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Comparative efficacy of two immunocontraceptive vaccines

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*As part of our research program to develop immunocontraception as a wildlife management tool, we compared the physiological responses of wild Norway rats (*Rattus norvegicus*) to two immunocontraceptive vaccines; one involved mouse zona pellucida peptide (MZPP); the other involved gonadotropin releasing hormone (GnRH). Efficacy was monitored by immune, hormonal, and natality responses. Both vaccines were effective, but GnRH was much more effective (100% sterility of both sexes vs. 50% sterility of MZPP-treated females). Breeding success of control rats was 88% with litters of 5–9 pups; breeding success of MZPP rats was 50% with litters of 2–8; GnRH rats produced no young. In GnRH-treated male rats monitored for up to 17 months, testosterone was nondetectable and testes were atrophied to about 10% of their original volume for 10–13 months. There were no notable differences in mortality or body weights among groups, and, with the exception of testicular regression, there were no changes in general appearance. The GnRH vaccine is potentially a good rat reproductive control agent that may be effective over the normal lifespan of a rat under natural conditions in the wild. Published by Elsevier Science Ltd.*

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Rats cause major damage world wide, yet attempts to control them by contemporary means (e.g. poisons or traps) are often less than satisfactory. The typical population response to lethal control is an increased rate of reproduction due to less competition among the animals remaining in the population. Knipling and McGuire[1] postulated a theory about the advantages of reproductive sterilization over killing. They concluded that if 70% of both sexes in a population could be sterilized for three generations, the entire population would be eliminated. A chief advantage of reproductive sterilization over removal of individuals is that these animals will continue to compete for food, nesting sites, and living habitat. Also, non-lethal methods, such as immunocontraception, are more apropos to current social attitudes about animals and animal treatment. Earlier attempts have been made to manage rodent populations via reproductive sterilization[2,3]. These attempts generally proved ineffective or impractical for numerous reasons; among them were that administration of synthetic steroids—a type of the so-called 'chemosterilants'—required daily or frequent repetitive doses and were ecologically unacceptable because of potential exposure of non-targets either directly or through the food chain. The immunological approach to contraception is more attractive than chemosterilants for several reasons.

For example, it does not have the food chain problems associated with chemosterilants, and it requires only limited periodic treatments.

Immunocontraceptive vaccines are directed against 'self' reproductive antigens (hormones or proteins) to which the recipient is immunologically tolerant. The antigens are made 'non-self' by coupling them to a carrier protein that is foreign to the animal[4]. In this study, we compared two immunocontraceptive vaccines in the wild Norway rat.

The first vaccine contained a synthetic mouse zona pellucida antigen coupled to a carrier protein, keyhole limpet hemocyanin (KLH). The zona pellucida is an acellular glycoprotein layer located between the oocyte and the granulosa cells on the outer surface of the mammalian egg; it provides species specificity and prevents multiple sperm penetration. Millar *et al.*[5] identified a 7-mer (amino acid) peptide containing the sperm receptor in the zona pellucida of the mouse. They demonstrated similarities between the mouse and rat zonae pellucida that do not exist in other mammalian species; this provided encouragement that the 7-mer peptide would be an effective immunocontraceptive in the rat. MZPP is too small to produce antibodies itself so it was coupled to a larger protein, KLH, to increase antibody production. The MZPP/KLH conjugate induces antibody responses to both KLH and MZPP. Antibodies to MZPP block conception by binding to the sperm-binding sites of the

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endogenous zona pellucida. This methodology is only effective in the female[6].

The second vaccine comprised gonadotropin releasing hormone (GnRH) made immunogenic by coupling to KLH[7]. GnRH is sometimes called the unisex hormone. It is produced by the hypothalamus and is responsible for the release of two pituitary hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the consequent release of other hormones that control the functioning of the ovaries and testes. Antibodies to GnRH produce infertility by binding to circulating endogenous GnRH; this precludes the GnRH from binding to its pituitary receptor, thereby interfering with its ability to effect the release of FSH and LH. The severe reduction or absence of these hormones leads to atrophy of the gonads and concomitant infertility in both sexes. GnRH has been previously used for immunocontraception[8–11]. Awoniyi *et al.*[12] demonstrated that GnRH-immunized rats selectively eliminate the release of FSH and LH without affecting other pituitary hormones; therefore other vital non-sexual functions are not impaired.

There is little data about the long-term duration of infertility caused by immunocontraception; hence, a portion of this study was designed to examine this aspect. Previous studies reported in the literature were done with inbred laboratory rats[13]; this study was conducted with the wild Norway rat.

METHODS

Eighty wild Norway rats were removed from our outdoor breeding colony, put into individual cages, and quarantined for 14 days. They were then randomly assigned to treatment groups as follows:

- **Group 1 (10♀)**—treated with 100 μg MZPP/KLH.
- **Group 2 (10♀)**—treated with 100 μg GnRH/KLH.
- **Group 3 (10♂)**—treated with 100 μg GnRH/KLH.
- **Group 4 (10♀, 20♂)**—untreated; controls bred to the treated rats.
- **Group 5 (10♀, 10♂)**—untreated; controls bred to each other.

Female rats ranged in weight from 200–305 g and the males ranged from 301–410 g. Environmental controls for the animal room maintained 21°C, and a 14/10 h light/dark schedule. The animals were allowed to acclimate to the light schedule for 30 days (including quarantine), and were fed a standard lab chow diet. Before vaccination or bleeding, rats were anesthetized by inhalation with methoxyflurane (Metofane®). The vaccines were administered subcutaneously at 6 sites along the back.

Antigen preparation

MZPP. The MZPP antigen was synthesized as described by Millar *et al.*[5] It consists of a 7-mer epitope with an 8-mer leader. The cysteine at the end of the leader provides an SH group for coupling to maleimide-activated KLH. Coupling was performed with reagents from Pierce Chemical, Rockford, IL. Two mg of MZPP were coupled with two mg of KLH resulting in 4 ml of conjugate; the conjugate was combined with 4 ml of complete Freund's adjuvant (CFA). One hundred μl of vaccine containing 100 μg of the conjugate were injected in each rat on Day 1. At Days 21 and 51, the rats were given the same dose as on Day 1 except with incomplete Freund's adjuvant (IFA).

GnRH. We purchased GnRH (a 10-mer hormone) containing a free carboxy terminus at the 10 position from Sigma Chemical Co, St. Louis, MO. Two mg of it were coupled to 2 mg of KLH using the EDC conjugation method from Pierce chemical. The rest of the vaccine formulation was the same as for MZPP. One hundred μl of vaccine containing 100 μg of the conjugate were injected in each rat on Day 1. At Days 21 and 51, the rats were each given the same dose as on Day 1 except with IFA.

Breeding studies

Rats were bred during the one month period starting 2 weeks after the last boost vaccination, the time when peak titers were expected. Breeding pairs were maintained for 19 days in breeding cages containing a section of PVC pipe as a nestbox; it was 30 cm long and 10.16 cm (4 in) in diameter. Inside the PVC pipe was a 15 cm square of burlap bagging fabric for nesting material. The females were considered pregnant if they made a nest of shredded burlap filling the diameter of the PVC pipe even though no pups were found. This is because, in our colony, they only made nests when pregnant and would sometimes consume the neonates.

The studies involving Group 1 (MZPP-treated females) and Group 2 (GnRH-treated females) were terminated after the breeding trials. Animals from Group 3 (GnRH-treated males) were kept for observation of long-term immunocontraceptive effects because of the ease of examining anatomical changes in the testes and measurement of testosterone.

Laboratory measurements

In addition to the breeding trials, the efficacy of the vaccines was assessed by measuring plasma progesterone, testosterone, and antibody titers. Rats were anesthetized, weighed, testicle size and general animal condition were noted, and blood samples were taken. Testicle size was determined by palpation and divided into four categories: (1) Normal, with turgidity and length representative of an adult rat; (2) Initial atrophy, not fully turgid with length shortened; (3) Progressive atrophy, with testis more flaccid than turgid and length continuing to decrease; (4) Complete atrophy with testis totally flaccid and length reduced to approximately half of normal. Blood samples were drawn immediately before treatment and at periodic intervals thereafter. Initially, blood was taken from the tail. However, due to inconsistent blood-draw success, we changed to taking blood from a scalpel nick on the side of a rear foot pad and used several 100 μl heparinized capillary pipettes to obtain 300–400 μl of blood. The repeated blood draws on each rat had no adverse effects; the nicks on the side of the foot pads heal with

no visible scarring and without the irritation apparently associated with multiple tail bleedings. Testes weights were taken when rats died.

Antibody titers were measured by enzyme-linked immunosorbent assay (ELISA). Plasma testosterone in the males and plasma progesterone in the females were assayed by the coat-a-tube RIA method (Diagnostic Products, Los Angeles, CA). Fifty μl of plasma were used for each assay.

Statistical analysis

Statistical analyses of testes weights and progesterone values were conducted with a *t*-test using SAS/STAT® software from SAS Institute Inc. Analyses of litters sired and litters born were performed using the McDonald test for comparing two proportions[14].

RESULTS

Breeding success

Because of mortality unrelated to treatment, the results are based on 8 rats per group. Seven of the eight control females (Group 5) produced normal-size litters ranging from five to nine pups. Four of eight MZPP females (Group 1) had litters ranging from two to eight pups (*Table 1*). Millar *et al.*[5] found a similar percentage of fertility using inbred mice; they reported that six of 12 MZPP-immunocontracepted mice gave birth and the litters were smaller-than-normal. None of the GnRH-treated females conceived (*Table 1*). The same results were seen when untreated females from Group 4 were bred to GnRH-treated males from Group 3; none of the males were fertile (*Table 2*).

Table 1 Plasma progesterone at breeding and whelping statistics of female wild Norway rats vaccinated with immunocontraceptives

Vaccine	Progesterone (ng ml^{-1}) $\bar{x}$	SD	No. Littered/ no. bred	Litter size
Control (Group 5)	7.1[a]	5.3	7/8[c]	5–9
MZPP (Group 1)	5.0[b]	2.8	4/8[d]	2–8
GnRH (Group 2)	1.9[a]	0.8	0/8[c]	0

[a]Significantly different from each other ($P = 0.04$). [b]Not significantly different from Control ($P = 0.39$). [c]Significantly different from each other ($P < 0.004$). [d]Not significantly different from control ($P > 0.05$)

Table 2 Plasma testosterone at breeding, testes weight, and fertility of male wild Norway rats vaccinated with GnRH immunocontraceptive

Vaccine	Testosterone (nmol l^{-1}) $\bar{x}$	SD	Testes weight (g) $\bar{x}$	SD	Litters sired/ total males
Control (Group 5)	9.1	8.1	3.0[a]	1.1	7/8[b]
GnRH (Group 3)	ND		0.8[a]	0.6	0/8[b]

ND: Not detectable, all rats. [a]Significantly different ($P < 0.001$). [b]Significantly different ($P < 0.004$)

Laboratory measurements

Behavior and physical condition. The gross behavior of animals in each of the three treatment groups did not deviate from that of the non-treated animals in the control groups. The overall physical condition of the animals appeared normal for wild Norway rats across all groups. The pelage of all animals in all groups improved in appearance during the study. These observations give credence to the hypothesis that immunocontraception does not impair other vital non-sexual functions. The testes of male rats injected with GnRH (Group 3) began to shrink after 60 days. After 90 days, the testes had regressed completely and the scrotums disappeared until 10–13 months after treatment. The mean testes weight (*Table 2*) of treated males was 0.8 g, SD = 0.5 and those of untreated males was 3.0 g, SD = 0.9.

Hormones. The drop in progesterone levels at breeding in MZPP-treated female rats was not significant ($P = 0.39$) (*Table 1*). A similar drop in progesterone levels following ZP treatment was reported by Skinner *et al.*[6]

Progesterone in the GnRH-treated females at breeding (*Table 1*) dropped significantly ($P = 0.04$) to levels comparable with those observed in normal males in our laboratory. This was expected since the lack of GnRH should result in complete atrophy of internal female reproductive organs. The progesterone that was present may have come from the adrenals.

Testosterone levels dropped to non-detectable within three months of the first injection in the GnRH-treated males of Group 3 (*Table 2*). This indicates a complete lack of GnRH biological activity.

ELISA antibody titers. At the time of breeding, the KLH antibody titers ranged from 4000 to 16000, the MZPP antibody titers ranged from 6000 to 12000, and the GnRH antibody titers ranged from 2000 to 8000.

Long-term immunocontraception studies (Group 3 GnRH/KLH males)

Peak KLH antibody titers were observed in the samples drawn 3 weeks after the 51-day boost and were similar to the other rats; they ranged from 6000 to 16000. KLH antibody titers remained high for 4 to 11 months; subsequently, they dropped gradually. In Rat V-10, the longest-lived animal in the study, there was a detectable titer at 17 months.

The GnRH titers peaked during the same time period as KLH titers and ranged from 2000 to 8000; the duration of the peak titer was 2–3 months, followed by a rapid drop with the exception of Male V-11 that peaked at 2000 for 8 months. Four rats (V-10, V-11, V-21 and V-23), remained alive after vaccination for periods ranging from 11 to 17 months. A curious—and previously unreported—development occurred in these four animals. The GnRH titers on the four rats went through a cycle of coming up, peaking, and going down to a low point that was still above prevaccination levels. Then, approximately 10 months after initial vaccination, the GnRH titers all began to rise again and reached titers, in three of four rats, higher than the previous peaks. During this period of renewed rising of GnRH titers, the KLH titers

continued to decline. Rats V-11 and V-21 died 13 and 15 months into the study; they each presented non-detectable testosterone levels from the third month of the study through the month before death, and complete atrophy of the testes up to death. Rat V-23 went from non-detectable testosterone level in January to a 2.8 nmol l^{-1} in April, a reading of 4.0 nmol l^{-1} in May, and 3.1 nmol l^{-1} in June. Also, by April the GnRH titer had risen from 4000 to 6000. This rat died at 16 months and necropsy immediately after death revealed small testes (1.5 g and 1.4 g) with formed but non-motile sperm.

The most interesting of the four rats was V-10, which lived the longest and provides the best example of the various vaccine effects (*Figure 1*). This animal lived for 17 months after the first vaccination; it was sacrificed because rapid weight loss indicated failing health. After the second boost, the testes atrophied and the plasma testosterone became non-detectable. Eleven months into the study the testosterone level began to rise and the testes began to develop. The testicular size reached normal by the 14th month, but then declined again by the 16th month. The arrested testicular development coincided with a rise in GnRH antibody titer that became higher than the original. V-10 was bred with several females during the time the testes had redeveloped to normal size, but conception did not occur.

DISCUSSION

The reduced number of females whelping, along with trends to smaller litter sizes and lower progesterone values in Group 1, may indicate that the antibodies to MZPP were effective in reducing ovulation as well as preventing sperm binding and penetration in the Norway rat. Skinner[6] suggested that a reduction in ovulation caused by ZP antibodies interfering with follicular development is the most obvious reason for the reduced plasma progesterone levels. This implies fewer ova available for fertilization and, therefore, smaller litters.

There is no question that GnRH was effective in sterilizing both sexes of the wild Norway rat. The results of the long-term study demonstrate that the sterilizing effect of GnRH antibody may last a lifetime in rats under natural conditions. The most fascinating aspect of the long-term study is the possibility that rats may have re-immunized themselves 11 to 12 months into the study. We hypothesize that after the concentration of circulating GnRH antibodies declined, free GnRH was allowed to circulate in the blood; the free GnRH then reactivated the testicular hormonal system producing a rise in testosterone levels and a concomitant regeneration of the testes. Then the free circulating GnRH was detected by GnRH-activated immune B-cells, precipitating a specific GnRH antibody response. This interfered with the activity of the newly released GnRH resulting in a second drop in testicular activity. In order to more thoroughly understand this interesting phenomenon, a longer-lived animal is needed to determine if this cycle can be repeated multiple times.

Although this study demonstrates the efficacy of immunocontraception in rats, in practice vaccination would have to be effected orally if it is to achieve Knipling and McGuire's[1] theoretical 70% of male and female sterilization for three generations to control populations.

Awoniyi *et al.*[12] demonstrated that the sexual activity in GnRH-immunized rats can be normalized by injecting testosterone. This use of testosterone as an antidote would be a valuable means of returning to full reproductive capacity any non-target animals that were accidentally vaccinated.

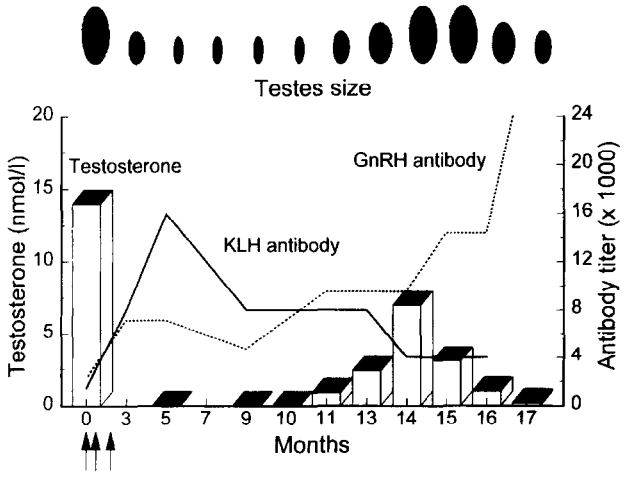

Figure 1 GnRH and KLH antibody titers, relative testes sizes, and testosterone levels in wild Norway rat V10 before and after vaccination with GnRH-KLH immunocontraceptive vaccine. Illustrated are the immunizations (↑) on Days 1, 21, and 51; complete cessation of testes activity; GnRH titer drop leading to regrowth of the testes; apparent self revaccination as the GnRH level rose; and subsequent high GnRH titer with testosterone reduction and testes regression

CONCLUSION

The immunocontraception trial with MZPP/KLH showed that the reproduction of Norway rats was reduced to only 50% of the females being fertile. Additional work with this vaccine will be needed to determine if this percentage can be improved to a more practical infertility rate of 80–100%. Treated rats that do conceive should have fewer pups. The main advantage of MZPP is that it is rodent specific; the disadvantages are that it only sterilizes the female and appears to be only partly effective as presently tested.

Immunocontraception with GnRH/KLH was 100% effective in precluding reproductive activity in both sexes. The major advantages of GnRH are that it can sterilize both sexes and that it has the potential of sterilizing rats for at least 1 year and perhaps as long as a lifetime; the main disadvantage is that GnRH is not species specific. We realize that in order for GnRH vaccine to be an effective rodent contraceptive, an oral delivery method must be available. Studies are underway at NWRC to develop an oral delivery system for vaccinating rodents with GnRH immunocontraceptive vaccine.

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