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Indenopyride derivative RTI-4587-073(l): A candidate for male contraception in stallions

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ABSTRACT

The objective of this study was to determine whether an indenopyridine derivative RTI-4587-073(l) was a good candidate for male contraception in horses. We hypothesized that a single administration of RTI-4587-073(l) causes significant suppression of testicular function in stallions without affecting sexual behavior. Three Miniature horse stallions received a single dose of 12.5 mg/kg RTI-4587-073(l) orally (group "treated"), whereas three other Miniature horse stallions received placebo only (group "control"). Semen was collected and evaluated from all stallions twice a week for three baseline weeks and 13 post-treatment weeks. Sexual behavior was video-recorded and analyzed. Testicular dimensions were measured using ultrasonography, and blood samples were drawn for endocrine evaluation once before treatment and once a week during the post-treatment period. Single administration of RTI-4587-073(l) caused severe oligoasthenozoospermia (low sperm number and low motility), shedding large numbers of immature germ cells in semen, and increased FSH concentrations in treated stallions. These effects were fully reversible within ~71 days. However, libido and copulatory behavior remained unchanged throughout the entire experiment. We concluded that RTI-4587-073(l) was a promising candidate for male contraceptive in domestic stallions. Further research should be performed to test this compound for fertility control in wildlife and humans.

1. Introduction

The wild horse overpopulation of the Western rangelands in the United States is a serious problem for the ecosystem of these territories. The Bureau of Land Management (BLM) estimated that, as of February 2012, ~31,500 wild horses were roaming on BLM-managed rangelands in 10 western states [1]. According to public records, the estimated current free-roaming population exceeds by nearly 11,000, the number that the BLM has determined can exist in balance with other public rangeland resources and uses. The main approaches currently used to control growth of these populations are adoptions and holding large numbers of these animals in captivity. Because wild horse adoptions have decreased in recent years, the numbers of horses and burros kept in corrals and pastures increased significantly. There is a lot of outcry from the public on how the BLM is handling this situation, which puts additional pressure on this

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institution to develop more humane methods of limiting overgrowth of wild horse population (e.g., contraception).

Recent advances in research on developing new methods of fertility control in humans can be helpful in finding new solutions for animal overpopulation [2]. Whereas the main target of contraception in humans and animals is a female, there is a growing interest in developing a "pill" or "shot" for males as well. Until recently, scientists focused their efforts on inventing a pill for men, which would disrupt male hormones, mainly testosterone. Unfortunately, due to poor efficiency of hormonal contraception (not effective in 10%–20% of men), and numerous side effects, this strategy has been mostly abandoned [3]. More recent investigations shifted from hormonal therapies toward more specific molecules targeting the testis *per se*. Among the currently studied candidates for nonhormonal male contraceptives, two groups are listed as leaders: disruptors of testes-specific genes and disruptors of Sertoli cell functions [3]. Indenopyridines are classified as a group of compounds that selectively perturb Sertoli-germ cell adhesion, causing massive exfoliation of germ cells from the seminiferous epithelium, without harming spermatogonia [4]. They were initially developed as antihistamines. However, the early derivative of hexahydroindenopyridine—(4aRS,5SR,9bRS)-2-ethyl-1,3,4,4a,5,9b-hexahydro-7-methyl-5-p-tolyl-2H-indeno(1,2-c) pyridine hydrochloride (Sandoz 20-438)—had strong anti-spermatogenic effects in rats [5], dogs [6], and mice [7]. There were no detrimental effects on an ability to ejaculate, and the profound anti-spermatogenic effects of the indenopyridines were reversible in most species.

The compound RTI-4587-073, formerly called l-CDB-4022, a newer indenopyridine derivative, is a mixture of L- and D-isomers. The L-isomer exhibits a particularly strong anti-spermatogenic activity. Therefore, RTI-4587-073(l) has recently generated an interest for its contraceptive effects. This compound was effective in suppressing spermatogenesis in monkeys, which were used to model contraception in humans [8]. The effects were robust but reversible, which were desirable characteristics for an ideal contraceptive for men and male animals.

Rapid, long-lasting, but reversible effects, as well as preservation of normal sexual behavior in various species, make RTI-4587-073(l) an ideal candidate for male contraceptive for wild horses. Therefore, the goal of this work was to investigate the effects of RTI-4587-073(l) on semen, sexual behavior, testicular volume, and reproductive hormones in stallions.

2. Materials and methods

2.1. Stallions

Six mature, Miniature horse stallions (7–12 years old) were used in this study. The stallions were housed in individual pens (11 × 14.6 m) placed in a large pasture located on the premises of the College of Veterinary Medicine, University of Florida. They had *ad libitum* access to water and were supplemented with hay and grain. This project was approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.2. Experimental design

This study was conducted from April to August, consistent with the physiological breeding season of horses in the northern hemisphere. Four weeks before treatment, the semen was collected from all stallions for five consecutive days (to remove extragonadal sperm reserves). Ejaculates collected on the fifth day were evaluated in order to establish a daily sperm output (DSO) for each animal [9]. After this period (DSO I), semen was collected and evaluated twice a week for three baseline weeks (BASELINE). Eleven days before treatment, an initial ultrasound evaluation of the scrotal testes was performed, and blood was drawn for baseline endocrine analysis. Stallions were randomly assigned to two groups: group "treated" ($n = 3$) and group "control" ($n = 3$). On Day 0, stallions in group treated were given a single dose of RTI-4587-073(l) (12.5 mg/kg, dissolved in 60 mL of 10% ethyl alcohol; Research Triangle Institute, NC, USA). Stallions from group control were given 60 mL of 10% ethyl alcohol (placebo treatment). All treatments were administered orally with a dose syringe. Semen was collected and evaluated twice a week from all stallions for 13 consecutive weeks (post-treatment), beginning on Day 1 after administration of the compound. Ultrasound evaluations of the testes and blood sample collections were performed once a week during this 13-week period. A second series of five daily semen collections (DSO II) was performed during the week 14 after treatment to evaluate ejaculates for any long-term effects of treatment on sperm output in stallions. Investigators, who examined semen, sexual behavior, and reproductive hormones, were blind to the treatment.

2.3. Semen collection and evaluation

Semen was collected from stallions using a dummy mount and a Har-Vet Mini artificial vagina fitted with a disposable liner (Har-Vet, Spring Valley, WI, USA). The gel fraction was separated from semen using nylon mesh filters (Animal Reproduction Systems, Chino, CA, USA). The initial sperm concentration was assessed using a densimeter standardized for equine semen (Model 534B, Mod. 1, Animal Reproduction Systems). A small amount of semen was extended with milk-based extender (EZ Mixin BF, Animal Reproduction Systems) to approximately 30×10^6/mL for motility analysis. Total and progressive sperm motility in extended semen was evaluated using a computer-assisted motility analyzing system (CASA; IVOS Version 10, Hamilton, Thorne Biosciences, Beverly, MA, USA). Standard values were established before analysis: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 70; minimum cell size, four pixels; straightness (STR) threshold for progressive motility, 75%; average path velocity (VAP) threshold for progressive motility, 50 μm/s; VAP threshold for static cells, 20 μm/s; cell intensity, 106. The following parameters of sperm motility were determined using CASA: progressive motility (PGM);

percentage of rapid (VAP $\geq$ 50 μm/s), slow (50 μm/s > VAP > 20 μm/s), and static spermatozoa (VAP $\leq$ 20 μm/s); curvilinear velocity (VCL); average path velocity (VAP); straight line velocity (VSL); beat cross frequency (BCF); and linearity (LIN) [10]. The accurate sperm concentration was determined using a hemacytometer (double Neubauer ruling, Fisher Scientific, Pittsburgh, PA, USA). A small aliquot of semen was fixed in buffered, 10% formalin solution (Formalin 10, Animal Reproduction Systems), wet mount slides were prepared, and sperm morphology was evaluated under ×1000 magnification using a phase-contrast microscopy (Olympus BH2, Olympus America Inc., Center Valley, PA, USA). A total of 100 individual spermatozoa were counted to determine the percentage of normal spermatozoa and all identifiable morphological abnormalities. If significant numbers of round cells were found in semen samples (more than 5 round cells/100 spermatozoa), stained semen smears were also prepared and analyzed in order to determine their origin. Semen smears were prepared, air-dried, and stained with hematoxylin and eosin stain (Fisher Scientific). Stained slides were analyzed using a bright light microscopy (Olympus BH2, Olympus America Inc.). The number of round cells per 100 spermatozoa was determined. The total number of sperm in each ejaculate was calculated. Furthermore, the efficiency of spermatogenesis was calculated for each DSO (total number of spermatozoa at DSO/total testicular volume) and expressed as 10^6 sperm/mL of testis.

A small sample of each ejaculate was frozen and stored in −70 °C for sperm chromatin structure assay [11]. Briefly, individual samples were thawed at ~35 °C. A 5 μL aliquot was combined with 195 μL of a buffered solution, which then was combined with a low pH (~1.2) solution for 30 seconds. Acridine orange solution (1.2 mL at 4.0 μg/mL) was added, and the sample was processed immediately on a flow cytometer. The term α_t was used to describe the relationship between the amounts of green (double-stranded DNA) and red (single-stranded DNA) fluorescence. Endpoints measured for the sperm chromatin structure assay included the mean$_{-\alpha t}$ SD (SD$_{-\alpha t}$), the percentage of cells outside the main population (COMP$_{-\alpha t}$) [11].

2.4. Sexual behavior

All semen collection sessions were recorded using a video camera equipped with a wide-angle lens (Sony HDR-XR550, Sony Comp., Japan), and submitted for analysis. Recorded material was viewed by a trained behaviorist. Numerous behavioral endpoints were derived as described [12]. Specific measures of precopulatory behaviors included the following: erection latency, latency to first mount with erection, latency to first mount without erection, number of mounts with erection, and number of mounts without erection. Latencies were measured in seconds, from initial exposure to stimulus mare to first occurrence of a specific response. Number of a response was a count of all occurrences of each response from entering the breeding shed by each stallion to ejaculation. Measures of ejaculatory efficiency included the following: number of sessions to ejaculation (for each subject for each semen collection day, the number of sessions in which semen collection was attempted before success or discontinuation of attempts), ejaculation latency (on ejaculatory mount, the interval from mount to first sign of ejaculation, such as rhythmic tail motion), and a number of thrusts (on ejaculatory mount, the number of organized, sweeping pelvic thrusts to ejaculation). Furthermore, the overall semen collection efficiency was ranked from the best to the worst for each collection session, as well as for each stallion, throughout the entire experiment. These ranks (daily between-subjects as well as within-subject ejaculation efficiency ranking) were based on the number of sessions required to achieve ejaculation, latency to mount with erection, ejaculation latency, mounts to ejaculation, and thrusts to ejaculation, as defined above.

2.5. Ultrasound evaluation of the scrotum

Both testes were evaluated using a portable ultrasound machine equipped with a 5- to 8-MHz curved-array transducer (Titan, SonoSite Inc., Bothell, WA, USA). Three dimensions of each testis were obtained from frozen images, using ultrasound calipers. For height and width, the cross-section of the testis was visualized by placing the transducer approximately in the middle of the testis, on its ventral aspect, perpendicular to the long axis. For measuring testicular length, the transducer was placed against the caudal pole of the testis, between the testis and the tail of the epididymis, with the ultrasound beam penetrating through the testis from caudal to cranial pole [13]. The testicular volume was calculated using the formula for ellipsoid volume [$V_t = (4/3\pi)\ (W/2)(H/2)(L/2)$, where W = width, H = height, and L = length] [14]. The volume of both testes were combined to obtain the total testicular volume.

2.6. Endocrine assays

A 10-mL sample of heparinized blood was collected from the jugular vein of each stallion to determine the concentrations of reproductive hormones. All blood samples were taken between 0700 and 0900. Plasma was separated and stored at −70 °C pending RIA for plasma concentrations of testosterone, estradiol, LH, FSH, and immunoactive inhibin [15–17]. The detection limits (sensitivity) and intra and interassay CVs for the assays were as follows—FSH: 0.5 ng/mL, 2.8% (intra only); LH: 0.25 ng/mL, 3.7% (intra only); immunoactive inhibin: 0.125 ng/mL, 2.1% (n = 6) and 4.8% (n = 2); estradiol: 10 pg/mL, 4.0% (n = 6) and 8.8% (n = 2); and testosterone: 0.10 ng/mL, 3.7% (n = 6) and 7% (n = 3), respectively.

2.7. Statistical analyses

Statistical analyses were performed using the analytical software package (Statistix 8, Analytical Software, Tallahassee, FL, USA). Normality of distribution of all variables was tested using a Shapiro-Wilk normality test. Logarithmic transformations of four variables (total number of spermatozoa, number of spermatozoa with detached heads, number of spermatozoa with abnormal midpieces, and BCF) were performed due to lack of normal

distribution. Significant effects of RTI-4587-073(l) or vehicle on semen parameters, endocrine concentrations, and behavioral endpoints within each group were determined using one-way ANOVA tests with comparisons to the last week before treatment (baseline). Differences between groups were determined using linear models ANOVA with two factors, and with interactions (groups and collection days), followed by Tukey's test.

All semen parameters, including efficiency of spermatogenesis, for both DSOs were compared between groups using a two-sample Student's *t*-test, and within each group using a paired Student's *t*-test.

For all statistical tests, $P < 0.05$ was considered significant.

3. Results

3.1. Semen parameters

There were significant differences between group control and group treated for almost all semen parameters analyzed in this study (main effects), except for bent midpieces and LIN. Furthermore, there were changes ($P < 0.05$) in most semen parameters after treatment with RTI-4587-073(l) within group treated. Specifically, sperm concentration and total sperm numbers decreased in group treated by Day 18 after treatment, and returned to pretreatment values by ~Day 71 after administration of RTI-4587-073(l) (Fig. 1A). These parameters were lower in group treated than in group control on Days 18 to 39 after administration of treatment. Total and progressive sperm motility also decreased by Day 18 after administration of RTI-4587-073(l) and returned to pretreatment levels by Day 46 after administration of the compound (Fig. 1B). Progressive motility transiently decreased in group control on Day 22 after treatment. The motility parameters in group control were higher than in group treated on Days 18 to 36 and on Day 43 after treatment. The percentages of the static sperm were increased in group treated on Days 15 to 53 after treatment, with the exception of Day 46 (Fig. 1C). This parameter had higher values ($P < 0.05$) in group treated than in group control on Days 18, 32, 36, and 43 after treatment. Increased percentages of sperm with tailless heads were present in semen collected from stallions from group treated on Days 22, 25, 36, 50, and 53 after treatment (Fig. 1D). These percentages were higher ($P < 0.05$) in group treated than in group control on Days 22, 25, and 36. The percentages of sperm with abnormal midpieces were increased in group treated on Day 36 after treatment only. Stallions from group control and group treated had small numbers of other morphological sperm defects, such as abnormal heads (0.7% and 1.2%, respectively), proximal cytoplasmic droplets (10.3% and 18.7%, respectively), distal cytoplasmic droplets (15% and 8%, respectively), bent midpieces (3.7% and 3.7%, respectively), bent tails (5.3% and 3.7%, respectively), and coiled tails (2.4% and 3.1%, respectively). There were no significant differences between groups or within groups in percentages of these defects or percentages of normal sperm. Large numbers of the immature germ cells appeared in ejaculates of treated stallions on Day 15 after administration of the compound, but were rarely present in control stallions (Fig. 1E). These numbers decreased slowly and reached the pretreatment levels by Day 43 after administration of the compound. Sperm chromatin structure was also affected by the treatment (Fig. 1F). $Comp_{\alpha t}$ gradually increased in semen obtained from treated stallions, to reach significance on Days 36 and 39 after treatment ($P < 0.05$, Fig. 1G). This parameter had higher values in group treated than in group control on Day 15, as well as on Days 36 and 39 ($P < 0.05$). There were no significant differences between both DSO trials in any of the semen parameters, within each group and between groups. However, there was a tendency ($P < 0.5$) for efficiency of spermatogenesis in group treated to be larger at DSO II than at DSO I (mean values: 73×10^6/mL and 26×10^6/mL, respectively).

Immature germ cells were identified as primary spermatocytes; degenerated spermatids with one, two, or more nuclei; and multinucleated giant cells (Fig. 2).

3.2. Sexual behavior

Selected endpoints of sexual behavior are presented in Figure 3A–D. There were no significant differences between groups or within any of the groups in the behavioral endpoints, including the overall efficiency of semen collection between both groups, as expressed by individual ranks (Fig. 3D). All stallions had adequate sexual interest and normal copulatory behavior throughout the study.

3.3. Ultrasound evaluation of the scrotum

There was a difference between groups in mean testicular volume (main effect; $P < 0.02$), with control stallions having larger testicular volume than treated stallions. However, there were no differences in testicular volume between groups on any given day ($P > 0.05$). Furthermore, testicular volume did not change ($P > 0.05$) within any of the groups after administration of RTI-4587-073(l) or vehicle (Fig. 4).

3.4. Endocrine assays

There were differences between group control and group treated (main effects) for concentrations of FSH ($P < 0.0001$) and LH ($P = 0.005$). These hormones had higher concentrations in group treated than in group control. There was a tendency for inhibin concentrations to be lower in group treated than in group control (main effect; $P < 0.1$). Mean serum concentrations of FSH increased ($P < 0.05$) in group treated on Day 17 after administration of RTI-4587-073(l), compared with pretreatment concentrations (Fig. 5A). However, concentration of this hormone in group treated was higher than in group control also on Days 10, 17, 45, and 52 after treatment. Concentration of inhibin did not change within any of the groups during the experiment ($P > 0.05$). Mean serum LH concentrations transiently increased ($P < 0.05$) in group treated on Day 3 after administration of RTI-4587-073(l), in comparison to pretreatment concentrations and in comparison to group control (Fig. 5C). Serum testosterone concentrations

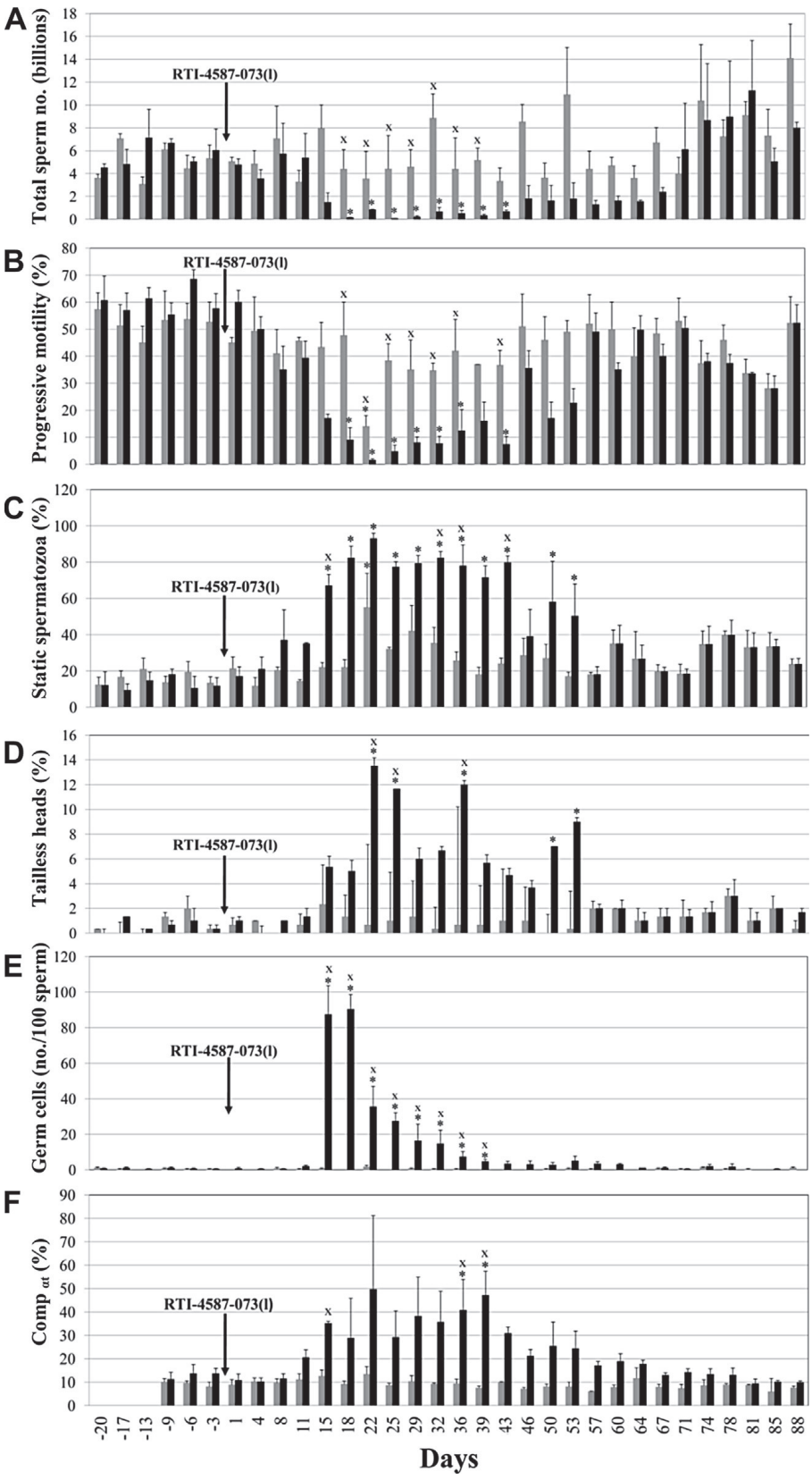

Fig. 1. Mean (± SEM) selected semen parameters in stallions treated with RTI-4587-073(l) (group treated) or with a vehicle (group control). Values with an asterisk (*), differed from the last value of the baseline period within each group ($P < 0.05$). X above a bar indicates a difference between groups on a given day ($P < 0.05$).

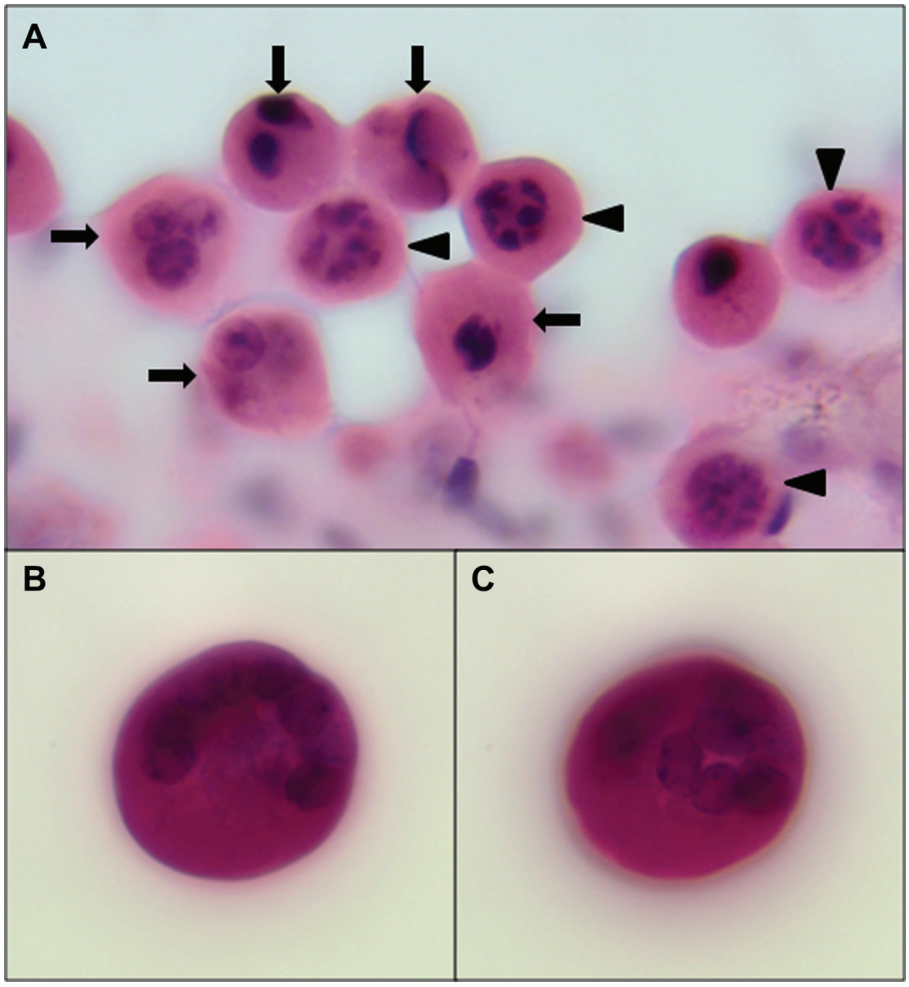

Fig. 2. Immature germ cells in semen collected from stallions treated with RTI-4587-073(l) (hematoxylin and eosin, ×1000). (A) Primary spermatocytes (arrow heads); spermatids with two three nuclei (thick arrows); (B) gigantic multinucleated cell; and (C) gigantic multinucleated cell.

increased on Day 24 after treatment in group treated ($P < 0.05$), but these values were not different than corresponding values in group control ($P > 0.05$). Serum concentrations of estradiol 17-β fluctuated throughout the experiment in group treated; it was increased on Days 24, 38, 66, and 80 after the administration of RTI-4587-073(l) ($P < 0.05$; Fig. 5E). Furthermore, the ratio of testosterone to estradiol 17-β increased on Days 3, 10, and 24 after treatment in group treated, in comparison to pretreatment concentrations ($P < 0.05$), but not in comparison to group control ($P > 0.05$). This ratio had a tendency to decrease on Days 52 and 80 after treatment in group treated ($P = 0.1$). There were no changes in serum concentrations of any of the reproductive hormones or in the ratio of testosterone to estradiol 17-β in group control during the study ($P > 0.05$; Fig. 5A–F).

4. Discussion

In the present study, administration of RTI-4587-073(l) had anti-spermatogenic activity in stallions. Severe oligoasthenozoospermia and a high number of the immature germ cells appeared in semen of treated stallions 2 weeks after administration of the single dose of this compound. These results agreed with those from the previous studies that a single dose of indenopyridine analogs cause significant germ cell loss from the seminiferous epithelium, leading to decreased sperm concentration and very low total sperm numbers. Interestingly, these changes were observed within a similar interval after drug administration in monkeys, dogs [9,18], and in stallions from group treated (by Days 14–18 after treatment). The detrimental effects of the compound on stallion semen were fully reversible within 71 days after a single administration of RTI-4587-073(l). Anti-spermatogenic effects of this indenopyridine were also reversible in monkeys, dogs, and mice, although they were usually, but not always, irreversible in rats [9,19,20].

Mechanisms of action of indenopyridines were extensively investigated in numerous experiments [9,20,21]. It was suggested that the main targets of indenopyridines were Sertoli cells, although spermatocytes and spermatids were also directly affected. Specifically, RTI-4587-073(l), formerly called l-CDB-4022, altered the expression of Sertoli-germ cell adherens junction proteins and disrupted Sertoli cell microtubule structure, which led to germ cell loss

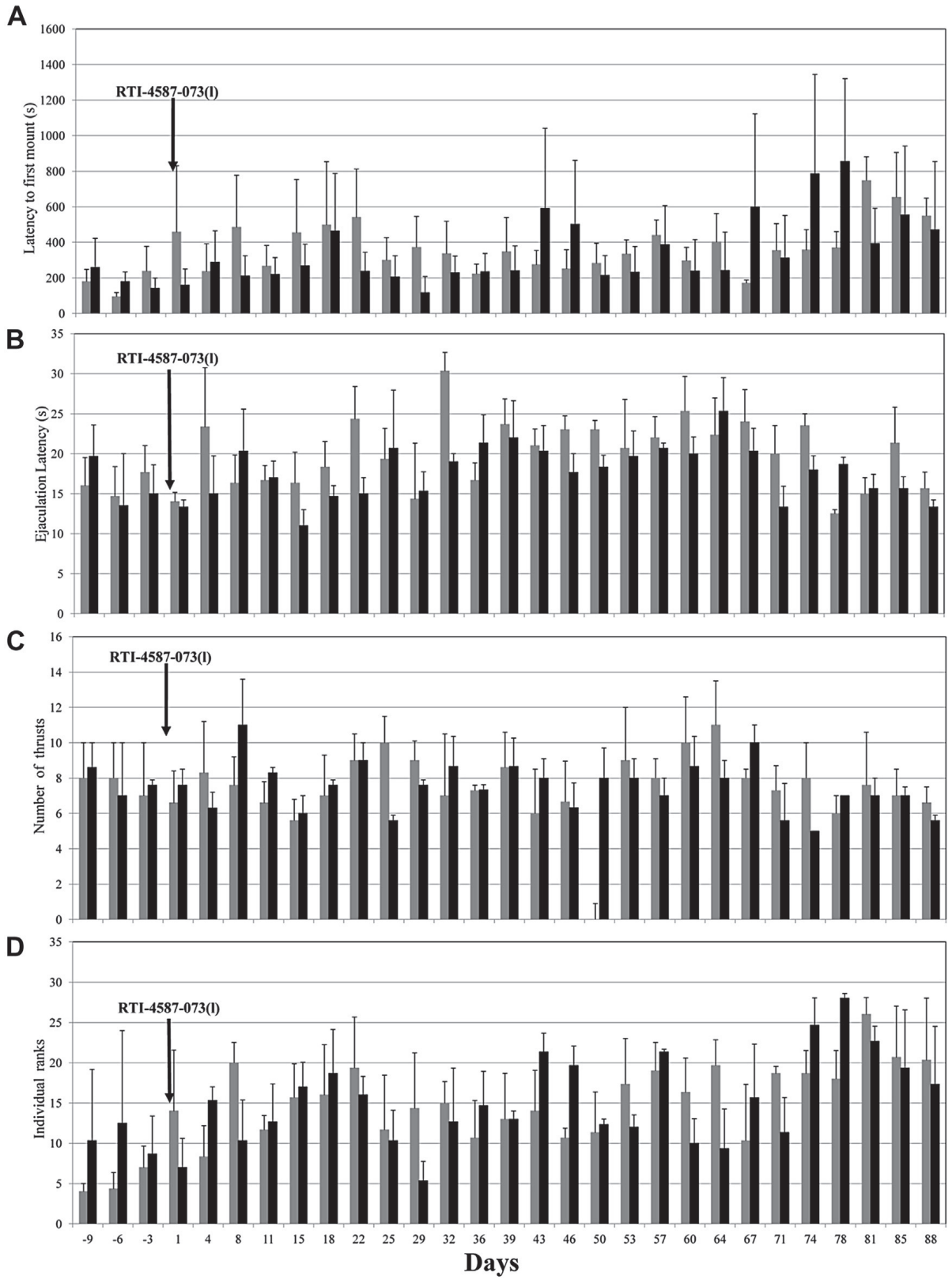

Fig. 3. Mean (± SEM) selected behavioral endpoints in stallions treated with RTI-4587-073(l) (group treated) or with a vehicle (group control).

from the seminiferous epithelium in the rat testes. In addition, the blood-testis barrier was compromised by disorganization of Sertoli cell tight junctions [20]. Apoptosis of germ cells was induced by activation of the pro-apoptotic factor, Fas, whereas expression of prosurvival factors was reduced. Effects on spermatocytes and spermatids included swelling of the nuclear envelopes in these cells, and fusion of spermatids, leading to formation of multinucleated giant cells.

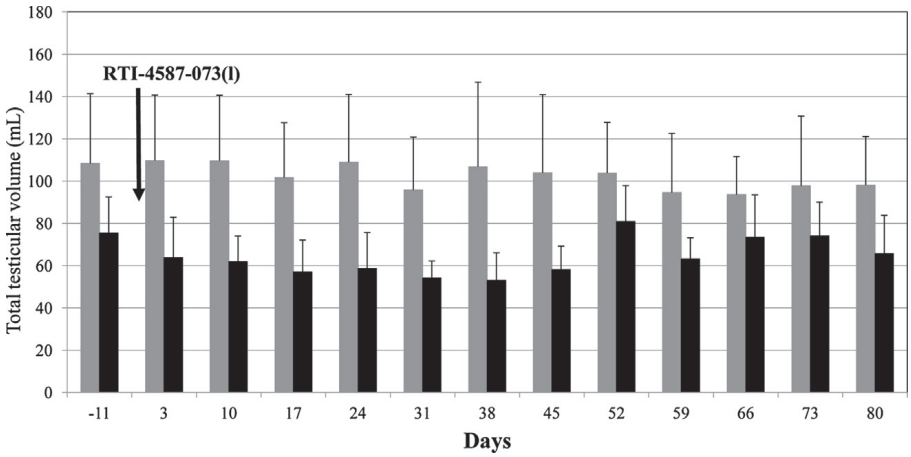

Fig. 4. Mean (± SEM) total testicular volume in stallions treated with RTI-4587-073(l) (group treated) or with a vehicle (group control).

Interestingly, spermatids in affected testes had abnormally diffuse and rarefied chromatin.

Similar effects occurred in stallions; that large numbers of round cells appeared in ejaculates of treated stallions in our study, we inferred that there was massive exfoliation of germ cells from the seminiferous tubules. Although primary spermatocytes looked relatively normal, the majority of spermatids had two or three nuclei or formed multinucleated giant cells with more than three nuclei located in the periphery of the cell. It has been established that low numbers of immature germ cells can be present in semen of fertile stallions, although high numbers of these cells in stallion semen are abnormal and are consistent with testicular degeneration, injury, treatment with anabolic steroids, or various toxins [22,23]. Unfortunately, exact reference values of these numbers in normal and abnormal stallions have not been established. The mean number of immature germ cells in ejaculates collected from control stallions in our study was very low (mean = 0.4 cells/100 sperm, range 0–1.6). Similar numbers of these cells were seen in stallions from group treated before treatment (mean = 0.6 cells/100 of sperm, range 0.3–1.0). It was noteworthy that large numbers of immature germ cells (mean = 90 cells/100 sperm) suddenly appeared in semen collected from all treated stallions 2 weeks after administration of RTI-4587-073(l). This acute and severe exfoliation of germ cells was very similar to previously described effects of various toxicants targeting the Sertoli cell in laboratory animals [24]. Furthermore, it was well documented that physical (e.g., heat) and chemical (e.g., PCBs, DDE) agents can disrupt condensation of sperm nuclear chromatin and nuclear shape [24]. In the present study, RTI-4587-073(l) also affected sperm chromatin structure in stallions. A similar effect was not reported in other species treated with indenopyridines; however, the susceptibility of sperm chromatin to denaturation was not evaluated in these studies. Interestingly, in our experiment, large numbers of sperm with altered sperm chromatin structure appeared in stallion semen 2 weeks after administration of RTI-4587-073(l). Because ~9 days are necessary for epididymal transport of spermatozoa in stallions [25], the compound must have directly affected the formation of a stable structure of sperm chromatin in the germ cells still residing in the seminiferous tubules, before epididymal maturation.

Specific changes in morphology of spermatozoa after administration of RTI-4587-073(l) were not investigated in previous studies. In our study, there was an increased percentage of tailess sperm heads in semen of treated stallions between Days 22 and 53 after administration of the compound. Small numbers of tailess sperm heads are often present in stallion semen (<0.5%); however, very high numbers are usually associated with sperm accumulation [26,27]. Our stallions were on a frequent semen collections schedule (initially daily collections, followed by two collections per week); therefore, semen accumulation was an unlikely cause of this abnormality. It was postulated that increased numbers of tailless sperm heads appear in semen of stallions with testicular degeneration, abnormal spermiogenesis, or abnormal epididymal function [28]. Malfunction of the Sertoli cells in RTI-4587-073(l)–treated stallions likely led to the abnormal spermiogenesis in our study. Furthermore, there might have been similar transient changes in the efferent ducts, as well as in the epididymal duct, as described in monkeys treated with l-CDB-4022 [10].

The detrimental effects of RTI-4587-073(l) on stallion semen were accompanied by the gradual increase in FSH serum concentrations, but without significant changes of circulating inhibin. There is a certain pattern of changes in circulating hormone values in an idiopathic subfertile/infertile stallion. Inhibin and estradiol decrease at first, FSH concentrations increase next, followed by an increase in LH concentrations [29,30]. Testosterone is usually the last hormone to decrease its concentration in blood samples [29,31]. Although it is somewhat surprising that there were no significant changes in concentrations of inhibin in treated stallions, the sampling frequency in our experiment was suboptimal and might have not reflected all endocrine changes that occurred after administration of the compound. Inhibin B decreased significantly at 6 hours

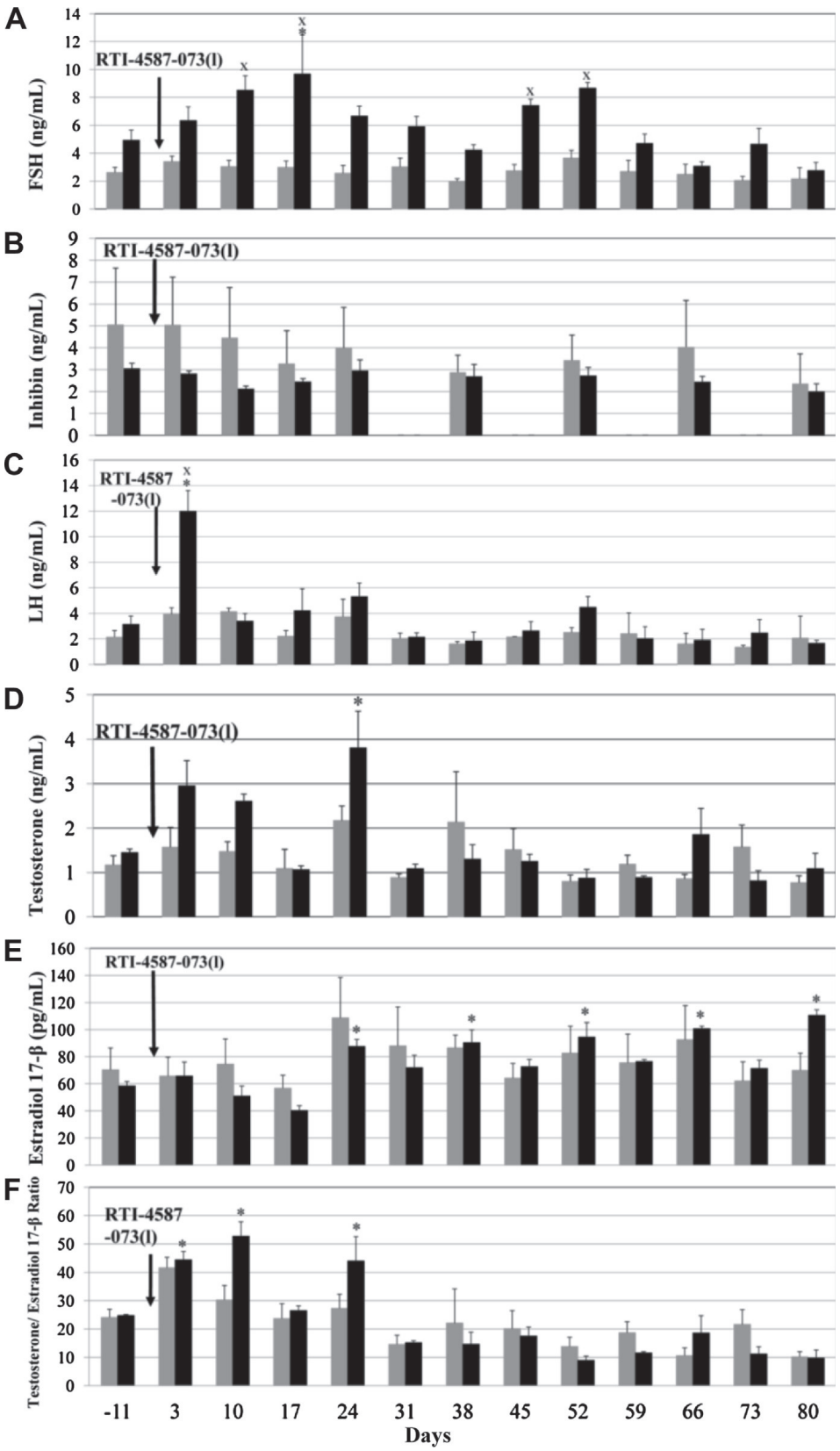

Fig. 5. Mean (± SEM) concentrations of reproductive hormones in stallions treated with RTI-4587-073(l) (group treated) or with a vehicle (group control). Values with an asterisk (*) differed from the last value of the baseline period within each group ($P < 0.05$), whereas X above a bar indicates a difference between groups on a given day ($P < 0.05$).

after a single dose of the compound in rats [32]. Therefore, another experiment on stallions with frequent sampling is needed to show if this acute effect occurs in horses. In contrast to other studies on RTI-4587-073(l), concentrations of LH transiently increased in treated stallions in our experiment (Day 3). Testosterone concentrations increased in treated stallions as well, but this effect appeared much later (Day 24), which may (delayed effect) or may not be correlated with the LH peak (independent event). Changes in LH and testosterone concentrations suggested that RTI-4587-073(l) may be affecting Leydig cells in stallions. Therefore, it appears that there are some species differences in actions of indenopyridines. Furthermore, it was somewhat surprising that testosterone to estradiol ratio increased in stallions treated with RTI-4587-073(l). Values of this parameter usually decline in men with idiopathic infertility [33]. However, there was a transient increase in LH concentration in treated stallions, which might have enhanced testosterone production, leading to the increased testosterone to estradiol ratio. One can speculate that LH concentration increased, as a response to an acute adverse effect of RTI-4587-073(l) on Leydig cells, and transient decline in testosterone production. Although we have not observed such an effect in treated stallions, more frequent collections of blood samples would be necessary to prove or disprove this theory.

Sexual behavior of control and treated stallions was assessed. Numerous behavioral endpoints were derived from the video-recorded sessions of semen collections. Statistical analysis of these values detected no significant changes in sexual responses of stallions within each group, and between both groups, throughout the experiment. This is an important finding, since preserving libido, and normal sexual behavior are crucial for harem stallions in the wild. Normal social structure of wild horses depends on continuous interactions among harem stallions, mares, and bachelor stallions [34]. Contraceptives, which suppress secretion of reproductive steroids, such as testosterone, have a potential of changing population dynamics, which is not desirable in free-ranging horses [35]. Therefore, indenopyridine RTI-4587-073(l) has superior qualities as a candidate for male contraceptive for animals and men in comparison to more traditional hormone-based contraceptives.

All findings in the current study provided strong evidence that a single administration of RTI-4587-073(l) induced severe but reversible testicular dysfunction in stallions, but did not significantly affect sexual behavior. We concluded that this compound is a good candidate for a male contraceptive and its applications should be further tested.

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