ANIMAL EXPERIMENTATION

Strategies for Ovulation Induction and Oocyte Retrieval in the Lowland Gorilla

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Purpose: Ovulation induction and oocyte retrieval were performed in a lowland gorilla in an attempt to propagate and potentially cryopreserve embryos from an infertile animal and to advance techniques to help preserve this endangered species.

Results: Following 34 days of leuprolide acetate suppression, human menopausal gonadotropins were administered for 14-days in a 32-year-old wild-born lowland gorilla. Ten oocytes were retrieved by transrectal ultrasound-guided aspiration. Other approaches to oocyte recovery were not feasible in this case. A serum estradiol concentration of 4700 pg/ml at the time of human chorionic gonadotropin administration did not induce ovarian hyperstimulation. Mature oocytes were recovered from follicles measuring 14 to 24 mm in diameter, with a corresponding average serum estradiol concentration of approximately 300 pg/ml for each mature follicle. Cryopreservation of a gorilla embryo was effected from cryopreserved gorilla spermatozoa.

Conclusions: Parameters for monitoring ovulation induction in the gorilla appear to be similar to those for humans. The results indicate that the use of a gonadotropin releasing hormone agonist and higher doses of gonadotropins than previously used in gorillas appear to improve oocyte recovery.

KEY WORDS: lowland gorilla; infertility; assisted reproductive techniques; in vitro fertilization.

INTRODUCTION

Because the worldwide gorilla population is threatened with extinction, procedures to treat infertility which would otherwise be considered unnecessary, expensive, and risky may be justified. One subgroup potentially amenable to such measures is the population of captive infertile female gorillas. In this group previous attempts have been made to achieve pregnancies using artificial reproductive techniques for oocyte retrieval; however, this effort has been unsuccessful. Not only would the success of this undertaking increase the number of gorillas, but it would perpetuate the genetic diversity of animals whose genetic contributions may otherwise be lost. This article reviews previous attempts at ovulation induction and oocyte retrieval in *Gorilla gorilla gorilla* and describes the methods used in a case that resulted in recovery of the most oocytes to date.

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REVIEW OF PREVIOUS ATTEMPTS

Nine previous attempts at ovulation induction and oocyte retrieval in gorillas have been described (1–5). The age of eight gorillas was reported at the time of oocyte recovery by either laparotomy or laparoscopy (mean age = 29). Two of the attempts were performed on a 34-year-old during a 4.7-month interval. The first seven attempts did not employ gonadotropin-releasing hormone agonist (GnRHa) suppression of endogenous gonadotropins (Table I). In two cases clomiphene citrate (CC) was used to induce ovulation. The other five procedures used 3 or 4 ampoules per day of human menopausal gonadotropins (hMG; Pergonal; 75 IU FSH and 75 IU LH per amp; Serono Laboratories, Inc., Norwell, MA;) for an average duration of 6 to 7 days. This resulted in an average oocyte recovery of 2.9 ± 2.5 (SD; range, 1 to 8). All mature oocytes were recovered from follicles measuring ≥ 14 mm in diameter determined by ultrasound at the time of aspiration. Evidence of premature luteinization was found in several cases (4).

In a preliminary study, the addition of a long-acting GnRHa, leuprolide acetate (Lupron Depot; TAP Pharmaceuticals, North Chicago, IL) avoided premature luteinization (3). However, hMG administration required a longer period of time (Table II). Preliminary experience suggests that, as in humans, the use of hMG, as opposed to clomiphene, and the addition of GnRH agonist suppression are advantageous for gorilla ovulation induction. A potential disadvantage is the development of antibodies against heterologous gonadotropins that could limit the number of ovulations induced by this procedure (6).

MATERIALS AND METHODS

Permission was obtained from the research review board of the Lincoln Park Zoo. A 32-year-old female gorilla caught in the wild in 1961 at the age of 3 was selected for an attempt at in vitro fertilization (IVF) and embryo transfer based upon a complete infertility assessment indicating severe tubal disease. Her first pregnancy at the age of 22 yielded a full-term vaginal delivery of a healthy female. Her only other pregnancy resulted in a stillborn male 6 years prior to the IVF attempt. In the intervening time the gorilla mated regularly with at least two males, which both subsequently fathered offspring with other females.

Information regarding ovulatory, tubal, peritoneal, cervical, and hormonal status had been collected from two prior annual exams performed under general anesthesia. A third examination included diagnostic laparoscopy. The animal was in good general health, although a detailed body composition analysis revealed her to be obese by gorilla standards, at a weight of 133 kg (7). Dietary management was instituted 30 weeks before the IVF procedure.

During the annual exams cultures of the cervix and uterus were obtained, cervical mucus was extracted for microscopic evaluation, and endometrial biopsies were taken using Pipelle biopsy curettes (Unimar, Inc., Wilton, CT). The curettes were guided into the cavity using a prostate probe (210 Aloka Ultrasound, 5 MHz; Corometrics Medical Systems, Inc., Wallingford, CT), for transrectal visualization.

Originally, the animal was isolated from her family group in a separate holding enclosure with the intent of relocating her to another facility. This isolation permitted daily collections of urine from the concrete

Table I. Summary of Gorilla Ovulation Induction and Oocyte Recovery Procedures Reported Without the Use of Gonadotropin-Releasing Hormone Agonist Suppression

Study Number	Age	Daily Dose of hMG ^a (Amps) or CC ^b	Duration of Treatment (Days of hMG)	Range of Follicle Diameter (mm) at Time of Oocyte Recovery	Method of Oocyte Recovery	Total Number of Oocytes Recovered	Reference
1	26	CC 100 mg	6	NR	Laparotomy	1	Burton & Ramsay, 1986
2	NR ^c	CC 100 mg	5	8–12	Laparoscopy	1	Wagner (reported in Loskutoff et al., 1991b)
3	33	4	6	10–>20	Laparotomy	3	Loskutoff et al., 1991a
4	32	4	6	12–15	Laparoscopy	4	Loskutoff et al., 1991a
5	34	4	7	12–20	Laparoscopy	1	Loskutoff et al., 1991a
6	18	3	6	NR	Laparoscopy	8	Loskutoff et al., 1991a
7	34	3	6	10–15	Laparoscopy	2	Loskutoff et al., 1991a

^a Human menopausal gonadotropins (75 IU FSH and 75 IU LH per ampule).

^b Clomiphene citrate.

^c Not reported.

Table II. Cases of Gorilla Ovulation Induction and Oocyte Recovery Procedures Reported Using Gonadotropin-Releasing Hormone Agonist Suppression

Parameters	Study Number		
	1	2	3
Age	20	33	32
Dates of GnRHa ^a administration (days prior to hMG ^b initiation)	18	19	34 and 8
Dose of GnRHa	3.75 mg	3.75 mg	7.5 mg
Daily hMG dose (amps)	3	3	6
Duration of treatment (days of hMG)	8	9	14
Range of follicle diameters (mm) on day of hCG ^c administration	11 to 14	16	12 to 22
Method of oocyte recovery	Laparotomy	Laparoscopy	Transrectal ultrasound-guided aspiration
Total number of oocytes recovered	1	3	10
Reference	Yee et al. 1990	Yet et al. 1990	Present case

^a Gonadotropin-releasing hormone agonist (Lupron Depot®).

^b Human menopausal gonadotropins (75 IU FSH and 75 IU LH per ampule).

^c Human chorionic gonadotropin.

floor. When it was decided that this animal was an appropriate candidate for an IVF attempt, plans to transfer the gorilla were postponed. Urine was collected for 27 weeks prior to the onset of ovulation induction. Samples were frozen at -20°C until assayed for estrone conjugates (EC) and pregnanediol conjugates (PC) using previously described methods (8–10). During immobilizations, venous blood was collected and centrifuged, and the serum was stored at -20°C until assayed. Serum estradiol (E_2) was assayed using antibody-coated tube kits (Diagnostic Products Corporation, Los Angeles, CA). Radioimmunoassays for serum prolactin, luteinizing hormone (LH), and progesterone were performed according to previously published methods (11). Assay of serum follicle-stimulating hormone (FSH) bioactivity was performed using a rat granulosa cell aromatase bioassay (12).

Lupron Depot (7.5 mg) was administered intramuscularly (im) by blowgun 51 days prior to oocyte aspiration without regard to the cycle day. The animal remained isolated, and daily urine collections were continued. A second dose (7.5 mg im) was administered 25 days prior to aspiration (Fig. 1). Six ampoules

of hMG were initiated im in a single daily dose by blowgun beginning on day 17 prior to follicle aspiration. On day 11 of hMG administration, the gorilla was anesthetized using ketamine hydrochloride (6 to 10 mg/kg; Ketaset; Bristol Laboratories, Syracuse, NY) by blowgun and then maintained in the anesthetized state by intravenous (iv) doses of ketamine hydrochloride as necessary. Constant airway and cardiovascular monitoring were employed.

The animal was placed in the dorsolithotomy position. An ultrasound machine with a multifrequency (5-, 6-, and 7.5-MHz) vaginal probe and a 5-MHz abdominal sector scanner (Sono Line, Model SL1, Siemens Medical Systems, Inc., San Ramon, CA) was used. A trial embryo transfer was readily performed by passing a transfer catheter through a guide (Catheter KNTS-50601, Cook OB/GYN Spencer, IN). During a second immobilization (15 days after beginning hMG), attempts were made to visualize the ovaries using a variety of equipment and approaches.

On the day prior to oocyte aspiration, the animal was started on oral clear liquids. Anesthesia was again induced using ketamine hydrochloride. Because of pre-

Fig. 1. Ovulation induction protocol.

vious difficulty maintaining this animal's airway using ketamine alone, the gorilla was intubated and maintained using isoflurane (Forane; Anaquest, Madison, WI). Body weight was measured and blood taken. In the operating room the animal was placed in the dorsolithotomy position and underwent repeated cleansing enemas using a human enema catheter set. Four liters of warm tap water enemas was followed by 3 L of normal saline. A 50:50 mixture of normal saline and betadine solution was then instilled in the rectum, followed by three liters of saline lavage and a final liter of phosphate-buffered saline at pH 7.2. Rigid sigmoidoscopy to 28 cm revealed healthy mucosa. Prophylactic cefotetan (2 g) and doxycycline (200 mg) were given slowly iv.

A 15-cm vaginal ultrasound probe was passed transrectally to 18 cm by including a portion of the narrow handle. A 33-cm, 17-gauge, single-lumen needle (Cook OB/GYN, Spencer, IN) was passed through the needle guide and all accessible follicles were aspirated. Flushing and reaspiration of follicles were not performed. Follicular fluid was aspirated into sterile tubes containing 1 ml of TALP-HEPES medium with 0.3% bovine serum albumin and heparin (10 IU/ml) at pH 7.4 and 37°C (13). An aspirator (Rocket USA, Ltd., Norwalk, CT) adjusted to 100 mm of Hg negative pressure was used. Oocytes were immediately identified from the aspirates, placed in culture and transported to the lab via incubator for in vitro fertilization. The IVF procedures for this case have been reported previously (14).

At the completion of follicle aspiration, the pelvis was inspected ultrasonographically for evidence of hemorrhage. Rectal hemorrhage was evaluated by passing swabs to 30 cm and examining them for blood. The animal was extubated after transport to her cage.

RESULTS

Infertility Assessment

During the first annual examination, performed 22 months prior to the IVF attempt, an hysterosalpingogram (HSG) showed evidence of an obstructed left tube with a moderate hydrosalpinx. A repeat HSG performed at the annual exam 10.5 months prior to the IVF attempt confirmed the previous findings. Because of possible tubal factor infertility amenable to surgical correction, the gorilla underwent a diagnostic laparoscopy 28 weeks prior to the initiation of ovulation induction to assess the feasibility of tubal repair.

Laparoscopy revealed such dense bowel and omental adhesions that none of the pelvic contents could be visualized. Methylene blue dye instilled transcervically could not be detected laparoscopically. It was determined that pelvic reconstructive surgery for infertility would be futile.

Based on several observations, the gorilla was believed to be ovulatory. The animal continued to exhibit cyclic copulation. Spot urine pregnanediol values obtained on the days subsequent to copulation were found to exceed 350 ng/ml. During an annual examination performed 8 months before hMG initiation, the presence of a right ovarian preovulatory follicle 3.1×2.1 cm was detected by transrectal ultrasonography. A simultaneous endometrial biopsy revealed late proliferative tissue, while the serum E_2 was in the follicular phase range at 34 pg/ml, and the serum progesterone was 2.1 ng/ml. Cervical mucus was clear, with a spinnbarkeit of 2 cm and a 2+ to 3+ ferning pattern. The animal was also found to be normoprolactinemic (17 ng/ml). The result of a bio-FSH (7.7 mIU/ml) performed on serum obtained at that exam was in the range of FSH values assayed simultaneously from five other female gorillas of reproductive age (<3 to 10.3 mIU/ml) collected at random cycle times. The same serum sample yielded an LH concentration of 12 mIU/ml.

At the time of laparoscopy 4 weeks later, transrectal ultrasonography revealed continued follicular activity, with follicles between 0.9 to 1.2 cm in both ovaries. Biopsy again revealed proliferative endometrium.

Period of Isolation

Neither the PC nor the EC urinary measurements during the animal's isolation period (beginning 27 weeks before hMG) demonstrated normal cycling. However, 6 days after the first administration of the GnRH agonist, the EC levels decreased to an undetectable level and remained undetectable thereafter (Fig. 2). No changes in the animal's behavior were observed following either the administration of the GnRH agonist or the addition of hMG.

Ovulation Induction

The serum E_2 level following 11 days of hMG (6 ampoules daily) was 910 pg/ml; the animal weighed 143.8 kg. Although the uterus was imaged readily, it was not possible to visualize the ovarian follicles transvaginally. Additionally, the 5-MHz transabdominal sector scanner failed to image ovarian follicles.

Fig. 2. Urinary estrone conjugate (EC) determinations corrected for creatinine excretion revealed no cyclic variation before the onset of ovulation induction (P = pergonal, hMG). However, the EC baseline responded to long-term GnRH agonist treatment (L = lupron, leuprolide acetate) with a significant drop, indicating successful suppression of endogenous gonadotropin activity. During hMG therapy EC rose from an undetectable baseline to 700 ng/mg creatinine by the eleventh day of hMG, when the serum E_2 concentration was 910 pg/ml. Urinary EC peaked at 1225 ng/mg creatinine on the day of hCG administration (day 15 of hMG, when the serum E_2 was 4700 pg/ml).

Therefore, the same dose of hMG was continued. On day 15 of hMG treatment, with a full bladder and positional manipulation, the gorilla's ovaries were noted and marginally visualized with the abdominal sector scanner; transvaginal visualization was impossible. A long prostate probe (Siemens Medical Systems, Inc., San Ramon, CA) passed to 26 cm per rectum achieved excellent visualization of the ovaries. No intervening structures existed between the bowel wall and ovaries. The E_2 rose to 4700 pg/ml, the body weight increased to 152.4 kg. Approximately 20 follicles ranging from 12 to 22 mm in diameter were observed within each ovary. Of these, approximately seven or eight follicles per ovary were >15 mm. Based upon the E_2 level and the appearance of the follicles, the fifteenth dose of hMG was withheld, and the animal was given 20,000 IU of human chorionic gonadotropin (hCG; Serono Laboratories, Inc., Norwell, MA) im by dartgun, with oocyte recovery planned 38 hr thereafter.

Oocyte Retrieval

On the day of oocyte retrieval, the animal weighed 157.4 kg. The vaginal ultrasound probe with an attached needle guide was inserted to a depth of 18 cm and revealed approximately 6 to 10 follicles on each ovary measuring between 14 and 24 mm in diame-

ter, with 8 to 12 smaller follicles per ovary. Multiple transrectal needle passes into each ovary were required to reach all possible follicles. Because of the narrow bony pelvis, it was impossible to angle the probe adequately toward the left ovary to aspirate four remaining follicles measuring greater than 15 mm; all follicles in the right ovary were aspirated. At the completion of the procedure, no reaccumulation of fluid was seen in either ovary, and no fluid collection was observed in the abdomen. The total procedure time was 120 min.

Initially, the gorilla exhibited a normal recovery from anesthesia and regained consciousness. However, approximately 1 hr following the completion of the procedure, she became unresponsive and expired despite vigorous and prolonged resuscitative efforts.

Postmortem Findings

Postmortem examination was performed immediately following demise. The uterus and ovaries were taken en bloc to the embryology lab within 45 min of demise in an attempt to recover more oocytes. The gross specimen revealed dense adhesions and fat encasing the uterus, tubes, and ovaries (Fig. 3). The fimbrial ends, which protruded from the fatty encasements, could not approximate the ovarian surfaces. There was a distal dilatation of the left tube.

Fig. 3. The uterus, tubes, and ovaries are encased in thick adhesions involving the omentum and bowel.

The endometrium was secretory and lush (0.8 cm in greatest thickness). At the aspiration site there was no evidence of intraabdominal hemorrhage, unexpected ovarian injury, or visible rectal damage; however, moderate edema was discovered within the abdominal wall. No evidence of ovarian hyperstimulation syndrome (OHSS) was discovered; intraabdominal ascites was absent, and the ovaries were not edematous or unusually enlarged (measuring 4.5×6 cm each). The cause of death was determined to be suffocation from laryngeal edema.

Bacteriologic Findings

Three organisms were identified in the aerobic and anaerobic cultures of the follicular fluids obtained transrectally: *Bacteroides fragilis fragilis*, *Bacteroides fragilis distasonis*, and *Clostridium difficile*. All of these are normally found in the human colonic tract in low numbers, and in the human *Bacteroides fragilis* may be responsible for intraabdominal infections. On culturing, the *B. fragilis fragilis* was resistant to both tetracycline and cephalothin, the *distasonis* subspecies was sensitive to tetracycline, and the *C. difficile* was sensitive to cephalothin. All of the organisms were sensitive to chloramphenicol. A single parasite in close association with one of the oocytes was identified morphologically by a parasitologist as probable *Strongy-*

loides stercoralis, which is a commonly encountered hookworm in the intestinal tracts of the great apes (15).

IVF Findings

Of the 10 oocytes that were recovered, four were in metaphase II and four were in metaphase I. One had an expanded cumulus but no polar body, and one contained a germinal vesicle (14). Attempts to procure fresh gorilla sperm by electroejaculation were unsuccessful, therefore cryopreserved gorilla sperm were employed. Initial fertilization was unsuccessful but a single embryo resulted from partial zona dissection. The embryo remains cryopreserved.

DISCUSSION

The development of assisted reproductive techniques for humans offers a strategy to increase the fecundity of non-reproductive gorillas. At present only about one-half of captive female lowland gorillas of reproductive age have reproduced, and of those, 30% had only a single birth (16). The reproductive efficiency of young (<age 23) captive lowland gorillas matches that of wild mountain gorillas (17). However, the fecundity of captive gorillas appears to decline significantly with age (18). By using assisted reproduc-

tive techniques, these nonreproductive gorillas may become valuable resources to maintain the proper genetic diversity of the limited populations in zoos (19).

Only after identifying and rectifying any physiological and behavioral causes of infertility may a gorilla be selected as an appropriate candidate for artificial reproductive techniques (4,20,21). Severe tubal disease made IVF the only feasible alternative in this 32-year-old study animal.

The collection of urine for EC and PC assay is practical for monitoring the menstrual cycle (22). In this case the animal appeared to be anovulatory during isolation, possibly from the inactivity and stress resulting from separation from her group. The animal's obesity may have further contributed to her anovulatory state. She was, however, ovulatory during the 6 preceding years while mating with fertile males.

The use of a GnRH agonist obviates the need to monitor LH frequently and to time hMG initiation by cycle phase. As in humans, GnRH agonists probably decrease the risks of premature endogenous LH surges and consequent cycle cancellations during hMG administration in gorillas (3). This adds reliability, convenience, and predictability to the timing of procedures. Doses administered 51 and 25 days prior to the day of oocyte collection prevented an endogenous LH surge and did not appear to impair follicular maturation.

A fundamental difference exists between the FSH profile during the menstrual cycle of gorillas and that of women. Two peaks of FSH have been observed in the follicular phase of the gorilla. Dahl *et al.* (13) suggested that the early peak may prime the development of ovarian follicles. If this is true, then the long duration of hMG administration in this case may have primed the follicles and contributed to the excellent ovarian response. Because measurements of urinary EC have not been previously correlated with follicular development during hMG ovulation induction, monitoring relied upon serum E_2 and ultrasound findings. In an effort to minimize the number of anesthesia episodes, only a single monitoring session under anesthesia (in addition to the aspiration) was initially planned. The day selected for the first immobilization (day 11 of hMG) was based upon the average number of days of hMG administration for human ovulation induction with the use of GnRH agonist suppression. If indications of incipient OHSS were noted during the monitoring sessions, the cycle was to be canceled by withholding further hMG/hCG.

Although laparoscopic oocyte retrievals are feasible (3,4), for unknown reasons, dense intraabdominal adhesions appear to be common in captive lowland gorillas (15) and may restrict laparoscopic visualization of the ovaries. Adhesions may also retain the ovaries high in the pelvis, making them inaccessible to vaginal ultrasonography. The long, narrow, bony pelvis of the great apes may further contribute to the potentially long distance from the apex of the vagina to the ovaries (Fig. 4).

An E_2 of 910 pg/ml indicated that the ovaries had not been adequately visualized by either abdominal or vaginal ultrasound scanning during the first monitoring session. Since it was felt that another monitoring session was critical to successfully time oocyte aspiration and to allow for cycle cancellation if necessary, the animal was immobilized again on day 15 of hMG administration.

Because neither laparoscopic, transvaginal, nor transvesicle approaches to oocyte recovery could be performed in this animal, the transrectal approach was designed. Twenty-four hours of a clear liquid diet was inadequate as judged by the abundant fecal matter evacuated by the enemas. While the transrectal approach was generally successful, the bony pelvis limited the approach angle of the rectal probe and resulted in incomplete aspiration of the ovaries. Furthermore, firm application of the probe against the rectal wall for needle insertion into the overlying ovaries was hampered by the lack of an anatomical stop within the rectum, causing slippage of the probe. Nonetheless, this approach resulted in the most gorilla oocytes yet recovered from a single attempt.

Ultrasound-guided recovery of oocytes has not been reported previously in a gorilla. Although a nonlaparoscopic technique helps to prevent the potential detrimental effect of CO_2 -induced pH shifts on the oocytes (23), the finding of an intestinal parasite in the presence of an oocyte following transrectal aspiration emphasizes that the consequences of this approach remain uncertain. The bacteriologic and parasitic findings in this case suggest that an attempt at helminthic eradication (15,24) before induction of ovulation accompanied by antibiotic prophylaxis, including chloramphenicol, may be prudent.

On the two occasions in this gorilla where serum E_2 concentrations were obtained, approximately 300 pg/ml of E_2 was measured for each mature follicle >15 mm in diameter. This corresponds to the expected human range. A generous dose of hCG (20,000 IU) was selected based on data indicating that a large LH surge correlates

Fig. 4. A comparison of the gross anatomy of the human pelvis (top) with that of the great ape pelvis (*pan*, bottom). Note the narrow outlet, the relative lack of the posterior cul-de-sac, and the greatly elongated diagonal conjugate (lower border of pubic symphysis to sacral promontory) of the great ape pelvis. The relative lack of lower uterine segment flexion and the larger overall size of the gorilla uterus combine to lengthen the distance from the vaginal apex to the ovaries.

with an enhanced luteal phase pregnanediol-3-glucuronide profile in the gorilla (25). Despite the high serum E_2 levels achieved, the gorilla did not appear to be overly susceptible to acquiring OHSS.

We conclude that the duration of hMG exposure may contribute to beneficial priming of gorilla ovarian follicles and that the use of a GnRH agonist permits extended hMG use. GnRH agonists also expedite follicular monitoring by minimizing the risk of premature luteinization and affording a minimal number of anes-

thesia interventions. Difficult access to the ovaries should be anticipated but successful transrectal oocyte retrieval is possible if necessary.

This effort poignantly demonstrates the substantial risk that accompanies attempts at artificial reproductive technologies. Nonetheless, the use of these techniques for gorillas should not be abandoned because selective assisted genetic propagation and embryo cryopreservation may help secure the preservation of this endangered species.

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