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Ovarian Stimulation of Squirrel Monkeys (*Saimiri boliviensis boliviensis*) Using Pregnant Mare Serum Gonadotropin

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The application of assisted reproductive technologies (ART) to nonhuman primates has created opportunities for improving reproductive management in breeding colonies, and for creation of new animal models by genetic modification. One impediment to the application of ART in *Saimiri* spp. has been the lack of an effective gonadotropin preparation for ovarian stimulation. Pregnant mare serum gonadotropin (PMSG) is inexpensive and readily available, but its repeated use in rhesus monkeys has been associated with induction of a refractory state. We have compared PMSG to recombinant human follicle stimulating hormone (rhFSH) for controlled ovarian stimulation in Bolivian squirrel monkeys. Groups of mature squirrel monkeys received rhFSH (75 IU daily) or PMSG (250 IU twice daily) by subcutaneous injection for 4 d during the breeding season (November to January) or nonbreeding season (March to September). Serum estradiol (E2) was measured daily. Follicular growth was monitored by abdominal ultrasound. During the breeding season, PMSG induced a higher E2 response than did rhFSH, with mean E2 levels being significantly higher within 3 d of stimulation. Superior follicular development in PMSG animals was confirmed by abdominal ultrasonography. During the nonbreeding season, PMSG elicited a similar increase in serum E2 levels despite the fact that basal serum E2 is typically low during the nonbreeding season. Repeated use of PMSG (≤3 cycles of administration) produced no attenuation of the E2 response. We conclude that PMSG is highly effective for repeated cycles of controlled ovulation stimulation in the squirrel monkey.

Abbreviations: ANOVA, analysis of variance; ART, assisted reproductive technology; E2, serum estradiol; IVF, in vitro fertilization; LH, luteinizing hormone; PMSG, pregnant mare serum gonadotropin; rhFSH, recombinant human follicle stimulating hormone

Advances in assisted reproductive technologies (ART) offer opportunities for in vitro fertilization (IVF), embryo transfer, and embryo and gamete cryopreservation. Sophisticated and efficient reproductive management reduces costs and increases the versatility of animals as research models in a variety of ways. For example, gamete and embryo freezing can reduce the number of animals needed, thus saving this valuable animal resource. IVF and related technologies can overcome problems of infertility and can allow production of many more gametes and embryos than can be produced in natural reproductive cycles. Embryos can be distributed to a large number of recipient females, thereby facilitating more rapid propagation of a desired trait. Finally, these technologies allow the introduction of new genes into the germline. This 'transgenic' technology has enormous utility for understanding fundamental mechanisms of mammalian development, for creating animal models of human disease, and for a variety of biotechnologic applications.[11] These techniques have been applied to nonhuman primates. For example, in the rhesus monkey, artificial insemination,[21] embryo transfer,[28] IVF,[27] and embryonic stem cell line derivation[5,22,25] are techniques that have been used successfully.

Because of the limited availability and expense of rhesus monkeys, the use of alternative nonhuman primate models has been proposed. Specifically, neotropical primates have been mentioned as probable alternative species.[2] Squirrel monkeys are the most commonly used New World primates in biomedical research.[1] In captivity, squirrel monkeys can live to be nearly 25 y of age. They reach reproductive age at approximately 3 y and can reproduce until 15 y. The reproductive biology of the squirrel monkey has been studied over the last 25 y and has been the subject of several publications primarily during the 1970s and 1980s.[8,12,13,16,17] Squirrel monkeys, unlike Old World primates, are seasonally polyestrus rather than menstrual cyclers. The breeding season occurs from November to January, and births occur from June through August. The breeding season consists of a cluster of ovulatory cycles that vary in length between 6 and 23 d.[7] Serum estradiol (E2) is the hormone most commonly measured for the purposes of cycle evaluation.[31] During the cycle, E2 values remain low until a midcycle peak. E2 ranges from <500 pg/ml during the luteal phase to >1500 pg/ml just prior to ovulation.[9,30] This peak follows spikes in follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations.[29,30] Serum progesterone is low (<50 ng/ml) at the beginning of the cycle and rises after ovulation (approximately 200 ng/ml). It remains high during the luteal phase of the cycle.[30] During the nonbreeding season, the levels of serum E2 are <100 pg/ml and those of progesterone are <20 ng/ml.[7] It has been reported that vaginal cytology can be used to evaluate the stage of the cycle in female squirrel monkeys.[12,14] However, we have noted in our colony that the presence and character of cervical mucous does not indicate a particular day in the cycle. In fact, copious vaginal discharge has been observed 2 d before and

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continuing through 2 d after ovulation.[10]

Despite advances in the understanding of the normal reproductive physiology of squirrel monkeys,[30] little has been done to develop this species as a model for ART. Indeed, we have only limited information in recent years on the best way to artificially induce follicle recruitment in squirrel monkeys. Pregnant mare serum gonadotropin (PMSG) is commonly used in the superovulation of other species, including rats,[15,20] mice,[26] pigs,[34] and rabbits.[33] In contrast, recombinant human FSH (rhFSH) has been successfully used for ovarian stimulation in marmosets,[18] baboons,[6] and rhesus macaques.[23,24]

The goal of the present study was to compare the effectiveness of PMSG and rhFSH on ovarian stimulation in squirrel monkeys. We investigated the effects of PMSG on E2 levels during both the breeding and nonbreeding seasons. To determine whether squirrel monkeys will become refractory to further stimulations, we evaluated PMSG use in the same animals for 2 and 3 stimulation attempts. Finally, we investigated whether ovarian stimulation with PMSG affected pregnancy rate or outcome once the previously stimulated females were returned to the breeding colony.

Materials and Methods

Animals. Adult female Bolivian squirrel monkeys (*Saimiri boliviensis boliviensis*) were housed at the University of South Alabama Center for Neotropical Primate Research and Resources. The animals were group-housed indoors with other females in a colony setting and exposed to a natural photoperiod that tracked local sunrise and sunset. Animals were fed a diet of LabDiet New World Monkey Diet (Richmond, IN) and seasonal produce. Peanuts and PRIMA-treats (Bio-Serv, Frenchtown, NJ) were offered as environmental enrichment. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of South Alabama and are in accordance with the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals.*[19]

E2 assay. Before the experiments, a serum sample was collected from each animal for determination of baseline serum E2. Serum E2 was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay using an automatic analyzer (Immulite 2500, Diagnostic Products Corporation, Los Angeles, CA). Samples were collected at 0800 by femoral venupuncture within 1 min of capture. Serum was frozen at –20 °C within 1 h of collection. Only females with baseline serum E2 of <250 pg/ml were included in these studies.

Drugs. Recombinant human FSH (rhFSH, Organon USA, West Orange, NJ) was reconstituted with sterile water according to the manufacturer's instructions. Pregnant mare serum gonadotrophin (PMSG, Sigma, St. Louis, MO) was reconstituted with sterile water.

Experimental procedure. The goal of the 1st experiment was to compare the efficacy of rhFSH and PMSG on stimulation of follicular recruitment in the breeding season. Follicular recruitment was determined by elevated serum E2 and was confirmed by abdominal ultrasonography (Image Point HX with 15-6L linear probe, Hewlett Packard, Andover, MA). The 1st study was performed in December or January, during the breeding season. We allocated 27 monkeys into 2 groups. In one group, 14 animals were injected daily (0800) for 4 d with rhFSH (75 IU subcutaneously). In the second group, 13 animals were injected for 4 d with PMSG (250 IU subcutaneously) twice daily (0800 and 1500). Serum was collected at 08:00 each day for determination of serum E2. All animals were examined via ultrasonography on day 4 of hormone administration, to determine follicular development. Transabdominal ultrasonography was performed on restrained, unanesthetized monkeys. Follicles were counted and measured on both ovaries in each animal. The presence of multiple follicles (diameter, ≥3 mm) was identified as evidence of follicular recruitment.

The goal of the 2nd experiment was to determine whether squirrel monkeys respond to PMSG during the nonbreeding season. Nine animals were injected with PMSG (250 IU subcutaneously) twice daily for 5 d during March or May. Serum was collected at 0800 each day for determination of serum E2. In addition, abdominal ultrasonography was performed on day 5 of hormone administration to determine follicular development, as described earlier.

The goal of the 3rd experiment was to determine whether multiple courses of PMSG would result in a loss of responsiveness to future stimulation attempts, thereby inducing a refractory state. Groups of animals were challenged with 1 (n = 9), 2 (n = 9), or 3 (n = 6) cycles of PMSG stimulation. Stimulation protocols (PMSG, 250 IU twice daily, subcutaneously) each were 4 d in length and occurred during both the breeding and nonbreeding seasons. Serum was collected each day for determination of serum E2, as described earlier.

After the 3rd study, 9 animals (6 stimulated for 3 cycles and 3 stimulated for 2 cycles) were returned to the breeding colony and were evaluated for pregnancy outcome. Live births, stillbirths, abortions, and failures to become pregnant were recorded. These numbers were compared with those of the general colony.

Statistical analysis. Where possible, two-way repeated-measures analysis of variance (ANOVA) was used to examine overall differences between groups. After these analyses, Holm–Sidak post hoc tests were run to determine specific differences between groups. Because we found significant interaction in the 1st study comparing rhFSH and PMSG, we ran a Kruskal–Wallis one-way ANOVA on ranks to look for overall effects of each variable independently (SigmaStat, Richmond, CA).

Results

rhFSH is the drug of choice for ovarian stimulation of many of the primates used in biomedical research.[6,18,24] However, in previous attempts, we have not been able to achieve adequate follicular recruitment in squirrel monkeys with the doses published for use in the marmoset (125 mg/kg body weight)[18] or rhesus macaque (approximately 12 mg/kg body weight).[24] In the 1st experiment, we sought to compare the effectiveness of rhFSH versus PMSG on follicular recruitment in the squirrel monkey during the breeding season (November to January). Serum E2 was measured daily during 4 d of treatment with rhFSH or PMSG (Figure 1). rhFSH did not significantly affect circulating E2 levels on any day of treatment. In contrast, PMSG resulted in a robust E2 response that exceeded the starting values (Figure 1). Follicular recruitment was confirmed by abdominal ultrasonography (Figure 2). The representative animal shown in Figure 2 demonstrated multiple follicles on both ovaries, with some follicles being approximately 3 mm in diameter. Serum E2 levels in this animal were >1000 pg/ml when this image was recorded.

Squirrel monkeys are seasonal breeders, and this characteristic has the potential to limit the application of ART to the breeding season. If these animals can be stimulated to produce oocytes during the nonbreeding season, ART studies could continue through-

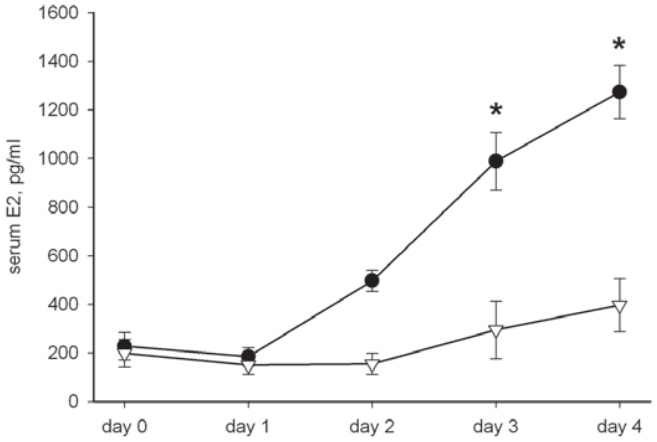

Figure 1. PMSG, but not rhFSH, stimulates ovarian development in squirrel monkeys. Squirrel monkeys were treated with PMSG (250 IU twice daily, n = 13; solid circles) or rhFSH (75 IU once daily, n = 14; open triangles) for 4 d. Blood samples were collected daily for determination of serum E2. Asterisks indicate significant ($P < 0.05$, two-way repeated-measures ANOVA) differences from day 0 E2 levels. Data are presented as mean ± standard error of the mean.

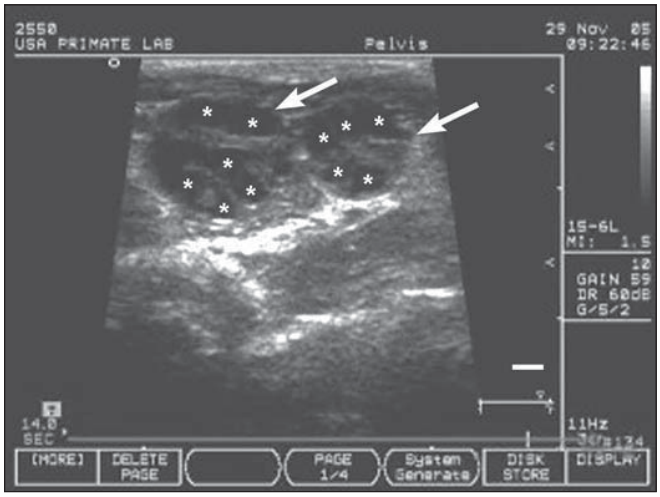

Figure 2. A representative transabdominal ultrasound image of the pelvis of a female squirrel monkey treated with PMSG for 4 d. Positions of the ovaries are indicated by arrows. The follicles within each ovary are indicated by asterisks. Scale bar, approximately 3 mm.

out the year. In the 2nd experiment, we evaluated the effect of PMSG on serum E2 levels in squirrel monkeys during March or May. PMSG was effective in stimulating E2 production in squirrel monkeys during this period. Serum E2 levels were elevated significantly ($P < 0.05$; two-way ANOVA) by day 3 of PMSG stimulation (Figure 3A). We compared nonbreeding season E2 values in these animals with those obtained when these same animals received PMSG during the breeding season; we noted no significant difference (Figure 3B). These results suggest that PMSG is as effective in stimulating serum E2 during the nonbreeding season as during the breeding season.

Repeated use of PMSG for multiple cycles of ovarian stimulation has been reported to induce a refractory state in rhesus monkeys.[3] In the 3rd experiment, we looked at repeated stimulation with PMSG in squirrel monkeys. Squirrel monkeys were challenged with PMSG for 4 days for 1 (n = 9), 2 (n = 9), or 3 (n = 6) cycles. All animals achieved high serum E2 levels after each cycle of PMSG (Figure 4). Serum E2 values on days 3 and 4 of treatment were significantly greater ($P < 0.05$) than those on day 0 for all 3 groups. Two-way ANOVA comparing previous stimulation and day of PMSG treatment revealed an overall significant ($P < 0.05$) effect of day but not of previous stimulation. These results suggest that squirrel monkeys do not become refractory when repeatedly challenged with PMSG.

In the rhesus monkey, repeated stimulation with rhFSH does not affect future reproductive outcome.[24] To determine whether similar success was achieved in squirrel monkeys, we evaluated pregnancy outcome in squirrel monkeys returned to the breeding colony after repeat challenges with PMSG. Of the 9 squirrel monkeys that were treated with PMSG for 2 cycles, 1 did not become pregnant, 1 had a stillbirth, and 7 had live births. This pattern of outcome is comparable with the reproductive statistics of a cohort of age-matched animals in the general colony. In the general colony, 67% of age-matched females became pregnant during the 2004–2005 breeding season; of those pregnancies, 12% ended in spontaneous abortion; 13% resulted in stillborn infants; and 75% yielded live births.

Discussion

In the present study, we attempted controlled ovarian stimulation with PMSG in female Bolivian squirrel monkeys. We confirmed follicular development with ultrasound examination and serum E2 measurements. We compared these results with those achieved using rhFSH and found PMSG during a 4-d protocol to be superior in the recruitment of multiple follicles. We then attempted nonbreeding season ovarian stimulation and repeat stimulation using PMSG. We found PMSG to be effective in inducing follicular growth under these circumstances. Finally, we found that the pregnancy rate of previously treated animals was not different from that of other group-housed Bolivian squirrel monkeys.

It was surprising that rhFSH was relatively ineffective in stimulating follicular development in squirrel monkeys, because a protocol for controlled ovarian stimulation of New World marmosets with rhFSH has been described.[18] However, Marshall noted that marmosets required high doses of rhFSH compared with those needed per kg in rhesus monkeys. Marmosets received 50 IU rhFSH daily for 5 or 6 d, translating approximately to a dose of 125 IU per kg body weight daily. In contrast, follicular recruitment is achieved in rhesus monkeys with a dose of only 12 IU per kg body weight daily.[24] Because adult female squirrel monkeys weigh approximately 600 g, we chose 75 IU rhFSH daily to achieve the same dose (per kg body weight) that was effective in marmosets. However, the dose of rhFSH for 4 d failed to increase serum E2 levels above baseline in squirrel monkeys. Longer periods of treatment with rhFSH (between 7 and 10 d) resulted in stimulation of serum E2 in some squirrel monkeys (data not shown), and it is possible that even higher doses might also be effective. However, the use of higher doses of rhFSH for longer periods of time is cost-prohibitive. Rather, we found that PMSG was extremely effective in stimulating follicular recruitment in squirrel monkeys over a 4-d period and is much more cost-effective (approximately 1/3 the cost of rhFSH).

It is not clear why PMSG is so much more efficacious than rhFSH in squirrel monkeys. It is possible that it results from the combination of trophic hormones in PMSG. PMSG has both FSH and LH activity.[4] Perhaps this mix of trophic hormones better mimics the

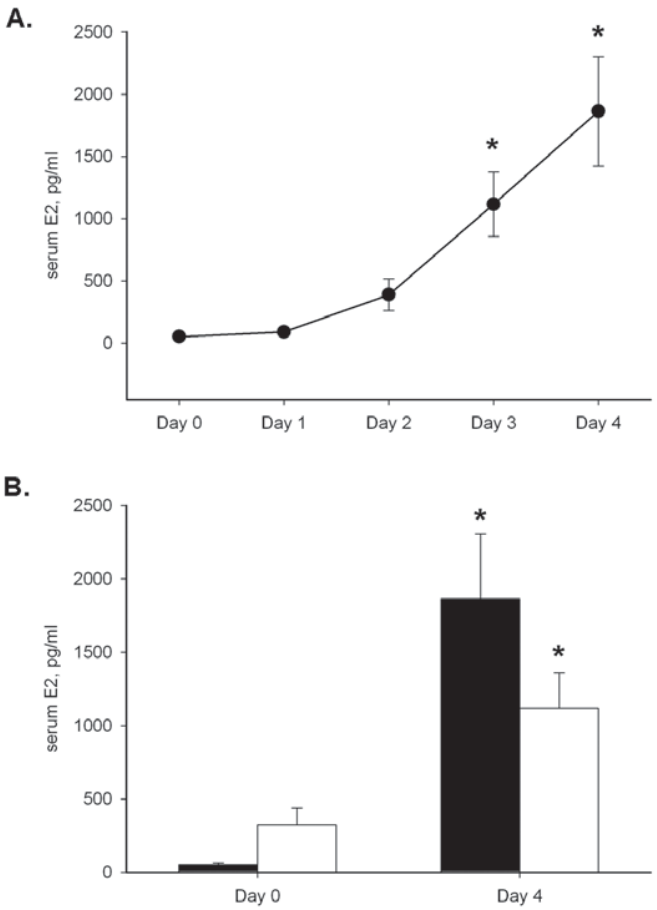

Figure 3. (A) Squirrel monkey ovarian stimulation with PMSG (250 IU twice daily) for 4 d (n = 6) during the nonbreeding season. Asterisks indicate E2 levels were significantly ($P < 0.05$, two-way repeated-measures ANOVA) elevated on days 3 and 4 of stimulation. Data are presented as mean ± standard error of the mean. (B) Comparison of E2 levels in squirrel monkeys given PMSG for 4 d during the breeding season (n = 6; open bars) compared with the nonbreeding season (n = 6; solid bars). Asterisks indicate that PMSG significantly ($P < 0.05$, two-way repeated-measures ANOVA) stimulated E2 levels during both seasons. Bar, mean ± standard error of the mean.

endogenous fluctuations of LH and FSH of the hypothalamo–pituitary–ovarian axis. When compared with purified ovine FSH, PMSG has 2/3 of the potency. However, when compared with purified ovine LH, PMSG has 3 times the biologic activity. This pattern suggests that much of the biologic effects of PMSG in squirrel monkeys may be due to a combination of FSH, LH, and chorionic gonadotropin-like activity. However, our results do not rule out the possibility that the FSH activity of PMSG is the sole contributor to the ovarian stimulation we observed in squirrel monkeys. Perhaps squirrel monkeys are more sensitive to equine FSH than to human FSH, and consequently, perhaps the FSH component of PMSG is more potent than rhFSH. Alternatively, the hypothalamo–pituitary–ovarian axis may be better activated by PMSG than rhFSH. Further study of the physiologic mechanisms of follicular recruitment by exogenous PMSG administration is warranted.

In the rhesus monkey, which also is a seasonal breeder, rhFSH can elevate serum E2 during the nonbreeding season. However, oocyte quality and blastocyst formation was reported to be reduced in the nonbreeding season.[32] We do not know whether

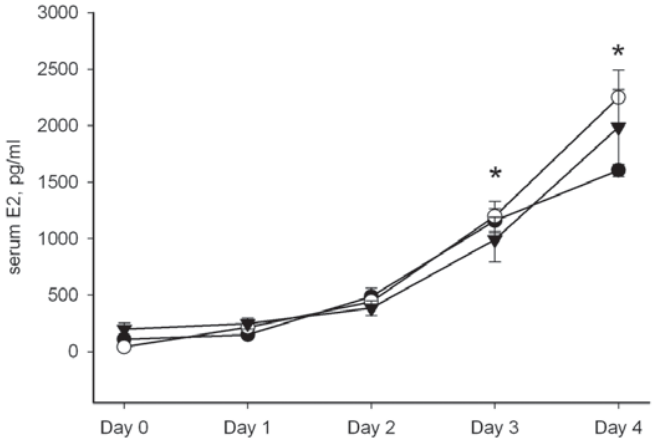

Figure 4. Repeat stimulation with PMSG (250 IU twice daily) for 4 d. Squirrel monkeys achieved high serum E2 levels with PMSG after all 3 cycles. Asterisks indicate E2 values at days 3 and 4 were significantly ($P < 0.05$, two-way repeated-measures ANOVA) greater than that of day 0 of PMSG treatment for all 3 groups. 1X, animals that received 1 cycle of PMSG stimulation (n = 9; solid circles); 2X, animals that received 2 cycles of PMSG stimulation (n = 9; open circles); 3X, animals that received 3 cycles of PMSG stimulation (n = 6; solid triangles). Data are presented as mean ± standard error of the mean. There was no significant difference between groups on any day.

we will observe the same reduction in oocyte quality in squirrel monkeys in the non-breeding season. As we extend our ART studies to IVF in squirrel monkey oocytes, it will be important to compare oocyte quality during the breeding with that during the nonbreeding season.

We were surprised to find that squirrel monkeys exposed to multiple courses of PMSG showed no refractoriness to subsequent PMSG challenges. Although we were able to produce a robust E2 response with PMSG administration, a refractory state to PMSG has been reported to occur in rhesus monkeys.[3] Bavister reported that this state is induced by the presence of nonprecipitating antibodies to PMSG.[3] To date, we have observed no reduction in responsiveness to PMSG by any female squirrel monkey, even after 4 courses of PMSG stimulation (data not shown). Finally, when we return previously PMSG-treated animals to the breeding colony, they are successful breeders.

In conclusion, we successfully used PMSG to stimulate squirrel monkey ovaries to produce follicles that elevated serum E2. PMSG can be used in this species during both the breeding and nonbreeding seasons. PMSG can also be used for repeated stimulations in the same animal. Our experiments indicate that PMSG is both more effective and more economical than rhFSH for controlled ovarian stimulation of female Bolivian squirrel monkeys.

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