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OVARIAN RESERVE TESTS AND THEIR UTILITY IN PREDICTING RESPONSE TO CONTROLLED OVARIAN STIMULATION IN RHESUS MONKEYS

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Abstract

Controlled ovarian stimulation (COS) is an alternative to natural breeding in nonhuman primates; however, these protocols are costly with no guarantee of success. Toward the objective of predicting COS outcome in rhesus monkeys, the current study evaluated three clinically used ovarian reserve tests (ORTs): day 3 (d3) follicle-stimulating hormone (FSH) with d3 inhibin B (INHB), the clomiphene citrate challenge test (CCCT), and the exogenous FSH Ovarian Reserve Test (EFORT). A COS was also performed and response was classified as either successful (COS+) or unsuccessful (COS−) and retrospectively compared to ORT predictions. FSH and INHB were assessed for best hormonal index in conjunction with the aforementioned tests. INHB was consistently more accurate than FSH in all ORTs used. Overall, a modified version of the CCCT using INHB values yielded the best percentage of correct predictions. This is the first report of ORT evaluation in rhesus monkeys and may provide a useful diagnostic test prior to costly follicle stimulations, as well as predicting the onset of menopause.

Keywords

inhibin; ovarian reserve; follicular response; peri-menopause; hormone predictors of fertility

INTRODUCTION

Ovarian reserve tests (ORTs) are used to give a relative measurement of the remaining follicular pool. ORTs have become more commonplace in human clinics to assist in counseling patients with regard to chances of pregnancy success with or without exogenous hormone treatment. Marked advances have been made using ORTs in human research, bypassing study using their animal model counterparts. Primarily, development of ORTs in

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nonhuman primate models would make it possible to identify the best animals for future research in artificial reproductive technologies. Secondly, controlled ovarian stimulation (COS) and *in vitro* fertilization are frequently an unavoidable alternative to natural breeding when animals are geographically isolated or are behaviorally incompatible. Finally, there is a great need to identify nonhuman primates entering menopause for use as subjects in basic aging research. Assisted reproductive technologies can be expensive and sometimes require moderately invasive animal procedures, with no guarantee of success. Therefore, use of ORTs could assist in focusing resources on animals with the best likelihood of successful pregnancies.

The monotropic rise in follicle-stimulating hormone (FSH) is often considered the hallmark event associated with declining fertility. Additionally, compared to young women, those that are peri-menopausal have reduced levels of inhibin B (INHB). Of note is the fact that these hormonal changes occur prior to any measurable change in estradiol, progesterone, or luteinizing hormone [Klein et al. 1996a]. Early follicular phase FSH (day 3) has been shown to correlate well with ovarian reserve [Wu et al., 2005]. Measurement of basal FSH alone, while useful, can be somewhat variable. Thus, a number of tests have been developed that, when used in conjunction with basal FSH levels have successfully predicted ovarian response. In general, most of these tests rely on assessing hormonal response to ovarian stimulation, whether at the level of the hypothalamus, pituitary, or direct action on the ovary.

The clomiphene citrate challenge test (CCCT) is another highly effective test of ovarian reserve based on disrupting the hypothalamic-pituitary-gonadal (HPG) axis' negative feedback. Clomiphene citrate (CC) is an orally active non-steroidal chemical composed of two isomers: zuclomiphene and euclomiphene. Zuclomiphene is the more active isomer [Speroff et al. 1999] and presumably acts by blocking estrogen receptors at the level of the hypothalamus and/or pituitary. When follicles are numerous, as in a young female, the ovary responds to increased levels of gonadotropins with an amplified production of ovarian hormones such as estradiol (E2) [Klein et al., 1998; Soules et al. 1998; Soules, 2005; Speroff et al. 1999]. The high levels of ovarian hormones then exert negative feedback to the hypothalamus and/or pituitary resulting in the inhibition of gonadotropin (FSH and LH) production. Conversely, a woman with poor ovarian reserve has low levels of E2 and higher or unchanged levels of FSH.

Administration of CC in mid follicular phase (days 5–9) blocks estrogen receptors, reduces negative feedback, and thus up-regulates FSH and LH production from the pituitary. Females with a large ovarian reserve respond to CC with a dramatic increase in E2, follicular growth, and a concomitant increase in granulosa cell production of inhibin B [Hoffman et al., 1998; Klein et al. 1998; Soules, 1998]. Older women with significantly reduced ovarian reserve respond to CC with little to no change in estradiol. In the peri-menopausal ovary, reduced follicle number results in a reduced inhibin response as well.

Typically, the CCCT predicts COS outcome based on d3 and d10 FSH values alone; however, in the present study INHB changes were measured to evaluate the relative responses at both the pituitary gland and ovary level. CCCT was 93% effective in predicting infertility in women and was more successful in identifying low ovarian reserve patients than basal FSH alone [Kahraman et al. 1997].

It has become clear that both FSH and E2 levels are important in assessing ovarian response. Recent development of the exogenous FSH ovarian reserve test (EFORT) significantly improved the reliability of using basal FSH for predicting in vitro fertilization (IVF) response [Kahraman et al. 1997]. EFORT involves measurement of basal FSH levels and E2

measurements taken at 24 hours following a single injection of FSH on cycle day 3. This testing regimen has proven more effective in predicting outcome to IVF than FSH measurements alone. Basically, these measurements predicted good and poor responders to IVF. Women with both low baseline INHB levels as well as reduced INHB response to EFORT were less responsive to IVF treatment or poor responders meaning that estradiol (E2) levels did not increase; whereas, women with higher INHB and a strong increase in E2 levels were good responders and excellent candidates for IVF [Kahraman et al. 1997; Soules et al. 1998].

The objective of this study was to evaluate three methods commonly used in humans to predict COS success. Two types of ORTs were performed: CCT and EFFORT. In addition, endogenous day 3 hormone levels were assessed in captive female rhesus macaques prior to beginning ORT treatment regimens. Endogenous hormone levels were compared to subsequent COS results. This is the first study to evaluate the use of ORTs in nonhuman primates and offers valuable information to clinical, laboratory, and captive settings.

METHODS

Animals

8 young (Y; 12 years) and 14 old (O; 19–26 years) female rhesus monkeys (*Macaca mulatta*) were identified for this study. Monkeys were singly housed and fed twice daily with water available *ad libitum* in a controlled environment on a photoperiod of 12L:12D. All the animal work and the experimental plan were conducted according to IACUC approved protocols of the Oregon National Primate Research Center (ONPRC) and the University of Maryland and were in compliance with the American Society of Primatologists' Principles for the Ethical Treatment of non Human Primates. All procedures were conducted on females in the same order.

COS

In a spontaneous menstrual cycle (first day of menses = day 1), Antide (Ares Serono; 0.5 mg/kg BW, sc, at 0800h) was administered on d1–d9 to inhibit endogenous gonadotropin production. Recombinant human FSH (r-hFSH; (Gonal-F; Ares Serono; 30 IU intramuscularly [IM] at 0800 and 1700)) was administered on d1–d6 followed by administration of both r-hFSH + r-h luteinizing hormone (Lahdi; Ares Serono; 30 IU each, IM at 0800 and 1700) on d6–d9 to stimulate the production of multiple preovulatory follicles. This regimen was followed by an injection of h-chorionic gonadotropin (Serono; 1000 IU, IM) on d10 or d11 to induce ovulatory maturation. Trans-abdominal ultrasonography was performed on d7 to assess the number and size of follicles. Blood samples were collected daily from the saphenous vein, and E2 levels were used to indirectly assess follicular response. COS was classified as successful (COS+), based on the following criteria (Table 1): peak E2 level greater than 1000 pg/mL and ≥ 3 follicles of 4 mm diameter on each ovary. Responses lower than these standards were considered unsuccessful (COS–).

Day 3 Hormones

During a spontaneous menstrual cycle, serum FSH and INHB were determined on day 3 (d3; menses=d1). Day 3 hormone value predictions of COS outcome were based on the following criteria (Table 2): day 3 FSH values less than or equal to 1.0 ng/mL were considered COS+ and day 3 FSH values greater than 1.0 ng/mL were predictive for COS–.

Clomiphene Citrate Challenge Test (CCCT)

During a spontaneous menstrual cycle, 50 mg clomiphene citrate was administered orally by inserting the suspension into a piece of banana on day 5 through day 9, and serum FSH and

INHB were determined on d3 and d10. The CCCT criteria for predicting COS outcome were based on both data from our laboratory collected in rhesus monkeys (data not shown) and human clinical protocols as described in Table 3. Day 3 and day 10 FSH values less than or equal to 1.0 ng/mL and INHB values greater than or equal to 50 pg/mL were predictive for COS+.

Modified CCCT (M-CCCT)

Results from the CCCT indicated that the addition of day 3 hormone values as a screening step could improve the prediction accuracy of the CCCT or eliminate the need to proceed with it. Therefore, d3 hormones were used to identify COS+ animals that were consequently excluded from further testing. Animals predicted for COS− based on hormone levels were administered 50 mg CC on days 5 through 9, and a blood sample was collected on day 10. Fold change in E2 greater than or equal to 2.0 predicted a COS+; while fold change in E2 less than 2.0 confirmed a COS−.

Exogenous Follicle Stimulation Hormone Ovarian Reserve Test (EFORT)

During a spontaneous menstrual cycle, Antide (Ares Serono; 0.5 mg/kg BW subcutaneous, at 0800) and r-hFSH (Gonal-F; Ares Serono; 30 IU; IM at 0800 and 1700) were administered. Whole blood was collected from the saphenous vein just prior to d1, and the first injection of r-hFSH was given approximately 24 hours following d2. The EFORT criteria for predicting COS outcome were based on both data that our laboratory collected in rhesus monkeys (data not shown) and human clinical protocols as described in Table 5. Fold change in FSH greater than or equal to 1.5 predicted a COS+; while fold change in FSH less than 1.5 predicted a COS−.

Hormone Assays

Monkeys were monitored for onset of menses (designated menses d1). Blood samples were collected from the saphenous vein in trained monkeys with no anesthesia. Serum was then stored at −20°C until assayed. Samples were assayed for E2 and FSH at the ONPRC Endocrine Core Laboratory.

Inhibin B was determined using the commercially available Active Inhibin B kit (Diagnostic Systems Laboratory; Webster, TX). This enzyme-linked immunoabsorbant assay (ELISA) was validated for use with rhesus monkey serum in our laboratory. Parallel displacement curves were obtained by comparing serially diluted (1:1 to 1:16) pooled rhesus monkey serum against known INHB concentrations. Recovery of known concentrations of unlabeled INHB added to pooled rhesus monkey serum was 100% with a coefficient of variation of 10%. Inter-assay coefficients of variation for two internal controls were 15% and 9%. The intra-assay coefficient of variation was always less than 10%.

Statistical Analyses

Descriptive statistics were conducted using SAS Statistical software (Cary, NC). For each ORT we examined the relationship between individual hormone measurements and COS outcome. Number of correct and incorrect predictions (% accuracy) for each ORT was determined overall for young and old monkeys. Chi-square analyses were performed to evaluate categorical data with ORT predictions compared to actual COS results. If more than two comparisons were made, the number of animals in each group are noted in the results; with a total of 8 young (Y; 12 years) and 14 old (O; 19–26 years) studied. The statistical criteria for X^2 used degrees of freedom of either 1 ($P=3.84$) or 2 ($P=5.99$) depending on the number of comparisons; minimal statistical significance was determined at $P < 0.05$.

RESULTS

COS

All young monkeys had a successful COS (COS+); however, old monkeys were split between COS+ (n=7) and COS- (n=7). Figure 1A describes the COS protocol used. Figure 1B shows a representative profile of a COS+ including measurements of the follicles as denoted on the ultrasound image. Peak E2 levels exceed 1000 pg/mL, and ultrasound visualization of the ovaries show multiple pre-ovulatory follicles; circulating concentrations of E2 reflect follicular development and steroid production by these follicles. Conversely, Figure 1C shows a representative profile of a COS- response. Peak E2 levels remain less than 600 pg/mL, and ultrasound visualization of the ovaries show an absence of developing large antral follicles (see measurements on the ultrasound image) despite treatment with exogenous gonadotropins.

Day 3 Hormones

Figure 2 depicts the percent accuracy for day 3 hormone values in young and old monkeys, respectively, with the COS outcomes listed. The protocol for blood collections is shown in Figure 2A. Figure 2B shows the ratios of correct to incorrect predictions of COS response using day 3 FSH values. Day 3 FSH was 59% accurate, but did not significantly predict COS outcome ($p > 0.05$). Figure 2C shows the ratios of correct to incorrect predictions of COS response using day 3 INHB values. Day 3 INHB was 77% accurate and also did not significantly predict COS outcome ($P = 0.06$).

CCCT

Figure 3 depicts the percent accuracy for the CCCT with the COS outcomes listed. The protocol for the CCCT is shown in Figure 3A. Figure 3B shows the ratios of correct to incorrect predictions of COS response using CCCT with FSH values. CCCT FSH was 45% accurate and did not significantly predict COS outcome. Figure 3C shows the ratios of correct to incorrect predictions of COS response using CCCT INHB values. The CCCT INHB was 59% accurate and also did not significantly predict COS outcome.

M-CCCT

Figure 4 depicts the percent accuracy for the M-CCCT with the COS outcomes listed. The flow chart protocol for the M-CCCT is shown in Figure 4A. Figure 4B shows the ratios of correct to incorrect predictions of COS response using M-CCCT with FSH values. CCCT FSH was 77% accurate and significantly ($P < 0.05$) predicted COS outcome. Figure 4C shows the ratios of correct to incorrect predictions of COS response using M-CCCT INHB values. The M-CCCT INHB was 82% accurate and also significantly ($P < 0.05$) predicted COS outcome.

EFORT

Figure 5 depicts the percent accuracy for the EFORT with the COS outcomes listed. The protocol for the EFORT is shown in Figure 5A. Figure 5B shows the ratios of correct to incorrect predictions of COS response using the EFORT with FSH values. EFORT (FSH) was 55% accurate and did not significantly ($p > 0.05$) predict COS outcome. Figure 5C shows the ratios of correct to incorrect predictions of COS response using the EFORT with INHB values. The EFORT (INHB) was 80% accurate and did not ($P = 0.06$) significantly predict COS outcome.

DISCUSSION

This is the first report using and comparing ORTs in monkeys. We evaluated the hormonal response to the selected ORTs in both young and old monkeys. We were surprised to find that both d3 FSH and d3 INHB alone had better predictive value than the CCCT. This differential outcome between humans and monkeys may be attributed to drug dosing issues. Clomiphene citrate has a bitter flavor and unlike with human patients, it must be mixed or hidden in a food source when administered to monkeys. With finicky eaters, compliance is difficult to assure, even in a laboratory setting. Secondly, data were not available from the literature to adjust the dosage for possible age-related differences in response. As such, it is possible that there may have been some difference in CC efficacy due to aging and responsiveness to the treatment. The rhesus monkeys in the present study exhibited variable hormone responses to CC similar to those reported by Littman and Hodgen that occurred in cynomolgus monkeys [Littman and Hodgen 1985]. In both cases, the monkeys responded in a widely varied manner and not always the same on subsequent cycles. Future studies may benefit from titrating the CC dose according to age. In a younger animal, levels of CC that are too high may result in reversing the desired effect: inhibition rather than stimulation of pituitary and ovarian hormones. Littman and Hodgen [Littman and Hodgen 1985] did not find any correlation between CC dose and peak levels of E2 or FSH; however the study was performed with cynomolgus monkeys and their highest dose was 30 mg (as compared to the 50 mg dose used in this study). Marut and Hodgen [Marut and Hodgen 1982] also observed declining levels of E2 in monkeys following administration of 25 mg CC daily for 5 days, despite elevated levels of FSH. This suggested that CC acted in an anti-estrogenic fashion, imparting ovarian refractoriness. Our dose of CC was higher (50 mg daily for 5 days), and we did not observe this same hormone pattern consistently, rather only in select females. Although CC is believed to act by blocking estrogen receptors in the hypothalamus, the exact mechanism of action is not fully understood and may include a direct action on the ovary, possibly via aromatase [Littman and Hodgen 1985]. Perhaps in future studies, a pure estrogen antagonist would provide more consistent results. With regard to continued use of the CCCT in rhesus monkeys, assessment of multiple hormones (day 3 INHB pre-screen and fold change in E2) greatly improves the prediction accuracy of this ORT.

EFORT with INHB showed a predictive accuracy of 80%, which was similar to the results, obtained using INHB levels with CCCT. The major advantage to the EFOR is the rapid result. Using the INHB assay kit (DSL, Webster, TX), results were available within 24 hours. Therefore, the EFOR may be performed conditionally converting to a full COS based on the early EFOR outcome, thus combining both the ORT and COS into one cycle. Additionally, the EFOR may provide an advantage in situations where oral medication is precluded and injections may be logistically more practical. The primary disadvantage to this method is the high costs associated with the FSH injections which must be weighed against the risk of waiting and risking the total loss of fertility in older or genetically valuable animals.

If EFOR predicts a COS-, the hormone injections would be purely diagnostic. This study was designed to evaluate various ORT regimens in the rhesus monkey. Our data consistently find INHB to be superior to FSH in predicting COS outcome, regardless of ORT (Table 6). In fact, our results show that the best diagnostic test for predicting COS outcome was the CCCT using INHB values in conjunction with fold change in E2 (82% accurate). These results are in agreement with human studies that have shown INHB to be better than FSH in predicting response to follicle stimulation. Figure 3 shows a flow chart depicting the modified CCCT with INHB measurements taken as part of the testing protocol.

Elevated FSH is often considered the hallmark effect of ovarian aging and is commonly used in clinical practices to make medical decisions with regard to both pregnancy and/or menopause. With the development of the INHB assay, various reports have suggested that INHB may actually be a more sensitive index for ovarian function and reserve [Klein et al. 1998; Santoro 2005; Soules et al. 1998]. Despite the publication of such reports, INHB levels are still not as widely used as FSH in clinical settings [Klein et al. 2004; Robertson and Burger 2002; Welt et al. 1999]. The results for d3 INHB alone warrant consideration even with the lower accuracy of 77% because this timing appears to be exquisitely predictive of follicular activity. INHB was more accurate than FSH in predicting COS outcome. This has been shown in human data as well [Danforth et al. 1998; Klein et al. 1996b; Welt et al. 1999]. INHB is an ovarian hormone and may be considered a more direct measure of ovarian function than pituitary FSH. Additionally, determining INHB production has the advantage of requiring only a single blood sample taken at appropriate timing and the INHB ELISA kits are commercially available and relatively easy to perform. Another potential hormone candidate, anti-mullerian hormone (AMH) has recently emerged as an index of ovarian reserve and predictor of the peri menopause transition because it is produced by granulosa cells [van Rooij et al. 2002; Al-Qahtani et al. 2005; Penarrubia et al. 2005; Themmen 2005; Tremellen et al. 2005; Visser et al. 2006]. We did not evaluate AMH and it will be interesting to see how this hormone changes relative to FSH and INHB.

Although ORTs have been in practice in human clinics for many years now, this is the first study addressing their potential use in nonhuman primates. It is important to acknowledge the value of finding and using the best hormone index with ORTs. Although FSH has long been known to be an early marker of ovarian decline, INHB may be a more sensitive and better predictor of COS outcome. The development of commercially available INHB ELISA kits has made it possible to easily measure INHB without the added precautions necessary when using radioactive isotopes (as with most FSH assays). Therefore, use of INHB may be more efficacious than FSH as a diagnostic tool both in humans and monkeys. Additionally, the statistical comparison did not always reveal significance, there is variation in the response of females within an age category. Young females responded to ORT in a more consistent manner that yielded high correct predictions. INHB was predictive of COS+ in 100% of the young females. Interestingly, this was the case for both CCCT-FSH and CCCT-INHB as well. Therefore, the choice of ORT method for use in conservation and research programs may rest on cost and time to conduct the test. In this respect, the cost for the clomiphene citrate in a CCCT for the rhesus macaque is approximately \$3 and the cost for the hormone injections necessary for a COS is approximately \$1,000, making this approach financially feasible and a highly effective diagnostic tool.

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All experiments were conducted in accordance with animal care regulations and under approved Institutional Animal Care and Use (IACUC) approval at ONPRC and University of Maryland, College Park and were in compliance with the American Society of Primatologists' Principles for the Ethical Treatment of non Human Primates.

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Figure 1. Representative hormone profiles of a controlled ovarian stimulation (COS)

A) COS protocol: onset of menses designated day 1; Antide administered on d1–9; r-hFSH administered on d1–6; r-hFSH and r-hLH administered on d6–9; hCG injected on d10 in induce ovulatory maturation of follicles. B) Representative estradiol (E2) profile of a successful COS (COS+) with accompanying ultrasound. C) Representative E2 profile of an unsuccessful COS (COS–) with accompanying ultrasound (numbers on the ultrasound denote follicular measurements).

Figure 2. Day 3 Hormone Values

A) Protocol for day 3 hormone values: blood was collected from the saphenous vein on menses day 3 and analyzed for FSH and INHB. B) Day FSH: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (59%). Day 3 FSH predictions did not significantly predict ($X^2 > 3.84$; $df=1$; $P > 0.05$) actual COS outcomes. C) Day 3 INHB: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (77%). Day 3 INHB predictions did not significantly predict ($P = 0.06$) actual COS outcomes.

Figure 3. Clomiphene Citrate Challenge Test (CCCT)

A) Protocol for CCCT: blood was collected from the saphenous vein on menses day 3 and day 10 and analyzed for FSH and INHB.. 50 mg clomiphene citrate was administered daily on menses days 5–9. B) CCCT using FSH values: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (45%). CCCT (FSH) predictions did not significantly predict ($X^2 > 3.84$; $df=1$; $P > 0.05$) actual COS outcomes. C) CCCT using INHB values: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (59%). CCCT (INHB) predictions did not significantly predict ($p > 0.05$) actual COS outcomes.

Figure 4. Modified Clomiphene Citrate Challenge Test (M-CCCT)

A) Flowchart of protocol for M-CCCT: Day 3 FSH or INHB served as an initial screen; FSH values ≤ 1.0 ng/mL or INHB values ≥ 50 pg/mL were excluded from further testing and predicted for a successful COS (COS+). Animals failing to meet the day 3 hormone criteria for a COS+ were further tested by the CCCT using fold change in E2 to determine COS+ or COS-. B) M-CCCT using FSH: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (77%). M-CCCT (FSH) predictions significantly predicted ($X^2 = 3.84$ or 5.99 ; $P < 0.05$; $df=1$ or 2 respectively) actual COS outcomes. C) M-CCCT using INHB: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (82%). M-CCCT (INHB) predictions

significantly predicted ($X^2 = 3.84$ or 5.99 ; $P < 0.05$; $df=1$ or 2 respectively) actual COS outcomes.

Figure 5. Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT)

A) Protocol for EFORT: blood was collected from the saphenous vein on menses day 1 and day 2 and analyzed for FSH and INHB. An intra-muscular injection of recominant human FSH (r-hFSH) was administered following the blood sample on day 1. B) EFORT using FSH values: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (55%). EFORT (FSH) predictions did not significantly predict ($X^2 > 3.84$; $df=1$; $P > 0.05$) actual COS outcomes. C) EFORT using INHB values: number of correct and incorrect COS outcome predictions with overall percent accuracy indicated (80%). CCCT (INHB) predictions did not significantly predict (X^2 ; $df=1$; $P = 0.06$) actual COS outcomes.

Table 1
COS Criteria for Stimulation Success

Controlled Ovarian Stimulation (COS) criteria for classifying a successful (COS+) or unsuccessful (COS-) response. Blood samples were collected daily during a COS and E2 was determined. Ultrasound was also performed to confirm the presence of multiple pre-ovulatory follicles. Peak E2 greater than 1000 pg/mL and greater than 3 follicles of 4 mm diameter on each ovary was designated a COS+. Peak E2 less than 600 pg/mL and less than 3 follicles of 4 mm diameter on each ovary was designated a COS-.

Hormone	Criteria	Ultrasound Criteria	Outcome
peak E2	> 1000 pg/mL	> 3 follicles of 4 mm diameter / ovary	COS+
	< 600 pg/mL	< 3 follicles of 4 mm diameter / ovary	COS-

Table 2
Day 3 Hormone Criteria for Predicting COS Outcome

Criteria for prediction outcome following controlled ovarian stimulation (COS). Day 3 FSH less than or equal to 1.0 ng/mL predicted a COS+; day 3 FSH greater than 1.0 ng/mL predicted a COS-. Day 3 INHB greater than or equal to 50 pg/mL predicted a COS+; day 3 INHB less than 50 pg/mL predicted a COS-.

Hormone	Criteria	Prediction
Day 3 FSH	≤ 1.0 ng/mL	COS+
	> 1.0 ng/mL	COS-
Day 3 INHB	≥ 50 pg/mL	COS+
	< 50 pg/mL	COS-

Table 3
CCCT Criteria for Predicting COS Outcome

Criteria for COS prediction using the clomiphene citrate challenge test (CCCT). 50 mg clomiphene citrate was administered daily on menses days 5–9. Day 3 (d3) and d10 FSH less than or equal to 1.0 ng/mL predicted a COS+; d3 and/or d10 FSH greater than 1.0 ng/mL predicted a COS–. D3 and d10 INHB greater than 50 pg/mL predicted a COS+; d3 and/or d10 INHB less than 50 pg/mL predicted a COS–.

Hormone	Criteria	Prediction
d3 and d10 FSH	≤ 1.0	COS+
d3 and/or d10 FSH	> 1.0	COS–
d3 and d10 INHB	≥ 50 pg/mL	COS+
d3 and/or d10 INHB	< 50 pg/mL	COS–

Table 4
M-CCCT Criteria for Predicting COS Outcome

Criteria for COS prediction using the modified clomiphene citrate challenge test (M-CCCT). D3 hormone values were used as an initial screen and monkeys with COS+ predictions were excluded from further testing. Monkeys with d3 hormone COS– predictions were subsequently treated with 50 mg clomiphene citrate daily on menses days 5–9. d3 to d10 fold change in E2 greater than or equal to 2 predicted a COS+; d3 to d10 fold change in E2 less than 2 predicted a COS–.

Hormone 1	Criteria 1	Hormone 2	Criteria 2	Prediction
d3 FSH	> 1.0 ng/mL	–	–	COS+
d3 FSH	< 1.0 ng/mL	d3 – d10 E2	≥ 2 fold change	COS+
d3 FSH	< 1.0 ng/mL	d3 – d10 E2	< 2 fold change	COS–
d3 INHB	≥ 50 pg/mL	–	–	COS+
d3 INHB	< 50 pg/mL	d3 – d10 E2	≥ 2 fold change	COS+
d3 INHB	< 50 pg/mL	d3 – d10 E2	< 2 fold change	COS–

Table 5
EFORT Criteria for Predicting COS Outcome

Criteria for COS prediction using the Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT). Human recombinant follicle stimulating hormone (h-rFSH) was administered on day 1 following blood sampling. D1 to d2 fold change in FSH greater than or equal to 1.5 predicted a COS+; d1 to d2 fold change in FSH less than 1.5 predicted a COS-. D1 to d2 fold change in INHB greater than or equal to 1.5 predicted a COS+; d1 to d2 fold change in INHB less than 1.5 predicted a COS-.

Hormone	Criteria	Prediction
d1 – d2 FSH	≥ 1.5 fold change	COS+
	< 1.5 fold change	COS–
d1– d2 INHB	≥ 1.5 fold change	COS+
	< 1.5 fold change	COS–

Table 6
Summary Table of Selected Ovarian Reserve Tests (ORTs)

Percent accuracy in predicting COS outcomes for d3 hormone values, clomiphene citrate challenge test (CCCT), modified CCCT (M-CCCT) and the exogenous FSH ovarian reserve test (EFORT). Chi-squared: P-values indicating significant predictions ($X^2 = 3.84$ or 5.99 ; $P < 0.05$; $df=1$ or 2 respectively) for ORTs relative to actual COS outcomes are noted; non-significance denoted as NS.

	% Accuracy in Predicting COS Outcome			
	FSH	p-value (FSH)	INHB	P-value (INHB)
Day 3	59%	NS	77%	$P = 0.06$
CCCT	45%	NS	59%	NS
M-CCCT	77%	$P < 0.05$	82%	$P < 0.05$
EFORT	55%	NS	80%	NS