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Refinement of a Protocol for the Induction of Lactation in Nonpregnant Nonhuman Primates by Using Exogenous Hormone Treatment

Shannon D Smith,[1] Joshua D Amos,[2] Krista N Beck,[1] Lisa M Colvin,[1] Kelly S Franke,[1] Brooke E Liebl,[2] and Sallie R Permar[2,*]

Obtaining sufficient quantities of milk from NHP is necessary for pharmacologic and immunologic studies required for the development and safety assessment of drugs and vaccines to be used in the maternal–infant setting. We previously induced lactation in nonpregnant female rhesus macaques (RM, *Macaca mulatta*) and African green monkeys (AGM, *Chlorocebus sabaeus*) for studies of immune responses in milk, but the volume collected was variable. To improve lactation induction protocols for nonbreeding nonhuman primates, we investigated serum hormone levels and collection protocols in AGM and RM. Here, we correlated milk volume with serum levels of endogenous and administered hormones: estradiol, prolactin, progesterone, and medroxyprogesterone in RM and AGM. We also investigated whether age, parity or the timing of milk collections were associated with the volume of milk collected from the AGM and RM in which lactation was induced by using exogenous hormones. We found an inverse correlation with serum estradiol and milk volume in the RM but no significant correlation between milk volumes and the remaining serum hormone levels in the induced RM or AGM. In addition, HIL AGM had higher peak estradiol levels than did naturally lactating AGM. A revised estradiol-sparing protocol increased milk volumes in the AGM. In addition, milk volume in RM was greater in the morning than the afternoon. In conclusion, we have refined a lactation induction protocol in nonpregnant primates, which is a needed alternative to using nursing primates for the assessment of drug levels and immune responses in milk.

Abbreviations: AGM, African green monkey; AGM1, first AGM lactation induction; AGM2, second AGM lactation induction; RM, rhesus macaque; NL, naturally lactating; HIL, hormone-induced.

The pharmacokinetics by which drugs and their metabolites or vaccine-elicited immune responses appear in the breast milk of lactating women is currently conducted only in aftermarket studies. Using lactating NHP for these studies would reveal pharmacokinetics, metabolism, and host responses in the milk compartment before the drug was marketed and used clinically in lactating women. However, the resources associated with using breeding female NHP for these types of studies are considerable. A much more time-efficient and cost-effective approach that spares the fetus or infant from exposure to experimental interventions is the induction of lactation in nonpregnant NHP. Moreover, this approach spares additional breeding solely for studies of lactation. In fact, this lactation induction protocol has been used successfully in several studies to explore immunologic and virologic mechanisms of postnatal virus transmission in SIV-infected and HIV1-vaccinated monkeys, with the goal of developing strategies to reduce breast-milk transmission of HIV1.[1,4,6,11,28,29]

We previously described our protocol for inducing lactation in RM and AGM by using exogenous hormone treatment (that is, hormone-induced lactation [HIL]).[22-25,28,29] Increasing doses of depot medroxyprogesterone and estradiol were administered intramuscularly for 2 mo to mimic the hormone fluctuations of natural pregnancy and achieve mammary tissue development. After 2 mo of hormone injections, haloperidol was administered orally to raise serum prolactin levels and induce milk production. Oxytocin was given prior to milk collection to stimulate milk let-down. We also reported a comparison of the cellular and immunoglobulin content in the milk of naturally lactating (NL) and HIL RM. The lymphocyte distribution was similar between the 2 types of milk, yet the IgG content was higher in HIL milk[22] but similar to that seen in less mature or early milk. Therefore, we concluded that this method was a good model for the assessment of immunologic parameters but that it may better represent the immunologic milieu of colostrum. The milk volumes obtained from the RM were quite variable (100 µL to 2 mL), and when this protocol was used in AGM, milk volumes collected were much less than expected (30 to 100 µL), often limiting the laboratory assays that could be performed. Therefore, optimizing the HIL protocol in the nonpregnant NHP would further facilitate pharmacologic and immunologic investigations in lactating NHP.

The aims of our current study were 1) to determine the relationship between serum hormone levels (progesterone, medroxyprogesterone, estradiol, and prolactin) and other clinical factors to milk volume collected from nonpregnant RM and AGM that underwent HIL and 2) to compare the hormone levels achieved during HIL to those of NL AGM. We hypothesized that age, parity, and serum hormone levels are associated with milk volume in each species after HIL, and we aimed to determine the combination that predicted the highest milk volume. Determining the clinical factors and hormone levels that best predict high milk volume in the HIL protocol would increase

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[1]Division of Laboratory Animal Resources and [2]Duke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina.
[*]Corresponding author. Email: sallie.permar@dm.duke.edu

the applicability of this technique, obviating the need to use breeding and infant animals in the assessment of drug levels and immune responses in milk.

Materials and Methods

Animals and housing. Female AGM and RM were sourced from the New Iberia Research Center (New Iberia, LA). All work and procedures were included in a Duke University IACUC protocol, and animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* and Public Health Service (PHS) expectations.[12,20] The NHP were housed in custom 9 ft^2 primate cages (Allentown, Allentown, PA) with internal dimensions of 31 in. width × 42 in. depth × 33 in. height and rotated access to an additional 9 ft^2 play cage (height, 66 in.) at Duke University Medical Center Division of Laboratory Animal Resources Vivarium. The AGM were fed LabDiet 5045 High Protein Monkey Diet (PMI Nutrition International, Brentwood, MO), and the RM were fed LabDiet 5038 Monkey Diet (PMI Nutrition International). The animals were kept at a temperature of 72 ± 2 °F, a 12:12-h light:dark cycle, and a room humidity of 30% to 70%.

The 6 AGM weighed 5.6 to 6.2 kg, they ranged in age from 14 to 15 y, and all were nulliparous. The 8 RM weighed 4.2 to 7.3 kg, they were 5 to 15 y old, and the parities of the RM ranged from 2 to 7 live births. In addition to having biannual sedation for health status checks, the AGM and RM underwent weekly abdominal palpation to detect uterine hyperplasia, and blood was collected every 2 mo for a CBC to ensure that there were no hematologic abnormalities due to hormone administration.

To address the study objectives, serum samples from hormone-induced lactating animals, 8 RM and 6 AGM, were obtained. The AGM were granted an IACUC exemption from pair housing due to the fact that SIV viruses, with which they were infected, may mutate and evolve over time so that cross-contamination might occur if the monkeys were socially housed.[21,29] The animals had never been socially housed, so the chances of viral cross-contamination greatly increased when pair-housing was attempted. The RM were pair-housed until interanimal aggression or end of study necessitated single housing. To aid in the comparison of the hormone levels found in our HIL AGM with those in NL AGM, banked serum samples were obtained from an indoor–outdoor colony of AGM housed at Wake Forest University Primate Center (Winston-Salem, NC) who were nursing infants younger than 6 mo, to most closely reflect serum hormone levels during NL.

HIL protocol. Figure 1 describes the timelines, drugs, and dosages used in the lactation induction protocols. On day 0, the start of first lactation induction in AGM (AGM1), the 6 nonpregnant female AGM were given medroxyprogesterone (150 mg/mL medroxyprogesterone acetate, injectable suspension, USP, Greenstone, Peapack, NJ) at a dose of 3 mg/kg IM and estradiol (5 mg/mL, estradiol cypionate injectable suspension USP, Depo-Estradiol, Pfizer, New York, NY) at a dose of 0.1 mg/kg IM (Figure 1). The estradiol injections continued as follows: week 2, 0.15 mg/kg; week 4, 0.2 mg/kg; week 6, 0.25 mg/kg; and week 10, 0.25 mg/kg. Haloperidol decanoate (2 mg/mL oral solution, Silarx Pharmaceuticals, Carmel, NY) was initiated on week 5 at a dose of 0.3 mg/kg PO twice daily, increased to 0.35 mg/kg PO twice daily at week 8, and increased to 0.4 mg/kg PO twice daily on week 11 for the remainder of the 14-wk lactation induction cycle. The monkeys received the liquid haloperidol mixed in a fruit-flavored drink, which they took readily.

For the second lactation induction in AGM (AGM2), the most notable change was that only 2 intramuscular estradiol injections were given, compared with the 5 of AGM1. AGM2 began 22 wks after the end of AGM1. On day 0 of AGM2, medroxyprogesterone and estradiol were given at a dose of 3 mg/kg and 0.1 mg/kg, respectively. The medroxyprogesterone dose was repeated on week 2. The intramuscular estradiol injections continued on week 2 (0.15 mg/kg) and week 4 (0.2 mg/kg). Haloperidol treatment was started on week 7 at a dose of 0.3 mg/kg PO twice daily (except the hormone was given orally once daily on weekends), increased to 0.35 mg/kg PO twice daily on week 10, again increased to 0.4 mg/kg PO twice daily on week 13, and continued at this dose for the duration of the lactation induction.

Because the induction protocol resulted in sufficient milk volumes from RM, a comparison study was not undertaken. On day 0, 8 female RM of various ages and parity were given medroxyprogesterone and estradiol intramuscularly at doses of 3 mg/kg and 0.1 mg/kg, respectively. On week 2, the medroxyprogesterone dose was repeated, and the estradiol dose was increased to 0.15 mg/kg. Oral haloperidol was started on week 3 at a dose of 0.3 mg/kg PO twice daily for the duration of the lactation cycle.[22-25,28,29]

Sedation and sample collection. The NHP were sedated by using ketamine (100 mg/mL, Ketaset Injection USP, Fort Dodge Animal Health, Fort Dodge, IA) at a dose of 10 to 20 mg/kg IM. Intramuscular diazepam (5 mg/mL, diazepam injection USP, Hospira, Lake Forest, IL) at a dose of 0.25 to 0.5 mg/kg was added to the regimen for AGM that needed additional muscle relaxation. The doses were tailored individually to each animal's response to the drugs. An injection of 10 U oxytocin (20 U/mL, oxytocin USP, Bimeda, Le Sueur, MN) was given intramuscularly at the time of sedation. When the NHP were deemed immobile, sterile lubricant was placed to protect the cornea, and their temperature, pulse, respiration, and weight were recorded. Blood was collected aseptically from the femoral vein by using a 22-gauge needle and a 10-mL syringe and placed into 10-mL serum separator tubes and 2-mL EDTA tubes. Hemostasis was achieved with cotton gauze and gentle digital pressure applied to the venipuncture site. To obtain milk samples, both nipple and mammary tissue were massaged manually for 2 to 3 min to stimulate milk let-down. The milk then was extruded from the tissue by manual stripping. The mammary tissue was massaged downward from the base to the nipple continuously, allowing for milk to spray into a 50-mL plastic conical vial at the end of each stripping motion. This action was repeated for as long as 5 min on each side or until no more milk sprayed from the duct.[22-25,28,29] In RM, on 3 different mornings, at least 3 d apart, milk was collected manually by the same person at either the same time as oxytocin administration, 5 min after oxytocin administration, or 10 min after oxytocin administration to determine whether the timing of oxytocin administration was associated with the volume of milk collected.

Hormone analysis. Serum hormone levels were determined on the same day and time as milk volumes were collected, by using commercial ELISA kits for medroxyprogesterone (Euro-Proxima, Arnhem, The Netherlands), estradiol, progesterone, and prolactin (ALPCO Immunoassays, Salem, NH). The medroxyprogesterone assay was performed according to the manufacturer's specifications except for elimination of the tissue extraction step. The serum samples were run in duplicate and analyzed on a plate reader (Victor X Multilabel Plate Reader, PerkinElmer, Waltham, MA) set to the ELISA kit manufacturer's specifications. The raw data were analyzed by using WorkOut 2.5 (Dazdaq, Brighton, England).

Statistical analyses. Because serum hormone levels and milk volumes were collected multiple times per monkey, associations

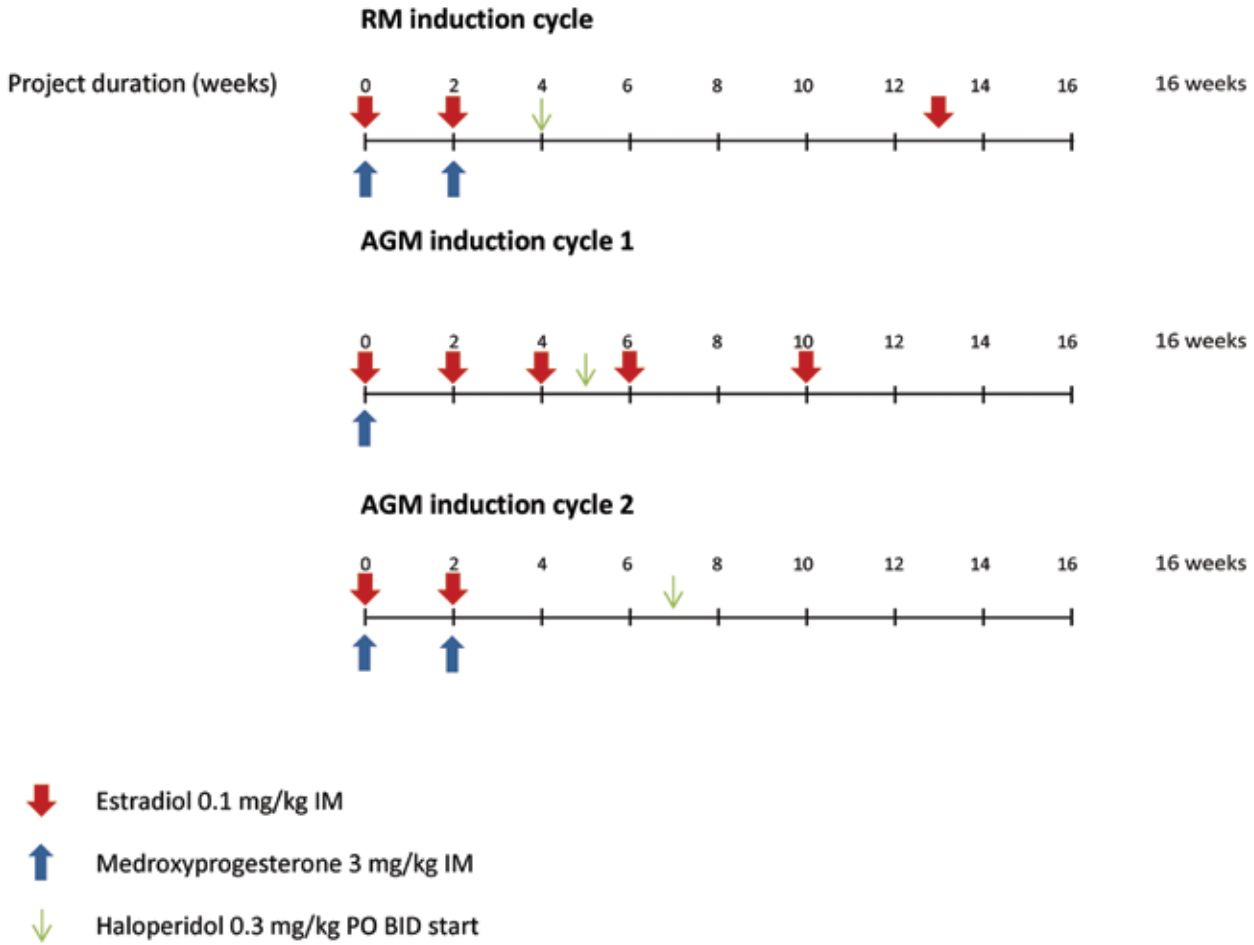

Figure 1. Lactation induction protocols: The lactation induction cycle and timing of doses of intramuscular medroxyprogesterone and estradiol and oral haloperidol are indicated. The numbers correspond to weeks of the lactation induction cycle. The red, blue, and green arrows denote doses of intramuscular estradiol and medroxyprogesterone and the start day of twice-daily oral haloperidol, respectively. The estradiol dose started at 0.1 mg/kg and was increased with each subsequent injection to a final dose of 0.25 mg/kg. The medroxyprogesterone dose remained the same at each injection (3 mg/kg). The haloperidol doses were increased to a maximum dose of 0.4 mg/kg in the AGM but remained at 0.3 mg/kg for the RM.

between the hormone levels (estradiol, medroxyprogesterone, prolactin, and progesterone) and milk volumes were tested by using repeated-measures analyses of covariance. (SAS Institute, Cary, NC). In these analyses, prolactin levels exhibited a marked right skew, so the logarithm of prolactin level was used. Spearman rank correlations were used to test the relationships of parity and age to mean milk volume. Mann–Whitney *U* tests were used to compare the peak hormone levels of the HIL AGM with those of NL AGM. Wilcoxon matched-pairs signed-rank tests were used to compare the mean volumes of milk collected from the same monkeys after 2 distinct lactation induction protocols and collected during the morning compared with the afternoon (GraphPad Prism versions 5 and 6, GraphPad Software, La Jolla, CA). Separate analyses were conducted on each group (AGM1, AGM2, and RM). *P* values are 2-sided and considered to be significant when less than 0.05.

Results

Comparison of initial AGM and RM lactation induction. In comparing the milk volumes and serum hormone levels between the AGM1 and the RM lactation inductions, we found some striking differences. Most notably, the RM produced higher milk volumes for a longer duration than did the AGM (Figure 2 A and B; $P = 0.03$, Mann–Whitney *U* test of peak milk volume for RM and AGM). In addition, the estradiol levels were elevated above background levels by the lactation induction in AGM1, whereas the estradiol level remained near prehormone treatment levels in the RM (Figure 2 C and D). Serum levels of prolactin were generally lower in the AGM than the RM, reaching peak values of only 40 to 60 ng/mL in AGM compared with 60 to 200 ng/mL in RM during the majority of the lactation induction cycle (Figure 2 E and F). Endogenous progesterone was suppressed to a lesser extent by medroxyprogesterone injections in the AGM1 than the RM lactation induction (Figure 2 G and H), likely due to the use of a single medroxyprogesterone dose in AGM compared with 2 doses in RM (Figure 2 I and J).

Comparison of serum hormone levels in NL and HIL AGM. We next compared the peak hormone levels in our HIL AGM to that of NL AGM who were less than 6 mo postpartum. Peak serum estradiol levels were significantly higher in our 6 HIL AGM (AGM1) than that of NL AGM (measured at a single time point; Figure 3 A, $P = 0.001$, Mann–Whitney *U* test). Despite the low volume of milk production, the HIL AGM had peak levels of prolactin that were similar to that of the NL AGM, with the range of values being generally, but not significantly, higher in the HIL AGM (Figure 3 B, $P = 0.49$, Mann–Whiney *U* test). Serum progesterone levels were similar between the NL and HIL AGM (Figure 3 C, $P = 0.15$, Mann–Whitney *U* test).

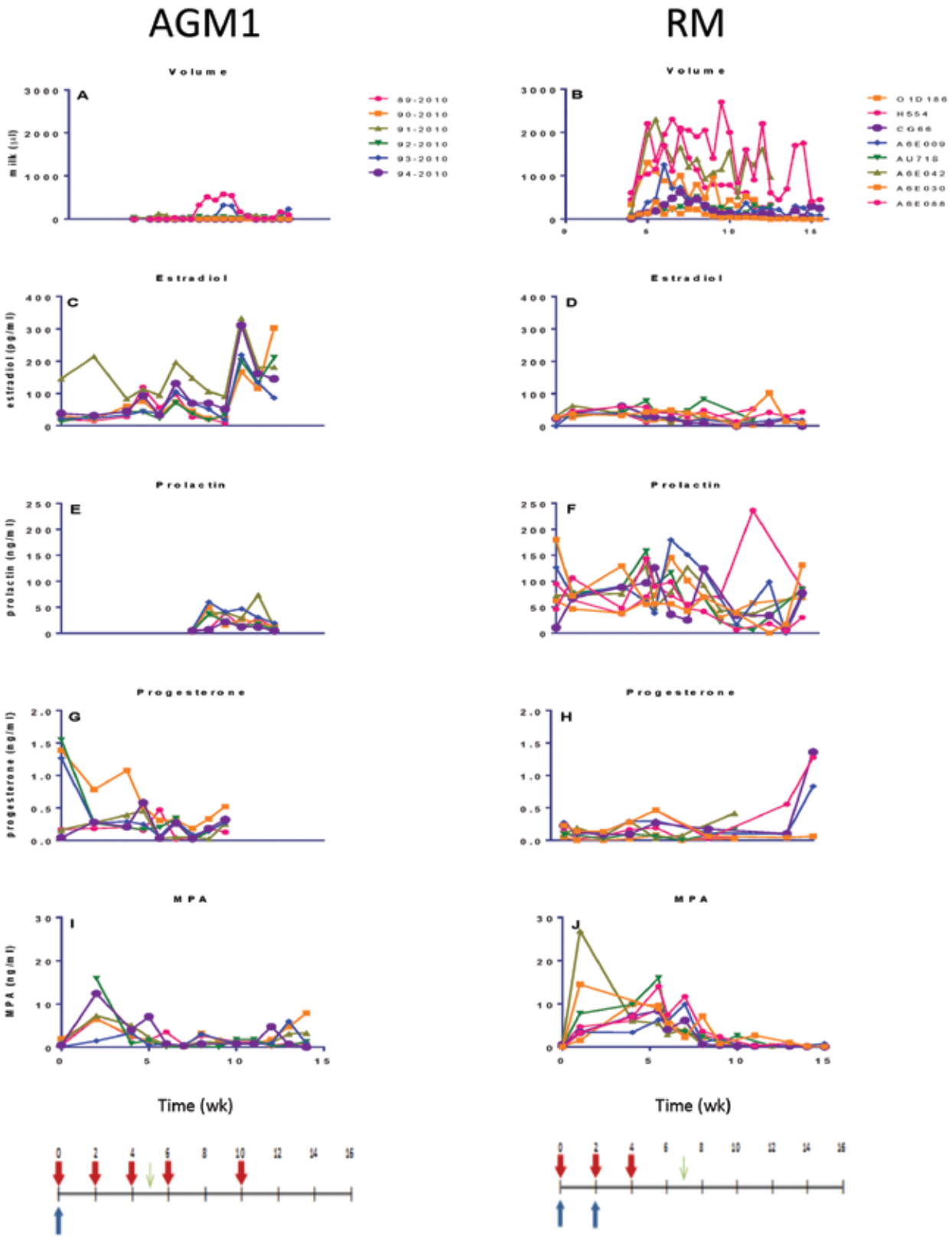

Figure 2. Comparison of milk volumes and hormone levels in AGM1 compared with RM lactation induction. (A) AGM1 milk volume. (B) RM milk volume. (C) AGM1 serum estradiol. (D) RM serum estradiol. (E) AGM1 serum prolactin. (F) RM prolactin. (G) AGM1 serum progesterone. (H) RM serum progesterone. (I) AGM1 serum medroxyprogesterone. (J) RM serum medroxyprogesterone. Each colored line represents a different animal. The red, blue, and green arrows denote estradiol and medroxyprogesterone doses and the start day for twice-daily haloperidol dosage, respectively.

Correlation of parity, age, and serum hormone levels with collected milk volumes. We next determined the relationship between parity or age and milk volume collected during the HIL protocol in the RM, because this group of animals varied in both parity and age. In this small cohort ($n = 8$), there was no significant correlation between milk volume and parity ($r = -0.15$, $P = 0.60$) or

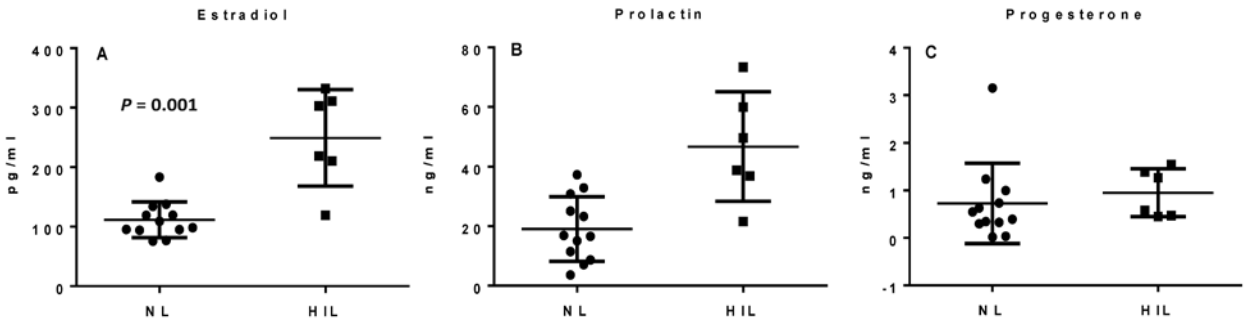

Figure 3. Serum estradiol levels are significantly (P = 0.001) higher in HIL than in NL AGM. The serum levels of (A) estradiol, (B) prolactin, and (C) progesterone in NL (circles) and HIL (squares) AGM. The center line indicates mean values, and the bars represent 1 SD. The data represent the peak serum hormone values obtained during the AGM1 lactation induction.

age (r = –0.23, P = 0.49). However, in the RM lactation induction, the estradiol level correlated inversely with milk volume according to repeated measures of covariance ($F_{1,48}$ = 7.52, P = 0.009). In contrast, no correlation was seen between milk volume and serum levels of prolactin, progesterone, or medroxyprogesterone in either species. The AGM in this study were nulliparous and older (mean age, 13.8 y) than the RM (mean age, 7.4 y), which may have contributed to the AGM having lower milk production. In addition, the AGM were slightly smaller (average weight, 5.1 kg) than were the RM (average weight, 5.9 kg). There was no correlation between age and milk volume in the AGM because all were 14 y old, except for one who was 13 y old.

Optimal timing of oxytocin administration and milk collection. According to the HIL research protocol, morning and evening milk collections were conducted only in the RM and provided the opportunity to compare milk volumes that were collected early in the day with those collected later. Mean milk volumes were significantly (P = 0.01, Wilcoxon matched-pairs signed-rank test) higher in the morning than in the afternoon (Figure 4 A).

Finally, we sought to determine whether the timing of oxytocin administration affected the milk volumes produced in the HIL RM. Given that the half-life of oxytocin is 3 to 5 min in humans,[7,13] we chose to compare the collection of milk at 0, 5, and 10 min after IM oxytocin administration. The pattern in milk volumes collected at any of the 3 time points after oxytocin administration was inconsistent across the cohort. However, in the 2 highest volume-producing RM (A6E042 and H554), the greatest milk volumes were collected at 5 min after oxytocin administration (Figure 4 B). However, milk volumes were highest at 5 min after oxytocin administration in only 4 of the 8 animals.

Estradiol-sparing lactation protocol in AGM. Because serum estradiol levels were significantly higher in our HIL protocol in AGM compared with NL AGM and because the estradiol level inversely correlated with milk volume in HIL RM, we devised an estradiol-sparing HIL protocol (AGM2) for AGM (Figure 1). AGM2 included only 2 intramuscular injections of estradiol at the beginning of the protocol as compared with the 5 estradiol injections throughout the AGM1 protocol. Moreover, the AGM2 protocol included 2 initial medroxyprogesterone injections to mimic the successful regimen used in the RM HIL protocol. With the exception of a single low milk-producing animal (91-2010, Figure 5 A and B), the animals produced a range of 44% to 1076% more milk per collection in AGM2 than during AGM1. The overall milk volume was significantly higher (P < 0.0001), Wilcoxon matched-pairs signed rank test) in the AGM2 induction compared with the AGM1 induction. At the end of AGM2, the serum estradiol values were significantly lower (P < 0.0001) than those found at the cessation of AGM1 (Figure 5 C and D).

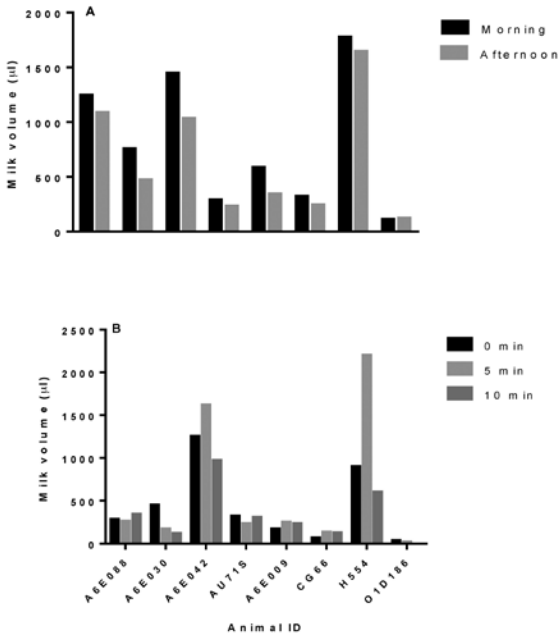

Figure 4. Greater milk volumes were obtained during RM morning collection. (A) Greater volumes were obtained during morning collections in 7 of 8 HIL RM. (B) Greater volumes were obtained when milk was collected 5 min after IM oxytocin administration in 4 of 8 HIL RM.

Unexpectedly, the AGM2 serum prolactin levels were also significantly lower (P < 0.0001) and more uniform among animals than were those of AGM1. The progesterone levels decreased over time in AGM1 but were more variable and trended toward higher levels (P = 0.48) in AGM2 (Figure 5 G and H). Finally, serum medroxyprogesterone levels were generally lower (P = 0.30) during AGM1, which included only a single medroxyprogesterone injection, but peaked at a higher level in AGM2, which included 2 doses of the depot hormone injection (Figure 5 I and J). Therefore, the estradiol-sparing HIL regimen seemed to achieve more stable hormone levels resulting in a higher volume and longer duration of milk production.

Discussion

The aims of this study were to increase milk volume collected during HIL protocols with RM and AGM. We examined the associations between milk volume and several factors, including serum hormone levels, age, and parity. We also compared the hormone levels of NL AGM with those of HIL AGM. The key findings of the study were that 1) except for the single inverse correlation between serum estradiol and

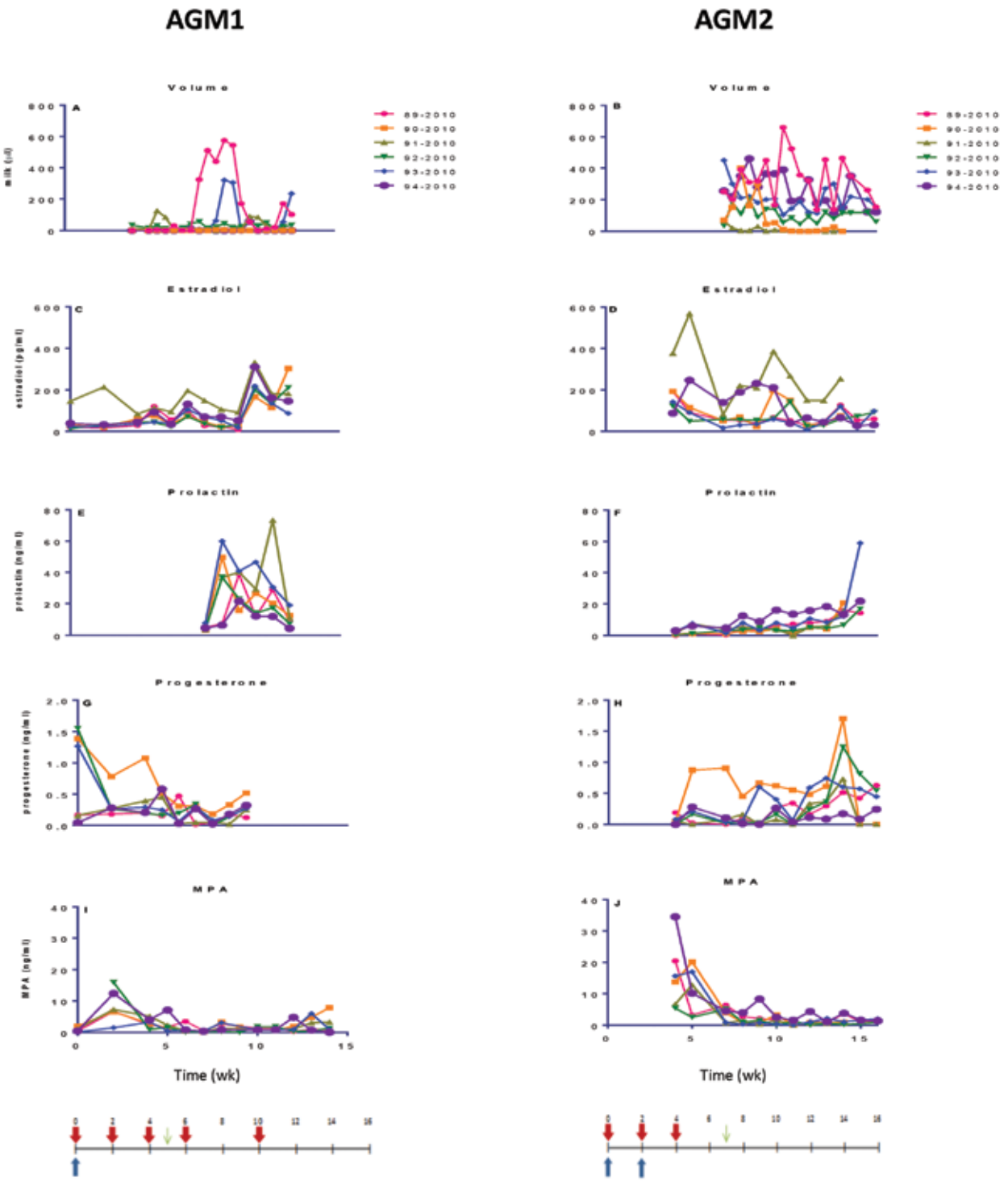

Figure 5. Increase in milk volume with estradiol-sparing HIL protocol in AGM. (A) AGM1 milk volume. (B) AGM2 milk volume. (C) AGM1 serum estradiol level. (D) AGM2 serum estradiol level. (E) AGM1 serum prolactin level. (F) AGM2 serum prolactin level. (G) AGM1 serum progesterone level. (H) AGM2 serum progesterone level. (I) AGM1 serum medroxyprogesterone level. (J) AGM2 serum medroxyprogesterone level. Each colored line represents a different animal. The red, blue, and green arrows denote estradiol and medroxyprogesterone doses and the start day for twice-daily haloperidol dosage, respectively.

milk volume in the RM, milk volume was not correlated with hormone levels in AGM or with progesterone, prolactin, or medroxyprogesterone in the RM, but the estradiol levels of NL AGM were significantly higher than those of the HIL protocol AGM; 2) the estradiol-sparing protocol used in AGM2 yielded significantly higher volumes and longer duration of milk collection; and 3) milk volumes were significantly higher in the morning compared with afternoon.

In our initial HIL protocol, we administered depot estradiol and medroxyprogesterone to mimic levels of those hormones in pregnancy and achieve mammary tissue development.[9,22,24,25,28,29] In the AGM1 study, we found that the peak estradiol levels were higher in our HIL AGM than in NL AGM. Moreover, estradiol is known to antagonize prolactin.[3] In addition, a second induction of lactation in the AGM by using an estradiol-sparing regimen (AGM2) resulted in higher milk

production in 5 of the 6 AGM. Therefore, future protocols may be modified further to reduce the administration of estrogen, by using only a single injection of medroxyprogesterone to bring about anestrus and injecting a dopamine antagonist to raise serum prolactin levels. Additional doses of medroxyprogesterone can be given to prevent the NHP from entering the estrous cycle again if serum prolactin levels are too low to prevent cycling. The mammary tissue development targeted by the estradiol injections may not be integral to the induction of lactation and may only be counterproductive to the effects of prolactin, especially if medroxyprogesterone alone can increase serum prolactin levels or result in lactational anestrus.[26] Although we previously compared the immune components of milk of NL and HIL,[22] future work should focus on the nutrient and microconstituent content of milk produced with this refined HIL protocol.

Although we expected that higher prolactin levels would result in higher milk volumes, we did not observe this pattern. It is possible that the estradiol injections in the AGM and RM induction protocols may have blocked the effect of prolactin.[17,19] Moreover, the considerable variability in the prolactin measurements might, in part, be explained by the different storage times of samples before batched assays were performed.[16] Anesthesia is reported to increase prolactin levels 5-fold in women[8] and 3 to 8 times in rats,[18] which is why some studies involving prolactin are conducted on awake animals by using an implanted vascular access port. The NHP in the current study were sedated with ketamine or ketamine–diazepam, so prolactin levels may have been elevated simply due to the sedation procedure. However, prolactin levels in the RM were much higher than the 40- to 60-ng/mL range that was measured in the AGM; this difference may explain why the milk volumes obtained in RM were so much higher than those of the AGM in both the AGM1 and AGM2 HIL protocols.[5,19]

Interestingly, we did not find a correlation between age or parity and milk volume in the RM, who varied in both age and parity in the current study. This lack of correlation is in contrast to previous work linking parity to the milk volume of NL in several species.[2,10,14,15,27] In the RM, the youngest and oldest NHP had the highest milk volumes, so age did not correlate with volume produced in this small cohort. The lack of correlation in the RM between milk volume and parity was likely due to individual variation in milk production and the small numbers of NHP included in this study. In addition, we were able to collect significantly higher milk volumes from the RM in the mornings than in the afternoons (Figure 5). This finding of increased morning volumes might be explained by overnight engorgement. Moreover, the oral haloperidol was given at the beginning and end of each work day, so the afternoon milk collections occurred before the second dose of the BID haloperidol was given. Finally, we observed that the milk volume from 2 high volume-producing monkeys seemed to be greater at 5 min compared with 0 and 10 min after oxytocin injection, likely due to the short half-life of oxytocin.[7,13] This timing indicates that intramuscular oxytocin can be injected after the removal of a sedated monkey from its cage, rather than while waiting for the NHP to become sedated after intramuscular ketamine injection.

In conclusion, hormonal induction of lactation in nonpregnant NHP is feasible and effective and spares the cost and resources associated with using breeding NHP and their infants in the study of experimental vaccines, drugs, and their metabolites in milk within the maternal–infant setting. This study refined a method of hormonal induction of lactation, and the milk volumes obtained by reducing exogenous estradiol administration were much improved. We hope that this protocol can be refined further and modified for use across lactating laboratory animal species in research studies where milk collection is needed.

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