Original Article

Assessing the feasibility of microneedle patches for GnRH immunocontraception in mixed-breed dogs (*Canis familiaris*)

Ai-Mei Chang[1,3,5] Hsiao-Hui Huang[2] Ding-Liang Hou[3] Lin Zi-Yu[4]
Mei-Chin Chen[4] Chen-Chih Chen[1,3*] Jai-Wei Lee[2*]

Abstract

The overpopulation of free-roaming dogs poses significant risks to endemic species, public health, and the environment through predation and disease transmission. While surgical castration is effective, it raises ethical concerns regarding animal welfare. Immuno-castration offers a humane and effective alternative for population control, but a more easily administered and cost-efficient method is needed to enable widespread vaccination efforts. In this study, we aimed to employ self-dissolving microneedle patches for a simple and potentially cost-effective immunization of a recombinant GnRH-based vaccine to control fertility. Eight mongrel dogs were randomly divided into 2 groups (n=4 each) and immunized with the recombinant GnRH-based vaccine through either intramuscular injection (IM group) or microneedle patches (MN group), respectively, as the primary immunization, followed by an intramuscular booster 4 weeks after. Production of GnRH-specific antibodies was detected 2 weeks after the primary immunization and lasted for at least 12 weeks. Moreover, the serum testosterone level was decreased in vaccinated males. Additionally, testicular atrophy and poor semen quality (concentration, abnormality, and viability) were found in all vaccinated dogs. Based on our results, the GnRH vaccine appeared to successfully elicit GnRH-specific antibody responses, which leads to reduced serum testosterone concentration, testicular atrophy, and poor semen quality in both IM+IM and MN+IM schemes. Self-dissolving microneedle patches may be a feasible approach to mass dog vaccination in the future.

Keywords: GnRH vaccine, immunocontraception, intramuscular injection, microneedle patch, mongrel dogs

[1]*Institute of Wildlife Conservation, College of Veterinary Medicine, National Pingtung University of Science and Technology, 1 Shuefu Road, Neipu, Pingtung, 91201, Taiwan*
[2]*Department of Tropical Agriculture and International Cooperation, International College, National Pingtung University of Science and Technology, 1 Shuefu Road, Neipu, Pingtung, 91201, Taiwan*
[3]*Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, 1 Shuefu Road, Neipu, Pingtung, 91201, Taiwan*
[4]*Department of Chemical Engineer, National Cheng Kung University, No.1, University Road, Tainan, 70101, Taiwan*
[5]*Menzies Institute for Medical Research, College of Health and Medicine, University of Tasmania, Hobart, 7000, Australia*
***Correspondence:** jaiwei@hotmail.com, ychih0502@gmail.com* (J.W. Lee, C.C. Chen)
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Introduction

One of the most prevalent and widely distributed terrestrial carnivores across the globe, the domestic dog (*Canis familiaris*), has an estimated global population of free-ranging dogs (FRDs) ranging from 700 million to 1 billion (Gompper, 2014). The lack of effective management strategies has led to a significant worldwide problem. This issue results in conflicts between dogs and humans/other animals due to aggressive dog behaviors, including biting, barking, and causing traffic accidents, as well as the transmission of diseases (Knobel *et al.*, 2014). Moreover, FRDs often suffer from poor health conditions (Abdulkarim *et al.*, 2021). Managing the population of FRDs is essential for the well-being of animals, public health, and the preservation of biodiversity.

The most common method for population control was the trap-neuter-vaccinate-return (TNVR) strategy, which involves surgical sterilization. Nevertheless, surgical castration has its drawbacks, including relatively high costs, time-consuming procedures, and ethical concerns (Massei, 2012). Furthermore, for the large-scale sterilization of FRDs, the administration of anesthesia and insufficient postoperative care can pose risks to animal welfare (Massei, 2012). Under this circumstance, immune-contraceptive vaccines can be an alternative or supportive approach to population control.

Immunocontraception is a method used to control animal fertility by stimulating the immune system to generate antibodies against reproduction-related hormones or antigens (Padodara *et al.*, 2022). Common antigens used in immune-contraceptive vaccines are the porcine zona pellucida protein (PZP) and gonadotropin-releasing hormone (GnRH) (Miller *et al.*, 2005; Shideler *et al.*, 2002). In comparison to the PZP vaccine, the GnRH-based vaccine has a broader range of applications as it can be used in both males and females (Siel *et al.*, 2016). GnRH-based vaccines can elicit the production of specific antibodies capable of inhibiting proteins or hormones associated with reproduction, thereby regulating fertility (Meloen *et al.*, 1994). GnRH immunocontraception has been applied in several animal species, including mice (Chang *et al.*, 2021), pigs (Einarsson *et al.*, 2011), bovine (Pérez-Linares *et al.*, 2017), donkey (Rocha *et al.*, 2018), caprine, ovine, rat (Siel *et al.*, 2016), feline (Lee *et al.*, 2019), canine (Chang *et al.*, 2023; Liu *et al.*, 2015), and white-tail deer (Miller *et al.*, 2008).

There are various methods for vaccine delivery, with intramuscular injection (IM) being one of the conventional approaches. However, this method may lead to complications such as axillary nerve damage (Kim *et al.*, 2017) and sciatic nerve injuries (Park *et al.*, 2019). Iatrogenic pain is a significant drawback of intramuscular injections, leading to decreased patient compliance (Taddio *et al.*, 2010). Additionally, disposable needles, such as those in insulin pens, generate waste with every injection, resulting in needle wastage (Cunha *et al.*, 2017).

In our previous study, we successfully developed a recombinant GnRH-based vaccine and demonstrated its effectiveness in reducing fertility in both male and female dogs (Chang *et al.*, 2023). To enhance animal welfare and simplify the process of administering injections, we hereby propose delivering the vaccine through a microneedle patch. The microneedle patch is an innovative, painless, and easily administered approach. With a needle length of less than 1 mm, it delivers vaccines to the skin's epidermis and dermis using a patch that can be applied to the skin with minimal training, making the process simpler than IM (Van Der Maaden *et al.*, 2012). Biodegradable polymers like poly (vinyl alcohol) (PVA), poly (vinylpyrrolidone) (PVP), and poly (methyl methacrylate) (PMMA) are used in the form of a self-dissolving microneedle patch that can be dissolved within min when applied to the skin (Waghule *et al.*, 2019). The benefits of a microneedle patch can simplify the vaccination process for dogs, particularly stray dogs in rural areas.

The objective of this study was to assess the effectiveness of self-dissolving microneedle patches compared to the intramuscular injection route for administering the recombinant GnRH-based vaccine in mongrel dogs. Parameters, including serum anti-GnRH IgG antibody and semen concentration, sperm morphology, sperm viability, testis measurements (length, width, and volume), concentration, and serum testosterone levels in males, were analyzed in vaccinated dogs.

Materials and Methods

Animals and Ethical Approval: A total of 8 healthy, uncastrated mongrel dogs that participated in this study were voluntarily registered by their owners, and they remained in their own respective households throughout the experimental period. The average age of dogs was 1.83 years (n=8), and the average weight was 14.28±1.41 kg (n=8). As for the dogs' reproductive index reference, both castrated (n=49, male) or uncastrated (n=57), they were all more than 1 year old and located in the "Animal Protection Shelters" in Pingtung County and Kaohsiung County, Taiwan. The sampling distribution of the control group was conducted evenly across all four seasons. The procedure for the animal experiment was approved by the Institutional Animal Care and Use Committee of the National Pingtung University of Science and Technology, Taiwan (Approval no. NPUST-109-103).

Vaccine preparation and Immunization scheme: The recombinant GnRH-based antigen contains eight linear repeated sequences of gonadotropin-releasing hormone I (GnRH I) and four T cell epitopes. The sequence of the antigen has been described in our previous study (Chang *et al.*, 2023). The dogs were randomly divided into two groups: intramuscular (IM) group (n=4, 3 males and 1 female) and microneedle patch (MN) group (n=4, 2 males and 2 females), respectively. The vaccination schedule is shown in Fig. 1. The GnRH vaccine for IM injection was prepared by emulsifying the recombinant GnRH antigen with an equal volume of a water-in-oil-in-water adjuvant (MONTANIDE ISA 206 VG) (1 mL per dose containing 500 μg of antigen) and is to be injected into the quadriceps muscle. On the other hand, the microneedle

patch was prepared by encapsulating 100 µg of recombinant GnRH antigen in hyaluronic acid (HA)-based self-dissolving microneedles (applied to the ears after carefully shaving with a razor to remove fur). The difference in antigen dosage between the IM injection and the microneedle patch (500 µg vs. 100 µg) can be attributed to the more efficient delivery and enhanced immune response associated with microneedle technology (Fig. 2). The microneedle patches were applied to the skin on the inner ear pinna by pressing down with the thumb, left on the skin for 15 min and then remove the patch. During the waiting period, rewards will be given to the dogs to encourage them to wait calmly and remain stable at that moment. Dogs in the IM and MN groups received the GnRH vaccine for IM injection and microneedle patch as the primary immunization, respectively. After 4 weeks, both the IM and MN groups were boosted with the GnRH vaccine for IM injection at the quadriceps muscle.

Sample collection: Blood samples were obtained via venipuncture from either the cephalic vein or external saphenous vein (2.5 mL) at a two-week interval. Samples were left to coagulate in BD Vacutainer Serum Collection Tubes (BD Biosciences, San Jose, CA, USA) for a minimum of 30 min at room temperature and then centrifuged at 1,000 x g for 10 min. The resulting serum was meticulously collected and preserved at −20°C until further analysis. Semen samples from the male dogs were collected at a 4-week interval (specifically at Weeks 4, 8, and 12) after the primary immunization, as per the study protocol.

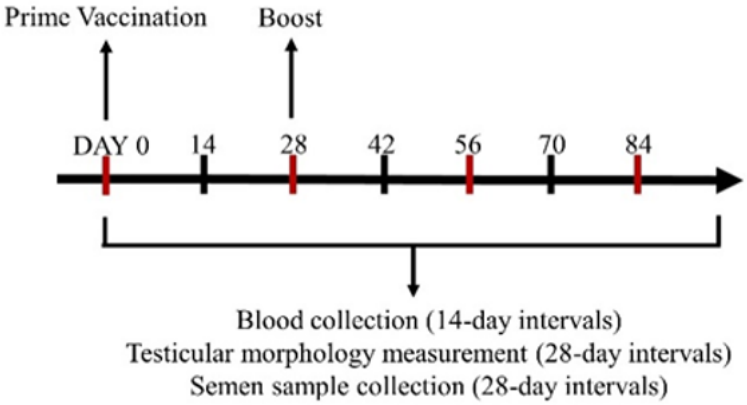

Figure 1 Outline of vaccination timeline.

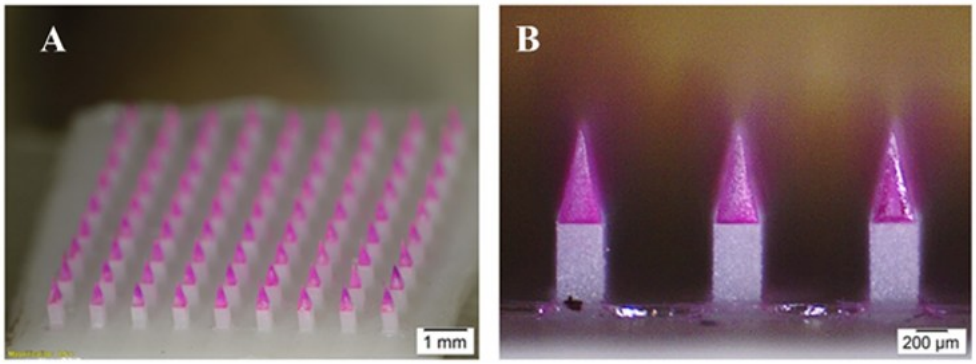

Figure 2 A: 9×9 microstructure array of microneedle patches. B: Microstructure tips coated with HA and Alexa-594-GnRH, the length of each tip was 599.1±8.4 µm.

Clinical evaluation of dogs: All dogs underwent regular medical examinations conducted by a licensed veterinarian to ensure their overall health and well-being throughout the study. These evaluations included a comprehensive assessment of their body condition, gait, and appetite to detect any potential signs of discomfort, illness, or adverse reactions. The veterinarian carefully monitored changes in body condition to identify weight loss or gain, evaluated gait to observe for any lameness or abnormal movements, and recorded appetite levels to assess general health. In addition, the injection sites for the vaccination in both groups were thoroughly examined. This included visual inspection for signs of swelling, redness, or bruising, as well as palpation to check for tenderness, induration, or abnormal masses. These checks were conducted at regular intervals following vaccination to identify and document any local reactions. The systematic and detailed clinical evaluation process ensured the accurate recording of any changes in the dogs' health status, contributing to the reliability and robustness of the study's findings.

Determination of complete blood count and blood chemistry profiles: The complete blood counts (CBC) and blood chemistry profiles (BCP) of all vaccinated dogs both before (Day 0) and after the primary immunization (Day 84) were analyzed. The CBC examination encompassed the analysis of nine different types of blood cells, including packed cell

volume (PCV) %, red blood cell count, total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets, using the Idexx ProCyte Dx Hematology Analyzer (Idexx Laboratories, Westbrook, ME, USA). The BCP analysis included five blood parameters: aspartate aminotransferase (AST), alanine transaminase, albumin, creatinine, and blood urea nitrogen, carried out using IDEXX Catalyst Dx chemistry analyzers.

Measurement of anti-GnRH IgG antibody using enzyme-linked immunosorbent assay ELISA: Flat-bottom 96-well Nunc 469,949 Immuno Clear Standard Module plates (In-termed–Nunc–Gibco, Burlington, Ontario, Canada) were initially coated with 1 µg of GnRH protein per well using a coating buffer (100 µL/well; 0.1 M carbonate bicarbonate buffer, pH 9.6; Sigma Aldrich, NSW, Australia). These coated plates were then incubated overnight at 4°C. Following this incubation period, the wells were subsequently blocked with BlockPRO buffer (200 µL/well; Visual Protein, Taipei, Taiwan) for 1 hr at 37°C. Once the blocking process was complete, the blocking buffer was removed, and the serum was diluted (I only see O.D.; there is no titration) in the same blocking buffer (50 µL/well; dilutions of 1:100) at 37°C for 1 hr. After the incubation with the serum, the wells were washed five times with 0.05% (v/v) Tween 20 PBS buffer (PBST), followed by adding horseradish peroxidase-conjugated goat anti-dog IgG antibody (1:5,000; MyBioSource, San Diego, CA, USA) in PBS containing 0.5% bovine serum albumin (50 µL/well) to each well. After incubation at 37°C for 1 hr, the plates were washed five times with PBST. Subsequently, 100 µL of 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate (Sigma Aldrich, NSW, Australia) were added to each well and allowed to react for 10 min. To stop the reaction, 100 µL of 2 M sulfuric acid solution was added to each well. Finally, the absorbance of each well was measured at 450 nm using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples with O.D. levels greater than the mean of Week 0 plus two standard deviations were considered positive.

Measurement of serum testosterone concentrations: To prevent interference from other steroid compounds, the serum samples underwent an extraction procedure. Hormone extraction and detection were carried out following the manufacturer's instructions for the testosterone-competitive ELISA kit (Cayman, United States). In brief, hormone samples were extracted using an organic solvent and evaporated by heating at 30°C under a nitrogen stream. The serum sample (500 µL) was mixed with 2.5 mL diethyl ether for testosterone extraction. To outline the competitive ELISA procedure, 50 µL of the sample, 50 µL of acetylcholinesterase conjugate (AChE, testosterone/progesterone tracer), and 50 µL of antiserum were added to a 96-well microplate coated with IgG. After incubation, the plate was washed five times, and 200 µL of Ellman's reagent was added to observe color development. The optical density was measured at a wavelength of 405 nm.

Analysis of scrotum and semen quality:

Scrotal size measurements: Scrotal length and width were assessed using an electrical vernier caliper at weeks 0, 4, 8, and 12. The testes were positioned side by side and pressed as deeply as possible into the scrotum for each measurement. These measurements were then employed to determine testicular volume using the following formula:

$$Volume(mm)^3 = 4 / 3\,\pi\left[0.5 \times {testis\ width}/{2}\right]^2\left[{testis\ length}/{2}\right]$$

Semen collection and analysis: The semen collection was implemented by the manual stimulation method, and semen samples were collected into 15-mL centrifuge tubes, warmed to 37°C, and subsequently divided into three fractions. The second fraction, the sperm-rich portion, was used for assessing semen quality, including sperm count (Neubauer hemocytometer), viability (wet mount preparation, 20 µm), and abnormality (Bartlett, 1962; Chang *et al.*, 2021). Sperm morphology was analyzed through two staining methods: air-dried Giemsa staining (Chima *et al.*, 2017) and eosin-nigrosin staining (Bartlett, 1962) in order to determine the proportions of normal and abnormal sperm. For Giemsa staining, an aliquot of semen (10 µL), diluted in PBS, was spread onto a microscopic slide and air-dried. The smear was fixed with methanol for 5 min, stained with Giemsa for 15 min, and then rinsed with tap water to remove debris. As for eosin–nigrosin staining, a 5 µL aliquot of diluted semen was mixed with an equal volume of eosin–nigrosin solution. The mixture was incubated at room temperature for 1 min and spread onto a new slide. The morphological abnormalities of approximately 200–500 sperm cells from each dog's semen sample were evaluated. Sperm head abnormalities were assessed based on the criteria established by Oettlé (1993). Sperm without tails or heads and those in contact with or overlapping other sperm or debris were excluded from the evaluation. The results obtained from the assessment of morphological abnormalities were presented as percentages.

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Statistical analysis: The data were presented as mean±S.D. Statistical analyses were carried out using various tests, including Student's t-test, Shapiro-Wilk test, Mann-Whitney rank sum test, and Welch's *t*-test, depending on the type of sample. The type of statistical method used for the analysis will be explained in both the text of the results and the figure captions. These statistical analyses were performed using Sigma Plot Version 14.0 (Systat Software, San Jose, CA, USA). Unless stated otherwise, the difference was deemed statistically significant at $P<0.05$. Significance levels are indicated as follows: * = $P<0.05$, ** = $P<0.01$, and *** = $P<0.001$.

Result

Clinical evaluation: Before the commencement of the experiment, all dogs underwent comprehensive health assessments and wellness procedures to ensure their suitability for participation in the study. These procedures included routine deworming and administration of core vaccinations for canine parvovirus, distemper, canine hepatitis, and rabies. Following these examinations, all dogs were confirmed to be in good clinical condition prior to the study. Throughout the study, all dogs received regular medical examinations conducted by a licensed veterinarian. These examinations included assessments of body condition, gait, and appetite to monitor overall health and detect any adverse reactions. Specific attention was given to monitoring the injection sites in both groups, with veterinarians performing visual inspections for swelling, redness, or bruising, as well as palpation to detect tenderness, induration, or abnormal masses. One day after the administration of the primary immunization of the GnRH vaccine, one dog in the intramuscular (IM) group exhibited swelling at the injection site. Additionally, mild limping was observed in this dog three days after the injection. In contrast, no physical abnormalities or side effects were observed in the dogs belonging to the microneedle (MN) group after receiving the booster vaccination. Moreover, no significant differences were observed in the average weight of the dogs between Week 0 (baseline) and Week 12 (final day). This systematic and thorough approach ensured that any changes in the health status of the dogs were accurately documented, contributing to the reliability of the study's findings.

CBC and BCP: The health indicators, CBC and BCP, were evaluated both before (Week 0) and after (week 12) the primary immunization, and no noticeable differences were observed between groups and time points (Table 1). In the CBC analysis, all dogs displayed normal levels of erythrocytes and thrombocytes. However, one dog in the MN group showed neutrophilia (26,625/μL) in Week 12. Nevertheless, no clinical abnormalities were observed.

Production of antibodies Specific to GnRH: Before the primary immunization (week 0), the serum IgG against GnRH was negative in all dogs (Fig. 3). Dogs received IM, but not MN, injection of the GnRH vaccine had serum IgG against GnRH increased 2 weeks after the primary immunization. However, after the booster at week 4, the levels of anti-GnRH IgG in serum peaked at Week 6 in both groups and were not different between IM and MN.

Testosterone concentrations in males: The changes in serum testosterone concentrations were illustrated in Fig. 4. Both IM and MN groups exhibited declined serum testosterone levels after vaccination. The testosterone concentrations in both vaccinated groups were significantly lower than those in the control group without castration at week 12 (Mann-Whitney U Statistic, $P=0.004$) and showed no significant difference compared to the control group with surgical castration (Mann-Whitney U Statistic, $P=0.288$).

Morphology of scrotum and semen quality analysis: The scrotal sizes in terms of length, width, and volume were compared among the IM group, MN group, and uncastrated dogs (n=57), as shown in Table 2. Regarding scrotum morphology, the length of the IM group exhibited a significantly smaller size compared to the control group after vaccination, while the MN group showed a significantly smaller size only in week 12. Semen samples were manually collected from all three fractions at Weeks 0, 5, 9, and 12. Prior to vaccination, the semen concentrations in the IM and MN groups were $4.22\pm2.68\times10^8$ and $1.13\pm0.28\times10^8$ sperms/mL, respectively (Fig. 5a). These values were not significantly different from those of the uncastrated dogs (n=43). Following Week 8, there was a slight decrease in sperm concentrations, and reaching their lowest point in Week 12. In terms of sperm viability, a similar pattern was observed in sperm concentration. However, the MN group exhibited low viability only in the last week (Week 12), while the IM group showed reduced viability since Week 8 (Fig. 5b and 5c). The percentage of sperm abnormalities in the IM and MN groups rose from 36.68%±5.66% and 33.24%±3.63% to 60.86% and 63.95%±0.25% at Week 12 (Fig. 5d). Notably, azoospermia was observed in two dogs within the IM group starting from Week 8 (Fig. 5e).

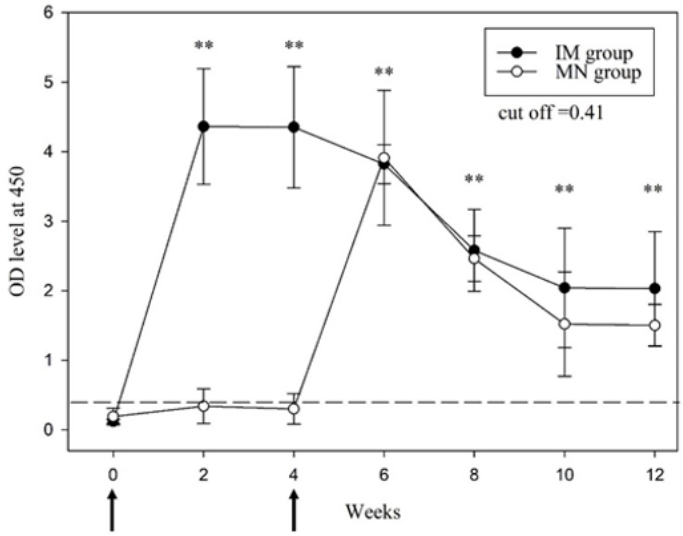

Figure 3 Serum anti-GnRH IgG titer profiles in the IM and MN groups are illustrated. Arrows on the x-axis indicate the primary vaccination (Week 0) and booster (Week 4). The data is presented as the mean ± standard deviation (S.D.). The dashed line represents the cutoff point, calculated from the O.D. value at Week 0±3×SD. The significant differences were compared with the uncastrated group. The significance levels for the Mann-Whitney U statistic are indicated as follows: * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

Table 1 CBC and BCP data for the volunteer dogs. Data are presented as mean ± standard deviation (SD.).

Group/Day Lab findings	References	Intra-muscular group		Micro-needle patches group	
		week 0	week 12	week 0	week 12
CBC					
P.C.V%	37.3-61.7	41.05 ± 12.23	43.43 ± 14.23	44.97 ± 11.23	47.08 ± 7.72
RBC (X10⁶/μL)	5.65-8.87	9.63 ± 2.8	9.48 ± 3.41	8.77 ± 0.64	9.96 ± 3.15
Total leukocytes (/μL)	5050-16760	11127.50 ± 5109.22	10707.42 ± 4012.24	17547.50 ±7856.12	12557.50 ± 4259.28
Neutrophils	2950-11640	10200 ± 7329.31	9505.60 ± 4807.17	8143.25 ± 7856.12	12524.50 ± 10160.88
Lymphocytes	1050-5100	1478.75 ± 1743.14	1478.46 ± 408.28	626.00 ± 4834.64	1759.75 ± 1181.37
Monocytes	160-1120	721.50 ± 894.97	229.47 ± 105.66	687.75 ± 146.31	327.50 ± 362.73
Eosinophils	60-1230	152.75 ± 157.79	350.92 ± 171.39	188.25 ± 564.76	396.00 ± 233.29
Basophils	10-260	195.75 ± 132.46	161.17 ± 194.54	249.50 ± 142.63	70.00 ± 60.55
Thrombocytes Number	148-484	334.05 ± 213.74	153.82 ± 262.74	370.75 ± 221.5	79.00 ± 27.35
BCP					
AST	0-50	40.00 ± 17.11	20.87 ± 15.38	41.00 ± 12.75	15.00 ± 4.83
ALT	10-125	47.00 ± 21.49	27.61 ± 12.79	70.50 ± 49.33	30.00 ± 3.46
Albumin	2.3-4	3.73 ± 0.22	3.21 ± 1.08	3.68 ± 0.37	3.58 ± 0.46
CREA	0.5-1.8	0.93 ± 0.18	0.75 ± 0.43	0.80 ± 0.16	0.66 ± 0.54
BUN		31.45 ± 7.36	28.06 ± 19.61	34.40 ± 5.17	33.73 ± 31.25

Reference: IDEXX; *Fuji-NX500i

Table 2 Correlations between parts of the spermatozoon (N=20)

Week (s)	IM group (n=3)			MN group (n=2)			Control group (n=57)		
	Length (cm)	Width (cm)	Volume (cm³)	Length (cm)	Width (cm)	Volume (cm³)	Length (cm)	Width (cm)	Volume (cm³)
0	4.02 ±0.31	2.46 ±0.11	12.79 ±1.56	3.72 ±0.14	2.38 ±0.19	11.10 ±1.82			
5	4.15 ±0.24	***2.00 ±0.26	**8.84 ±2.51	4.29 ±0.17	2.18 ±0.12	10.64 ±1.29			
9	**3.67 ±0.16	***1.91 ±0.12	***6.97 ±0.78	3.94 ±0.22	2.19 ±0.16	9.83 ±0.90	4.22 ± 0.55	2.59 ± 0.38	15.51 ± 5.79
12	***3.25 ±0.14	***1.71 ±0.40	***5.19 ±2.37	3.85 ±0.26	**2.06 ±0.18	**8.60 ±1.71			

Data are presented as mean ± S.D. Scrotum morphology was analyzed using Kruskal–Wallis one-way analysis. The dogs were compared with the uncastrated group to evaluate significant differences. Significance levels are indicated as follows: * = $P<0.05$, ** = $P<0.01$, and *** = $P<0.001$.

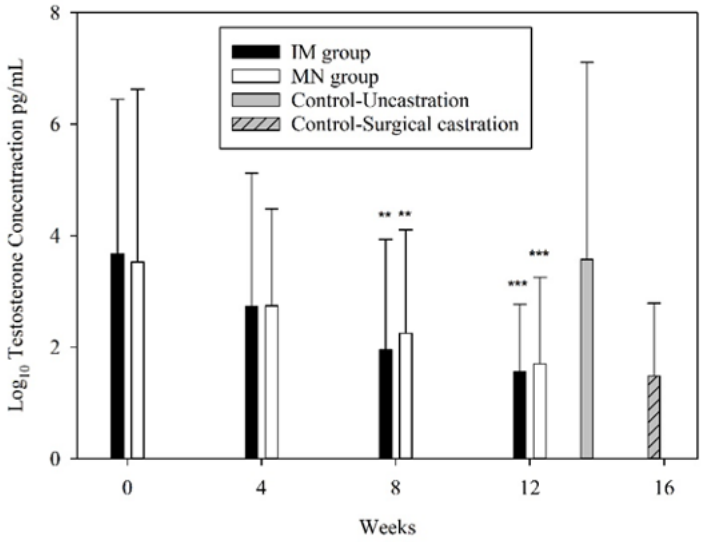

Figure 4 Serum testosterone concentrations (pg/mL) were measured in the IM group (n=3) and MN group (n=2) at various time points and compared to that of surgically castrated (n=49) and uncastrated (n = 89) dogs. The results are reported as the mean ± standard deviation (S.D.). The significant differences were compared with the uncastrated group (Mann-Whitney U Statistic). Significance levels are indicated as follows: * = P<0.05, ** = P<0.01, and *** = P<0.001.

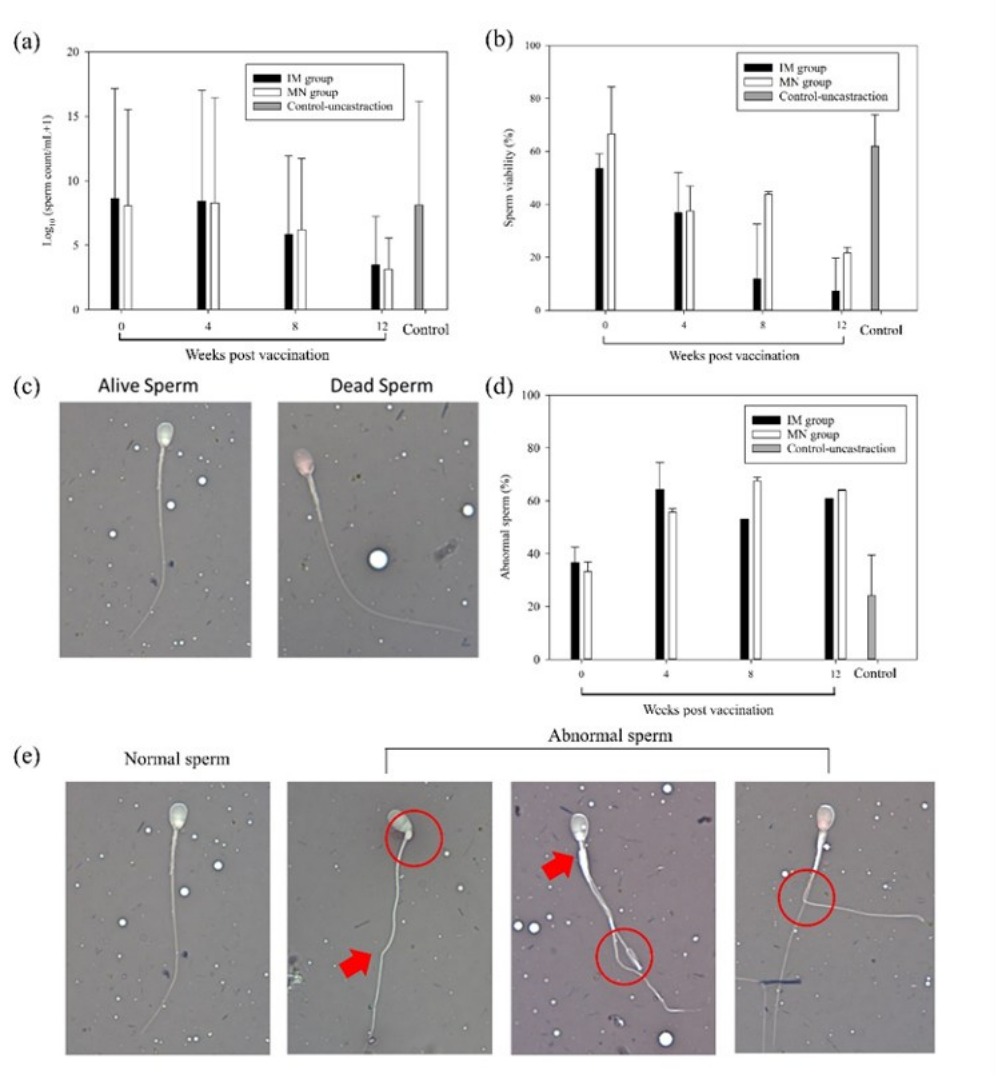

Figure 5 Sperm parameters, including concentration, abnormality, and viability, were evaluated in the IM group (n=3), MN group (n=2), and uncastrated dogs (n=43) at various time points post-vaccination. The figures depict (a) Sperm concentration, (b) Percentage of sperm viability, (c) Morphological identification of live and dead sperms using eosin–nigrosin staining, (d) Percentage of abnormal sperms, and (e) Morphology of normal and defective sperms. The data are presented as the mean ± standard deviation (S.D.).

Discussion

Dissolving microneedle patches is a painless, sharp-free, and easy-use device for large-scale vaccination (Zhao *et al.*, 2022). Although microneedle patches have been widely used in human medicine for diseases such as polio, influenza, and measles, they have rarely been utilized in animals (Arya and Prausnitz, 2016; Edens, Collins, *et al.*, 2015; Edens, Dybdahl Sissoko, *et al.*, 2015; Kim *et al.*, 2010).

Dogs have typically received drugs or vaccines using a syringe. However, using a syringe for administration causes numerous challenges, such as inconvenience, poor compliance, and discomfort. In this study, we aimed to compare the vaccine effectiveness through microneedle patches and the intramuscular route to increase animal welfare by assessing the efficacy, safety, and overall well-being of the vaccinated animals.

In our study, microneedle patches were applied to dog ears and left in place for 15 min. Then, the patch was removed, leaving the microneedles inside the skin. During the experiment, the dogs tolerated microneedle patches well without any side effects, and they were gradually acclimated to the patches using positive reinforcement with treats, in comparison with intramuscular injection, which caused noticeable side effects, including swelling, reddening, and mild limping in both this study and our previous research (Chang *et al.*, 2021; Chang *et al.*, 2023). The safety of using microneedle patches has been proven in numerous studies, which highlight their reliability for vaccination purposes (Zhu *et al.*, 2020). The ease of administration without needle injections is compliant with the preferences of both dogs and owners.

Regarding the production of anti-GnRH IgG in serum in response to the vaccination, we observed that using microneedle patches as a priming vaccination can lower the concentration (100ug) of the antigen and yield similar results to IM injection with a high dose (500ug) after the booster. A similar finding of microneedle patches with the potential to reduce the usage of antigens was also reported in the context of adenovirus vaccine administration using microneedle patches (Flynn *et al.*, 2021). Consequently, the vaccination strategy of employing microneedle patches for priming and subsequently boosting with IM might prove to be cost-effective.

Testosterone is the sexual hormone that exerts influence on various aspects of male dog behavior and the development of the reproductive system (Taha and Noakes, 1982). Vanderstichel *et al.* (2015) mentioned that after chemical and sterilization for 6 months, the dogs showed no long-term changes (<1 ng/mL) in blood testosterone concentrations. We found similar results with immunocastration; since week 4, testosterone concentrations decreased over time in both the IM and MN groups. In addition to lower testosterone concentrations, we observed testicular atrophy and poor sperm quality in our research in both vaccinated groups. Specifically, concerning changes in testicular morphology, the MN group exhibited a slower transformation until week 12, in contrast to the IM group, which showed changes by week 5. The delay in testis atrophy can be a limitation of microneedle patch administration. This situation may be attributed to the delayed increase in antibodies in the MN group, which in turn slowed the atrophy of the tests. In this study, the intramuscular vaccine contains ISA 206 as the adjuvant, while the microneedle patch is based on HA, which has poor immunogenicity, possibly affecting the immune responses. HA is the most popular material for producing microneedle patches due to its advantageous properties, including strong water absorption, biocompatibility, viscoelasticity, and cost-effectiveness. Future work should explore more immunostimulant materials for microneedle patch manufacturing to directly enhance the immune responses. The application of microneedle patches in animals has thus far been limited to dogs for preventing rabies and canine influenza, excluding the use of the GnRH vaccine for fertility control (Almalik *et al.*, 2017; Arya *et al.*, 2016; Choi *et al.*, 2020). However, for women's fertility control, a microneedle patch containing the contraceptive hormone levonorgestrel has been quite successful (Li *et al.*, 2019). With the effect of self-administration and long-acting capabilities, the product has successfully passed the preclinical phase of development. Due to the limitations of using dogs as experimental animals, it was not possible to include an adequate number of female dogs in the experiment. Consequently, the effect of this vaccine on female hormones is missing, which is a limitation of the research.

Overall, microneedle patches offer a promising technology for effective and accessible vaccination in dogs for fertility control. In this study, both the IM group and MN group demonstrated the ability to stimulate the production of antibodies specific to GnRH, reduce serum testosterone levels, and impair sperm quality. We acknowledge the limitations imposed by the small sample size; however, this study was designed as a proof-of-concept investigation to assess the feasibility of microneedle patches for GnRH immunocontraception. Despite the small sample size, the findings provide valuable preliminary evidence supporting the potential of microneedle patches for large-scale immunocastration.

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References

Abdulkarim A, Khan MA and Aklilu E 2021. Stray animal population control: methods, public health concern, ethics, and animal welfare issues. World Vet J. 11(3): 319-326.

Almalik A, Benabdelkamel H, Masood A, Alanazi IO, Alradwan I, Majrashi MA, Alfadda AA, Alghamdi WM, Alrabiah H and Tirelli N 2017. Hyaluronic acid coated chitosan nanoparticles reduced the immunogenicity of the formed protein corona. Sci Rep. 7(1): 10542.

Arya J and Prausnitz MR 2016. Microneedle patches for vaccination in developing countries. J Control Release. 240: 135-141.

Arya JM, Dewitt K, ScottGarrard M, Chiang Y and Prausnitz MR 2016. Rabies vaccination in dogs using a dissolving microneedle patch. J Control Release. 239: 19-26.

Bartlett D 1962. Studies on dog semen. Reproduction. 3(2): 173-189.

Chang A-M, Chen C-C, Hou D-L, Ke G-M and Lee J-W 2021. Effects of a Recombinant Gonadotropin-Releasing Hormone Vaccine on Reproductive Function in Adult Male ICR Mice. Vaccines. 9(8): 808.

Chang A-M, Chen C-C, Lee J-W, Hou D-L, Huang H-H and Ke G-M 2023. Effects of a novel recombinant Gonadotropin-Releasing Hormone-1 vaccine on the reproductive function of mixed-breed dogs (*Canis familiaris*) in Taiwan. Vaccine. 41(13): 2214-2223.

Chima UM, Abu AH, Dawuda PM, Kisani AI and Ahemen T 2017. Effect of storage time on cauda epididymal sperm parameters of Nigerian local dogs. Open J Vet Med. 7(11): 151-161.

Choi I, Na W, Kang A, Ahn M, Yeom M, Kim H, Lim J-W, Choi S, Baek S and Song D 2020. Patchless administration of canine influenza vaccine on dog's ear using insertion-responsive microneedles (IRMN) without removal of hair and its in vivo efficacy evaluation. Eur J Pharm Biopharm. 153: 150-157.

Cunha GHD, Barbosa RVA, Fontenele MSM, Lima MAC, Franco KB and Fechine FV 2017. Insulin therapy waste produced in the households of people with diabetes monitored in Primary Care. Rev Bras Enferm. 70: 618-625.

Edens C, Collins ML, Goodson JL, Rota PA and Prausnitz MR 2015. A microneedle patch containing measles vaccine is immunogenic in non-human primates. Vaccine. 33(37): 4712-4718.

Edens C, DybdahlSissoko NC, Weldon WC, Oberste MS and Prausnitz MR 2015. Inactivated polio vaccination using a microneedle patch is immunogenic in the rhesus macaque. Vaccine. 33(37): 4683-4690.

Einarsson S, Brunius C, Wallgren M, Lundström K, Andersson K, Zamaratskaia G and Rodriguez Martinez H 2011. Effects of early vaccination with Improvac® on the development and function of reproductive organs of male pigs. Anim Reprod Sci. 127(1-2): 50-55.

Flynn O, Dillane K, Lanza JS, Marshall JM, Jin J, Silk SE, Draper SJ and Moore AC 2021. Low adenovirus vaccine doses administered to skin using microneedle patches induce better functional antibody immunogenicity as compared to systemic injection. Vaccines. 9(3): 299.

Gompper ME 2014. The dog-human-wildlife interface: assessing the scope of the problem. Free-ranging dogs and wildlife conservation. 9-54.

Kim HJ, Park SK and Park SH 2017. Upper limb nerve injuries caused by intramuscular injection or routine venipuncture. Anesthesia and Pain Medicine. 12(2): 103-110.

Kim Y, Quan F, Compans RW, Kang S and Prausnitz MR 2010. Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity. J Control Release. 142(2): 187-195.

Knobel DL, Butler JR, Lembo T, Critchlow R and Gompper ME 2014. Dogs, disease, and wildlife. Free-ranging dogs and wildlife conservation. 144-169.

Lee YJ, Jo EJ, Lee HW, Hwang BR, Kim YH, Park BJ, Cho YJ, Lee YA, Choi IS and Han JS 2019. Evaluation of infertility efficacy of the *E. coli* expressed STF2-GnRH vaccine in male cats. J Vet Sci. 20(3): e30.

Li W, Tang J, Terry RN, Li S, Brunie A, Callahan RL, Noel RK, Rodríguez CA, Schwendeman SP and Prausnitz MR 2019. Long-acting reversible contraception by effervescent microneedle patch. Sci Adv. 5(11): eaaw8145.

Liu Y, Tian Y, Zhao X, Jiang S, Li F, Zhang Y, Zhang X, Li Y, Zhou J and Fang F 2015. Immunization of dogs with recombinant GnRH-1 suppresses the development of reproductive function. Theriogenology. 83(3): 314-319.

Massei G 2012. Catch, inject and release: immunocontraception as alternative to culling and surgical sterilisation to control rabies in freeroaming dogs. Compendium of the OIE global conference on rabies control. Incheon-Seoul (Republic of Korea) OIE.

Meloen R, Turkstra J, Lankhof H, Puijk W, Schaaper W, Dijkstra G, Wensing C and Oonk R 1994. Efficient immunocastration of male piglets by immunoneutralization of GnRH using a new GnRH-like peptide. Vaccine. 12(8): 741-746.

Miller LA, Gionfriddo JP, Fagerstone KA, Rhyan JC and Killian GJ 2008. The single-shot GnRH immunocontraceptive vaccine (GonaCon™) in white-tailed deer: comparison of several GnRH preparations. Am J Reprod Immunol. 60(3): 214-223.

Miller LA, Johns BE and Killian GJ 2005. Immunocontraception of white-tailed deer with GnRH Vaccine. Am J Reprod Immunol. 44(5): 256-273.

Oettlé E 1993. Sperm morphology and fertility in the dog. J Reprod Fertil Suppl. 47(4): 257-260.

Padodara R, Singh V, Odedara A, Vasava A, Sharma A and Mehta V 2022. Modern approaches to contraception in domestic and wild animals: a review. J Global Ecol Environ. 16(1): 14-25.

Park C, Cho W and Son B 2019. Latrogenic injury to the sciatic nerve due to intramuscular injection: a case report. Korean J Neurotrauma. 15(1): 61.

Pérez-Linares C, Bolado-Sarabia L, Figueroa-Saavedra F, Barreras-Serrano A, Sánchez-López E, Tamayo-Sosa A, Godina A, Ríos-Rincón F, García L and Gallegos E 2017. Effect of immunocastration with Bopriva on carcass characteristics and meat quality of feedlot Holstein bulls. Meat Sci. 123: 45-49.

Rocha JM, Ferreira Silva JC, Neto H, Moura MT, Ferreira HN, Silva Junior V and Oliveira M 2018. Immunocastration in donkeys: clinical and physiological aspects. Pferdeheilkunde Equine Med. 34(1): 12-16.

Shideler S, Stoops M, Gee N, Howell J and Lasley B 2002. Use of porcine zona pellucida (PZP) vaccine as a contraceptive agent in free-ranging tule elk (*Cervus elaphus nannodes*). Reprod Suppl. 60: 169-176.

Siel D, Vidal S, Sevilla R, Paredes R, Carvallo F, Lapierre L, Maino M, Pérez O and Sáenz L 2016. Effectiveness of an immunocastration vaccine formulation to reduce the gonadal function in female and male mice by Th1/Th2 immune response. Theriogenology. 86(6): 1589-1598.

Taddio A, Appleton M, Bortolussi R, Chambers C, Dubey V, Halperin S, Hanrahan A, Ipp M, Lockett D and MacDonald N 2010. Reducing the pain of childhood vaccination: an evidence-based clinical practice guideline. Can Med Assoc J. 182(18): E843-E855.

Taha M and Noakes D 1982. The effect of age and season of the year on testicular function in the dog, as determined by histological examination of the seminiferous tubules and the estimation of peripheral plasma testosterone concentrations. J Small Anim Pract. 23(6): 351-357.

Van Der Maaden K, Jiskoot W and Bouwstra J 2012. Microneedle technologies for (trans) dermal drug and vaccine delivery. J Control Release. 161(2): 645-655.

Vanderstichel R, Forzan M, Perez G, Serpell J and Garde E 2015. Changes in blood testosterone concentrations after surgical and chemical sterilization of male free-roaming dogs in southern Chile. Theriogenology. 83(6): 1021-1027.

Waghule T, Singhvi G, Dubey SK, Pandey MM, Gupta G, Singh M and Dua K 2019. Microneedles: a smart approach and increasing potential for transdermal drug delivery system. Biomed Pharmacother. 109: 1249-1258.

Zhao B, Jin Z, Yu Y, Li Y, Wang J, Wan W, Hu C, Li X, Li Y and Xin W 2022. A thermostable dissolving microneedle vaccine with recombinant protein of botulinum neurotoxin serotype A. Toxins. 14(12): 881.

Zhu D, Zhang X, Zhang B, Hao Y and Guo X 2020. Safety assessment of microneedle technology for transdermal drug delivery: a review. Adv Therap. 3(8): 2000033.