Vaccine: X 10 (2022) 100138

Contents lists available at ScienceDirect

Vaccine: X

journal homepage: www.elsevier.com/locate/jvacx

Longevity of an immunocontraceptive vaccine effect on fecundity in rats

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ARTICLE INFO

Article history:
Received 26 May 2021
Received in revised form 25 November 2021
Accepted 21 December 2021
Available online 27 December 2021

Keywords:
Anti-GnRH vaccine
Fertility control
Human-wildlife conflicts
Immunocontraception
Wildlife management
Rats
Litter size

ABSTRACT

- Increases in human-wildlife conflicts alongside cultural shifts against lethal control methods are driving the need for alternative wildlife management tools such as fertility control. Contraceptive formulations suitable for oral delivery would permit broader remote application in wildlife species.
- This study evaluated the contraceptive effect and immune response to two novel injectable immunocontraceptive formulations targeting the Gonadotropin Releasing Hormone (GnRH): MAF-IMX294 and MAF-IMX294P conjugates, both identified as having potential as oral contraceptives. The study also explored whether in multiparous species immunocontraceptives may either totally prevent reproduction or also affect litter size.
- Female rats, chosen as a model species, were given three doses of either MAF-IMX294 or MAF-IMX294P to compare anti-GnRH immune response and reproductive output up to 310 days post-treatment.
- Both formulations induced anti-GnRH antibody titres in 100% of rats and significantly impaired fertility compared to control animals. Following treatment with MAF-IMX294 and MAF-IMX294P 0 of 9 and 1 of 10 females respectively produced litters following the first mating challenge 45 days post-treatment, compared to 9 of 9 control animals.
- Across the whole 310 day study period 7 of 9 females from the MAF-IMX294 group and 10 of 10 females in the MAF-IMX294P group became fertile, producing at least one litter throughout six mating challenges.
- No significant differences were found between the two formulations in antibody titre response or duration of contraceptive effect, with an average time to first pregnancy of 166 days for MAF-IMX294 and 177 days for MAF-IMX294P for all females that became fertile.
- Following treatment with MAF-IMX294 and MAF-IMX294P the first litter produced post-infertility in treated females was significantly smaller than in control animals. This indicates treatment with immunocontraceptives may induce an overall suppression of fecundity extending past an initial infertility effect. This increases the potential long-term impact of these immunocontraceptives in multiparous species such as commensal rodents.

Introduction

Human-wildlife conflicts are increasing worldwide in parallel with the need to manage wildlife populations efficiently [1–3]. Historically, the default method for managing these conflicts has been lethal control [4,5]. However, increasing public antipathy towards lethal methods has resulted in a cultural shift towards non-lethal population management alternatives. This shift is driven by concerns about animal welfare and humaneness of options such as shooting, but also due to an increased awareness of the environmental impact of methods such as poisoning [6–8].

Public surveys commonly report that non-lethal methods are more publicly acceptable than lethal control, particularly for cases involving iconic species, or for wildlife issues arising in urban and populated areas [9–11]. Amongst non-lethal methods, fertility control is often rated as highly acceptable [9,11–13] and is therefore

Abbreviations: MAF, Mycobacterium avium fragments; GnRH, Gonadotropin-releasing hormone; IM, Intramuscular; NT, No detectable titre.

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https://doi.org/10.1016/j.jvacx.2021.100138
2590-1362/

being increasingly advocated for use in wildlife population management as a humane population control. Compared to lethal methods, fertility control could provide multiple benefits [13]. For instance, it may reduce the horizontal transmission of diseases by causing less social disruption than culling and decreasing contact rates between males and females, and also decrease vertical transmission through removing the parent-offspring infection pathway [14–16]. Importantly, fertility control can be used for population management where removal of animals is not an option (e.g. in elephants [17–19]), or in areas where wildlife or feral animal populations are valued for religious or cultural reasons, such as free roaming cattle and macaques in Hong Kong [20,21].

One method of long-term wildlife fertility control is the use of immunocontraceptive vaccines. These contraceptives induce infertility by stimulating an immune response to proteins or hormones essential for reproduction such as the Gonadotrophin Releasing Hormone (GnRH). GnRH is a key regulator in the reproductive system and suppressing the GnRH prevents the production of hormones responsible for ovulation and spermatogenesis, inducing infertility in both males and females [22]. An injectable GnRH-based immunocontraceptive vaccine, GonaCon (USDA, Pocatello, ID, USA), has proven effective in many species including wild boar, cattle, kangaroos, goats, badgers, and feral horses [13,23–27]. However, for fertility control to have wider applications at population level or at large scale there is a need for oral contraceptives that can be administered without the physical handling of animals.

Subsequent development of new vaccines has focused on smaller recombinant proteins containing GnRH. Significant immune response in domestic pigs was demonstrated following a single vaccination by injection of a GnRH-recombinant protein named IMX294 [28]. IMX294 comprises a heptameric protein (50,000 MW) containing seven copies of GnRH. A heptameric protein structure was chosen as it is a larger structure, more visible to the immune system, and proven to increase immune response compared to monomeric constructs [29].

When combined with the novel adjuvant *Mycobacterium avium* cell wall fragments (MAF), this recombinant protein IMX294 was proven to induce anti-GnRH antibodies high enough to cause infertility in rats when delivered as six oral doses [30].

Building on these results, this study tested two candidate injectable immunocontraceptive vaccine formulations. The first using IMX294, as in Massei, Cowan [30]; the second utilised a modified version of the same recombinant, named IMX294P. IMX294P includes a modification of the seven C-terminal acids in comparison to IMX294 which better enables purification of the protein and is thought to enhance immunogenicity and increase the T-cell specific immune response via increased adhesion to cell surfaces [31].

Both formulations employed the novel adjuvant *Mycobacterium avium* cell wall fragments (MAF), conjugated to either IMX294 or IMX294P (named MAF-IMX294 and MAF-IMX294P) administered as an emulsion.

This study explored the immune response to MAF-IMX294 and MAF-IMX294P injectable formulations in laboratory rats, used as a mammalian model species. In addition, the study explored the effect on reproduction and whether in multiparous species like rats, immunocontraceptives may act by either totally preventing reproduction or by also suppressing litter size.

The aims of this study were: 1. To evaluate and compare the immune response and related anti-fertility effect over multiple breeding cycles of two formulations of an anti-GnRH vaccine, MAF-IMX294 and MAF-IMX294P when administered to rats by intramuscular injection; 2. To determine potential effects on litter size in animals that became fertile after a period of contraceptive-induced infertility.

Methods

Subjects

The laboratory rat was used as a model mammalian species. Thirty Wistar strain nulliparous female rats were obtained (Envigo, UK), weighing between 185 g and 220 g. The rats were housed in pairs or trios in cages (56 × 38 × 25 cm) placed in temperature and humidity controlled rooms on a 12 h light: 12 h dark cycle. Each cage had woodchip litter, corner housing, cardboard tubes and chew sticks for enrichment (Datesand, UK). Rats were provided with *ad libitum* water and rat pellet diet (5LF2, IPS Ltd., London, UK). On arrival, animals were weighed, before being randomly assigned to Treatment group (n = 10/group, three treatment groups, Table 1) and all were left to acclimatise for nine days. Rats were then microchipped with Passive Integrated Transponder (PIT) tags for individual identification and a baseline blood sample (maximum 0.5 ml) was obtained (21G needle) from the tail vein of each animal under brief anaesthesia induced *via* facemask using sevoflurane. Adult Wistar strain males (Envigo, UK) of proven fertility, given a tail marking for identification, were used for breeding purposes only.

The use of animals in this study was approved in the UK by the Animal and Plant Health Agency's Animal Welfare Ethical Review Body and carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986.

Treatment

The vaccine consisted of *M. avium* fragments (MAF) conjugated to the Gonadotropin releasing hormone (GnRH) recombinant protein IMX294 or IMX294P to form MAF-IMX294 and MAF-IMX294P. IMX294 is a GnRH recombinant construct (expressed in *E. coli*), comprising a heptameric protein (50,000 MW) in which each of the seven subunits has a single GnRH molecule fused at the N terminus. IMX294P is identical to IMX294 except that the seven C terminal amino acids have been modified (Fig. 1).

Fragmentation of *M. avium* whole cells was accomplished using a microfluidizer (Model 110L, equipped with Model G10Z ceramic interaction chamber, 87 μm; Microfluidics™). Fragmentation yielded a bimodal MAF particle size distribution, the first peak (mean ± SD) ranging from 0.23 μm ± 0.075 μm to 0.75 μm ± 0.32 μm, max = 0.421 μm ± 0.15 μm, and the second peak ranging from 1.2 μm ± 0.51 μm–4.01 μm ± 2.5 μm, max = 2.11 μm ± 0.74 μm. The fragments of *M. avium* were coupled to IMX294 and IMX294P, to form the MAF-IMX294/MAF-IMX294P conjugate in phosphate-buffered saline (PBS) solution as described in Massei, Cowan [30]. The conjugation was achieved using a two-step EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide): N-hydroxysuccinimide ester coupling method as in Hermanson [32].

The formulations for intramuscular (IM) injection were obtained by combining the vaccine conjugates with mineral oil and surfactant (mineral oil: 90% w/w Sigma M1180 USP light grade mineral oil, surfactant: 10% w/w Sigma M8819 mannide monooleate), and made into an emulsion. Injectable conjugates were prepared at the National Wildlife Research Center (NWRC, United States).

To reduce the number of animals used in the trial in line with the NC3Rs principles (Replacement, Reduction, and Refinement), the negative control group (n = 10) consisted of rats that were part of a joint study on oral dosing not reported here. Rats were given six oral doses of empty *Lycopodium clavatum* exines suspended in phosphate buffered saline that had previously shown no effect on reproductive output.

Table 1
Treatment protocol for testing the effectiveness of putative immunocontraceptive vaccine formulations MAF-IMX294 and MAF-IMX294P to induce infertility in laboratory rats.

Group	n	Formulation	Route	Dose	Vaccine concentration	Number of doses	Dosing schedule
1	10	MAF-IMX294	Intramuscular injection (IM)	0.2 ml	200 μg	3	Day 1, 15, 31*
2	10	MAF-IMX294P	Intramuscular injection (IM)	0.2 ml	200 μg	3	Day 1, 15, 31*

* At third dose two rats per group dosed on day 29 to evaluate injection site reaction before remaining animals were dosed on day 31.

IMX294: MEHWSYGLRP GGSKKQGDAD VCGEVAYIQS VVSDCHVPTA ELRTLLEIRK LFLEIQKLKV ELQGLSKE**

IMX294P: MEHWSYGLRP GGSKKQGDAD VCGEVAYIQS VVSDCHVPTA ELRTLLEIRK LFLEIQKLKV EGRRRRRS**

Fig. 1. Primary amino acid structure of IMX294 and IMX294P. Differences are underlined.

Protocol

Two treatment groups, Group 1 – MAF-IMX294, and Group 2 – MAF-IMX294P, were used to evaluate the contraceptive effect of the vaccine *via* intramuscular (IM) injection. The vaccine was administered into the back thigh muscle under general anaesthesia, as a prime dose followed by two boosters at approximately 15 day intervals (Table 1). Brief anaesthesia was induced by administration of sevoflurane by facemask. Both treatment groups were administered three vaccine doses of 0.2 ml (one initial dose and two booster doses) given as 0.1 ml in each leg (23G needle). Following the second treatment dose, swelling at injection site was seen in all females in Group 1 and in eight of ten in Group 2. This reaction is similar to those reported for equivalent immunocontraceptive vaccines with a combination of antigen, mineral oil and surfactant emulsion delivered intramuscularly [33]. Seven of 20 treated females were administered an analgesic (Metacam 0.1 ml) following exhibition of transient lameness in one leg. Lameness lasted no longer than 72 h post-dose in six of seven, one female in Group 1 was removed following lameness at 72 h post-dose. The third treatment dose was administered into the front of the thigh with two animals from each group injected on day 29 to evaluate any injection site reaction before all remaining animals were dosed. Animals in the negative control group (Group 3) were used as a baseline for reproductive output. One female died due to a blockage of the trachea following the first oral dose.

The first mating challenge began two weeks after completion of dosing on study day 45. One male was introduced into each cage. Males were then removed after ten days. After a further 14 days females were housed singly. Pups were counted upon parturition and removed at ten days old. Females were then returned to original pairs or trios.

Subsequently, females of all three groups (MAF-IMX294, MAF-IMX294P, and controls) were bred for five further mating challenges, commencing on study day 112, 163, 206, 249 and 291, to evaluate the long-term vaccine efficacy.

Post-treatment blood samples (max 0.5 ml) were obtained (21G needle) from the tail vein of all females under conscious restraint at 15, 31 and 45 days after the first treatment dose. Blood samples were then taken subsequently from treated females after each mating challenge (day 100, 155, 205, 248 and 290) to measure antibody titre levels.

Through mating challenge numbers 2–6, females were retained within the study until the mating challenge following their first successful pregnancy. For the subsequent mating challenge females were mated and removed prior to parturition with litter size data and blood samples collected post-mortem (carried out on day 179, 224, 266 and 310).

Male:female mating groups were maintained in mating challenges 1–3. To reduce the likelihood that variations in male fertility impacted upon female breeding success, during mating challenges 4–6 female rats were housed for five days with original mated male and males were then rotated one mating group forward and housed with the new mating group for the remaining five days.

Analysis

The effectiveness of immunocontraceptive vaccination was measured by: 1. Quantification of blood serum anti-GnRH antibody titres; 2. Reproductive output in terms of both proportion of females giving birth and litter sizes.

Following blood sampling, serum was separated by centrifugation and stored at −20 °C. Anti-GnRH antibody titres in serum samples were quantified using an indirect enzyme-linked immunosorbent assay (ELISA). The ELISA was based on that used by Miller, Johns [34] and specifically adapted for the laboratory rat as described in Massei, Cowan [30], using rabbit anti-rat IgG, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO, USA). A post-treatment serum sample was considered positive for anti-GnRH antibodies if the optical density value was greater than the mean optical density plus two standard deviations of the control values (pre-treatment sample) for each respective dilution.

Statistical analyses were undertaken using SPSS for Windows (Version 25, IBM Corp., 2013) and R version 3.4.3 [35]. Between groups differences in the proportions of females giving birth after each mating challenge were analysed using pairwise chi-squared tests. Differences in litter sizes were examined using independent samples *t*-test. Differences in anti-GnRH antibody titre levels between experimental groups were examined by ordinal logistic regression with individual peak titre (ordered category) as the response variable and group (MAF-IMX294 vs. MAF-IMX294P) as the explanatory variable using the package "MASS" [35] in R (proportional odds ratio, POR and their 95% confidence intervals, CI are reported). An alpha level of 0.05 was used for all statistical tests.

A receiver operating characteristic (ROC) analysis was used to quantify how accurately the anti-GnRH antibody titre level can be used as a threshold to determine infertility. A ROC curve was created based on the trade-off between sensitivity (true positive rate: True Positives/(True Positives + False Negatives)) and specificity (false positive rate: False Positives/(True Negatives + False Positives)) at all anti-GnRH antibody titre levels, compared to observed positive rate (whether the female was later observed to give birth). A sensitivity $\geq$ 95% was used as the criterion to derive the threshold of anti-GnRH antibody titres above which rats were predicted to be infertile.

The second part of the study evaluated the long-term effect of the two vaccine formulations on immune response and fertility across all six mating challenges over a total period of 310 days. A Cox-proportional hazard model was fitted to the data to estimate

the mean time to first pregnancy for both treatment groups. Over the whole study period group breeding success was evaluated in relation to proportions of individuals in each group which had bred at least once up to each time point (using each mating challenge as a time point), to account for the reducing sample sizes following individual removal post-successful pregnancy.

Results

Forty-five days after administration of the first treatment dose, anti-GnRH IgG antibody titres were generated in 100% of females in both treatment groups. There were no detectable anti-GnRH titres generated in the negative control group. Individuals treated with MAF-IMX294 (Group 1) had titres ranging between 512 k and 2048 k. MAF-IMX294P (Group 2) generated titres ranging between 128 k and 2048 k (Table 2).

Breeding in relation to treatment

Overall, both MAF-IMX294 and MAF-IMX294P induced infertility in treated female rats up to 56 days post-first treatment (Table 3). No litters were produced from the group injected with MAF-IMX294 following the first mating challenge (days 46–56 following the first treatment dose). This was significantly different to the control group where all nine rats became pregnant ($P < .001$). One out of ten females bred in the MAF-IMX294P group, which was also significantly different to the control group ($P = .001$). There was no significant difference found in the proportion of females breeding between the two treatment groups ($P = 1.0$).

Breeding in relation to titre

As a previous study estimated that an anti-GnRH titre threshold of 256 k or above is associated with infertility in rats [30], the likelihood of breeding for all females in this study was evaluated in relation to this titre threshold. The results of the first mating challenge output supported previous research as none of the 18 females exhibiting titres $\geq$ 256 k prior to mating produced a litter. The one female exhibiting a titre below this level (128 k) produced a litter, in addition to all nine control females breeding successfully (all with no detectable titre).

Long-term effect on fertility

Treatment with either MAF-IMX294 or MAF-IMX294P induced infertility of varying duration over ten months (Fig. 2). While all nine control females and all ten females in Group 2 (MAF-IMX294P) bred at least once during the study period, two of the nine females in Group 1 (MAF-IMX294) remained infertile throughout all six mating challenges. Overall, mean time to first pregnancy for females that did breed was 177 days for MAF-IMX294 treated animals and 166 days for MAF-IMX294P treated animals, with no difference ($P = .55$) between treated groups. Mean time to first pregnancy for the control group was 49 days. The number of females breeding successfully in the control group for the six mating challenges was 100%, 71%, 86%, 100%, 100% and 100% respectively.

In the second mating challenge, 112–122 days post-first treatment dose, the cumulative number of females breeding per group until this time point in both treatment groups (four out of nine in Group 1, three out of ten in Group 2) was significantly lower than cumulative fertility in the control group ($P = .05$ and $P = .02$ respectively), where nine out of nine females had previously produced a litter (Fig. 2). There was no significant difference found in fertility level between the two treatment groups at this time point ($P = .86$).

There was no significant difference found between the treatment groups and the control group in the total proportion of fertile females in the group (calculated cumulatively) from the third mating challenge onwards, days 163–301 (challenge 3- $P = .13$; challenge 4- $P = .29$).

The results of the long-term effect of the two vaccine formulations on immune response and fertility in rats showed that treatment Group 1 (MAF-IMX294) appeared to reach a peak titre response marginally faster than individuals in Group 2 (MAF-IMX294P) (Fig. 3). All females in Group 1 exhibited their highest titres in blood samples taken at either 31 or 45 days following the first treatment dose. Both of these blood samples were collected before the first mating challenge, in which none of the Group 1 females produced a litter. In Group 2, two females did not exhibit their peak titres until the blood sample at 100 days post-first treatment dose, which was taken after the first mating challenge and in which one of the pre-peak females produced a litter.

In the overall titre response, the two vaccine formulations did not statistically differ in the peak level of anti-GnRH antibody titre reached during the whole study period (POR = 0.22, 95% CI −3.4 to 0.2).

Using breeding data and anti-GnRH titre levels across all time points, for all individuals in all treated groups, the ROC curve indicates that antibody titres greater than 512 k were necessary to maintain infertility over the course of the study (true positive rate >95%, Fig. 4).

Effect of treatment on return to fertility

As females in both treatment groups were observed to regain fertility (produce a litter) after a period of vaccine-induced infertility, the effect of treatment on fecundity following infertility was evaluated for both treatment groups. Litter size of the first litter produced and the litter size of second litter produced from treated animals were compared to the size of first and second litter in all control females. One treated female (Group 2) was not included in litter size analysis as this female remained fertile throughout the post-treatment period and one treated female (Group 1) was not included in first litter analysis as size was not recorded for first litter.

There was no difference found in either first ($P = .52$) or second litter sizes ($P = .75$) produced between the two treatment groups. Litters sizes were significantly reduced for the first litter post-infertility in both Group 1 ($P = .003$) and Group 2 ($P < .001$) com-

Table 2
Anti-GnRH antibody titre levels detected 45 days following the first treatment dose of putative immunocontraceptive vaccine (MAF-IMX294 and MAF-IMX294P). NT = no detectable titre. n = Sample size.

Group	Treatment (route)		Titre (1:X,000)									% with titre
		n	NT	16	32	64	128	256	512	1024	2048	
1	MAF-IMX294	9							1	2	6	100
2	MAF-IMX294P	10					1	2	2	2	3	100
3	Control	9	9									0

Table 3
Number of females breeding, with mean litter size (+SD) for females that bred in the first mating challenge 45–55 days after the first treatment.

Group	Treatment	n	n bred	% bred	Mean (SD) litter size for females that bred
1	MAF-IMX294 (IM)	9	0	0	–
2	MAF-IMX294P (IM)	10	1	10	10 (±0)
3	Control	9	9	100	10.6* (±2.2)

* n = 8, litter size unknown for 1 female.

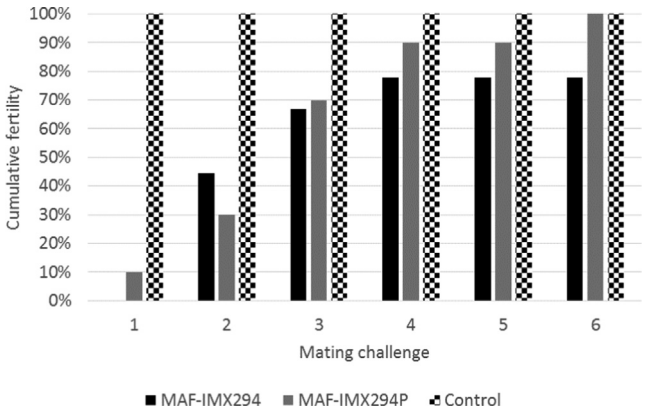

Fig. 2. Cumulative fertility in female rats following completion of treatment with either MAF-IMX294 or MAF-IMX294P compared to control females across all mating challenges. Percentage indicates total number of females in each group breeding at least once up to and including each time point. Mating challenges (10 days) corresponded to 45, 112, 163, 206, 249, and 291 days respectively following the first treatment dose.

pared to the control group (Table 4) indicating that females produce fewer offspring per litter upon returning to fertility than untreated females. The difference in litter size was not significant in the second litter post-infertility between controls and Group 1 (P = .083) and controls to Group 2 (P = .084). However, the second litter size was also significantly lower in treated than in control females when the treatment groups were pooled, although the difference was less pronounced than for the first litter (P = .04).

The average first litter size of five pups per litter in the pooled treated females was less than half of the average first litter size for control females at 10.56 per litter (Table 4). This difference was highly significant (P < .001).

Discussion

This study showed that treatment with MAF-IMX294 and MAF-IMX294P can induce infertility in rats for approximately 4 months after the end of treatment, with a longevity of effect that thereafter varied widely between individuals. Crucially, fecundity was affected by both contraceptive formulations post-infertility, with litter size suppressed for at least one breeding cycle. This suggested that the effects of these drugs could extend beyond simple infertility in multiparous species. This study also provided evidence of the "gold standard" titres that an oral GnRH-based contraceptive vaccine should induce to maintain infertility in this species.

These results indicate that there are no substantial differences between MAF-IMX294 and MAF-IMX294P in terms of immunogenicity and contraceptive effect. However, there were some minor differences, such as the earlier peak in antibody titre levels, and the maintenance of infertility in two individuals, which indicate that the MAF-IMX294 may be marginally more effective than MAF-IMX294P.

In both formulations anti-GnRH antibody titres of 256 k or above induced infertility and titres of greater than 512 k were associated with long-term infertility across the entire treated period of 300 days. This titre threshold (>512 k) for sustained infertility is higher than that indicated from the titre levels recorded at the first mating challenge post-treatment, and from the anti-GnRH titre threshold in rats suggested in previous comparable research, which found that titres of 256 k or above were sufficient to prevent pregnancy [30]. However, unlike previously, it is important to note that in this study the titre threshold calculation used measurements across a long time period in which titre levels were reducing as time progressed. If titres were still rising during the mating challenge period, the time interval between titre measurement and mating may have led to an assumed titre lower than that of the actual titre. Conversely, if titre levels were decreasing over the

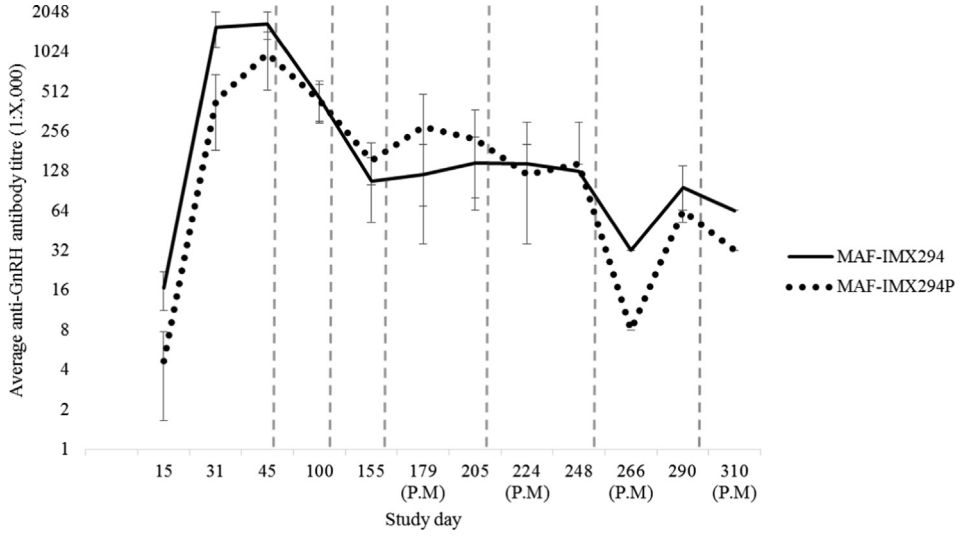

Fig. 3. Average anti-GnRH antibody titres produced over time following treatment with either MAF-IMX294 (Group 1) or MAF-IMX294P (Group 2). Error bars show confidence intervals. P.M - post-mortem blood sample, taken from females removed prior to parturition. Dashed vertical bars indicate mating challenge periods.

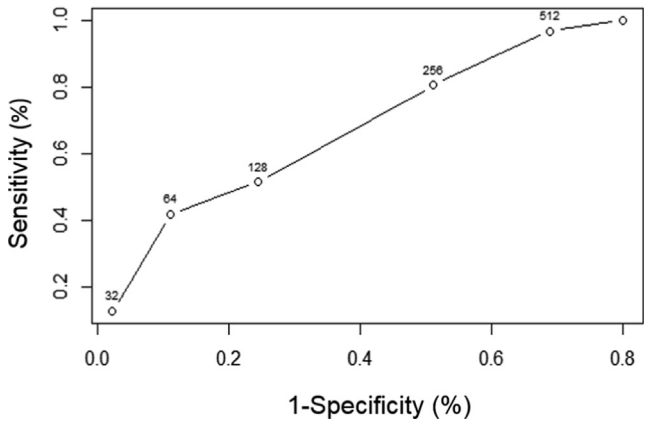

Fig. 4. Receiver operating characteristic (ROC) plotting true positive rate (Sensitivity) against the false positive rate (Specificity) for pregnancy in relation to anti-GnRH antibody titre level (1:X,000) following treatment with MAF-IMX294 and MAF-IMX294P.

mating challenge period, as in mating challenges two to six, the actual titre at conception may have been lower than the titre measured pre-mating depending on the rate of titre drop-off. Therefore, the threshold indicated here of greater than 512 k is more likely to be a titre necessary to maintain a sustained long-term infertility in this species. However, infertility was maintained in some individuals at titres lower than 256 k, which suggests that whilst relatively high titres are associated with infertility, relatively low titres are not necessarily predictive of infertility, as previously found [30].

Importantly, the results showed that litter sizes were significantly reduced for subsequent litters in females returning to fertility following treatment with either MAF-IMX294 or MAF-IMX294P. The comparison of reproductive output between treated and control females is partially limited by the experimental design and use of a negative control group without a corresponding injectable treatment. However, the reproductive output in the Control group (proportion of females breeding and mean litter size) was within the range of previously observed untreated Wistar strain females kept under equivalent conditions [30] and equivalent to mean litter sizes found in published literature on Wistar females [36]. Therefore, the breeding success observed in the Control group can be considered a reliable data comparison from which to draw the stated conclusions. The observed delay in recovery of reproductive output will have implications for the effects of contraception at population level, and suggests that the contraceptive effect is not a simple on/off switch in multiparous species. Instead, this indicates that there may be an association between litter size and immune response to GnRH. Similar results following the administration of a single injection of the GnRH-based vaccine GonaCon were obtained in cats where the litter sizes in treated females that started breeding after a period of infertility were significantly lower for the first litter produced than that of control animals [37,38].

One possible reason for the lower reproductive output would be a partial suppression of the Follicle Stimulating Hormone (FSH) and the Lutenising Hormone (LH) surge by the GnRH vaccine. This may cause disruption of folliculogenesis and result in lower rates of ovulation, or may affect the maintenance of the *corpus luteum* during the early luteal phase, causing reduced numbers of developed embryos [39–41]. The difference in litter sizes in treated compared to control animals was less pronounced for the second litter post-infertility, which indicates that fecundity may return to normal levels if antibody titre levels continue to decrease. This difference in size at second litter was significant only when treatment groups were pooled. As the group sizes were small it is possible that a statistically significant difference would be found between treated and control groups with a higher sample size. Future research monitoring treated females for multiple litters post-infertility would be needed to quantify the longevity of any fecundity suppression.

If the overall effect of vaccination against GnRH includes both a period of total infertility followed by a period of reduced fecundity, this increases the longevity of effect of the contraceptive, particularly for multiparous species. To fully evaluate the length of overall contraceptive effect of these formulations in this species in a practical context, the breeding success of individuals would need to be monitored on a constant basis rather than with artificial periods of challenge as in this study. Particularly in the case of rats, where females are able to cycle and breed continuously, the length of effect may be dependent on the number of breeding cycles rather than on a fixed length of time post-treatment.

Rodent pest-species can cause substantial negative economic impacts [42,43] and there are constant calls for novel methods of population control as methods based on rodenticides are considered inhumane by many stakeholders [44]. For rats that are prolific breeders, infertility would need to be maintained at a high level to have a sustained impact on population growth and in most r-selected species any contraception longevity will need to be sufficient to counter any resulting density-dependant reproduction. For example, modelling estimates that over 70% of a rat population would need to be sterilized for more than two successive generations to induce population eradication [45]. However, rates of infertility of between 50% and 66% may be sufficient in some rodents to achieve some level of population reduction [46,47].

Furthermore, it is possible that any suppression of fertility proven in rats could be translated to a greater population limiting effect in seasonally breeding species. Where animals reproduce only once per-year, a long-term contraceptive would have a significant impact on yearly recruitment levels [48]. Alternatively, even a short-term fertility suppression could translate to a significant

Table 4
Average litter size and standard deviation (SD) for the first and second litters from control females and in females re-acquiring fertility after intramuscular treatment with either MAF-IMX294 or MAF-IMX294P.

		First litter		Second litter	
		N females	Average litter size (SD)	N females	Average litter size (SD)
Treated	MAF-IMX294	6	5.5 (±2.59)[a]	7	9.43 (±2.64)
	MAF-IMX294P	9	4.67 (±2.24)[b]	6	9.83 (±1.72)
	Pooled	15	5 (±2.33)[c]	13	9.62 (±2.18)[d]
Control		9	10.56 (±2.07)[a,b,c]	7	11.86 (±2.12)[d]

[a] $P = .003$.
[b] $P < .001$.
[c] $P < .001$.
[d] $P = .04$.

limiting influence in some species if delivery of treatment could be aligned with seasonal population fluctuations or seasonal peaks in breeding. As modelled by Shi, Wan [49], application of fertility control in Brandt's voles prior to winter had a greater effect in the following year as it enhanced the population limiting effect of natural winter mortality.

Most previous studies were focussed on wildlife that generally breed once per year such as deer, elk, and cattle [20,23,50]. However, due to the practicality of re-capturing treated animals to collect blood samples, only a small proportion of studies have reported both reproductive output and immune response (e.g. [23,24]). This study was the first to monitor the effects of immunocontraceptives on reproduction and on the immune response to the vaccines for multiple consecutive breeding cycles in a multiparous, r-selected species. To move towards practical applications of fertility control for r-selected species, such as commensal rodents, IMX294 should be tested further as a candidate oral contraceptive. If the longevity of oral contraceptives utilising IMX294 could match that of the injectable IMX294 formulations tested in this study, population control of wildlife with significant economic and environmental impact could become feasible.

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.

Acknowledgements

Funding for this study was provided by the UK Squirrel Accord, to whom we are very grateful. We would also like to thank the staff of the FERA Animal Unit for technical support and Dr. Matt Brash MRCVS who provided veterinary support. The funders had no role in study design, data collection and analysis, or preparation of the manuscript but were consulted on, and are in agreement with, the decision to publish.

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