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Overriding follicle selection in controlled ovarian stimulation protocols: Quality vs quantity

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Abstract

Selection of the species-specific number of follicles that will develop and ovulate during the ovarian cycle can be overridden by increasing the levels of pituitary gonadotropin hormones, FSH and LH. During controlled ovarian stimulation (COS) in nonhuman primates for assisted reproductive technology (ART) protocols, the method of choice (but not the only method) has been the administration of exogenous gonadotropins, either of nonprimate or primate origin. Due to species-specificity of the primate LH (but not FSH) receptor, COS with nonprimate (e.g., PMSG) hormones can be attributed to their FSH activity. Elevated levels of FSH alone will produce large antral follicles containing oocytes capable of fertilization in vitro (IVF). However, there is evidence that LH, probably in lesser amounts, increases the rate of follicular development, reduces heterogeneity of the antral follicle pool, and improves the viability and rate of pre-implantation development of IVF-produced embryos. Since an endogenous LH surge typically does not occur during COS cycles (especially when a GnRH antagonist is added), a large dose of an LH-like hormone (i.e., hCG) may be given to reinitiate meiosis and produce fertilizable oocytes. Alternate approaches using exogenous LH (or FSH), or GnRH agonist to induce an endogenous LH surge, have received lesser attention. Current protocols will routinely yield dozens of large follicles with fertilizable eggs. However, limitations include non/poor-responding animals, heterogeneity of follicles (and presumably oocytes) and subsequent short luteal phases (limiting embryo transfer in COS cycles). However, the most serious limitation to further improvements and expanded use of COS protocols for ART is the lack of availability of nonhuman primate gonadotropins. Human, and even more so, nonprimate gonadotropins are antigenic in monkeys, which limits the number of COS cycles to as few as 1 (PMSG) or 3 (recombinant hCG) protocols in macaques. Production and access to sufficient supplies of nonhuman primate FSH, LH and CG would overcome this major hurdle.

Review

In many primate species, ranging from humans to great apes to Old World monkeys, the endocrine and local interactions between and within components of the hypothalamic-pituitary-ovarian axis result in the selection and maturation of a single "dominant" follicle and its timely release of one oocyte capable of fertilization near the middle of the menstrual cycle (Fig. 1). Knowledge of the processes involved in the growth, selection, maturation, ovulation and luteinization of the primate follicle

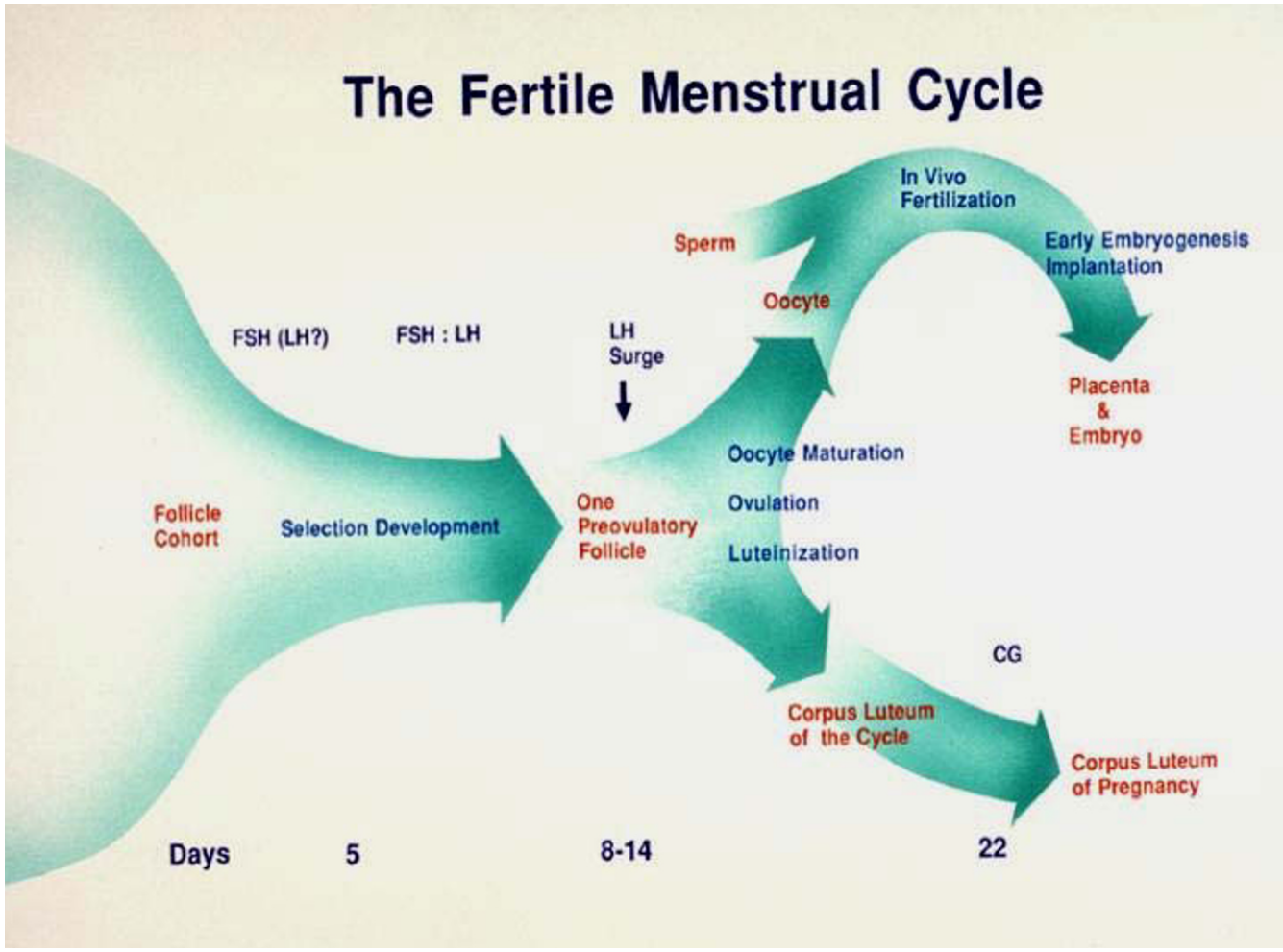

Figure 1
Diagram of the events occurring in the ovary and reproductive tract during the initial three weeks of the fertile menstrual cycle leading to natural reproduction in primates.

has increased substantially in recent years, particularly from experimental studies in macaque monkeys (for review, see [1]). The importance of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in follicular/oocyte development in the primate ovary was recognized almost 70 years ago [1,2], but recent efforts to experimentally manipulate gonadotropin support are providing new knowledge of the cellular processes controlled by FSH and LH (see preceding chapter, [3]). It is clear that methods which increase circulating levels of gonadotropins will override the usual mechanism that selects a single dominant follicle, and stimulate the development of multiple large antral follicles whose enclosed oocytes have the potential for procreation (Fig. 2).

A major factor in the development and application of ARTs to basic and applied aspects of primate reproduction was the use of controlled ovarian stimulation (COS) protocols. These COS cycles generate numerous large antral follicles and hence many oocytes that are available for such ART procedures as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), nuclear transfer (NT), and resultant embryos for transfer (ET) into the reproductive tract, in vitro culture and embryonic stem (ES) cell development, or for genetic evaluation and manipulation (see following chapters). The authors have addressed the development and use of COS protocols in ART research in earlier reviews over the past decade [4-6]. This chapter will review the current status of the field, with particular emphasis on the limitations and controversies associated with follicular stimulation protocols.

Follicular stimulation protocols

In theory, methods which increase the levels of endogenous gonadotropic hormones or administer exogenous gonadotropins should stimulate multiple follicular growth in primates. The former approach is used clinically

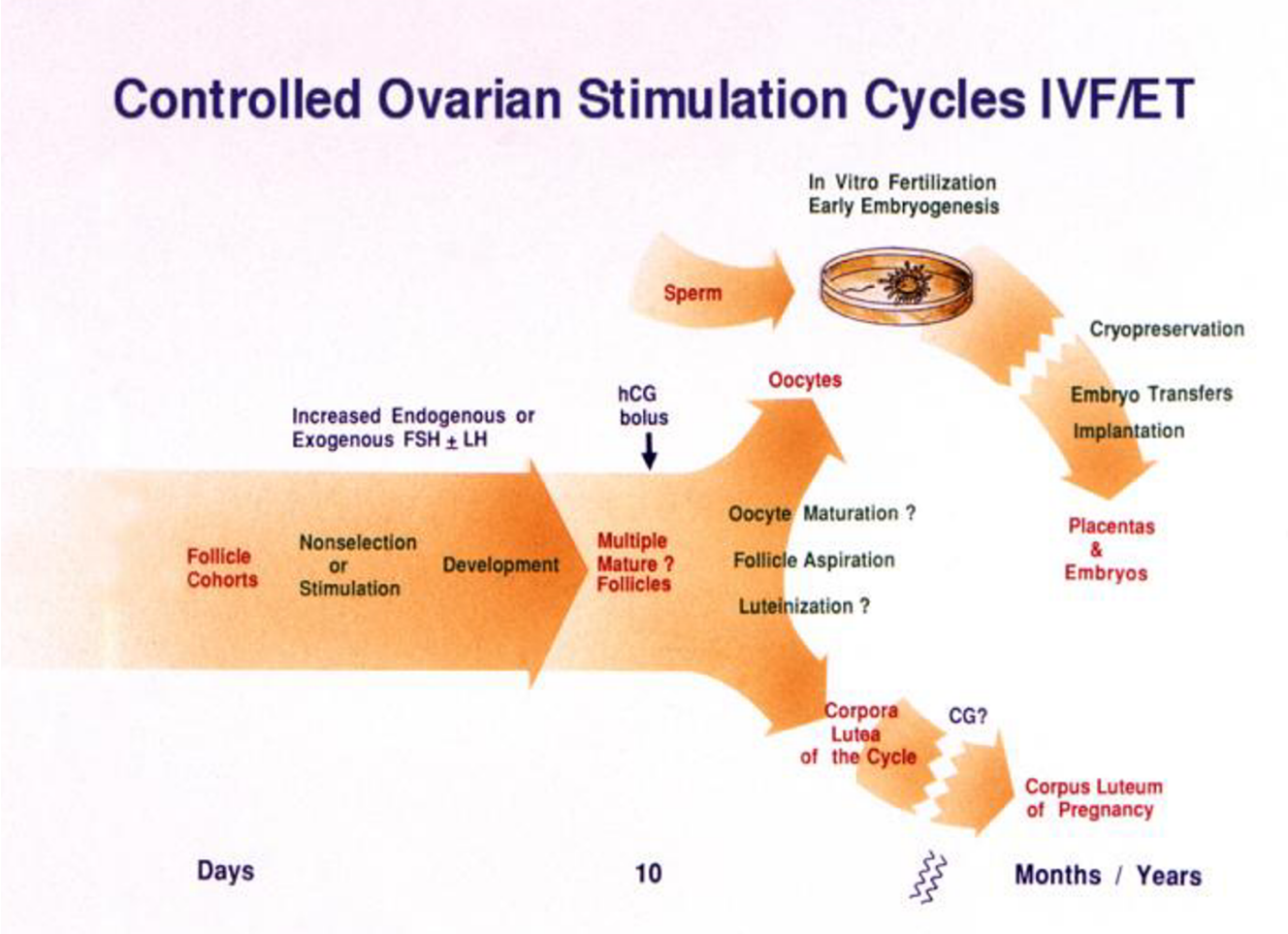

Figure 2

Diagram of events occurring in the ovary and in vitro during controlled ovarian stimulation cycles leading to assisted reproduction in primates. This chapter will discuss the methods and their limitations for increasing circulating levels of gonadotropins (FSH, LH, CG) to override the typical selection and maturation of a single "dominant" follicle in the natural menstrual cycle, thereby stimulating the development and maturation of multiple large follicles whose oocytes can be collected for in vitro manipulation (e.g., in vitro fertilization, IVF) prior to return to the reproductive tract (embryo transfer, ET) for pregnancy initiation.

in women, wherein an anti-estrogen (e.g., clomiphene [7]) or, more recently, an aromatase inhibitor (i.e., letrozole [8]) is administered to antagonize or eliminate estrogen's negative feedback control of pituitary gonadotropin secretion, thereby raising endogenous FSH and LH levels. Although clinically successful in ovulation induction (few follicles) and COS (many follicles) cycles, this approach is rarely used in nonhuman primates (NHPs, e.g., [9]) except to consider the possible local role(s) of estrogen in the primate follicle. Estrogen is believed to promote FSH-stimulated folliculogenesis in some species, notably rodents [10,11], but there is considerable controversy regarding its actions, if any, in the primate follicle [12]. The reported lack of estrogen receptor (ER)-α in primate follicles supported a minimal role, but the subsequent discovery of the ER-β isoform [13] and its presence in primate follicles has renewed this controversy [14,15]. Limited studies employing steroid (including selective estrogen) ablation during gonadotropin-stimulated antral follicle development suggest that oocyte maturation and fertilizability could be suboptimal in rhesus monkeys [12], but this has not been rigorously addressed in any NHP species or women.

Because of the greater potential for supraphysiologic response (higher gonadotropin levels and larger follicle numbers), investigators have preferred to administer exogenous gonadotropins, either of nonprimate or primate origin. Following the discovery of two distinct pituitary gonadotropins in the 1940's, the efforts of van Wagenen [16] and Knobil [17] demonstrated that follicular growth and ovulation could be stimulated in intact

and hypophysectomized monkeys, respectively, using purified preparations of macaque gonadotropins. Nevertheless, because of more general availability, investigators also initially used nonprimate gonadotropins, typically but not exclusively, pregnant mare serum gonadotropin, which resulted in 1984 in the first rhesus monkey infant born after COS, follicle aspiration, IVF and ET [18]. Indeed, investigators around the world continue to use PMSG, now termed equine chorionic gonadotropin (eCG) for COS protocols in NHPs, such as African green monkeys [19].

With the emergence of clinical ART programs, investigators began to use commercially available preparations of human gonadotropins, initially urinary preparations, such as human menopausal gonadotropin (hMG; containing both FSH and LH) and a more purified preparation of hFSH. With the advent of recombinant (r) DNA technology in the mid-1990s, pure r-hFSH (devoid of LH activity) and r-hLH (devoid of FSH activity) became available for testing in rhesus macaques, and is now the preparation(s) of choice for many physicians treating infertile women in ART clinics. However, a standard or optimal protocol of human gonadotropins has not emerged from clinical protocols in women, or from COS procedures in any NHP species. In macaque species, for example, investigators have employed gonadotropin regimens of hFSH alone [20-22], a combined treatment of hFSH plus hLH [23], and a sequential protocol of hFSH alone followed by an interval of hFSH plus hLH [24-26]. Despite the issues described in subsequent sections, these protocols can successfully generate multiple large antral follicles with fertilizable oocytes both in adult primates and, more recently, in prepubertal monkeys [6,27]. The latter is analogous to the immature, PMSG-treated rodent model that is extensively used in basic and applied research [28,29].

It is noteworthy that a timely LH surge of normal magnitude and duration does not usually occur during COS protocols, presumably due to the supraphysiologic levels of estrogen having a predominantly negative-, rather than positive-, feedback effect at midcycle [5]. Indeed, if one unexpectedly occurs, oocyte collection is usually disrupted or cancelled (see subsequent section). Oocytes from FSH/LH-stimulated follicles may be collected at the immature (germinal vesicle or GV-intact) stage for attempts at in vitro maturation (IVM [20,30]). However, generally, the actions of the LH surge, notably resumption of meiosis to generate a metaphase II oocyte capable of fertilization, are mimicked by administering a bolus of the LH-like hormone, human chorionic gonadotropin (hCG). Typically, urinary preparations of hCG were employed [18,31], but more recently, pure r-hCG became available for inducing ovulation events in women and NHPs [21,32].

Preparations of hCG have been the hormone of choice, particularly because of its general availability and much longer half-life than hLH; hence, one injection is sufficient to maintain surge levels over a 27–36 hr interval to collect a large percentage of maturing (metaphase I or II) oocytes by follicle aspiration prior to ovulation [32]. However, it is possible to produce surge levels of endogenous or exogenous LH for various intervals in women and NHPs by administering either a gonadotropin releasing hormone (GnRH) agonist or urinary/recombinant hLH [33,34]. Although GnRH agonists are used successfully in some clinical ART programs, and may be indicated in some patients at risk for developing ovarian hyperstimulation syndrome (OHSS [35]), macaque species appear less sensitive to such GnRH regimens. Up to 3 injections of GnRH or a GnRH agonist only produced a short LH surge of ≤ 14 hrs and was insufficient to reinitiate meiotic maturation of oocytes [33]. In contrast, one injection of hLH produced LH surges of approximately 18–24 hrs that reinitiated oocyte development, but failed to sustain the development and/or function of the macaque corpus luteum. Only after two injections of hLH were administered at 18 hr intervals did one achieve surge levels of LH for 36–48 hrs accompanied by oocyte maturation and corpus luteum development/function comparable to that observed in hCG-treated animals [34]. Although these and other [36] studies are providing needed information on the strength-duration requirements for ovulatory processes in primate follicles, COS regimens attempting to induce an endogenous LH surge or providing exogenous LH as an ovulatory stimulus have been rare in NHPs [37].

A standardized regimen of human gonadotropins has not evolved, but treatment generally begins in the early follicular phase (prior to natural selection of the dominant follicle, which occurs as early as day 5 of the menstrual cycle in macaques and women) and continues for 6–11 days. At ONPRC, the authors currently employ the following regimen for COS cycles after comparing three different protocols in rhesus monkeys [38]. Beginning around menses, adult, cycling females receive twice daily IM injections of 30 IU r-hFSH for 6 days, followed by 30 IU r-hFSH and r-hLH for 3 days. On day 10, the animals then receive a single IM injection of 1000 IU r-hCG to induce ovulatory events. Although ovulatory follicles develop, aspiration by laparoscopy is typically performed ≥ 27 hr after hCG injection to retrieve maturing (M I and II) oocytes before follicle rupture. This regimen can be individualized per animal, based on criteria for desired numbers/size of antral follicles and circulating estrogen levels, by varying the interval of FSH + LH exposure [5]. However, this requires labor-intensive efforts to regularly perform transabdominal ultrasonography and rapid estradiol assays, usually daily from day 7 of treatment. Also, based

on their effectiveness and reversibility in macaques, a GnRH agonist [22,23] or antagonist [32,39] can be administered concomitantly throughout or during the last part of the gonadotropin stimulation protocol to assure prevention of an endogenous LH surge (see later section).

It should be noted that the functional luteal phase that follows the exogenous gonadotropin treatment (FSH ± LH, followed by hCG) in COS cycles is abnormal as noted in clinical ART [40] and NHP [32,41] protocols. Although circulating progesterone levels are often supraphysiologic, due to the presence of multiple luteinized follicles/corpora lutea, the length of the luteal phase is typically shortened. This is likely due to the suppression of circulating pituitary LH levels by the supraphysiologic levels of ovarian steroids and/or the residual action of GnRH analogs administered during multiple follicular development [40]. Thus, once the circulating levels of administered hCG decline to baseline, luteotropic support for luteal structure-function is lost, progesterone secretion declines and early menstruation results [41]. Clinically, luteal phase support in the form of progesterone supplements is the method of choice to allow embryo transfer in COS cycles [40]. However, embryo transfer during COS cycles in NHPs is not routine. The typical approach to date is to cryopreserve embryos and to transfer thawed embryos into monkeys (either the egg donor or a surrogate mother) during the luteal phase of a natural menstrual cycle [4,42]. This eliminates any potential problem during the luteal phase in COS cycles.

Major limitation – availability and antigenicity of gonadotropins

Availability of suitable gonadotropin preparations for follicular stimulation protocols is the most critical limitation to the use of ARTs in NHPs. Although nonprimate preparations, e.g., eCG, are readily available, these are by far the least desirable gonadotropins for two reasons: species-specificity of action and antigenicity. Following evidence of species specificity of growth hormone action in primates, Van Wagenen speculated from her experience that a similar species specificity applied to LH, but not FSH, action in the primate ovary (see review [2]). Subsequent studies appear to support this premise; e.g., primate LH and hCG were 500–1000 times more efficient than nonprimate gonadotropins in inhibiting ^{125}I-hLH binding to macaque LH-CG receptors, whereas all gonadotropins were equipotent for rodent LH receptors [43]. These results emphasize the need for primate gonadotropins, at least LH-CG, in studies in NHPs. Investigators should realize that any activity of nonprimate gonadotropins in NHPs is likely due solely to FSH in the preparations, unless very large quantities are used. However, their use especially in large amounts is further contraindicated by the antigenicity.

The gonadotropic hormones are species-unique glycoproteins that elicit production of neutralizing antibodies in NHPs. This is well-documented in macaques, where nonprimate gonadotropins can produce ovarian refractoriness to further gonadotropin therapy after one COS cycle [44]. Since human gonadotropins are more homologous to those of NHPs, one would expect a lesser immune response, but use of urinary preparations typically produced significant titers of anti-gonadotropin antibodies (as detected by protein A-precipitable ^{125}I-hCG in serum) and failure of further gonadotropin treatment to promote multiple follicular development after two COS cycles [45]. The use of recombinant human gonadotropins appears to delay the immune response, allowing three or more COS cycles per macaque before modest levels of anti-LH/CG and anti-FSH antibodies were detected. Table 1[46] summarizes evidence that following COS protocols employing r-hFSH; -hLH, and -hCG as described in our standardized regimen: (a) only a few animals (2 of 11) display borderline levels of antibodies (i.e., 4–9% of added ^{125}I-labeled hCG or FSH is antibody-bound) after **two** protocols, but (b) most animals (6 of 10) have borderline levels and a few monkeys have high (>10% of bound radioactive hCG or FSH) after **three** protocols. Due to hCG's longer half-life (resulting in continued albeit declining levels of hCG in the circulation for 7 days post-injection in COS cycles [41]), it appears that animals produce anti-hCG antibodies prior to anti-FSH antibodies. Therefore, COS protocols that eliminate the hCG bolus as the ovulatory stimulus, e.g., during oocyte collection for IVM, likely can be repeated more than three times in macaques. Since the antibodies generated by human gonadotropins during COS cycles do not disrupt normal menstrual cyclicity, fertility or successful pregnancy in macaques [45], it appears that these anti-gonadotropin antibodies do not neutralize endogenous pituitary or chorionic gonadotropins. Thus, these animals can still be valuable in the colony, e.g., as natural breeders or as ET recipients, even after elimination from further COS protocols due to generation of antibodies to nonprimate or human gonadotropins.

Clearly, the availability of nonhuman primate – especially macaque – gonadotropins would overcome the limited ability to perform repeated COS protocols and greatly facilitate experimentation. In the late 1980s, the National Institutes of Health contracted for the production of cynomolgus macaque FSH and LH by recombinant DNA technology. However, the small amounts generated serve primarily as antigen or reference hormone for gonadotropin assays (distributed by the U.S. National Hormone and Peptide Program). Although in rare instances they can be used for in vivo studies in macaques [47], the limited supply precludes their use in COS protocols. Generation of ample supplies of recombinant macaque gonadotropins

Table 1: Antihuman gonadotropin antibodies in macaque serum prior to and following three consecutive controlled ovarian stimulation (COS) cycles with recombinant human gonadotropins [46].

Protocol	Number of animals	^{125}I-hCG bound[a] Before[b]	^{125}I-hCG bound[a] After[b]	Number of animals	^{125}I-FSH bound[a] Before[b]	^{125}I-FSH bound[a] After[b]
First	12	3.1[c]	2.9	12	2.6	2.6
Second[d]	9	3.4	2.9	9	2.6	2.8
	2	3.8	7.0	2	3.0	6.6
Third[d]	3	3.4	3.3	4	2.6	2.7
	6	3.1	7.0	4	2.8	6.5
	1	18.1	25.5	2	7.2	12.1

[a]Represents protein A precipitation of antibody-bound ^{125}I-hCG or ^{125}I-FSH in serum. Nonspecific binding was 2.5% and 2.3%, respectively. See [45] for methodologic details. [b]"Before" and "After" represents serum samples collected seven days prior to the first injection of COS cycle and the last two days of the luteal phase of the COS cycle, respectively. [c]Baseline levels of antibody were defined as ≤ 4% of bound radioactivity (negative response), borderline responses were represented by 4–9% of bound radioactivity, and positive responses were present if values ≥ 10%. [d]One animal was ovariectomized prior to the second protocol; one animal did not exhibit an ovarian response to COS during the third protocol.

to permit current and future use of COS protocols in ART programs would clearly facilitate research in the NHP model, e.g., permit repetitive use of optimal or "genetically-selected" monkeys for oocyte/embryo production, including sequential experimental protocols on individual animals. NHP gonadotropins would permit ART-related procedures to preserve or modify genetic-defined animals and to maintain endangered species or genetic lines of macaques or other primates.

Until NHP gonadotropins are available, programs are largely dependent on the sale or donation of human gonadotropins from a few pharmaceutical companies (e.g., Ares Serono, Organon). Their product donations to NHP ART programs in the past decade were critical to many research and development efforts; the authors estimate that the ART program at ONPRC annually consumes human gonadotropins (r-hFSH, r-hLH, r-hCG) having a commercial value of over $200,000. During the development of clinical ART programs and the testing of recombinant preparations, companies recognized the value of research efforts in NHPs as preclinical trials for their products. However, with the world-wide approval, use and great demand for r-hFSH, LH and CG now established, it is not clear that this source of materials will continue to be available and is unlikely to allow expansion. The unsettling scenario of limited availability of human gonadotropins as a product donation for NHP research provides further impetus for the creation of ample supplies of macaque gonadotropin preparations.

Ongoing controversy – the need, or lack thereof, for LH in COS protocols

One of the major reasons that a standard regimen of gonadotropin hormones has not evolved for promoting multiple follicular development in COS cycles is the unresolved issue regarding the need for LH in the protocol. It is generally recognized that LH secreted during the follicular phase of the menstrual cycle is essential for the steroidogenic function of the dominant follicle destined to ovulate at midcycle in primates [1]. This is exemplified by the two-cell, two-gonadotropin model for estrogen production by the follicle, wherein (a) theca interna cells contain LH receptors and respond to circulating LH with increased production of androgen, whereas (b) granulosa cells contain FSH receptors and respond to FSH by increasing the conversion of androgen to estrogen. The rising levels of circulating estradiol act on various target tissues, including the hypothalamic-pituitary axis to elicit the midcycle gonadotropin surge which causes periovulatory events in the mature follicle. However, it is less clear whether LH has additional vital roles in the developing follicle in primates [3], either independent of its steroidogenic actions or via local steroid effects analogous to androgen or estrogen actions in rodent follicles [12].

With the advent of pure recombinant gonadotropins, notably r-hFSH and r-hLH, it became possible to evaluate follicular stimulation protocols consisting of either exogenous FSH alone or in combination with LH, in NHPs [39,42]. The authors chose to directly compare follicle, oocyte, and embryo parameters in rhesus monkeys following protocols with r-hFSH (30 IU, 2× per day) alone or with an equivalent amount of r-hFSH and r-hLH (30 IU each, 2× per day). Prior to treatment, animals received a GnRH antagonist for 90 days to maintain an LH-deficient and hypo-estrogenic state throughout the proposed interval of follicular growth from the preantral to mature antral stage [48]. Morphologic assessment of ovaries removed after GnRH antagonist treatment revealed the absence of any follicles larger than the small (≤ 1 mm diameter) antral stage (Fig. 3, left panel).

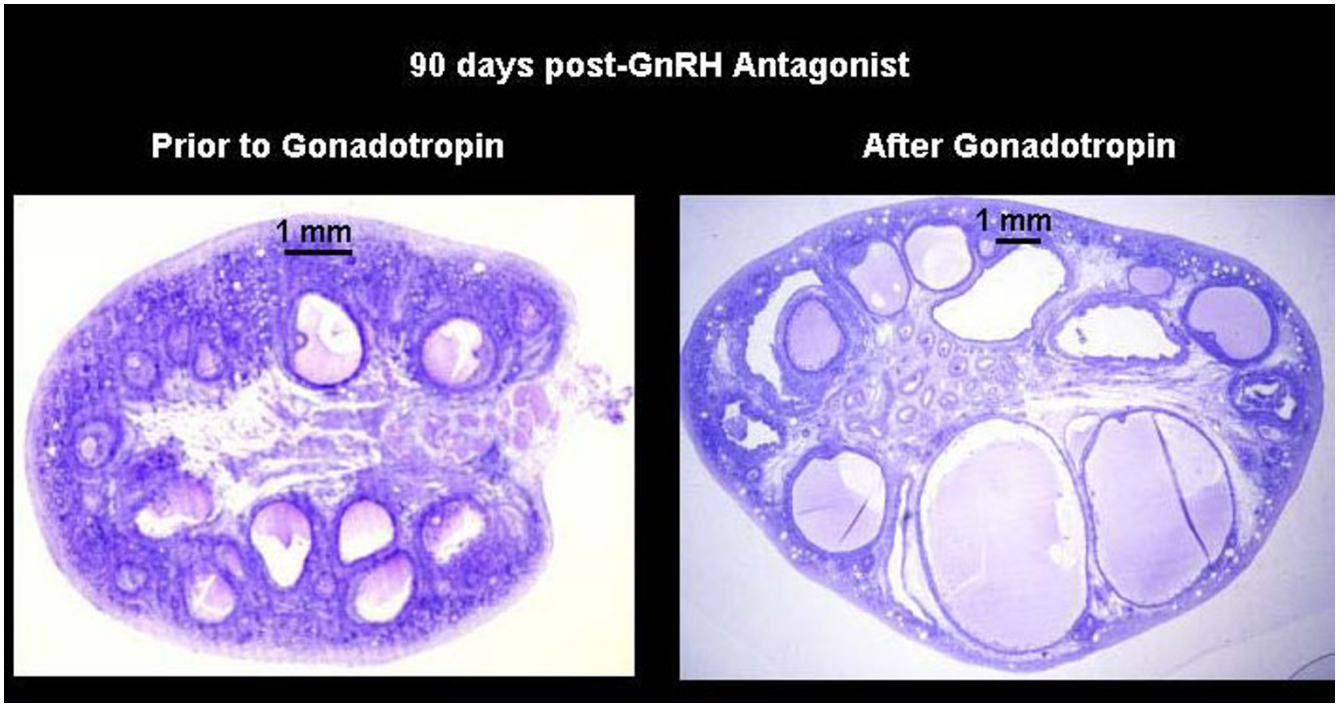

Figure 3
Histologic sections of ovaries. Ovaries were removed from rhesus monkeys after 90 days of treatment with GnRH antagonist prior to (left panel) and following administration of r-hFSH and r-hLH (right panel). Note the absence of any large (>1 mm diameter) antral follicles following GnRH antagonist exposure, versus the development of 2–6 mm antral follicles after 9 days of gonadotropin treatment. See text, and ref [39] for further details.

As expected, based on the two-cell, two-gonadotropin model, the levels and patterns of circulating estradiol differed during the two treatment protocols [39]. Serum levels remained at baseline (<20 pg/ml) during the first five days of r-hFSH treatment, then increased and plateaued at levels (~200 pg/ml) that were markedly less ($p < 0.05$) than those in r-hFSH + r-hLH-treated animals. In contrast, serum estradiol levels rose steadily following initiation of r-hFSH + r-hLH treatment, and peaked at levels (~1000 pg/ml) that were 5-fold higher than those in r-hFSH-treated animals. Nevertheless, either gonadotropin treatment regimen could stimulate the growth of numerous antral follicles (~24 follicles ≥ 2 mm diameter; Fig. 3, right panel), and a greater proportion of mature (metaphase II), fertilizable eggs were obtained at 27 hrs post-hCG injection from FSH- versus FSH + LH-treated animals. These findings support the concept that in pharmacologic COS protocols, FSH alone is adequate for the folliculogenic and gametogenic events required to produce viable embryos in NHPs. This finding is consistent with retrospective meta-analyses finding little if any difference in ovulatory and/or pregnancy rates between hFSH and hFSH + hLH protocols for ovulation induction or ART-ET in women [49-51].

Nevertheless, there are indications that addition of LH has some positive effects in COS protocols. In our macaque study, the FSH + LH treatment regimen required a shorter interval than FSH alone (9 vs 12 days, $p < 0.05$) to stimulate follicles to the stage of administering the ovulatory hCG bolus. Also, all FSH + LH-treated animals achieved the follicular development required for hCG administration, whereas 2 of 7 monkeys receiving FSH alone failed to display adequate folliculogenesis. Although fertilized oocytes from both treatment regimens were capable of in vitro development to hatched blastocysts and in vivo development to normal offspring after ET, there were some differences [42]. Notably, embryos from FSH-only treatment protocols were less likely to survive cryopreservation and thawing, and required longer to develop to the morula-to-hatched blastocyst stage than those from FSH + LH protocols. It is intriguing to note that the slower preimplantation development rate in vitro correlated with evidence of delayed rescue of corpus luteum function (16

days post-LH surge) following ET of embryos derived from FSH-only protocols. These data suggest that inclusion of LH in COS protocols improves the efficiency and rate of preovulatory follicle development, embryo "viability" and the rate of preimplantation embryo development in macaques. Whether these parameters are influenced by the greater estrogen milieu provided by LH exposure is unknown. These results are consistent with several published reports from clinical programs, notably those of Filicori and colleagues [52,53], that inclusion of LH has practical (e.g., shortens treatment and therefore hormone costs) and theoretical (e.g., reduces heterogeneity in follicle size) benefits in ovarian stimulation protocols.

Nevertheless, this issue remains controversial, as well as the related question regarding how much LH is sufficient for optimal folliculogenesis. It seems likely that less LH than FSH is required; studies in hypogonadotropic, hypogonadal women suggest that a ratio of 2 IU r-hFSH:1 IU r-hLH is optimal for promoting follicular development [54]. Likewise, our recent study evaluating LH requirements for final ovulatory maturation of the naturally selected dominant follicle during the menstrual cycle in macaques indicates that a 2:1 ratio (but not 1:0 ratio) is as capable as a 1:1 ratio of FSH-LH in producing an ovulatory follicle [55]. It is likely that some of the controversy in this field is related to the lack of control or analysis of endogenous LH levels during protocols, and that endogenous LH combined with exogenous FSH is sufficient for follicular development. It is important that researchers employing NHPs are aware that different GnRH analog/gonadotropin treatment regimens do not necessarily produce similar follicles, oocytes or embryos. Moreover, their similarity to those generated in the natural menstrual cycle awaits rigorous analysis.

Ongoing problem – heterogeneity of animal and follicle response

Despite the success in developing COS protocols in NHPs, it is apparent the response in terms of multiple follicular development is quite variable. We reported earlier [5] that rhesus monkeys displayed four types of responses to our gonadotropin treatment protocols in terms of patterns and levels of circulating estradiol: (a) **classical responders** with continuously rising estradiol levels throughout treatment, (b) **biphasic responders** with estradiol levels transiently declining by >20%, but rebounding thereafter, (c) **abbreviated responders** with estradiol declining after more than five days of treatment, and (d) **nonresponders** with estradiol levels never rising above those observed in spontaneous cycles. Our standard sequential regimen of hFSH followed by hFSH + hLH resulted in the greatest frequency (17 of 25 protocols or 67% of animals) of classical responders. However, a significant percentage of animals (8 of 25 or 33%) fell into categories b-d and either did not reach follicle aspiration (e.g., nonresponders) or provided oocytes that fertilized and cleaved in vitro at a much lower percentage than those from classical responders (13% vs 41%). However, even in classical responders the variation in peak estrogen levels (e.g., 4480 ± 1012 pg/ml, mean ± SEM, n = 17) and numbers of oocytes retrieved (which is positively correlated with peak estradiol levels; $p < 0.05$) is remarkable.

If researchers are monitoring daily estradiol levels and follicle numbers/diameters, it is possible to individualize the treatment regimen, as in clinical ART protocols, to reduce variability in follicular stimulation in NHPs [5]. However, an individualized approach does not eliminate the occurrence of nonclassical responders. Many of the abbreviated and biphasic estradiol responses in monkeys appear associated with a spontaneous LH surge (>100 ng/ml) or "mini-surge" (<100 ng/ml) on the day before declining estrogen levels [5]. The addition of GnRH analogs (first agonists, and more recently, antagonists) is used clinically to prevent endogenous LH surges during COS protocols. In addition, ART patients are often treated with these drugs prior to starting gonadotropin treatment to permit arbitrary initiation of protocols independent of the menstrual cycle, thereby projecting follicle aspiration for a convenient time during the work week. With the development of second- and third-generation GnRH analogs, these drugs have been administered to macaques prior to [22], throughout [23,32] or in the latter part (unpublished) of the gonadotropin treatment regimen for these purposes. However, effective methods are needed to identify potential nonresponders prior to initiating follicular stimulation protocols. Attempts in the clinic include evaluation of basal FSH levels and ultrasound monitoring of the pool of small antral follicles [56] in ART patients. However, these are not easily monitored in macaques, and one report suggests that FSH levels are not predictive of a poor response to gonadotropin stimulation in cynomolgus monkeys [57]. Anecdotal reports suggest that estradiol levels below those expected at the onset of the follicular phase (or a poor estrogen response to GnRH agonist [57]) portend a poor follicular response in NHPs, but this has not been rigorously evaluated.

Another issue is the increasing realization that COS protocols in NHPs and women result in the development of a heterogeneous population of antral follicles that differ in size (Fig. 4), health and perhaps maturity and oocyte quality. For example, gonadotropin stimulation protocols in macaques [39] can generate a cohort of antral follicles prior to hCG injection that vary in size between 2 mm diameter (30% of total cohort), 3 mm diameter (40%), and 4–6 mm diameter (30%). It is unclear how this size distribution relates to the cytoplasmic or nuclear maturity of oocytes collected after the hCG bolus, e.g., in the above

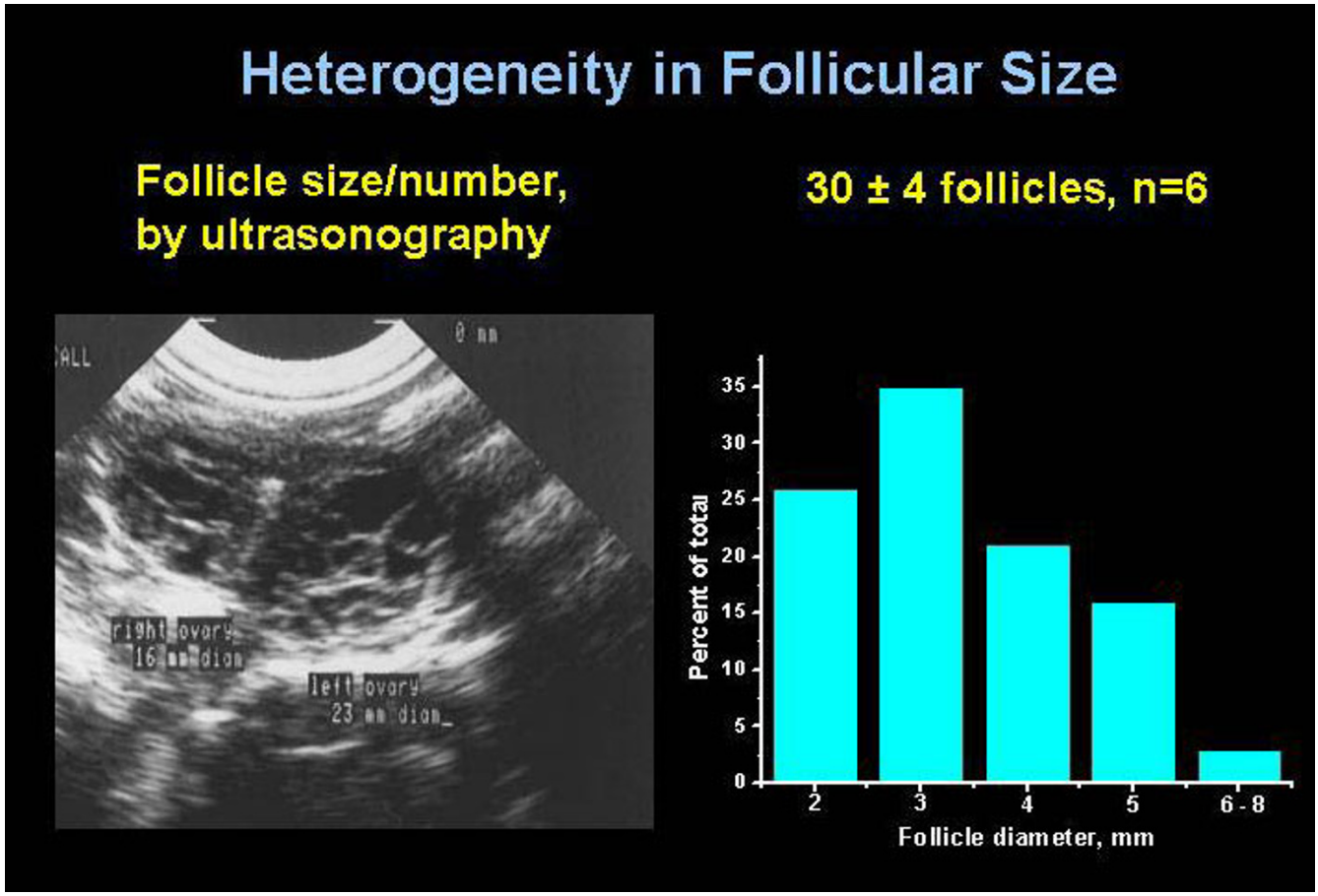

Figure 4
Illustration of the heterogeneity in follicle size following controlled ovarian stimulation in rhesus macaques. The number and size of antral follicles that develop on the ovaries after daily treatment with exogenous gonadotropins can be estimated by transabdominal ultrasonography (left panel). The percent of the total follicle cohort at various sizes ≥ 2 mm diameter on day 7 of our standard COS protocol (6 days of r-hFSH, 30 IU 2× per day, then r-hFSH + r-hLH, 30 IU each 2× per day; plus daily GnRH antagonist treatment [15]) are illustrated in the right panel. Typically, in this and prior [39] protocols, follicles vary in size between 2 to 6–8 mm in diameter.

study at 27 hrs post-hCG, 24 follicles ≥ 2 mm diameter yielded 25 oocytes with approximately 20% not resuming meiosis, 70% at metaphase I and 10% at metaphase II. Moreover, only 52% of the mature oocytes (MII at collection or after 8 hrs in vitro) were successfully fertilized by IVF [39]. Likewise, a recent study [58] examining follicular histology in macaque ovaries at various intervals after administration of the hCG bolus in COS cycles determined that (a) many of the follicles displayed the expected features of luteinization and neovascularization between 12 and 36 hrs post-hCG, but (b) a significant (30–40%) percentage of follicles display features of gross degeneration (e.g., unadhered, pyknotic granulosa cells in the antrum) indicating follicle atresia (Fig. 5). It is tempting to speculate that this subgroup of follicles correlates with the 30–40% of follicles that do not ovulate following an hCG bolus in COS cycles [59]. Since follicles are typically aspirated prior to rupture, the collected pool of oocytes would contain those from luteinizing as well as degenerating follicles. How this relates to the heterogeneity in maturation state, fertilizability, and embryonic potential of individual oocytes is unknown. This heterogeneity may be a lesser issue in clinical fertility programs where 2–3 of the "best looking" fertilized eggs/early embryos are selected for ET in patients. However, it is a greater issue in NHP studies where every oocyte/embryo is a valuable commodity for basic and applied research. It is important that researchers recognize the heterogeneity of follicles, oocytes and embryos derived from COS protocols and the potential impact, particularly in relating

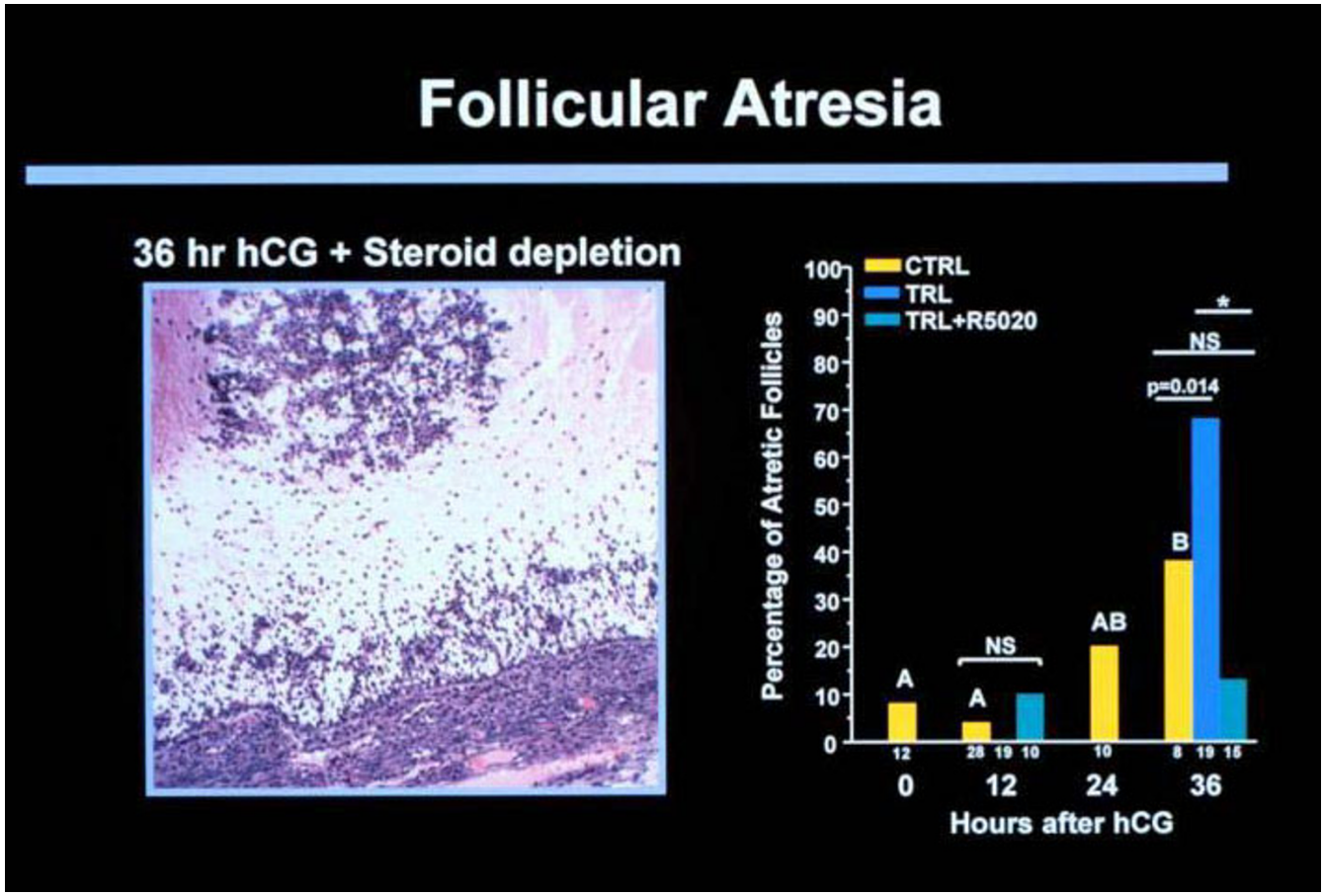

Figure 5

Morphology (left panel) and percent (right panel) of atretic follicles. Follicles were exhibiting histologic evidence of atresia (pyknotic, unadhered granulosa cells in the antrum; left panel) following administration of the hCG bolus in COS protocols in rhesus monkeys. Although relatively few (<10%) appear atretic prior to hCG injection (0 hr; small number below bar indicates sample size), the number increases significantly by 36 hrs post-hCG (40%; controls, CTRL). Moreover, the percentage of atretic follicles was influenced by the steroid milieu, since administration of a steroid synthesis inhibitor (trilostane, TRL) produced a cohort of 70% atretic follicles and co-administration of a progestin (R5020) reduced the percentage to pretreatment (0 hr hCG) levels of 10%. Thus, oocytes collected prior to follicle rupture could originate from degenerating, as well as healthy (i.e., luteinizing, ovulatory) follicles. See ref [59] for further details, including statistical (X^2) analyses. Different letters above bars indicate significant differences over time within controls (CTRL; hCG alone). Asterisk, or NS indicate significant or nonsignificant differences between treatment groups at one timepoint.

experimental results to those occurring in the ovarian cycle, during pregnancy initiation and embryogenesis in untreated NHPs.

Conclusions

Over the past 15 years, the remarkable increase in use of COS-ART protocols in clinical practice to treat infertile couples [60] has been paralleled by applications of this technology to numerous NHP species, from great apes [61] to baboons [62,63], and various Old World monkeys [19,22,64-66] to New World monkeys [67]. Reports from zoological settings [61,65] as well as many NHP research centers (see also following chapters) illustrate the potential value of this approach to preserve and foster reproduction of endangered primate species or primates of a known genetic character that are valuable for applied research of direct relevance to human health. A large supply of competent gametes (notably oocytes) and embryos will also facilitate basic and applied research on primate gametogenesis, fertilization, early embryogenesis and pregnancy initiation – areas that logistically and ethically are difficult or cannot be performed in humans. However,

limitations remain, including the lack of availability of NHP gonadotropins which seriously curtails current ovarian stimulation protocols in the predominant research model, the Old World macaque. Also, the heterogeneity of response between and within COS protocols, in terms of the antral follicle population, oocyte quality and embryo potential, should be recognized by primate researchers. The latter is a significant issue for NHP studies where every oocyte/embryo is a valuable commodity and distributed arbitrarily between treatment groups in research protocols. A standard gonadotropin treatment regimen may never by generally accepted, either clinically or experimentally, due to the controversial need for LH in antral follicle maturation. Nonetheless, further progress in the described research areas is likely – especially if adequate sources of NHP gonadotropins become available for in vivo studies, including COS protocols.

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