranslated regions of 114 and 117 bp, respectively, and a common open reading frame of 855 bp (Fig. 1). However, they differed in the length of their 3'-untranslated regions, corresponding to 630 and 1946 bp in the short and long forms, respectively (Fig. 1).

The coding region of equine StAR cDNA encodes a 285 amino acid protein, which is identical in length to human (14), pig (21) and bovine StAR (15, 21), but one amino acid longer than that of the mouse (8), rat (17-19) and hamster (22) protein (Fig. 2). Comparison across species indicates that the amino acid sequence of equine StAR is

highly similar to that of other mammalian homologs, being 90%, 89%, 88%, 87%, 87%, 86% and 88% identical to the human, porcine, bovine, murine, rat, hamster and ovine StAR. Computer analysis of the StAR protein sequence using Prosite software (Oxford Molecular Group Inc) identified several potential phosphorylation sites, including two cAMP- and cGMP-dependent protein kinase (Ser 56 and Ser 195), three protein kinase C (Thr 5, Ser 13, Ser 186) and four casein kinase II (Ser 61, Ser 69, Thr 204, Thr 263) phosphorylation sites. Also, a putative mitochondrial transit peptide was predicted in position 1 to 55.

4.4.2. Length of the StAR 5'-untranslated region

Primer extension analysis was used to determine the full length of the StAR 5'-untranslated region. One major extension product was produced with RNA extracted from a corpus luteum, whereas none was generated when negative control spleen RNA was used (Fig. 3). The size of the extension product, as determined by comparisons with an unrelated sequencing reaction run in adjacent lanes, was 85 nucleotides. Therefore, our longest StAR cDNA clone (clone 1-2) appears to lack 10 nucleotides of the primary transcript, suggesting a full-length 5'-untranslated region of 127 nucleotides.

4.4.3. Inverse regulation of StAR mRNA in granulosa cells and theca interna

To study potential changes in StAR mRNA levels during equine follicular luteinization, preovulatory follicles were isolated between 0 and 39 h post-hCG and Northern blot analyses were performed on RNA extracted from intact follicle wall (theca interna with attached granulosa cells). StAR transcripts of approximately 3.0 kb were

detected in walls of follicles isolated at 0 h, and administration of hCG had no significant effect on relative transcript levels (Fig. 4A). However, higher levels of StAR mRNA were observed in two corpora lutea obtained on day 8 of the estrous cycle (Fig. 4A).

To determine the relative contribution of each steroidogenic cell type in follicular StAR mRNA expression, Northern blots were prepared from isolated preparations of granulosa cells and theca interna. Results revealed an unexpected reciprocal regulation of StAR transcripts by hCG in each cell type. In granulosa cells, StAR was undetectable or present at very low levels between 0 and 12 h post-hCG, but the transcript increased between 24 and 39 h (Fig. 5A). In contrast, levels of StAR mRNA in theca interna were high prior to hCG injection (0 h), remained relatively constant until 33 h post hCG, and then dropped dramatically at 36 and 39 h post-hCG (Fig. 6A). Uniform RNA loading in all Northern blots was confirmed by hybridization with a cDNA encoding the rat elongation factor Tu (Figs. 4B, 5B and 6B).

To provide a quantitative estimate of the relative changes in StAR mRNA during the gonadotropin-induced luteinization process, Northern blots were prepared from follicle wall, theca interna and granulosa cells of four separate preovulatory follicles isolated at each time point between 0 and 39 h post-hCG, and results were quantified by densitometric analyses. No significant changes were observed in the relative levels of StAR transcripts in follicle wall (Fig. 7, panel A). However, this apparent constant expression of follicular StAR concealed a significant increase in message levels in the granulosa cell layer, first detected at 30 h post-hCG (panel B, Fig. 7), and a significant drop in StAR transcripts in the theca interna layer at 36 and 39 h post-hCG (panel C, Fig. 7).

4.4.4. Follicular fluid concentrations of progesterone

Concentrations of progesterone were measured in follicular fluid of preovulatory follicles isolated between 0 and 39 post-hCG to assess whether the regulation of StAR mRNA in granulosa and theca interna related to changes in progesterone biosynthesis. Progesterone levels were relatively low in follicles isolated prior to gonadotropin treatment (0 h post-hCG; 53.6 ± 21.1 ng/ml), and remained unchanged at 12 and 24 h (Fig. 8). However, follicular fluid concentrations of progesterone were significantly increased at 30 h and reached maximal levels at 39 h post-hCG (783.0 ± 246.1 ng/ml).

4.5. DISCUSSION

Previous reports have used the eCG/hCG primed immature rat model to study the regulation of StAR in follicles *in vivo* (17, 33), and cultures of granulosa cells to investigate *in vitro* some of the molecular mechanisms of StAR expression (16, 27, 29, 33-36, 47). This study is the first to document the precise time-course and cell-specific regulation of StAR mRNA *in vivo* in preovulatory follicles of a large monoovulatory species, and of sexually mature animals. The mare was selected as an animal model for several reasons: the preovulatory follicle reaches a relatively large size (40-45 mm in diameter), its development can be precisely monitored *in vivo* by ultrasound imaging, and the process of equine follicular recruitment, selection and dominance is similar to that of other species (40, 48). Results provide evidence for a novel inverse regulation of StAR transcripts in theca interna and granulosa cells of equine preovulatory follicles in response to an ovulatory dose of hCG. Also, the study reports for the first time the

cloning of equine StAR from which the complete primary structure of the StAR protein is deduced.

Comparative analyses underscore the highly conserved nature of StAR across species, with the amino acid sequence of the equine protein being more than 86% identical to that of other species (12, 13). However, although the equine protein appears to contain a putative mitochondrial transit peptide within the first 55 amino acids, it does not have the consensus motif for mitochondrial two-step cleavage identified in mouse StAR (8). This divergence from the murine sequence is also observed in human (14), bovine (15) and porcine StAR (21), and argues against a critical role for this region in StAR action. Indeed, mounting evidence in the mouse clearly show that the steroidogenic action of StAR rather involves C-terminal domains (49, 50). The activity of the protein appears to lie outside of the mitochondria, and mitochondrial import is not required for StAR action (49, 50). Computer analysis of the equine StAR amino acid sequence also revealed several potential phosphorylation sites that could modulate the activity of the protein. While the functional role of each site remains to be established, results indicate that the potential phosphorylation site located at serine 195, shown in human and mouse to regulate StAR activity (51), is conserved in the equine protein.

Northern blot analyses revealed the presence of one major StAR transcript of about 3.0 kb in follicular extracts, and a minor band of about 1.8 kb in a few samples. The finding of multiple equine StAR transcripts is in agreement with results in other species. Three mRNAs have been observed in mice and rats, including two major bands of 3.4 and 1.6 kb (17, 52). One major transcript of 1.6 kb and two minor mRNAs of 4.4 and 7.5 kb have been reported in human (14, 53), two transcripts of 3.0 ad 1.8 kb were

detected in bovine tissues (15, 21, 34, 53), and up to three transcripts have been reported in the pig (21, 34). Only one StAR mRNA of 2.8 kb has been observed in sheep (20). Our cloning results suggest that differences in length of transcripts is attributable to variations in the 3'-untranslated region, as the short form equine StAR measuring 1.6 kb appeared derived from an internal polyadenylation signal (5'-AATAAA-3') located 22 bp from the end of the clone. However, the functional significance, if any, of multiple StAR transcripts remains unknown.

One key finding of the present study is the reciprocal regulation by gonadotropins of StAR transcripts in theca interna and granulosa cells of equine preovulatory follicles. This result clearly highlights the importance of defining the contribution of each steroidogenic cell type, as Northern blots from whole follicular wall extracts could have erroneously lead to the conclusion that hCG had no effect on StAR expression in equine follicles. The pattern of induction of StAR mRNA in equine granulosa cells compares with that observed in the immature rat model after hCG administration *in vivo* (17, 33), and with the ability of agonists of the protein kinase A pathway to upregulate StAR expression in granulosa cells *in vitro* (16, 27, 29, 33-36). Also, absence of the transcript in equine granulosa cells isolated prior to gonadotropin treatment is consistent with results obtained in cattle (54). Interestingly, the increase in steady state levels of StAR mRNA by hCG paralleled a significant rise in follicular fluid concentrations of progesterone, suggesting a link between StAR expression in granulosa cells and the onset of follicular luteinization *in vivo*. A more precise understanding of its relative role in the equine follicle should result from further studies on the characterization and gonadotropin-regulation of key enzymes involved in equine

follicular steroidogenesis. However, a relationship between StAR and steroid hormone production has been clearly established in various systems (11, 14, 23).

In contrast to granulosa cells, high levels of StAR mRNA were observed in theca interna of equine preovulatory follicles isolated prior to hCG. This observation is not surprising, considering the hypertrophied and highly steroidogenic appearance of the theca interna layer in equine preovulatory follicles isolated during early estrus, as characterized under light microscopy (55). Also, elevated levels of StAR transcripts have been reported in theca interna of large follicles in rats (17, 33), cows (54) and humans (29, 30). However, whereas StAR mRNA remained relatively constant until 33 h post-hCG, a dramatic loss in the transcript occurred thereafter in theca interna. This loss is cell-type specific since a concomitant increase of StAR is observed in the neighboring granulosa cell layer. To our knowledge, this is the first time that such a reciprocal regulation of StAR mRNA is simultaneously observed in distinct cellular compartments of ovarian follicles, or of any other steroidogenic tissue. The loss of StAR transcript in equine theca interna occurred 6-9 h prior to the expected time of ovulation (37). While the biological significance of the loss of StAR transcript remains to be precisely established, we believe that it could represent a first biochemical consequence of the degenerative process undergone by theca interna of equine follicles just prior to ovulation (55, 56). Indeed, Van Niekerk *et al.* (55) showed that, in contrast to other species, the theca interna degenerates at the time of ovulation in mares, and therefore does not contribute to the formation of the corpus luteum. Interestingly, the abrupt disappearance of StAR between 33 and 36 h post-hCG could suggest a timing for

the onset of the degenerative process, thereby providing a paradigm to study its molecular regulation.

In summary, this study describes for the first time the primary structure of equine StAR, and reports the cloning of two transcripts that differ primarily in the length of the 3'-untranslated region. The equine protein is composed of 285 amino acids, and its sequence is highly homologous to that of other species. The gonadotropin-dependent and cell-specific regulation of StAR mRNA *in vivo* was studied in a series of preovulatory follicles isolated before and after hCG treatment. Results revealed a unique inverse regulation of StAR mRNA in equine follicular cells, with hCG causing an induction of StAR transcripts in granulosa cells and the disappearance of the message in theca interna. While these changes are believed to relate to the luteinization of granulosa cells and the degeneration of theca interna prior to ovulation in mares, future studies are needed to better understand the role of StAR during equine follicular steroidogenesis.

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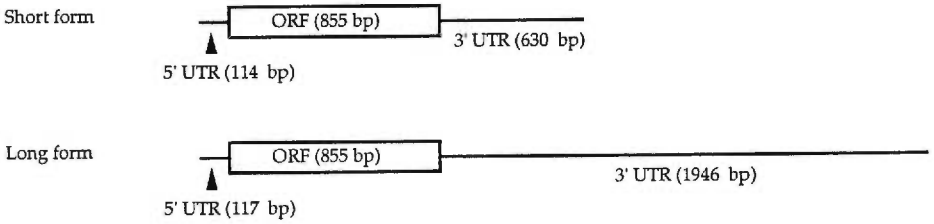
Fig. 1. Primary structure of equine StAR cDNA. *A*, Schematic representation of two forms of equine StAR; the short and the long form differ in the length of their 3'-UTR. *B*, Complete nucleotide sequence of the equine StAR long form obtained from clone 1-2 as described in *Materials and Methods*. The ORF is indicated by uppercase letters, the translation initiation (ATG) and stop (TAA) codons are highlighted in bold, the 5'-UTR and 3'-UTR are shown in lowercase letters, and numbers on the left refer to the first nucleotide on that line. The first (c) and last nucleotide (c) of the equine StAR short form cDNA are underlined and bolded. Nucleotide sequences were submitted to GenBank (accession numbers AF031696, AF031697).

A



B

```
1       ggagccagag gcagatcctg acaggctcag gaggtctgga agaagctccc cttgagagta ggagcaatag cagcagcagc aacagcactg ctacatttgc
101     acccgcggct ggaaacgATG CTCCTCGCGA CGTTTAAGCT GTGCGCTGGG AGCTCCTACA GACATGTGCG CAATATGAAG GGGCTGAGGC ACCAAGCTGC
201     CTTGGCCATT GGCCAGGAGC TGAACTGGCG GGCACCTGGG GGCCCGACCC AGAGTGGGTG GATCAACCAG GTTCGGCGTC AGAGCTCTCT GCTTGGCTCT
301     CAGCTAGAAG ACACTCTCTA CAGCGACCAG GAGCTGGCCT ATATCCAGCA GGGAGAGGAG GCAATGCAGA AGGCCCTGGG CATCCTCCGC AACCAGGAGG
401     GCTGGAAGGA GGAGAACCAG CAGGCAAACG GGGACAAAGT GCTGAGTAAA GTGGTCCCAG ACGTGGGCAA GGTATTCCGG CTGGAGGTGG AGGTGGACCA
501     GCCCATGGAG AGGCTTTATG AAGAGCTTGT GGAACGCATG GAGGCCATGG GAGAGTGGAA CCCAAATGTC AAGGAGATCA AGGTCCTGCA GAAGATTGGA
601     AAAGATACCG TCATCACCCA TGAGTTGGCT GCAGAATCAG CAGGAAACCT TGTGGGGCCC CGAGACTTTG TGAGTGTGCG TTGTGCCAAG CGCCGAGGCT
701     CCACCTGTGT GTTGGCTGGC ATGGCCACAC AGTTTGAGGA GATGCCCGAG CAGAAAGGTG TCATCAGAGC TGAGCATGGT CCCACTTGTA TGGTGCTCCA
801     TCCCCTGGCT GGAAGTCCCT CAAAGACCAA ACTCACTTGG CTGCTCAGCA TTGACCTCAA GGGATGGCTG CCAAAGACTA TCATCAACCA GGTCCTTTCG
901     CAGACCCAGG TGGATTTTGC CAACCATCTG CGCAAGCGCC TGGAGTCCAG CCCTGCTCCT GAAGCCAGGT GTTAAagacc aacttgctgc tcccaactgt
1001    tcctagctgc attggcttgc atgctcatca ggaaaatccc tgccagaagc ctcaacatct gtttaagatc tttatctgag gatagtgtga tggggtggta
1101    gtatgtttag agtatggtaa taggactcag actggtaaaa ttttactacc aagaaaatgg ggacaaggct cttagaagaa acttctagct tcttcactga
1201    ttagctacga aatgaaggtt aagggtccca aaatatttgt aaaacttttt cctgggtcta atgtctacct gaaaacatct taaaaatgct actggctgat
1301    acggggtgca gaggtgctaa atacaaggac tgggacctca tgctttacgg gctcaagagc tccattctct gcaggcagtg tgtatggaca ttcagggtct
1401    tacaaagggg ctccggcaac cctcccctcc tacaccaagt aggtagagag ctgctccact ggacaagcaa ctccccacag caggtgcctg ctaaagcct
1501    agtccaagtt ttcttgatga aaaagtacaa aactaattat tagactgctt cccttacttc ttagacgagc aagtcagaat aaagaatcat aactaacaga
1601    caaacttcag cctgtgcatg ttttaaaatt ctgctcctag aaaaaaaaaa tctagtaaat tgcaactgca tactaaagga gtcctggaca aagattttaa
1701    ttaaactaaa tcccctagct cattaaaagg aaaaactact gggtagttat gtcattcgac tagaatcagt taccagaatc tttcataaaa atttaatttc
1801    cacgatatgc acaagccttc atcagttctt aaactctaca actggtaaca tttcaagatg aagattatga ctgaggattt ttatttacct aattaaaagg
1901    aaggacagta ccaaatatga gagaacgaac ggcattacta agataaaaac aaattttatt ttcccgtagt gattgtttag acctaaaaat ggaactgaat
2001    ctttagttca gattctatta tatcagcttt cagcttggct tgggctgcca ctttatctgc ttagtaggtc agtgttttac tattttgagg agatggctgg
2101    aagaaggggt aatatggtgt tagcaaatcc ctttccaaga ttcagagact ttttgctttg aaaatataaa tcgggatcgg attattttgt taaaatgtgc
2201    aagtaggatg aaaaaccaac attaccactt gaagcttttc cattttagtt ctaagagcat tatgaacata atttggaaac atagctctag cttattaaac
2301    aaattagcag tagtgggaat gctcatgttt actgaggtat tgaaatttta caattggtag aggtattatt atttattctg attgtttgtt agaatagtct
2401    atttttggca tagatcaaac tgtttatctt tccaggagct tcagtgtgag agagagagct gtagaggtgt ttctagaaca gaattaccat ccactcccag
2501    ctaagaaaca tggtcacttg tttgaagatg ggtaaagcaa tacatactct ggtttcaaaa gtaaagaaat ggtctttcaa cacacaccat ggttttgagc
2601    tcaattataa tttgccttct tcaaactacg cactgtgtct ttggggtgac tggagagata ttttcccact tgtttaattt catattggaa taaaataagt
2701    tgtcactgat gatctgttta aagaaaccat tttatacagg actgtatggg gcgtggatat tctttgagcc cttcaaatga acttctcaaa agacagacac
2801    ttcgaatatg aagtataaag aggcaaaatc attatttctg taacctctaa tttatgaaag ccaaatgtac aaaaacctaa ttttatcatt caaattaaat
2901    gagtgtacaa gttaaaaa
```

Fig. 2. Deduced amino acid sequence of equine (equ) StAR and comparison with human (hum), pig, bovine (bov), mouse (mou), rat, hamster (ham) and ovine (ovi) homologs. Ovine StAR has not been fully characterized and only a partial sequence is shown. Identical residues are indicated by a printed period. Potential phosphorylation sites for cAMP- cGMP-dependent protein kinase (**A**), protein kinase C (**B**) and casein kinase II (**C**) are highlighted in bold uppercase letters.

```
          B         B
equ   MLLATFKLCA GSSYRHVRNM KGLRHQAALA IGQELNWRAP GAPTQSGWIN    50
hum   .......... ......M... ....Q..... .S.......L ....P.T...    50
pig   .......... .......... .......... L.........L ....SGS...    50
bov   .......... .......S.. ...QQ..... ..........L ...APAA...    50
mou   .F........ ......M... .......... ......W..L .DSSPG-.MG    49
rat   .F........ ......M... .......V.. ......R..L .D.SPG-.MG    49
ham   .......... ......L... .......... ..........L .DSSPG-..S    49
ovi

          A     C       C
equ   QVRRQSSLLG SQLEDTLYSD QELAYIQQGE EAMQKALGIL RNQEGWKEEN   100
hum   ....R..... .R..E..... .....L.... .......... S......K.S   100
pig   ....R..... ......F... .D........ ....R..D.. S......K.S   100
bov   ....RG.... .....P.... ....H..... ....R..... KD.....K.S   100
mou   ....R..... ....A..... ...S...... V......... N......K.S    99
rat   ....R..... ....A..... ...S...... .......... N......K.S    99
ham   ....R..... ....AA...E ...S...... V......... S......K..    99
ovi                                                      .K..

equ   QQANGDKVLS KVVPDVGKVF RLEVEVDQPM ERLYEELVER MEAMGEWNPN   150
hum   ..D.....M. .......... ....V..... .......... ..........   150
pig   R.E...E... ..I....... ....V..... .......... .........S   150
bov   R.....E... ..I....... ....V..... .......... ..........   150
mou   ..E...E... .M........ ....V..... D.......D. ..........   149
rat   ..E...E... ....G..... ...LVL.... D.......D. ..........   149
ham   ..E...E... .......... ....V..... D...A...D. ..........   149
ovi   R.....E... ..I....... ....V..... .......... .........S
                                              B         A
equ   VKEIKVLQKI GKDTVITHEL AAESAGNLVG PRDFVSVRCA KRRGSTCVLA   200
hum   .......... ....F..... ...A...... .......... ..........   200
pig   .....I.... .......... ...A...... .......G.T .....V....   200
bov   .......... .......... ...V...... .........T .....M....   200
mou   ........R. .......... ..AA...... .........T ..........   199
rat   .......K.. .......... R.AA...... .........T ..........   199
ham   ........K. .......... ..AA...... .......... ..........   199
ovi   .......... ....I..... ...A...... .....R...T .....M....
        C
equ   GMATQFEEMP EQKGVIRAEH GPTCMVLHPL AGSPSKTKLT WLLSIDLKGW   250
hum   ....D.GN.. .......... .......... .......... ..........   250
pig   ....D.G... .......... .......... .......... ..........   250
bov   ....LY.... Q......... .......... .......... ..........   250
mou   ....H.G... ..S....... .......... .......... ..........   249
rat   ....H.G... ..S....... .......... .......... ..........   249
ham   ....H.G... ..S......Q .......... ........F. ..........   249
ovi   .T.TLY.... Q......

                C
equ   LPKTIINQVL SQTQVDFANH LRKRLESSPA PEARC    285
hum   ...S...... .......... .......H.. S....    285
pig   .......... .......... .......R.. L....    285
bov   .......... .......... .......C.. L....    285
mou   .......... ....IE.... ......A... S..Q.    284
rat   .......... ....IE..S. .......... S..Q.    284
ham   ...S...... ....ME.... .......... S....    284
ovi
```

Fig. 3. Primer extension analysis of equine StAR mRNA. A labeled 30-mer antisense oligonucleotide complementary to the region from base pairs 46 to 75 of the StAR long form cDNA (Fig. 1) was hybridized to RNA samples containing (corpus luteum) and not containing (spleen) StAR, and primer extension was performed as described in *Materials and Methods*. Reactions were analyzed on a 6% polyacrylamide gel and the size of the extended product was determined by comparison with the products of an unrelated sequencing reaction shown on the left. Results showed a 85-nucleotide extension product corresponding to a major transcription initiation site. No extension product was detected with RNA isolated from spleen (negative control).

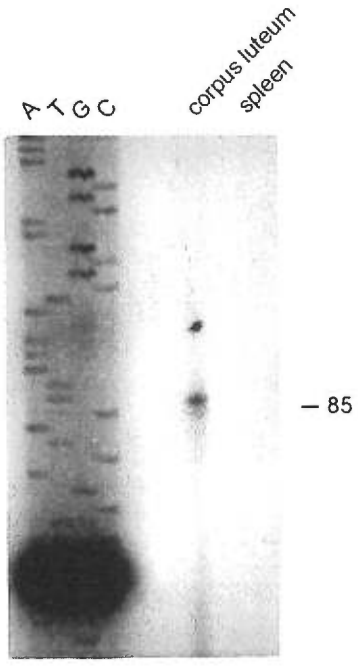

A T G C
corpus luteum
spleen
— 85

Fig. 4. Regulation of StAR mRNA by hCG in equine preovulatory follicles. Preparations of follicle wall (theca interna with attached granulosa cells) were obtained from preovulatory follicles isolated 0, 12, 24, 30, 33, 36 and 39 h after hCG, as described in *Materials and Methods*. In addition, two corpora lutea (CL) were isolated on day 8 of the estrous cycle. Samples of total RNA (10 μg/lane; 2 follicles/time point) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (*panel A*). The same blot was stripped of radioactivity and hybridized with a cDNA encoding the rat elongation factor Tu (*EFTu*) as a control gene for RNA loading (*panel B*). *Brackets* on the left show migration of 28S and 18S ribosomal bands, and markers on the right indicate migration of RNA standards. Filters in *panels A* and *B* were exposed to film at -70 C for 4 and 2 h, respectively.

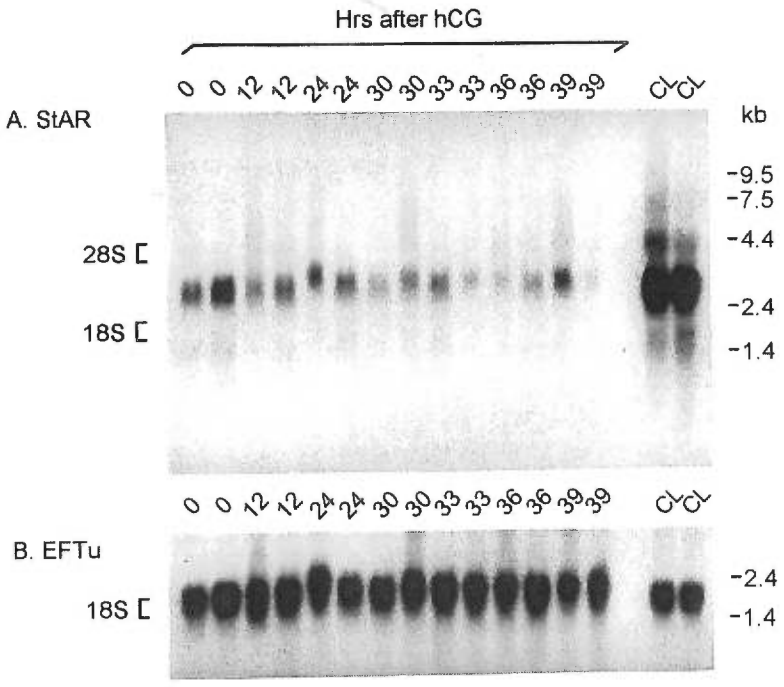

Hrs after hCG
0 0 12 12 24 24 30 30 33 33 36 36 39 39 CL CL
A. StAR
kb
-9.5
-7.5
-4.4
-2.4
-1.4
28S
18S
0 0 12 12 24 24 30 30 33 33 36 36 39 39 CL CL
B. EFTu
18S
-2.4
-1.4

Fig. 5. Regulation of StAR mRNA by hCG in granulosa cells of equine preovulatory follicles. Isolated preparations of granulosa cells were obtained from equine preovulatory follicles isolated between 0 and 39 h after hCG, as described in *Materials and Methods*. In addition, preparations of theca interna (TI, 0 h) and of a corpus luteum (CL, day 8 of cycle) were isolated. Samples of total RNA (10 μg/lane; n=2 follicles/time) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (*panel A*). The same blots were stripped of radioactivity and hybridized with a cDNA encoding the rat elongation factor Tu (*EFTu*) as a control gene for RNA loading (*panel B*). *Brackets* on the left show migration of 28S and 18S ribosomal bands, and markers on the right indicate migration of RNA standards. Filters in *panels A* and *B* were exposed to film at -70 C for 6 and 2 h, respectively.

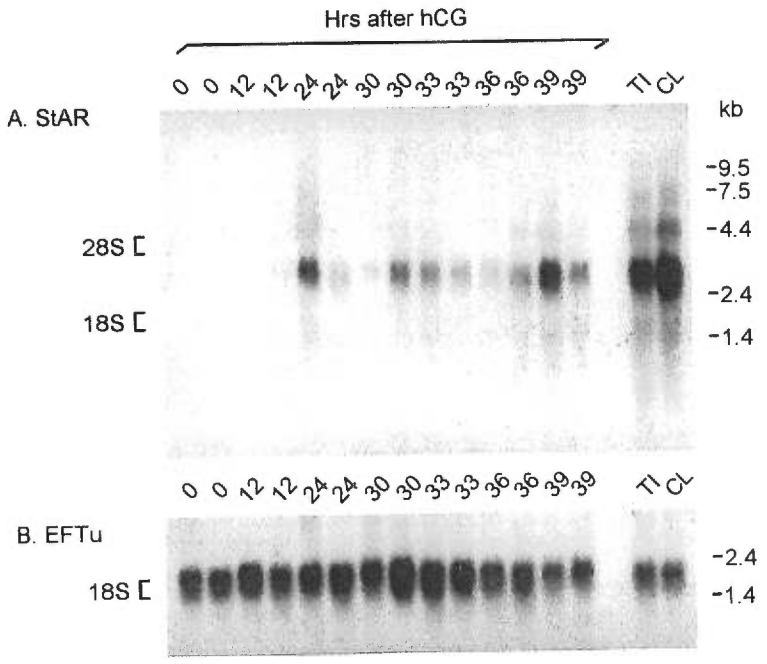

Hrs after hCG
0 0 12 12 24 24 30 30 33 33 36 36 39 39 TI CL
A. StAR
kb
-9.5
-7.5
-4.4
28S
-2.4
18S
-1.4
0 0 12 12 24 24 30 30 33 33 36 36 39 39 TI CL
B. EFTu
-2.4
18S
-1.4

Fig. 6. Regulation of StAR mRNA by hCG in theca interna of equine preovulatory follicles. Isolated preparations of theca interna were obtained from equine preovulatory follicles isolated between 0 and 39 h after hCG, as described in *Materials and Methods*. In addition, samples of granulosa cells (GC, 39 h) and of a corpus luteum (CL, day 8 of cycle) were isolated. Samples of total RNA (10 μg/lane; n=2 follicles/time) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (*panel A*). The same blots were stripped of radioactivity and hybridized with a cDNA encoding the rat elongation factor Tu (*EFTu*) as a control gene for RNA loading (*panel B*). *Brackets* on the left show migration of 28S and 18S ribosomal bands, and markers on the right indicate migration of RNA standards. Filters in *panels A* and *B* were exposed to film at -70 C for 4 and 2 h, respectively.

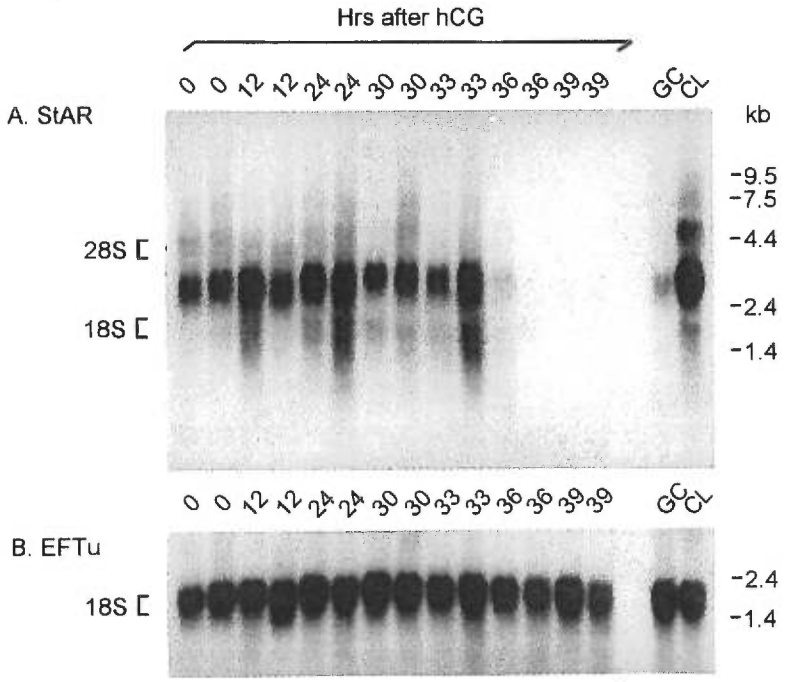

Hrs after hCG
0 0 12 12 24 24 30 30 33 33 36 36 39 39 GC CL
A. StAR
kb
-9.5
-7.5
-4.4
28S
-2.4
18S
-1.4
0 0 12 12 24 24 30 30 33 33 36 36 39 39 GC CL
B. EFTu
-2.4
18S
-1.4

Fig. 7. Relative changes of StAR mRNA levels in equine follicle cells isolated between 0 and 39 h after hCG treatment. Samples (n=10 μg) of total RNA extracted from follicle wall (*A*), granulosa cells (*B*) and theca interna (*C*) were analyzed by Northern blotting with the equine StAR cDNA, and subsequently with the rat elongation factor Tu (*EFTu*) cDNA as a control gene for RNA loading. After autoradiography (films not shown), the StAR signal intensity was quantified by densitometric analysis, and normalized with the control gene EFTu. Results are presented as StAR mRNA levels relative to EFTu (mean ± SEM; n=4 follicles/time point). Columns marked with an asterisk are significantly different ($P<0.05$) from 0 h post-hCG.

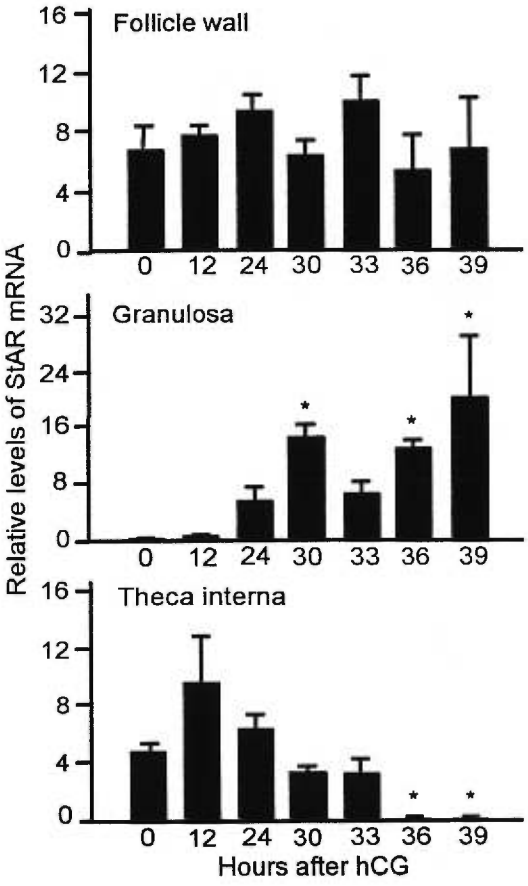
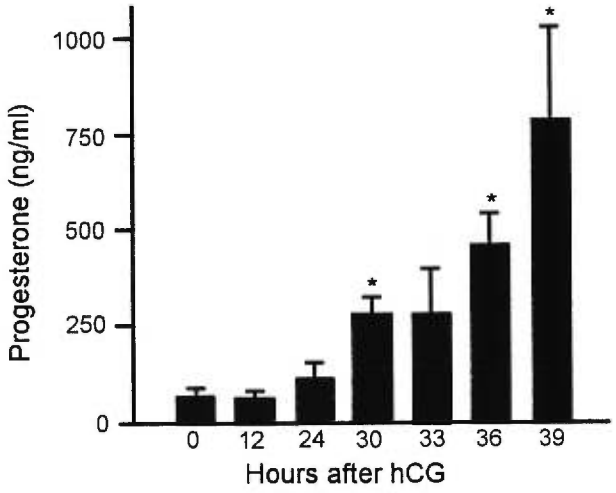

Follicle wall
Granulosa
Theca interna
Relative levels of StAR mRNA
Hours after hCG
0
12
24
30
33
36
39
4
8
16
32
*

Fig. 8. Follicular fluid concentrations of progesterone in equine preovulatory follicles. Preovulatory follicles were isolated between 0 and 39 h after hCG, and follicular fluid concentrations of progesterone were determined by specific RIAs. Results are shown as mean ± SEM (n = 5 follicles/time point, 0-30 h and 36 h after hCG; n = 6 follicles, 33 h after hCG; n = 3 follicles, 39 h post-hCG). Columns marked with an asterisk are significantly different ($P<0.05$) from 0 h post-hCG.


Progesterone (ng/ml)
1000
750
500
250
0
*
*
*
0
12
24
30
33
36
39
Hours after hCG

5. GENERAL DISCUSSION

This project describes, for the first time, the cellular and vascular changes in equine follicles during the ovulatory process. The administration of hCG induced marked changes in granulosa cells, including an expansion of the granulosa cell layer which was caused by the accumulation of acid mucosubstances between granulosa cells. Although loosening of the granulosa cells has been observed in follicles isolated just before ovulation in other species (Priedkalns et al., 1968; Parr, 1974; Cherney et al., 1978), the degree of expansion observed in equine follicles appears unique. The precise nature of these mucosubstances remains to be determined, but would likely include members of the glycosaminoglycan family such as hyaluronan, heparan sulfate and dermatan sulfate (Jackson et al., 1991; Chen et al., 1993; Tirone et al., 1997).

Induction of ovulation by hCG in equine preovulatory follicles caused profound changes in the morphology of the theca layers. These changes include a marked thinning of the interna layer and the detection of occasional pyknotic cells at 36 to 39 h after hCG. This finding is consistent with the observation made by Van Niekerk et al., (1975) that theca interna cells in equine follicles degenerate at the time of ovulation and do not contribute to the formation of the CL

Another dramatic finding in the equine theca interna layer is the infiltration of eosinophils during the ovulatory process. The infiltration of eosinophils near the time of ovulation has been observed in preovulatory follicles of pigs (Standaert et al., 1991) and sheep (Cavender and Murdoch, 1988). There seems to be species differences in the leukocyte subtypes attracted in preovulatory follicles, as macrophages and neutrophils are the major leukocyte population present in the wall of the preovulatory follicles in

rats (Brannstrom et al., 1993) and humans (Brannstrom et al., 1994). The function of these dramatic alterations in ovarian leukocyte populations remains largely unexplored. The infiltration of leukocytes in other parts of the body occurs under the influence of chemotactic factors (Norman and Brannstrom, 1994). Several chemotactic substances have been found in follicular fluid of equine, bovine, ovine and human (Herriot et al. 1986, Seow et al., 1988, Murdoch and McCormick 1989; 1993, Castilla et al., 1990; Watson et al., 1991; Runesson et al., 1996; Sirotkin and Luck, 1995).

A series of vascular changes was observed in theca interna and theca externa of equine preovulatory follicles after hCG-induced ovulation. These changes include an increase in numbers of blood vessels, and induction of hyperemia, vasodilation, oedema and hemorrhages. Histamine, bradykinin and products of the cyclooxygenase and lipoxygenase pathways are potent vasoactive agents believed to play an important role in the ovulatory process (Piacsek et al., 1971; Guraya and Dhanju, 1992; Murdoch et al., 1993; Priddy and Killick, 1993; Tsafriri et al., 1993). The follicular hyperemia and increased vascular permeability is likely to be initiated by histamine (Piacsek et al., 1971; Morikawa et al., 1981; Murdoch and Cavender, 1987), and propagated by prostaglandins (Espey, 1980; Murdoch and Cavender, 1987; Espey, 1994). Each agent causes vasodilatation and enhanced capillary/venule permeability by relaxing vascular smooth muscle (Espey, 1980; Kaley et al., 1985). LH stimulates PGHS-2 expression, which lead to synthesis of prostaglandins from arachidonate (Sirois et al., 1992; Sirois, 1994; Sirois and Doré, 1997; Boerboom and Sirois, 1998). The role of prostaglandins during the ovulation process was highlighted in recent gene targeting studies in which female mice deficient for PGHS-2 were infertile and failed to ovulate (Dinchuk et al.,

1995; Lim et al, 1997). In the mare, PGHS-2 mRNA and protein are induced in granulosa cells about 10 h prior to ovulation (Sirois and Doré, 1997; Boerboom and Sirois, 1998).

This study also characterized the primary structure of equine StAR, and identified two isoforms of StAR that differ only in their 3'-UTR. The ORF encodes a 285 amino acid protein that is more than 86% identical to StAR of other species. In contrast to the murine StAR sequence (Clark et al., 1994), the equine StAR sequence has only one mitochondrial targeting sequence within the first 55 amino acids, and does not have the consensus motif for mitochondrial two-step cleavage. This divergence was also observed in humans (Sugawara, et al., 1995), cows (Hartung et al., 1995), and pigs (Pilon et al., 1997). Interestingly, recent studies showed that the activity of the StAR protein lies outside of the mitochondria, and is independent of mitochondrial import (Arakane et al., 1996; Arakane et al., 1998). The putative site for cAMP phosphorylation at S-195 is conserved in equine StAR. This site plays an important role in StAR activity, as a mutation at S-195 resulted in 50% reduction in pregnenolone production (Arakane et al., 1997).

Northern blot analysis revealed the presence of two StAR mRNAs, a major band of 3.0 kb and a minor band of 1.6 kb. The presence of multiple transcripts have been observed in other species. Four StAR transcripts have been observed in hamsters (Fleary, et al., 1997). Three StAR mRNAs have been reported in mice, including two major bands of 3.4 and 1.6 kb, and a minor band of 2.7 kb (Clark, et al., 1995). In humans, there is one major transcript of 1.6 kb and two minor transcripts of 4.4 and 7.5 kb (Sugawara et al., 1995; Gradi et al., 1995). In rats, two major bands of 3.4 and 1.6 kb,

and a less abundant transcript of 1.2 kb were observed (Sandhoff and McLean, 1996a). Two StAR mRNAs were reported in cows, a major band of 3.0 kb, and a minor band of 1.8 kb. (Hartunge et al., 1995). In pigs, a major hybridizing band of 1.8 kb and a fainter band of about 1.1 kb were reported in one study (Pilon et al., 1997), whereas a major band of 2.8 kb and two weaker bands of 1.6 and 0.8 kb were observed in another study (Pescador et al., 1997). Only one band of 2.8 kb of StAR mRNA was reported in sheep (Juengel et al., 1995). These differences in size of StAR transcripts are attributable to variations in the 3'-untranslated region. However, the functional significance of multiple messages for StAR is not known.

The regulation of StAR mRNA in vivo was studied in equine preovulatory follicles isolated during estrus after hCG-induced of ovulation. Results revealed the presence of an inverse regulation of StAR mRNA in granulosa and theca interna cells prior to ovulation. In the follicular wall (theca interna with attached granulosa cells), levels of StAR transcripts remained unchanged throughout the ovulatory process, giving the impression that StAR was not regulated prior to ovulation. However, northern blots preformed on isolated follicular cells revealed an unique inverse regulation of StAR mRNA in granulosa cells (upregulation) and theca interna (down regulation). The induction of StAR in granulosa cells has been observed after gonadotropin treatment in vivo (Sandhoff and McLean, 1996; Soumano and Price, 1997; Ronen-Fuhrmann et al., 1998), and after stimulation with agonists the protein kinase A pathway in vitro (Selvaraj et al., 1996; Pescador et al., 1996, Kiriakidou et al., 1996; Pescador et al., 1997; Balasubramanian et al., 1997; Thompson et al., 1997; Ronen-Fuhrmann et al., 1998). In contrast to granulosa cells, high levels of StAR mRNA were observed in theca cells

isolated prior to hCG. Although high levels of StAR mRNA have been observed in theca interna of rats (Ronen-Fuhrmann et al., 1998; Sandhoff and McLean, 1996), cows (Soumano and Price, 1997), and humans (Kiriakidou et al., 1996), the loss StAR mRNA in equine preovulatory theca interna just prior to ovulation has not been reported in other species. The biological significance of the loss of the StAR transcript remains undetermined, but it could represent a first biochemical consequence of the degenerative process occurring in theca interna of equine follicles just before ovulation (Van Niekerk et al., 1975; Ginther, 1992)

6. GENERAL CONCLUSIONS

The objective of this project was to characterize some aspects of the final maturation of the equine preovulatory follicle. My conclusion are that

1. Dramatic cellular and vascular changes occur in equine follicles during gonadotropin-induced ovulation, including a marked expansion of the granulosa cell layer, a thinning of the theca interna layer, a massive migration of eosinophils into theca interna and theca externa, and a progressive increase in congestion, edema, and hemorrhage as ovulation approches.

2. Two forms of equine StAR transcripts have been cloned, a long form (2918 bp) and a short form (1599). The two forms differ only in the length of their 3'-UTR. The equine StAR has an ORF encoding a 285 amino acid protein that is more than 86% identical to StAR of other animal species.

3. Human CG induces a unique inverse regulation of StAR mRNA in theca interna and granulosa cells of equine preovulatory follicles. StAR mRNA was undetectable in granulosa cells isolated prior to hCG treatment, but the transcript was induced 24h post-hCG. In contrast, high levels of StAR mRNA were detected in the theca interna of equine preovulatory follicles isolated prior to hCG, but a dramatic decrease in StAR mRNA was observed 36h and 39 h post-hCG.

7. GENERAL REFFERNCES

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