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Injection of Antiangiogenic Agents into the Macaque Preovulatory Follicle

Disruption of Corpus Luteum Development and Function

Timothy M. Hazzard,[1] Richard M. Rohan,[4] Theodore A. Molskness,[1] John W. Fanton,[2] Robert J. D'Amato,[4] and Richard L. Stouffer[1,3]

[1]Division of Reproductive Sciences, [2]Animal Resources, and [3]Department of Physiology and Pharmacology, Oregon Regional Primate Research Center/Oregon Health & Science University, Beaverton, OR; and [4]Surgical Research, Children's Hospital, Boston, MA

Ovulation and conversion of the follicle into the corpus luteum involve remarkable changes in vascular permeability and neovascularization of the luteinizing granulosa layer. To evaluate the importance of these vascular events in follicle rupture and luteal development, sequential experiments were designed in which vehicle or angiogenic inhibitors (TNP-470 or angiostatin) were injected directly into the preovulatory follicle of rhesus monkeys during spontaneous menstrual cycles. After control injections, 13 of 14 animals exhibited serum levels of progesterone (P) during the subsequent luteal phase that were comparable to untreated animals in our colony. Following low-dose (400 pg/mL) TNP-470, serum P levels increased normally until d 8 of the luteal phase, but then declined prematurely by d 9 ($p < 0.05$ compared to controls) and remained below controls until menses. Following high-dose (2 μg/mL) TNP-470, serum P levels were diminished in the early luteal phase (d 3–5; $p < 0.05$ compared to controls), but reached typical levels at mid luteal phase, only to decline prematurely by d 9 ($p < 0.05$) and remain low until menses. Control ovaries displayed indices of follicle rupture (protruding stigmata) and luteinization. TNP-470-treated ovaries exhibited signs of distension (torn surface epithelium/ tunica albuginea) and luteinization; however, a well-formed stigmata was not observed. A "trapped" oocyte was not observed in serial sections of developing corpora lutea from control or TNP-470-treated animals. However, the early corpus luteum of TNP-470-injected ovaries contained pockets of excessive numbers of blood cells that were absent in controls. Angiostatin did not alter serum P levels or ovarian morphology compared to controls. These data suggest that acute exposure to the antiangiogenic agent TNP-470 impairs the development and functional capacity of the primate corpus luteum in a dose-dependent manner. The results are consistent with a critical role for angiogenesis in cyclic ovarian function in primates.

Key Words: Angiogenesis; TNP-470; angiostatin; ovary; reproduction.

Introduction

The development and maintenance of the corpus luteum, as an endocrine gland that secretes the steroid hormone progesterone (P), is essential for implantation and embryonic survival during early pregnancy. The physiological events that occur in the transformation of the follicle to the corpus luteum include remarkable vascular changes *(1–3)*. A high degree of endothelial cell proliferation is associated with early corpus luteum development *(3–5)*. In fact, the intensive angiogenesis that occurs during luteal formation establishes a capillary network in the mature corpus luteum where a majority of steroidogenic cells are adjacent to one or more capillaries *(6)*. Although angiogenesis during luteal development is thought to be critical for the provision of steroid precursors to luteal cells, as well as transport of P into the general circulation *(7)*, a cause–effect relationship remains to be established. One approach to testing this concept is to determine if antiangiogenic agents disrupt luteal development or function.

Two such angiogenic inhibitors are TNP-470 and angiostatin. TNP-470 is a semisynthetic analog of (*O*-chloroacetylcarbamoyl) fumagillol [also known as AGM-1470 *(8,9)*], which selectively inhibits capillary tube formation *(9)* by acting on endothelial cells at a number of sites in the angiogenic pathway *(10)*. Alternatively, angiostatin, an endogenous protein identified as a proteolytically derived fragment of plasminogen *(11)*, selectively targets proliferating endothelial cells and induces mitotic cell death *(12)*. Although angiostatin is an effective tumor-growth suppressor *(11,13)*, its

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Author to whom all correspondence and reprint requests should be addressed: Dr. Richard L. Stouffer, 505 NW 185th Avenue, Beaverton, OR 97006. E-mail: stouffri@ohsu.edu

effects on luteal function have not been examined. However, administration of TNP-470 (AGM-1470) to female mice resulted in fewer and smaller corpora lutea than controls *(14)*, suggesting that luteal growth is angiogenesis dependent. However, because TNP-470 was administered throughout the ovarian cycle, it is unclear whether the observed effects are the result of disruption of angiogenesis in the growing follicle or the subsequent corpus luteum. In contrast, Fraser et al. *(15)* concluded that systemic administration of TNP-470 to nonhuman primates had no effect on luteal function in the marmoset and variable effects in the stump-tailed macaque. Nevertheless, vaginal administration of TNP-470's parent compound fumagillin during the luteal phase in rhesus macaques inhibited P production and pregnancy establishment *(16)* although it remains unclear whether the latter effect was via the corpus luteum or uterus. The reported differences may be species dependent or, alternatively, the result of different routes of administration, as TNP-470 is metabolized rapidly in the blood and its pharmacodynamic properties are incompletely understood *(17, 18)*. These limitations may be overcome by direct administration of antiangiogenic agents into ovarian compartments to promote local effects at a specific stage of folliculogenesis or luteal development.

Therefore, this study was designed to *(1)* validate a technique for injecting agents directly into the preovulatory follicle during the menstrual cycle of the rhesus monkey and *(2)* determine if local administration of the antiangiogenic agents, TNP-470 and angiostatin, impairs periovulatory events and the development/functional life-span of the corpus luteum.

Results

In Vitro Studies: Dose and Toxicity Trials

Compared to control vehicle (dimethyl sulfonate/phosphate-buffered saline [DMSO/ PBS]), incubation with 0.1–2 μg/mL TNP-470 did not alter P secretion by luteinized granulosa cells or cell number at 24 (not shown) or 48 h of culture (Fig. 1). However, P levels ($p = 0.56$) and cell number ($p < 0.05$) declined during incubation with higher doses (5 and 10 μg/mL) of TNP-470. Thus, two doses of TNP-470 (400 pg and 2 μg/mL) were chosen to achieve intrafollicular concentrations, which are similar to (400 pg/mL) or higher (2 μg/mL) than the reported IC_{50} for endothelial cell proliferation *(8,19)*.

Incubation with 5–60 μg/mL angiostatin did not alter progesterone secretion by luteinized granulosa cells or cell number at 24 or 48 h of culture compared to controls (data not shown). Thus, a dose was chosen to achieve an intrafollicular level (60 μg/mL), which was similar to the systemic concen-tration reported to be successful in reducing tumor volume *(20)*, and greater than that resulting in half-maximal inhibition of bovine corneal endothelial (BCE) cell proliferation *(21)*.

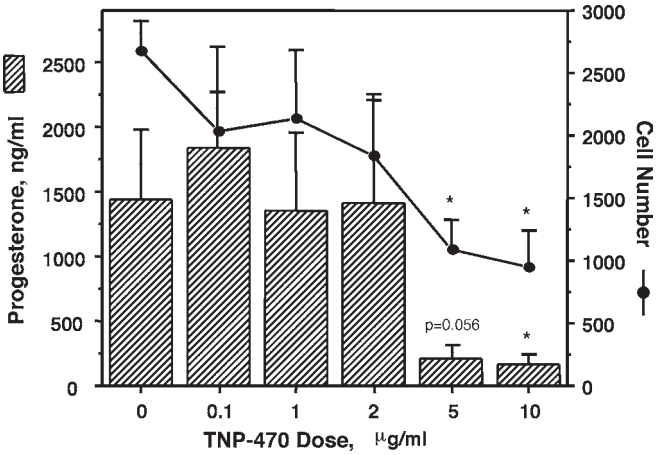

Fig. 1. Changes in luteinized granulosa cell number and progesterone concentrations after culture with 0–10 μg/mL of TNP-470 for 48 h. Asterisks indicate significant differences ($p < 0.05$) compared to DMSO/PBS alone.

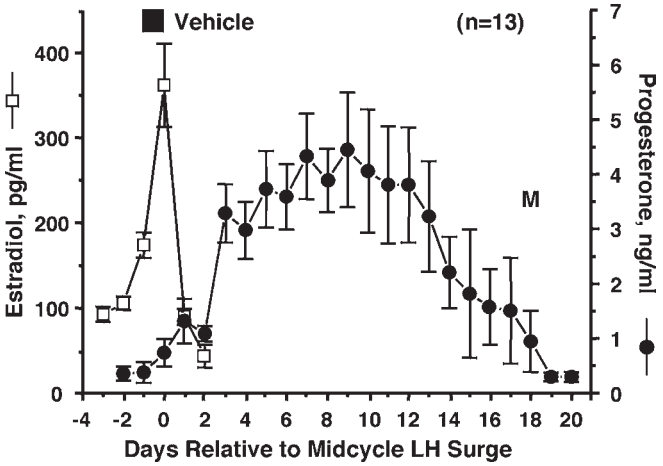

Fig. 2. Changes in serum estradiol (E) and progesterone (P) concentrations beginning just prior to the midcycle LH surge until the day of menstruation in vehicle-treated (control; $n = 13$) animals. Each point represents the mean ± SEM. The rectangle represents the day of DMSO/PBS injection into the preovulatory follicle. M = the average day of menstruation.

In Vivo Studies: Intrafollicular Injections

The pattern and levels of serum E and P in vehicle-treated (control) animals were typically comparable to those observed during natural menstrual cycles in untreated animals in our colony *(22)*. Vehicle treatment in both the TNP-470 (PBS: DMSO) and angiostatin (PBS) groups, resulted in 13 of 14 animals displaying typical hormonal patterns (Figs. 2 and 5). E levels peaked during the ovulatory surge of luteinizing hormone (LH), which is designated as d 0. P levels began to increase at d 0, and continued to rise until peaking at mid luteal phase (d 6–8). P concentrations remained elevated until around d 11–12 and then declined throughout the late luteal phase until menstruation. In both groups, one vehicle-treated animal did not have hormone patterns typical of untreated animals (not shown) and was dropped from further protocols.

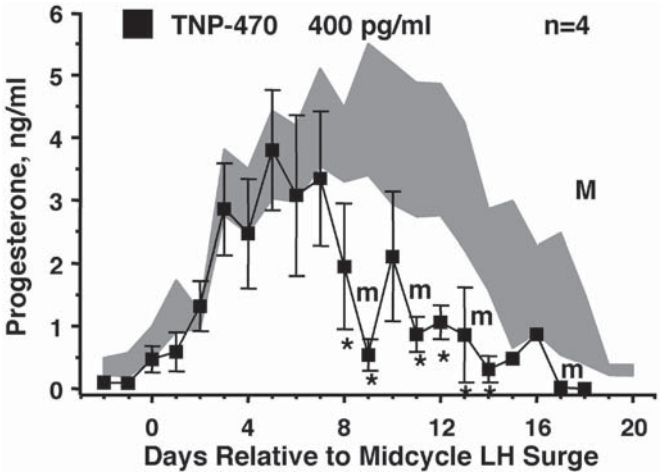

Fig. 3. Changes in serum P concentrations in animals receiving an intrafollicular injection of low-dose TNP-470 (squares) compared to the experimental controls (shaded area = mean ± SE). m depicts the day of menstruation for each TNP-470 treated animal and M denotes the average day of menstruation for the controls. The rectangle represents the time of injection. Asterisks indicate days of significant differences compared to controls ($p < 0.05$).

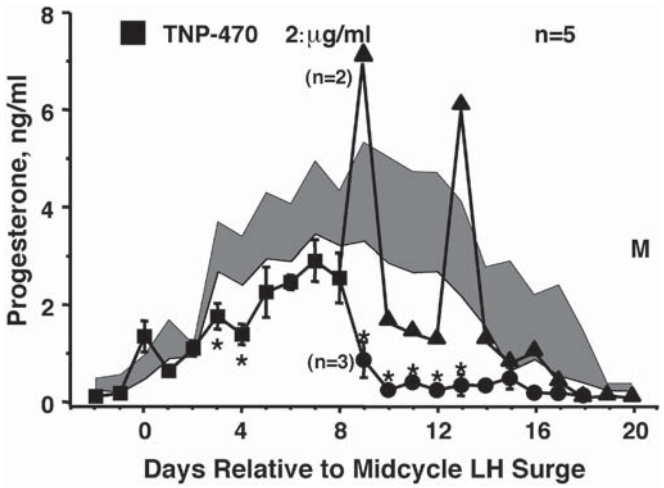

Fig. 4. Serum P changes after intrafollicular injection of high-dose TNP-470 compared to the experimental controls (shaded area = mean ± SE). On d 8, TNP-470-treated animals were separated into two groups (triangles, $n = 3$; circles, $n = 2$) to denote the variation of animals receiving the high-dose treatment. For other details and abbreviations, see the legend to Fig. 3.

Figure 3 represents serum P concentrations after injection of low dose TNP-470 into the preovulatory follicle, compared to control animals. Although serum progesterone concentrations increased normally during the early stages of the luteal phase, TNP-470 treatment caused a premature decline ($p < 0.05$) in progesterone levels by d 8 post-LH surge, and values remained low until menstruation in these animals. Further, the onset of menstruation occurred prematurely in three of four animals compared to controls.

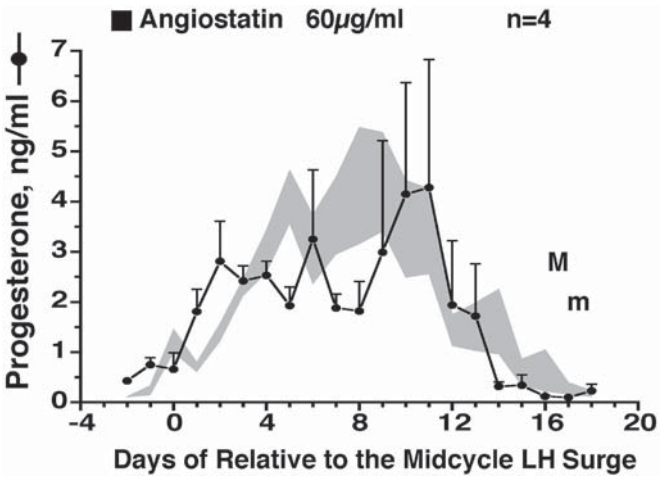

Fig. 5. Pattern of serum P concentrations after intrafollicular injection of angiostatin (circles) compared to experimental controls (PBS; shaded area = mean ± SE). For all other details and abbreviations, see legend to Fig. 3.

Administration of a high dose of TNP-470 resulted in a slightly different hormonal profile (Fig. 4). In all five animals injected, serum P concentrations increased in the early luteal phase, but were significantly lower than controls at d 3–4 post-LH surge. P levels then increased normally toward the mid luteal phase, only to decline prematurely in three of five animals by d 9 and remain low ($p < 0.05$) until menstruation. In 2 of 5 animals, progesterone values fluctuated greatly with intermittent peaks comparable to control levels, before declining prior to menstruation. The day of menstruation was not different between TNP-470-treated animals and controls.

Figure 5 summarizes serum P concentrations after intrafollicular injection of angiostatin. The P levels in all four animals treated with angiostatin were not different from values measured in control animals at any stage of the luteal phase. Further, the day of menstruation was not different between control and treated animals.

All ovaries of control and treated (TNP-470 doses and angiostatin) animals were observed 3 d after the intrafollicular injection. Vehicle-injected follicles displayed indices of rupture (protruding stigmata; Fig. 6B) and luteinization (yellowish tissue with prominent capillaries). TNP-470-treated follicles exhibited signs of distension (torn surface epithelium/tunica albuginea) and luteinization, but not necessarily of follicle rupture (malformed or missing stigmata; Fig. 6C). Because it was unclear if ovulation occurred, another laparoscopy was performed on d 5 in TNP-470-treated animals, which revealed an increased vascular presence, but signs of follicular rupture remained enigmatic. In contrast, angiostatin-injected follicles exhibited no gross differences in any indices of rupture/luteinization (Fig. 6D) compared to controls.

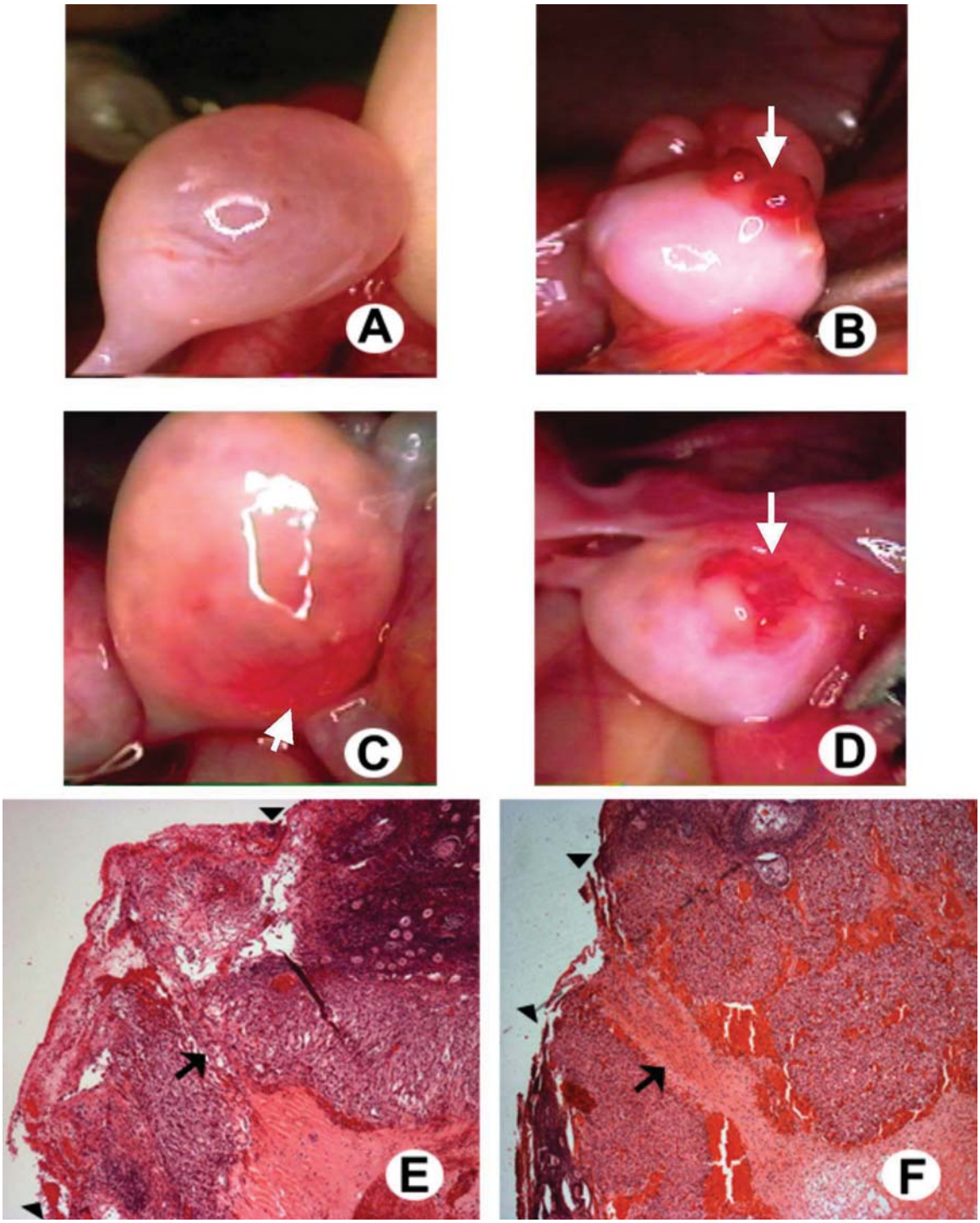

Fig. 6. Indices of follicular rupture after intrafollicular injection of vehicle or antiangiogenic compounds. (**A**) For comparison, a typical preovulatory follicle on the injection day (the follicle size comprises more than half of the ovary); (**B**) stigmata formation (arrow) 3 d after injection of vehicle; (**C**) possible ovulation site with minimal stigmata (arrow) observed 3 d after injection of high-dose TNP-470 (note vasculature at the apex of the follicle); (**D**) stigmata formation (arrow) 3 d after injection of angiostatin. (**E,F**) (at 2.5× magnification) represent the site of follicular rupture observed in serial sections of ovaries collected after treatment with vehicle or high-dose TNP-470. (**E**) Site of oocyte extrusion in a vehicle-injected ovary (arrowheads mark outcropping of tissue [stigmata] on the surface of the ovary; arrow denotes the ovulatory canal); (**F**) the ovulatory canal (arrow) from a TNP-470 injected ovary with no apparent stigmata associated with the exit point; note the extensive pockets of blood cells throughout the luteal tissue.

Evaluation of serial sections of the ovary from control and TNP-470 treated animals revealed that there were no trapped oocytes by 3 d after the LH surge, regardless of treatment (Fig. 6E,F). Importantly, a channel or pathway by which the oocyte was expelled from the ovulatory follicle (Fig. 6E,F) was observed in all animals. However, the raised stigmata was only apparent in the controls (Fig. 6E), as no outcropping of tissue was evident after TNP-470 treatment (Fig. 6F). Notably, TNP-470-treated tissues appeared to contain excessive numbers of blood cells located in various pockets throughout the luteal tissue that were absent in control sections (Fig. 6E,F). Because of the lack of any changes in follicle rupture or luteal function, ovarian tissues in angiostatin-treated animals were not analyzed.

Discussion

Intrafollicular injection is an effective technique by which follicular fluid can be collected and exogenous factors can be tested *in situ* with minimal trauma- or stress-related disruption of the ovarian cycle. Further, a sequential design, that first tests vehicle injections in treatment groups, allows removal of the occasional animal that exhibits ovarian dysfunction during this protocol (i.e., 1 of 14 animals). Previously, follicular sampling was successfully used in cows without negatively affecting follicular development *(23)*. In our study, disturbance of the follicle by needle penetration did not result in significant leakage, which is most likely the result of the use of a small-diameter needle, plus penetration of the ovarian capsule and follicle opposite to the follicular apex, as well as expediting penetration and removal of the needle. Moreover, vehicle injection resulted in typical periovulatory patterns of E and P, and functional life-span of the corpus luteum observed in untreated monkeys in our colony *(22)*. Thus, our study provides a primate model in which various compounds (e.g., antiangiogenic factors) can be administered locally into the preovulatory follicle to examine the direct effects on ovulation and corpus luteum development.

Intrafollicular injection of the low-dose (400 pg/mL) of TNP-470 impaired subsequent luteal function in the rhesus macaque as determined by a decline in serum P concentrations 8 d after the gonadotropin surge. Although levels increased normally during early luteal development, they dramatically declined to basal levels during the mid luteal phase and remained low until menstruation. Our data support evidence in the stump-tailed macaque showing that systemic treatment of TNP-470 administered prior to ovulation can alter serum P levels during the subsequent luteal phase; however, these results were variable, as diminished luteal function was achieved in only 3 of 12 animals studied *(15)*. Although our low dose of TNP-470 was more successful in disrupting normal luteal function, this may be the result of the different routes of administration. Alternatively, differences may be dose related as a higher dose (mg/kg) of TNP-470 decreased luteal growth in mice *(14)*.

In an effort to determine if TNP-470 could completely eradicate circulating progesterone levels in the luteal phase, a high-dose (2 μg/mL; the maximal dose resulting in no adverse effects on luteinized granulosa cells in culture) was administered. Indeed, the early luteal rise in serum P concentrations was diminished, but only temporarily, as levels were similar to controls by the mid luteal phase. Although the subsequent decline in mid to mid-late luteal P concentrations was similar to those from low-dose-treated animals, two animals displayed transient rises in P levels no different from controls. Thus, the higher dose of TNP-470 may not be more efficacious in altering luteal phase P concentrations.

Although the decline in serum P concentrations at the mid luteal phase was significant in both the low and high-dose TNP-470-injected animals, the limited effect of this angiogenic inhibitor in the early luteal phase is intriguing. Intraluteal P concentrations are reportedly highest in the early luteal phase and do not display the typical biphasic pattern characteristic of the serum levels during the luteal phase *(24)*. This discrepancy between intraluteal and serum concentrations may offer a clue as to why TNP-470 does not reduce circulating P levels in the early luteal phase. Although intense angiogenesis occurs during early luteal development *(3)*, the developing vasculature may be inadequate to transport P away from the corpus luteum, as the network of capillaries is not uniformly organized until the mid luteal phase *(25)*. Therefore, the effect of TNP-470 may only be realized in the mid luteal phase, when in the absence of a fully developed capillary network, luteal P cannot be optimally transported into the systemic circulation. Further studies are warranted to determine the effects of periovulatory TNP-470 exposure on the structure–function of the corpus luteum in the early luteal phase, as well as at the mid luteal phase during premature functional regression of the corpus luteum.

Although the decline in circulating P at the mid luteal phase altered the onset of menstruation, the effect was dose dependent. Monkeys injected with low-dose TNP-470 generally underwent premature menstruation, whereas onset of menstruation was more typical of untreated animals after high-dose TNP-470 treatment. Characteristically, the onset of menstruation in the rhesus macaque is preceded by approx 3 d of serum P concentrations less than 1 ng/mL (present study and ref. *22*). Further, removal of the corpus luteum by ovariectomy is followed by menstruation in 3 d *(26)*. Therefore, low-dose TNP-470 was capable of shortening the menstrual cycle in most animals. However, animals receiving a high dose of TNP-470 demonstrated suboptimal serum P concentrations, but the duration of the menstrual cycle was unaltered. Lalitkumar et al. *(16)* reported that 6 d of exposure to fumagillin between d 5 and 17 after ovulation did not alter the menstrual cycle lengths in monkeys. In contrast, Klauber et al. *(14)*, demonstrated that treatment of mice with

AGM-1470 disrupts endometrial maturation and decreases the number of proliferating vessels. Although the current study did not address effects of TNP-470 administration on uterine endometrium, we cannot rule out the possibility that our high dose may have been sufficient enough to locally or systemically affect the uterine environment and thus delay menstruation in the face of low serum P levels.

Laparoscopic observation of control and TNP-470-injected follicles 3–5 d after treatment revealed apparent physical differences in ovulatory features. Interestingly, the follicular rupture site on ovaries observed 3 d after administration of TNP-470 appear comparable to rupture sites observed very early after ovulation in natural cycles *(25)*. Later observation of an ovary 5 d postadministration of TNP-470 continued to reveal signs of limited stigmata development. Nevertheless, ovulation appears to occur in the presence of TNP-470, as no "trapped" oocytes were observed in ovarian serial sections and an ovulatory channel was apparent. Likewise, TNP-470 did not prevent hypertrophy or luteinization of the follicle wall. However, the presence of excessive blood cells in the luteinizing follicle suggests a lack of vessel integrity. Interestingly, venule dilation and red blood cell extravasation was observed in the endometrium of monkeys receiving intravaginal TNP *(27)*. Our data indicate that intrafollicular administration of TNP-470 does not prevent ovulation or luteinization of the follicle wall, but it may disrupt stigmata formation and/or repair of the follicular rupture site possibly by interfering with vascular integrity. Further studies are warranted to examine the effects of TNP-470 on cellular markers of follicle rupture/repair and luteal development.

Unlike TNP-470, there was no apparent effect of intrafollicular injection of angiostatin on follicle rupture and the pattern or levels of serum P throughout the luteal phase in rhesus monkeys. Further, the normal decline in P levels observed at the end of the luteal phase was followed by timely menstruation. These results suggest that angiostatin is unable to alter the development or functional lifespan of the corpus luteum during the menstrual cycle. However, the lack of effect may be related to limitations in drug delivery or bioactivity *(11)*. Importantly, the frequency of administration may be inefficient, as a comparison of continuous versus bolus injections of angiostatin demonstrates that continuous infusion dramatically improves this compound's antiangiogenic effect *(28)*. Finally, we cannot dismiss the possibility that angiostatin would be effective if administered at a higher dose, as doses up to 50 mg/kg/12 h inhibit tumor growth in mice *(29)*.

In summary, this is the first report of direct injection of agents into the preovulatory follicle in primates with the goal of investigating the effects of antiangiogenic compounds on ovulation and luteal development. The injection technique described here did not alter ovulatory timing or subsequent development and function of the corpus luteum. However, intrafollicular injection of the angiogenic inhibitor TNP-470 significantly impairs the function and lifespan of the corpus luteum and possibly the development/repair of follicular rupture in the primate ovary. This novel approach to testing pharmacologic agents in the preovulatory follicle could provide useful information on their potential antifertility action in primates.

Materials and Methods

Animals

The general care and housing of rhesus monkeys at the Oregon Regional Primate Research Center was described previously *(30)*. Animal protocols and experiments were approved by the ORPRC Animal Care and Use Committee, and studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals *(31)*.

In Vivo Studies Employing Intrafollicular Injection of TNP-470 or Angiostatin

Adult, female rhesus monkeys exhibiting normal menstrual cycles of approx 28 d were bled daily by saphenous venipuncture beginning 6–8 d after the onset of menses. Serum concentrations of estradiol (E) and P were measured by radioimmunoassays validated for nonhuman primates *(32,33)*. Estradiol values were used to estimate the day of the midcycle gonadotropin surge and time of ovulation as previously established in our laboratory *(34)*. Briefly, d 1 of the luteal phase is designated as the day when preovulatory serum E levels (range: 250–600 pg/mL) decline to less than 100 pg/mL *(34)*. In order to administer the antiangiogenic compound prior to ovulation, intrafollicular injections were performed when serum estradiol levels exceeded 150 pg/mL.

Intrafollicular injections [as modified from Ginther et al. *(23)*] were performed on anesthetized *(35)* animals during surgery to expose the ovary bearing the dominant follicle. An insulin syringe containing 50 μL of solution was inserted through the stroma of the ovary contralateral to the apex of the follicle before penetrating the follicular wall. Then 50 μL of follicular fluid was aspirated into the syringe, diluting the injectable by half, before injecting 50 μL of this mixed solution into the follicle. The syringe was then withdrawn from the ovary, and the follicle was observed to note if any deflation occurred. Ovaries were viewed by a second laparoscopy 3 d postinjection for evidence of follicle rupture and luteinization. Blood samples were collected on a daily basis until the first day of menses and analyzed for serum P levels. The day of the LH surge was confirmed, using a mouse Leydig cell bioassay for bioactive LH validated previously for macaque serum *(36)*, and verified that all animals were injected on the day before (d –1) or of (d 0) the midcycle gonadotropin surge.

A sequential experimental design was applied in which animals ($n = 14$) received an intrafollicular injection of vehicle (PBS w/wo DMSO, Control) during the first experimental cycle (protocol 1) and the antiangiogenic substance

of choice in the second experimental cycle (protocol 2). After menses in protocol 1, animals were permitted to recover for at least one menstrual cycle before blood samples were taken to start protocol 2. In protocol 2, animals in the TNP-470 study received 400 pg/mL (80 pg in a 200 μL follicle [follicular volume determined from aspiration studies]; $n = 4$) designated as a low dose or a high dose of 2 μg/mL (400 ng in a 200 μL follicle; $n = 5$), whereas animals in the angiostatin study received a dose of 60 μg/mL (12 μg in a 200 μL follicle; $n = 4$). Lyophilized TNP-470 (Takeda Abbot Pharmaceuticals, Osaka, Japan) and human FC-angiostatin fusion protein [gift from Dr. K. Javaherian *(37)*] were diluted in PBS as described in the in vitro studies.

In further protocols 3 and 4, animals again acted as their own controls but were assigned randomly to receive an intrafollicular injection of either vehicle, or one of the antiangiogenic compounds on d –1 or d 0. On d 3 after the injection, the ovary bearing the injected follicle was removed by laparoscopy and fixed in formaldehyde overnight before embedding in paraffin.

Tissue Analysis

All paraffin blocks were serially sectioned by the Imaging and Morphology Core Laboratory, ORPRC, using an American Optical (Southbridge, MA) microtome and mounted on Superfrost plus slides (Fisher, Santa Clara, CA). All slides were stained with hematoxylin and eosin and viewed to determine if the oocyte was released from the follicle or trapped within the luteinized tissue.

In Vitro Studies of Drug Toxicity Using Macaque Follicle Cells

Luteinized granulosa cells were obtained during controlled ovarian stimulation cycles in monkeys by follicle aspiration as previously described *(30)*. After the oocytes were removed for other studies *(38)*, somatic cells were harvested from follicular aspirates by centrifugation at 277*g* for 15 min (4ºC). The cell pellet was resuspended in TALP-HEPES *(39)*, and enriched for granulosa cells as described by Chaffin and Stouffer *(40)*. In brief, cells were centrifuged at 190*g* (10 min, 4ºC) and resuspended in Hams F-10 medium (Life Technologies, Grand Island, NY) plus 0.1% bovine serum albumin (BSA). The resuspension was layered onto a gradient of 40% Percoll (Sigma Chemical, St. Louis, MO) and 60% Hank's balanced salt solution with 0.1% BSA and centrifuged at 470*g* for 30 min at 4ºC. The resulting layer of granulosa cells was resuspended in Hams F-10, cell numbers were determined using a hemacytometer, and cell viability was assessed by trypan blue exclusion. Cells were resuspended in DMEM F-12 with LDL (25 μg/mL; Sigma, St. Louis, MO), ITS (insulin, 5 μg/mL; transferrin, 5 μg/mL; sodium selenite, 5 μg/mL; Sigma), aprotinin (10 μg/mL; Sigma) and approx 50,000 cells per 500 L were plated in each well (48-well plate; Corning Costar Corp., Corning, NY). Lyopholized TNP-470 was resuspended in DMSO (1 mg/mL) and diluted with PBS and added to the wells in final concentrations of 0.1, 1, 2, 5, and 10 μg/mL. Human FC-angiostatin fusion protein was resuspended directly in PBS and added to wells in final concentrations of 5, 10, 20, 40, and 60 μg/mL. The range of testing was based on similar studies with tumor cells *(41)*. Cells were incubated at 37ºC in duplicate either alone (controls), with diluted DMSO (1:50; TNP-470 study only) or one of five doses of the antiangiogenic compound of interest for 24 and 48 h. Incubation media was removed and stored for P analysis. Cell number was determined by crystal violet staining *(42,43)*.

Statistics

Progesterone concentrations and cell numbers in luteinized granulosa cell cultures were analyzed using a one-way analysis of variance (ANOVA) (STATPAK, Northwest Analytical, Portland, OR) to determine any dose-dependent effects of the antiangiogenic substances in vitro. Serum P levels were analyzed using a repeated measures ANOVA (STATPAK) to characterize differences between controls and the low and high doses of TNP-470 or angiostatin. All differences were considered significant at $p < 0.05$ and values are presented as mean ± SEM.

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