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Preliminary investigation: Down-regulation of testosterone by an anti-GnRH immunotherapeutic (Improvest®) in a Southern White Rhinoceros (*Ceratotherium simum simum*) Bull

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ABSTRACT

The ability to maintain bull white rhinoceros (*Ceratotherium simum simum*) in breeding groups while preventing unwanted reproduction could be beneficial for bull wellbeing compared to solitary housing. To date, no contraception has been investigated for male rhinoceros species, but anti-GnRH immunotherapeutics have been used in female white and greater one-horned rhinoceros, as well as other wildlife species, to reduce reproductive steroid hormone concentrations. This study investigated the efficacy of Improvest® in down-regulating testosterone concentrations in a genetically well-represented bull white rhinoceros. Improvest® (0.6 mg; Distributed by Zoetis, Inc., Kalamazoo, MI 49007) was administered in the left triceps muscle, followed by boosters at 4, 12, 26, 42, 51, and 67 weeks. Serum testosterone was measured by enzyme immunoassay in blood samples collected voluntarily approximately weekly before and during the initial treatment, then monthly for long-term monitoring. No adverse effects from Improvest® were observed with the exception of mild swelling at the injection site. A five-fold decrease in testosterone concentrations was measured within the fourth week after the second dose, 8 weeks following initial treatment, and basal concentrations (17.3 ± 1.1 ng/mL) were maintained until a break-through increase in concentrations occurred 16 weeks after a booster injection. This case study demonstrates that testosterone concentrations can be suppressed in white rhinoceros bulls treated with Improvest®. These findings suggest anti-GnRH immunotherapeutics could be promising tools for bull rhinoceros management, and further studies of consistency in responsiveness, effects on behavior and fertility, and reversibility are merited.

1. Introduction

At facilities where white rhinoceros (*Ceratotherium simum simum*) reproduction is highly successful, it is sometimes necessary to pause breeding to prevent genetic over-representation of individuals in the Species Survival Plan® [1] and to address limitations on space available for housing weaned offspring (A. Eyres, personal communication). Separating the breeding bull from the group prevents reproduction. However, because white rhinoceros are social [2–4], the ability to maintain group housing for the bull could promote wellbeing, and effective contraception would allow this without unwanted offspring production.

Methods of contraception used for wildlife, and ungulate species in particular, include surgical procedures, hormone therapy, and immunocontraceptives [5,6]. Surgical methods are not practical for rhinoceros because of risks associated with their large size. Synthetic progestins (oral, injectable, or implants) and gonadotropin releasing hormone (GnRH) super-agonists (injectable or implants) are among the hormonal treatment options for down-regulating the hypothalamic-pituitary-gonadal (HPG) axis [5,6]. Although short-term treatment with progestins [7,8] and GnRH agonists [8–11] has been used in estrus synchronization and ovulation induction protocols in female rhinoceros, there are no studies of their use in male rhinoceros. To the best of our knowledge no contraception has been investigated in rhinoceros species. Improvac® (0.3 mg/mL gonadotropin releasing factor analog-diphtheria toxoid conjugate; Zoetis, Inc., Germany), an anti-GnRH immunotherapeutic, was used in female white and greater one-horned (GOH) rhinoceros (*Rhinoceros unicornis*) to decrease the size

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of reproductive tract tumors by down-regulating steroid hormone secretion [12]. Understanding whether an anti-GnRH immunotherapeutic might down-regulate testosterone production in a male rhinoceros would be a useful first step for understanding whether such treatments might merit future investigation as a contraceptive.

Several anti-GnRH immunotherapeutics are commercially available (e.g., Improvest®, Improvac®, Bopriva®). Improvest® (0.2 mg/mL gonadotropin releasing factor analog-diphtheria toxoid conjugate; Zoetis, Inc.) is approved in the United States (NADA 141–322) in male pigs and gilts (*Sus scrofa domesticus*) and is an alternative to surgical castration with reversibility [13]. Improvest® induces production of anti-GnRH antibodies, which bind to endogenous GnRH and prevent binding to pituitary receptors, resulting in the cessation of gonadotropin secretion and gonadal steroid hormone secretion [14]. In the domestic horse (*Equus caballus*), considered a model species for rhinoceros, stallions treated with an anti-GnRH immunotherapeutic generally showed decreased testosterone, libido, and semen quality after two injections [15], but the effect of anti-GnRH immunotherapeutics on testosterone concentrations and sperm motility varied depending on the type of adjuvant used [16]. In wildlife, anti-GnRH immunotherapeutics have been used to reduce aggressive behavior and suppress musth and fertility in African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephant bulls [17–19]. Improvest® has been used to reversibly suppress testosterone in male giraffe (*Giraffa camelopardalis*) [20] and to reduce testosterone and aggression in large flying foxes (*Pteropus vampyrus*) [21]. As a prerequisite to understanding the potential for Improvest® to be used as contraception in male white rhinoceros, this preliminary study investigated the efficacy of Improvest® in down-regulating testosterone concentrations in one genetically well-represented bull.

2. Methods

A 2250 kg, 11yo, proven fertile white rhinoceros bull was housed individually in a 0.2 hectare enclosure for four months of monitoring before housing with a female/sub-adult herd comprised of 14 individuals in a 10 hectare day pasture and 0.3 hectare night pasture. He was fed 3.2 kg Mazuri® Browser Rhino pellets (Mazuri Exotic Animal Nutrition, St. Louis, MO 63166 USA) supplemented with vitamin E (3.735 IU/kg Emcelle® tocopherol, Stuart Products^{inc} Bedford, TX 76022 USA) and *ad libitum* coastal hay daily. Supplementation with oral vitamin A (0.036IU/kg micellized vitamin A palmitate, SFI Health™, Reno, NV 89521 USA) began four months into the study.

Administration of Improvest® in boars per manufacturer instructions includes an initial injection followed by a booster injection one month later, then slaughter within 3–10 weeks. Maintenance boosters were given at 3-month intervals in giraffe [20]. In this study, Improvest® (0.6 mg, 3 mL; Distributed by Zoetis, Inc., Kalamazoo, MI 49007USA) was administered in the left triceps muscle with the rhinoceros's voluntary participation, followed by a booster after 1 month and thereafter at 12, 26, 42, 51, and 67 weeks. The goal was to introduce the male to the female/sub-adult herd after testosterone had decreased and the male was presumably contracepted or his libido suppressed. Serum testosterone was measured in blood samples collected voluntarily from the left auricular vein approximately weekly before and during the initial vaccination regimen. Two weeks after the third Improvest® injection, the frequency of blood sample collection was decreased to monthly for long-term monitoring. Serum was stored frozen at −20°C until analysis.

An enzyme immunoassay (EIA) using a polyclonal anti-testosterone-6-carboxymethyl oxime antiserum (R156/7; Munro, University of California, Davis, CA, 95616) and testosterone conjugate (testosterone-3-carboxymethyl oxime:horseradish peroxidase, hereafter HRP), previously validated for use with white rhinoceros serum [22], was used to measure serum testosterone according to a previously published protocol [20]. In brief, microtiter plates (#655081, Santa Cruz Biotechnology, Inc., Dallas) precoated with 10 μg/mL goat anti-rabbit immunoglobulin G (#A009–25MG; Arbor Assays, Ann Arbor, MI) were maintained at 4°C in sealed pouches with desiccant until use. Serum samples were diluted 1:30 in assay buffer (0.04 M NaH_2PO_4, 0.06 M Na_2HPO_4, 0.15 M NaCl, 0.1% BSA, pH 7.0) immediately prior to assaying in duplicate. Reagents and plates were brought to ambient temperature (~24°C) prior to use, and 50 μl testosterone standard or diluted serum sample was added to precoated wells, followed immediately by 50 μl of HRP, then 50 μl of antibody, excluding two blank wells for a nonspecific binding control. Plates were incubated (2 h) at ambient temperature before washing four times with 0.008% Tween 20 (# P1379–100 mL; Millipore Sigma, St Louis, MO) in distilled water. After washing, 100 μl High Kinetic Tetramethylbenzidine (#TMBHK-1000; Moss, INC., Pasadena, MD) colorimetric substrate was added to each well, and the plates were incubated at ambient temperature until an optical density of 1.0 at 450 nm was attained for the zero hormone standards, whereon 1 M HCl (#320331; Millipore Sigma, St Louis, MO) was added. Plates were read with a BioTek ELx808 microplate reader (BioTek Instruments, INC, Winooski, VT). Assay sensitivity was 0.02 ng/mL. Inter-assay variation of two blood samples run on each plate was 17.1%, and intra-assay variation was 4.5 ± 1.1%. Hormone concentrations are reported as ng/mL and plotted over time.

Two females in the herd delivered calves within one month of the bull's second dose of Improvest®; one female delivered a calf eight months after his treatment began. Thus, although these three females were open during the male's Improvest® treatment, they were also lactating and might not have been available for the male to breed. One previously parous female was neither pregnant nor lactating and thought to be cycling based on observations of regular courtship behavior, including chin-resting and mounting, by the bull. Three blood samples were voluntarily collected from each female over a 13–15-day period during month 17 of the male's Improvest® treatment to determine if they were pregnant. An EIA using a monoclonal antibody produced against 4-pregnen-11-ol-3,20-dione hemisuccinate:bovine serum albumin (Quidel clone number 425; Munro, University of California, Davis, CA, 95616) and progesterone:horseradish peroxidase conjugate, previously validated for use with white rhinoceros serum [23], was used to measure progesterone concentrations. Serum samples were diluted 1:2 in assay buffer immediately prior to assaying in duplicate on a single plate according to a previously published protocol [24]. Assay sensitivity was 0.08 ng/mL; intra-assay variation was 5.9 ± 0.8%. Hormone concentrations are reported as ng/mL.

3. Results

No adverse effects from the anti-GnRH immunotherapeutic were observed with the exception of mild swelling at the injection site. Anecdotally, the bull's behavior was described by keepers as less intense and antagonistic toward other rhinoceroses. Two lactating females' progesterone concentrations were consistently < 2 ng/mL during month 17 of the bull's Improvest® treatment, suggesting they were in lactational anestrus throughout his treatment and unable to provide evidence of the efficacy of Improvest® as a contraception for the bull. The third lactating female ovulated during the 13-day monitoring period, evidenced by an increase in progesterone from 0.2 ng/mL to 8.6 ng/mL, but no mounting behavior was observed. The non-lactating female's progesterone concentrations were consistently < 2 ng/mL, suggesting she was not cycling during that 13-day period; however, the bull was observed chin-resting and mounting her at 26, 30, 51, and 67 weeks of treatment. Intromission was not observed.

Serum testosterone concentrations initially ranged from 91 to 203 ng/mL pre-treatment (mean ± SEM = 140.1 ± 15.9 ng/mL) and began to decrease after the week 4 booster (Fig. 1). Concentrations were approximately five-fold lower (27 ng/mL) by 8 weeks following the initial treatment and remained between 16 and 25 ng/mL (17.3 ± 1.1 ng/mL) for the duration of monitoring until week 67 (Fig. 1). Testosterone concentrations increased to 58.2 ng/mL at week 67,

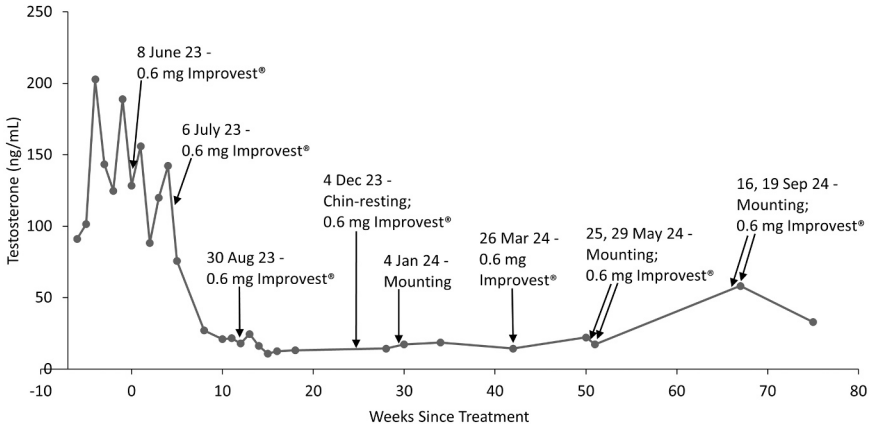

Fig. 1. Serum testosterone concentrations and sexual behavior in a bull white rhinoceros (*Ceratotherium simum simum*) treated with Improvest®.

3.4 × higher than average concentrations during weeks 8–51, prompting administration of another booster (Fig. 1). Thus, testosterone concentrations remained less than pre-treatment concentrations up to 16 weeks after the last booster once basal concentrations had been achieved. The longest interval that basal testosterone concentrations were maintained without a break-through increase in testosterone between boosters was 13.5 weeks, based on the serum samples (from 22 December 2023–26 March 2024) collected between the 26- and 42-week boosters.

4. Discussion

This preliminary investigation documents the successful down-regulation of testosterone secretion by Improvest® in a white rhinoceros bull. Keepers noted this bull's behavior was subjectively less intense and antagonistic toward other rhinoceroses once testosterone concentrations decreased. Objective data on the frequency of aggressive interactions and dominance merits investigation in a future study to understand if the effects of Improvest® on white rhinoceros bulls might be similar to observations of reduced aggression and dominance in African elephants administered an anti-GnRH immunotherapeutic [17,19].

Testosterone concentrations measured in this study prior to Improvest® treatment were higher than those reported in some bull white rhinoceros exposed to adult females [22,25,26], which might be accounted for by differences in assay protocols and reagents between labs [27]. The decrease in testosterone concentrations measured in the bull post-treatment was unequivocal, and his post-treatment concentrations were similar to the concentrations measured in the same lab for single samples from three young bulls (9–10 yoa) that were not housed in breeding situations (3.6–11.4 ng/mL). Suppressed testosterone concentrations were noted within the fourth week after the second dose, 8 weeks following initial treatment. Testosterone concentrations in a male giraffe [20] and in male elephants [18,19] also decreased after the second dose. In female white and GOH rhinoceros administered Improvac® at 0, 4 and 16 weeks, ovarian luteal activity did not decrease in white rhinoceros until 4 months after the initial dose, whereas luteal activity decreased 2 months after the initial dose in GOH rhinoceros [12]. Age appears to affect anti-GnRH immunotherapeutic efficacy in horses and mules (*Equus caballus* × *asinus*) with young males more consistently showing reduced testosterone, libido, testes size, sperm production and quality [16,28] than older males [28,29]. However, even among young horses, treatment effects on hormone concentrations and semen quality can vary depending on individual variation in GnRH antibody titers despite receiving the same formulation and dosing [30]. Additional investigation will be required to determine the consistency of responsiveness of bull rhinoceros to Improvest® and whether responsiveness is affected by age.

In this study, basal testosterone concentrations had not increased by 13.5 weeks after a booster. Although testosterone concentrations remained lower than pre-treatment values up to 16 weeks after a booster, concentrations had risen above baseline, presumably because of the longer duration since the last booster. Duration of efficacy appears to be slightly longer in elephants administered an anti-GnRH immunotherapeutic, whose testosterone concentrations are suppressed for 5 months [19]. Return to pre-treatment testosterone concentrations was not tested in this study prior to the bull's relocation to another facility. In horses, reversal was achieved within 2–5 months following cessation of GnRH immunotherapeutic injection [29].

Although testosterone was decreased in the white rhinoceros, the impact of Improvest® treatment on spermatogenesis, as well as the duration of any such effects following discontinuation, was outside the scope of this study and remains unknown. Mounting behavior by the bull continued to be observed despite low testosterone, though no intromission was observed. Mounting behavior also persisted in a bull giraffe treated with Improvest® [20]. Although the three lactating females were not necessarily expected to resume cycling within about one year of calving, at least one of them appeared to be cycling by 14 months post-partum. A fourth female was neither pregnant nor lactating during the bull's treatment and was observed in courtship with the bull yet did not become pregnant. Thus, although two females were available for breeding, only one was available throughout the bull's treatment. In the absence of any observations of intromission and ejaculation, these observations are insufficient to assess contraception efficacy of Improvest® in white rhinoceros. In horses, stallions administered an anti-GnRH immunotherapeutic might produce enough sperm to impregnate a mare despite diminished semen quality [29], possibly due to continued FSH secretion, which is not suppressed as effectively as LH secretion [31]. Future studies on white rhinoceros could include testicular measurements and semen collection to determine sperm quality and contraceptive efficacy, though these are unable to be performed in a non-anesthetized animal. Additional areas of future study include investigating whether contraception using Improvest® in white rhinoceroses is reversible, how long after the last dose reversibility is achieved, and whether reversibility is affected by the number of doses received.

This case study demonstrates that testosterone concentrations can be suppressed in white rhinoceros bulls administered Improvest®. Suppressed testosterone concentrations might have implications for reduced fertility and aggressive behavior, which if demonstrated by future studies, could indicate utility of an anti-GnRH immunotherapeutic in the management of rhinoceros bulls when group housing without reproduction is desired.

CRediT authorship contribution statement

Elizabeth E. Hammond: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Linda M. Penfold:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Lara C. Metrione:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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