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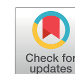
Patchless administration of canine influenza vaccine on dog's ear using insertion-responsive microneedles (IRMN) without removal of hair and its *in vivo* efficacy evaluation



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A R T I C L E I N F O

Keywords:
Insertion-responsive microneedles
Canine influenza vaccine
Hair removal
Compliance
Less pain

A B S T R A C T

Microneedles provide the advantages of convenience and compliance by avoiding the pain and fear of needles that animals often experience. Insertion-responsive microneedles (IRMN) were used for administration to a hairy dog without removing the dog's hair. Canine H3N2 vaccine was administered with IRMN attached to the dog's ears *ex vivo* and the conventional microneedle system (MN) was administered for 15 min to compare puncture performance and delivery efficiency. The vaccine was also administered to compare antibody formation using IRMN with the use of intramuscular injection. The veterinarian observed the behavior of the dog during the course of the administration and compared the response to IRMN with that of intramuscular administration.

The tips of IRMN were separated from the base and delivered into the hairy skin successfully. Puncture performance of IRMN were the same as that of coated microneedles (95%), but delivery efficiency of IRMN were 95% compared to less than 1% for coated microneedles. The H3N2 vaccine inoculated into the dog's ears showed the same antibody formation as the intramuscular injection. The dog appeared to be more comfortable with IRMN administration compared to syringe administration.

IRMN are the first microneedle system to deliver a canine vaccine successfully into a hairy dog without removal of the dog's hair. The use of IRMN can provide both convenience and compliance for both the dog and the owner.

1. Introduction

Various formulations and delivery systems have been developed to provide for the delivery of drugs and vaccines into animals, including dogs, via oral, intramuscular, subcutaneous, and topical administration [1–6]. Among these delivery methods, intramuscular administration has been the most widely used [6–8]. Currently, most companion animals receive vaccines for diseases such as Distemper, Hepatitis, Parvovirus, Parainfluenza infection, and Leptospira (DHPPL), corona virus, kennel cough, rabies, and influenza virus via injection into the subcutaneous skin layer or muscle [9–11]. These needle-based injections require a high volume of the drug to produce sufficient immunity, and such high-volume administration can evoke pain responses and the formation of a lump when the vaccine is inoculated using the wrong route, which in turn can trigger a hypersensitivity reaction that may require application of additional fluid or immunosuppressive treatment

Abbreviations: MNs, microneedles; MAP, microneedle array patch; CIV, Canine influenza virus; IRMN, insertion-responsive microneedles; HA, Hyaluronic acid; HAU, Hemagglutinin unit (dose)

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[12–15].

Canine influenza virus (CIV) is a serious pathogen that causes respiratory disease and hemorrhagic pneumonia, and it can also provoke secondary infection of bacteria, all of which can elevate the animal death rate [16]. H3N2 CIV originated from avian host, and it was isolated for the first time in dogs in South Korea in 2007, spreading to the US in 2015 [16,17]. Intramuscular (IM) administration of H3N2 CIV vaccine stimulates serum antibody production, but this method of administration has significant limitations, including the need for trained health staff, a cold chain system, and a large storage space and facilities [15,18–21]. Dangerous waste, cross-contamination, and thermal instability of the vaccine are additional problems involved in the IM administration of liquid formulations [22–25]. The administration of the vaccine using microneedles can overcome the limitations of IM administration.

Microneedle systems have been introduced to overcome the limitations of administration using large needles such as syringes [24–27]. Microneedles (MN) are a drug delivery system using microstructures with a length of several hundred micrometers. MN can deliver active pharmaceutical ingredients (API) into the skin layer with minimal pain regardless of the molecular weight or polarity of API [28–32]. Thus, MN allow a variety of drugs to be delivered into animals such as dogs with minimal pain and fear [15,32–34]. In our study, a vaccine was delivered into dogs using microneedles, and successful antibody formation was obtained. Vaccine microneedles have the advantages of skin immunization because there are immune cells in the skin layer such as dermal dendritic cells (DDCs) and Langerhans cells (LCs) [6,35–39]. For this reason, It can be applied to intradermal application with less dose and can induce to similar or more immune responses compared with intramuscular injection [6,36,40–43]. In order to deliver the necessary amount of drug with dissolving microneedles (DMNs) or coated microneedles (CMNs), sufficient attachment time is needed, which requires the use of a patch. Therefore, the final MN product, called microneedle array patch (MAP), consists of MNs and a patch. In the case of humans with little hair, there is no problem attaching patches, but MNs cannot be attached to the hairy skin surface of animals, so hair must be removed in order to insert the MNs into the skin. [27,44–46]. A microneedle array patch (MAP) has been developed for human use that can be attached to relatively hairless arms or hands, but it is impossible to attach the MAP to a hairy site of the human body [47–49]. Therefore, a new microneedle system needs to be developed for attachment to hairy animal skin without removal of hair, and instructions for implementing the new system for animals are necessary.

In this study, we utilized a new system of insertion-responsive microneedles (IRMN) [25,50] to administer H3N2 CIV vaccine to a dog's ear without removing the dog's hair. IRMN are similar to dissolving microneedles (DMNs), but DMNs must be attached for a sufficient time for the drug to dissolve in the skin after insertion. IRMN are MNs that separate the drug-coated water-soluble tips from the non-soluble base when they are inserted into the skin. The adhesion force at the interface of the water-soluble tips and the non-soluble base is controlled by the design and kind of material used to manufacture the MNs. Even if there is hair on the skin, IRMNs can be inserted into the skin by passing between the hairs, and the drug coated water-soluble tips are immediately separated from the base by the insertion force applied to the interface of the tips and the base (Fig. 1). After the water-soluble tips coated with the drug are separated in the skin, both the drug and the tips are rapidly dissolved in body fluid. Therefore, since there is no necessity of attaching a patch, 100% of the loaded drug can be delivered by pressing on the patch with a finger for a few seconds.

In this study, the water-soluble tips were separated immediately after administration, reducing administration time to a few seconds (as with syringe administration). Because the administration time is very brief, 100% of the loaded drug can be delivered by pressing on the patch with a finger without actually attaching the patch. The possibility and achievements of patchless administration of IRMN were proved by a canine vaccine experiment. The H3N2 CIV vaccine formulation was loaded on IRMN, and the geometries, mechanical properties, and drug delivery properties of IRMN were investigated by *ex vivo* experiments. H3N2 CIV vaccine–insertion-responsive microneedles (H3N2CIV-IRMN) were inoculated into dogs to evaluate the efficacy of H3N2CIV-IRMN. Then the antibody formation by H3N2CIV-IRMN was observed, and the efficacy of H3N2CIV-IRMN was compared with that of IM administration (H3N2CIV-IM).

2. Materials and methods

2.1. Preparation of virus and vaccine

Madin-Darby canine kidney (MDCK, ATCC CRL-2936) cells were obtained from the American Type Culture Collection (ATCC). The MDCK were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and antibiotics. The 293 T cells were cultured in Opti-MEM (Gibco) supplemented with 5% FBS and antibiotics. All cells were maintained at 37 °C in 5% CO_2.

A/canine/VC378/2012 (H3N2) and A/canine/Korea/01/2007 (H3N2) were propagated in MDCK cells with 1 μg/mL of TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 1% antibiotic–antimycotic in DMEM at 37 °C and 5% CO_2.

Inactivation of virus was conducted by 0.2% final concentration of formalin for 18 h at 37 °C and purified by ultracentrifugation at 100,000 × g, 4 °C for 4 h. Then, measurement of the pelleted whole viral protein content was detected by a colorimetric protein assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocols with albumin standard. The absorbance optical density was measured at 560 nm using a microplate reader (SpectraMax® i3x; Molecular Devices, San Jose, CA, USA).

2.2. Preparation of coating formulation of H3N2 CIV vaccine and H3N2 CIV vaccine-insertion-responsive microneedles

2.2.1. Preparation of coating solution of H3N2 CIV vaccine

The vaccine coating solution was prepared according to the following procedure. Inactivated A/Korea/VC378/2012 (H3N2) vaccine solution was mixed with Adjuvant Montanide™ gel (SEPPIC Animal Health, France) at a 9:1 ratio (vaccine to adjuvant). The coating solution was prepared by mixing an aqueous solution of 13% (w/v) polyvinyl alcohol (Sigma-Aldrich, St. Louis, MO, USA) as a thickener and an aqueous solution with 7.8% (w/v) sucrose (Sigma-Aldrich, St. Louis, MO, USA) as a stabilizer.

2.2.2. Manufacturing process of insertion-responsive microneedles

The master structure of a microneedle array has 97 microneedles that are 800 μm high and 370 μm wide, with 400 μm of space between the microneedles situated in a 1-cm diameter disk. The polydimethyl siloxane (PDMS, SYLGARD 184, Dow Corning, MI, USA) mold was prepared from master structure.

The microneedle array was manufactured in three steps [25,50] : (1) preparation of tip, (2) preparation of base and integration of tips and base and (3) coating the vaccine formulation on the IRMN. Step 1 involved preparation of the tip portion: An aqueous gel was prepared by dissolving hyaluronic acid (HA, SK Bioland, Cheonan, South Korea), sucrose and Tween 80 (10%, 5%, and 0.1% [w/v], respectively). Gentian violet was added in the tip formulation for visual confirmation of successful administration of the tips. The aqueous gel was centrifuged at 3000 rpm for 2 hr to remove bubbles. The prepared gel was placed on the mold and pressurized with a force of 30 N for 5 s using a press machine to fill the cavities of the tips and then remove the excess solution. This process was repeated three times and the tips were dried at room temperature for 10 min. Step 2 involved preparation of the base

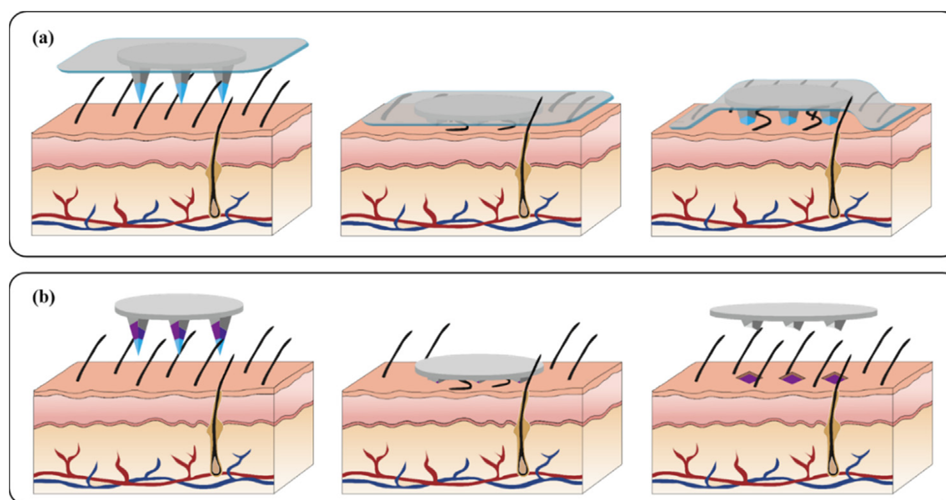

Fig. 1. Descriptive image to illustrate the comparison of Insertion-responsive microneedles (IRMN) and coated microneedles at a hairy site. (a) A coated microneedles patch was applied on the hairy site, but the patch was lifted by the hair. (b) IRMN were pressed on the hairy site and the base was removed after 10 s.

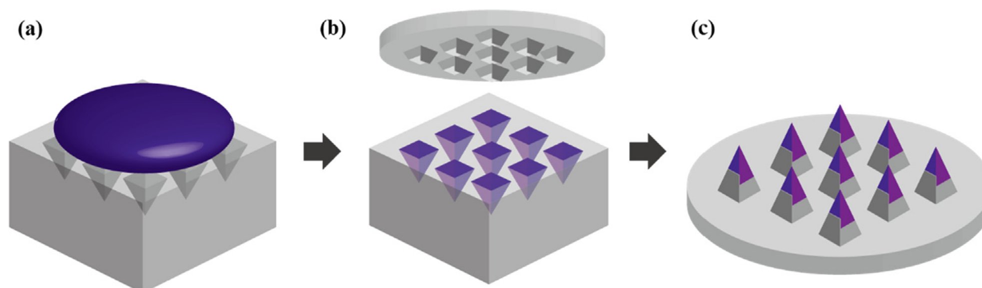

Fig. 2. The fabrication process of insertion-responsive microneedles. (a) Filling hyaluronic acid (HA) and sucrose in the cavities of the mold. (b) Placing PCL pellets in the cavities with a guide of another PDMS mold and melting. Then placing the solidified PCL stand on the PDMS mold and pressing the PCL base over cavities filled with HA/sucrose at 70 °C. (c) Release of microneedles from PDMS mold.

and integration of the tips and the base. The base of the IRMN with a guide was made of polycaprolactone (PCL, Average Mn 80,000; Sigma-Aldrich, St. Louis, MO, USA) by molding from cavities of another mold with a guide 200 µm in height, and a PCL base of the IRMN 200 µm thick was placed on a circular PCL disk 1 cm in diameter and 500 µm thick. After aligning the PCL base and the cavities of the PDMS mold, the PCL base was pressed over the cavities of the mold with a force of 30 N for 5 s using a press machine at 70 °C. After cooling for 10 min at room temperature, the IRMN were peeled off of the PDMS mold (Fig. 2).

Polylactic acid (PLA, Lactel, Birmingham, AL) pellets were loaded into a PDMS mold and placed in a vacuum oven under 70 kPa at 190 °C for 30 min. After cooling at room temperature, the base was peeled off of the PDMS mold. Then the gentian violet formulation was coated with the same solution and following the same procedure, the final microneedle assay was fixed on the patch (MAP).

2.2.3. Process of coating H3N2 CIV vaccine on insertion-responsive microneedles

Step 3 involved coating the vaccine formulation on the IRMN: The coating solution containing the vaccine formulation was poured into a micro-well with 400 µm of depth. To prevent the water-soluble tips from becoming blunt as a result of being dipped in the coating solution during the coating process, the IRMN were rapidly frozen by spraying liquid nitrogen on the sample [25]. The IRMN were then brought close to the coating solution and immersed in the well containing the coating solution at a rate of 1 mm/s. One second after immersion, the coated IRMN were removed from the solution. The coated IRMN were dried at room temperature for about 10 min. This coating process was repeated twice. The H3N2 CIV vaccine–insertion responsive microneedles were stored in a desiccator at 22 °C (Fig. 3).

The shapes of the IRMN and the H3N2CIV-IRMN were observed using an optical microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) and a scanning electron microscope (SEM, JSM-7500F, JEOL).

2.3. Antigen distribution in the IRMN

To label the IRMN with lipophilic dyes (DiD), the viruses were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) for 30 min at room temperature. Unbound dye was removed using dialysis (MWCO 5 K). The viral aggregates were then removed using 0.45-µm pore size filters. Fluorescent images of antigen-coated IRMN were collected using a laser scanning confocal microscope (LSM700, Carl Zeiss, Jena, Germany).

2.4. Insertion tests of IRMN into dog ears (Ex vivo)

In order to investigate whether IRMN can be administered to a dog's hairy ear without a hair removal process, dog ears (Cronex Co. Ltd., Hwaseong. South Korea) were obtained and tested. For visual observation of the successful delivery of IRMN, a coating solution containing Gentian violet was used instead of a vaccine coating solution, and the coating was performed by the same coating method as described above. A patch-type microneedle system was prepared to compare with a conventional microneedle system. The microneedle patch was made of only PLA in the same mold, the gentian violet formulation was coated with the same coating procedure, and the final microneedle array was fixed on the patch (MAP).

The dog ears (without the hair removed) were pinned onto the fixation plate, and IRMN and a MAP were administered separately to dog ears. Gentian violet-coated IRMN were pressed on the dog's ear for 10 s with the thumb and removed. A gentian violet-coated MAP was attached to the ear for 15 min after pressing the thumb on the dog’s ear for 10 s. The intradermal delivery of the coating material was then confirmed by images under a camera (DSC-RX100M3, Sony, Tokyo,

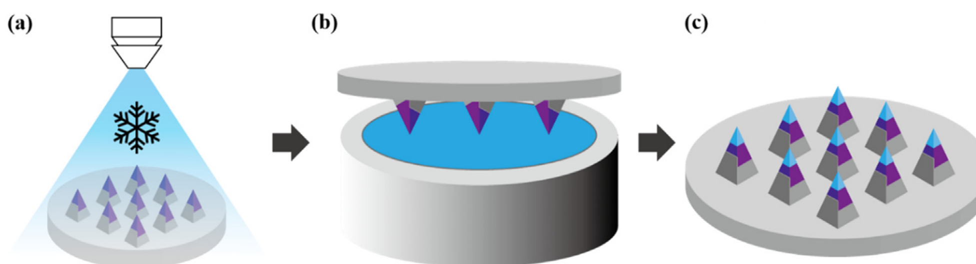

Fig. 3. Coating process of vaccine on IRMN. (a) To prevent the water-soluble tips from becoming blunt as a result of being dissolved by the coating solution, the tips were frozen using liquid nitrogen. (b) Vaccine formulation coating on frozen IRMN. (c) Vaccine-coated IRMN were dried.

Japan). When the coating material is delivered into the skin, the colored formulation should not be left on the tips. Delivery efficiency was shown by comparing the number of dots left by the microneedle tips with the total number of microneedles.

2.5. Cellular uptake efficacy of IRMNs' antigen

To analyze antigen internalization in macrophage cells, ovalbumin (OVA) labeled with fluorescein isothiocyanate (FITC) released from IRMN was used. RAW 264.7 cells were seeded into a 35-mm m-dish (Ibidi, Madison, WI, USA) at a density of 4×10^5 cell/dish and incubated overnight at 37 °C. The cells were washed twice with phosphate-buffered saline (PBS, 10 mM, pH 7.4) and incubated with antigen and OVA-FITC released from IRMN for 6 h at 37 °C. To observe nucleus localization, the cells were rinsed twice with PBS and the nucleus was stained with 50 nM of DAPI (Invitrogen, Carlsbad, CA, USA) for 15 min at 37 °C. The cells were then washed with PBS and fluorescent images were collected using a laser scanning confocal microscope (LSM700, Carl Zeiss, Jena, Germany).

2.6. Vaccination in dogs

Seven-week-old male Beagle dogs were purchased from CLSbio (Bucheon, South Korea) with each dog being serologically negative for influenza viruses and housed in isolation cages within the BSL-2 facility at Green Cross Veterinary Products (Yongin, South Korea) for the study. Animal care and treatment were conducted in accordance with guidelines establishment by Institutional Animal Care and Use Committee of Green Cross Veterinary Product (GCVP-2018-02-R).

Forty beagles (n = 10 per group) were vaccinated two times at 2-week intervals. The vaccination was performed using a previously described vaccination method [25]. The VC378-IM group was vaccinated by intramuscular (IM) administration and the VC378-IRMN group was vaccinated using IRMNs. These two groups each received 2^7 hemagglutinin unit (HAU) of VC378 antigen. A third group was vaccinated with one dose (500 µl vol.) of CaniFlu-Max (Bionote, Inc., Hwaseong, South Korea). The fourth group (placebo) was inoculated with phosphate-buffered saline (PBS). For IM administration, 0.5 ml of volume of vaccine was administrated into the caudal thigh muscles by using 22G needle. For microneedle vaccination, IRMN were placed on the skin without hair removal, pressed by gentle thumb pressure, held in place for 10 s, and detached from the skin. Serum was collected at the day of initial immunization and 14 days after the second vaccination.

The Canine Acute Pain scale of the Colorado State University (CSU) School of Veterinary Medicine, Fort Collins, CO, was used for the evaluation of the dogs' pain. This scale was developed as a teaching tool to help students recognize specific behaviors that may be indicative of pain. Two veterinarians monitored the dogs' behavioral and physiological responses during and after vaccination and determined appropriate pain scores ranging from 0 to 4, where 0 is comfortable, 1 is slightly unsettled, 2 is uncomfortable, 3 is unsettled, and 4 is screaming.

2.7. Hemagglutination inhibition assay

The HI assay was performed using a previously described method according to the WHO Manual (WHO Manual on Animal Influenza Diagnosis and Surveillance, http://www.who.int/iris/handle/10665/68026). Briefly, serum samples were treated with receptor-destroying enzyme (RDE, Denka Seiken Co., Japan) in a 1:3 ratio (vol/vol) at 37 °C overnight and heat-inactivated at 56 °C for 30 min. After RDE treatment, six volumes of phosphate-buffered saline (PBS) were added to the RDE-treated serum samples. RDE-treated sera were serially diluted by 2-fold in a 25 µl volume in a 96-well plate. Virus was adjusted to 4 HAU/25 µl and added to each well. The HI titer was measured with 0.5% packed chicken red blood cells. The limit of detection was 1:10 for the HI assay.

2.8. Microneutralization assay

Serum neutralizing antibody titers were measured in MDCK cells as described previously by Rowe et al. (1), with modifications. Briefly, a monolayer of MDCK cells was cultured in minimal essential medium (MEM) supplemented with 10% FBS. Heat-inactivated sera were serially diluted 2-fold and mixed with 100 times the TCID50 of the VC378 virus. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of serum that was able to neutralize 100 times the TCID50 of the virus in cells.

3. Results

3.1. Vaccine-coated insertion-responsive microneedles

Fig. 4(a) shows the image of the manufactured vaccine-coated insertion-responsive microneedles (IRMN). IRMNs consist of water-soluble tips made of HA/sucrose and a non-soluble base made of PCL. When the IRMNs penetrate the stratum corneum of the skin, the tips encounter the external force of hard tissue such as bone. Separation of the IRMN tips from the base occurs when the external force applied to the MNs is greater than the adhesion force at the boundary between the tips and the bases. Blue-colored gentian violet was added to the vaccine formulation for visual investigation. The IRMN consisted of a base portion (1) that caused separation, a water-soluble tip portion (2) where separation occurred, and a tip (3) coated with the vaccine formulation (Fig. 4(b)). The application of IRMN to the skin generated the separation of the tip (2) from the base (1) due to external forces caused by hard bone tissue, and the vaccine formulation was delivered together with the tip after separation. The remaining tip in the skin was made of HA, a biocompatible, water-soluble, and biodegradable polymer. When the water-soluble vaccine formulation was coated on the IRMN, the coating solution could dissolve the tip. The tip could also become blunt as a result of dissolution, so that the IRMN could not penetrate into the skin. To overcome this difficulty, the IRMN were rapidly frozen to lower the dissolution rate of the tip during coating. This coating method allowed the water-soluble vaccine formulation to be applied on the IRMN without changing the geometry of the IRMN. As

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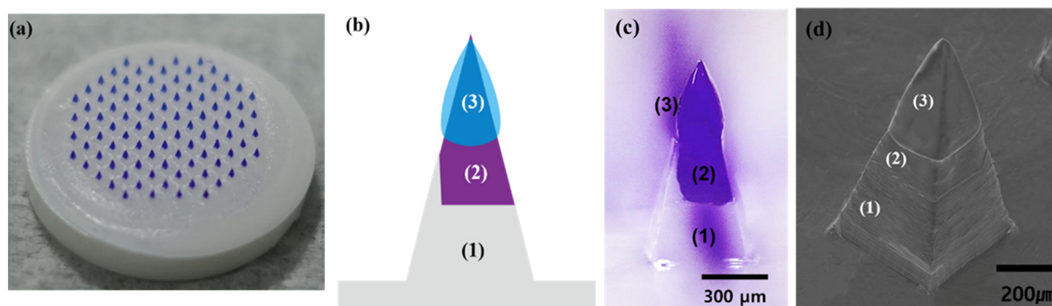

Fig. 4. (a) Optical image of insertion-responsive microneedles with vaccine formulation, gentian violet, and additives. (b) Schematic description of vaccine-coated IRMN (c) optical image of vaccine-coated IRMN and (d) SEM image of vaccine-coated IRMN : (1) base, (2) tip with gentian violet, and (3) vaccine formulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shown in Fig. 4(c), the vaccine formulation was located on the sharp tip of the IRMN. The geometries of a PLA microneedle array had the same characteristics as IRMN.

3.2. Antigen distribution in the IRMN

To investigate the distribution of the vaccine formulation on the IRMN, A/canine/VC378/2012 (H3N2) virus was labeled with lipophilic dyes (DiD) and then the formulation was coated on the IRMN using the freezing method (Fig. 5). Confocal laser scanning microscopy images of the resulting multilayer-coated microneedles showed local distribution of the vaccine formulation on the tip ends of the IRMN.

3.3. Ex vivo insertion of vaccine-coated IRMN into hairy dog ears

Coated microneedles were placed on the patch (Fig. 6(a)) and pressed on a dog's hairy ear *ex vivo* for 10 s and then attached to the ear using a patch for 15 min (Fig. 6(b)). IRMN shown in Fig. 6(a’) were pressed on the dog's ear for 10 s and removed (Fig. 6(b’)). As shown in the Fig. 6(b), a patch was not suitable for fixing microneedles on the ear. The puncture performance for both coated MNs (CMNs) and IRMNs was 100%. Also, the insertion efficiency was also 100% for both CMNs and IRMN. When the coated MN on the patch were administered to the skin, all the MN penetrated into the skin. However, the delivery efficiency of coated MN was about 0.6%, indicating that most of formulation was left on the MN. The term delivery efficiency means the amount of drug delivered compared to the total amount of drug contained in the MNs. The coated formulation should be dissolved fully for successful delivery into the skin. But the patch was lifted by the hair, which caused the release of MN off of the skin. The non-uniform attachment of the MAP resulted in low delivery efficiency. As shown in the image of the coated microneedles removed after 15 min of attachment (Fig. 6(c)), most of the coating formulation remained on the surface of the MN. This indicated that a conventional MN system could not achieve successful delivery without hair removal. However, in the case of IRMN, the blue dots on the tip ends could not be seen by visual investigation (Fig. 6(c’)). The tips of the IRMN were successfully delivered through the hairy skin. *Ex vivo* results showed (Table 1) that IRMN are a successful system for delivering drugs through hairy skin. IRMN are the first system to deliver drugs successfully without hair removal. CMNs and IRMNs had different insertion times for achieving 100% delivery of the drug into the skin: < 10 s for IRMNs and 10–30 min for CMNs. Thus, the insertion time for IRMN and CMNs made a difference in the amount of drug delivered into the skin.

3.4. Cellular uptake efficacy of IRMNs’ antigen

Antigen cellular uptake by antigen-presenting cells (APC) is the basis of the immune response mechanism in vaccinations. Furthermore, cytoplasmic delivery of antigens is crucial for the induction of antigen-specific immune responses. In this study, OVA-FITC released from IRMN was investigated to determine whether antigen cellular uptake occurred in RAW264.7 cells in the same way as in the direct addition of OVA-FITC to the cells. OVA-FITC is used for surrogate antigen for studying antigen cellular uptake by monitoring the fluorescent signal and OVA position inside of cells. For this reason, OVA-FITC was used to check the cellular uptake of the OVA released from IRMNs. As shown in Fig. 7, OVA-FITC released from IRMN showed cellular uptake, and this suggests that the antigens in coating formulation were absorbed normally into the cells.

3.5. Pain and comfort of IRMN

Based on the Canine Acute Pain scale, the responses of the dogs in the animal experiments were recorded and summarized as numerical values. As shown in Fig. 8(a), the average pain rating with IRMN was very low (0.2). In other words, the dogs felt little to no discomfort when IRMN were attached on the inside of the dogs’ ears. On the other hand, the pain ratings with IM administration ranged from about 1.0 to 4.0 depending on the dog regardless of the type of vaccine, and some dogs showed anxiety or discomfort.

Fig. 8(b) shows that all the MN tips were attached and the vaccine formulation was delivered into the skin successfully when the IRMN were pressed and removed from the dogs’ ears for 10 s. Even with hair on the ears, the IRMN were attached successfully. In addition, dogs’ ears are relatively hairless and can be visually checked to determine whether the IRMN have been attached successfully. Therefore, when drugs including vaccines are administered to a dog with IRMN, we recommend the inside of the dog's ear as a suitable site for successful administration and easy visual investigation.

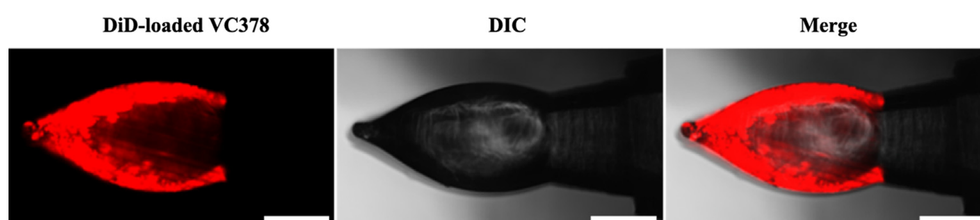

Fig. 5. Confocal laser scanning microscopy images of DiD-loaded vc378 distribution in the microneedle. Representative confocal images of microneedles coated with DiD-loaded vc378 (left, DiD-loaded vc378; middle, DIC; right, merge). The scale bars are 200 μm.

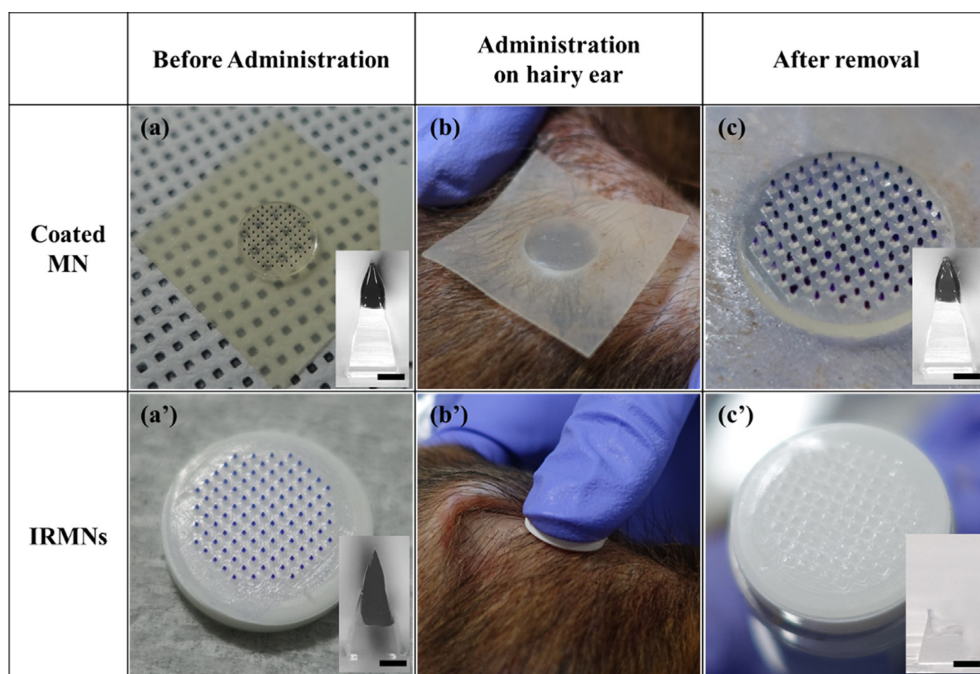

Fig. 6. Comparison of delivery efficiency of insertion-responsive microneedles and coated microneedles after insertion into a dog's hairy ear. (a, a') Vaccine-coated microneedle array patch (MAP) with magnified image and vaccine-coated insertion-responsive microneedles (IRMN) with magnified image. (b, b') Administration of MAP and IRMN, respectively. (c, c') Images of MAP and IRMN with magnified image after removal from skin. Scale bar in magnified image is 150 µm.

Table 1
Comparison of puncher performance and delivery efficiency of insertion-responsive microneedles with coated microneedles on the hairy site (n = 5).

	Coated microneedles	IRMN
Puncher performance	96 ± 1.4	95 ± 1.3
Delivery efficiency	0.6 ± 0.8	95 ± 1.9

3.6. Vaccination and antibody response (in vivo)

To assess the immunogenicity of the IRMN vaccine in dogs, beagle dogs were immunized twice with the commercial canine influenza vaccine by administering inactivated VC378 vaccine either intramuscularly (i.m.) or with IRMN 14 days apart. Both vaccines induced HI titers against homologous strains (HI GMT of 21 to 30) (Fig. 9(a)). However, the IRMN vaccine induced statistically higher SN titers after prime vaccination in sera from immunized dogs, showing mean SN titers of > 80 against the homologous VC378 strain at 14 days post vaccination (Fig. 9(b)). IRMN were successfully administered to hairy dogs without hair removal, resulting in the same antibody formation as with IM administration. In addition, administering IRMN inside the dogs' ears successfully induced antibody formation.

4. Discussion

Traditionally, drugs or vaccines have been administered to dogs by a syringe. However, administration by syringe has many difficulties, including inconvenience, low compliance, and pain. In this study, IRMN were administered inside dogs' ears without removal of hair, and vaccine-coated IRMN induced antibodies the same as those induced by IM injection.

Microneedles are a delivery method that facilitates the compliance of dogs because they cause less pain. However, administering a conventional microneedle array patch requires the removal of hair, which is not easy and can result in grooming-related stress. IRMN required only a few seconds of administration time without the use of a patch because they were composed of dissolvable hyaluronic acid tips and biocompatible polycaprolactone bases, and the tips were instantly separated from the base during microneedle insertion. The dogs used in our study showed less anxiety and discomfort as well as less pain

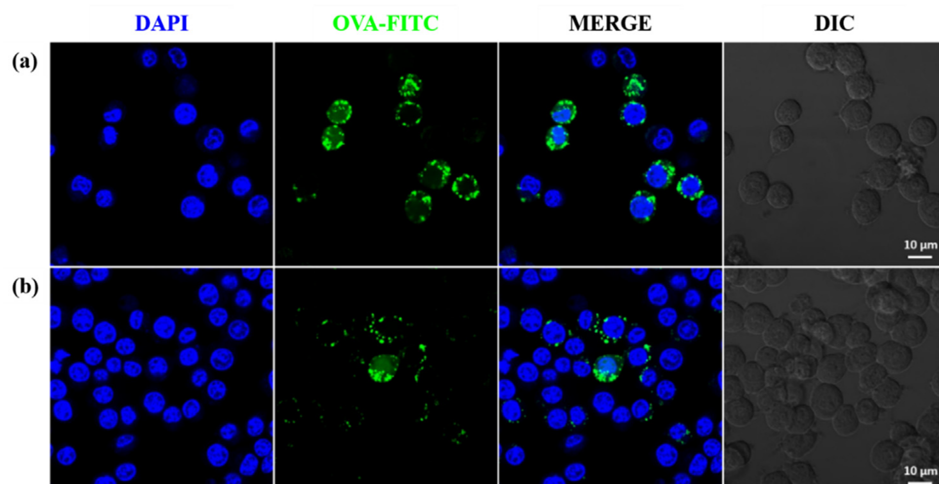

Fig. 7. Fluorescent images of RAW264.7 cells treated with a mixture of (a) OVA-FITC only and (b) OVA-FITC released from IRMN for 6 h at 37 °C. Scale bar = 10 µm. Blue and green fluorescence represents nuclei and OVA-FITC, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

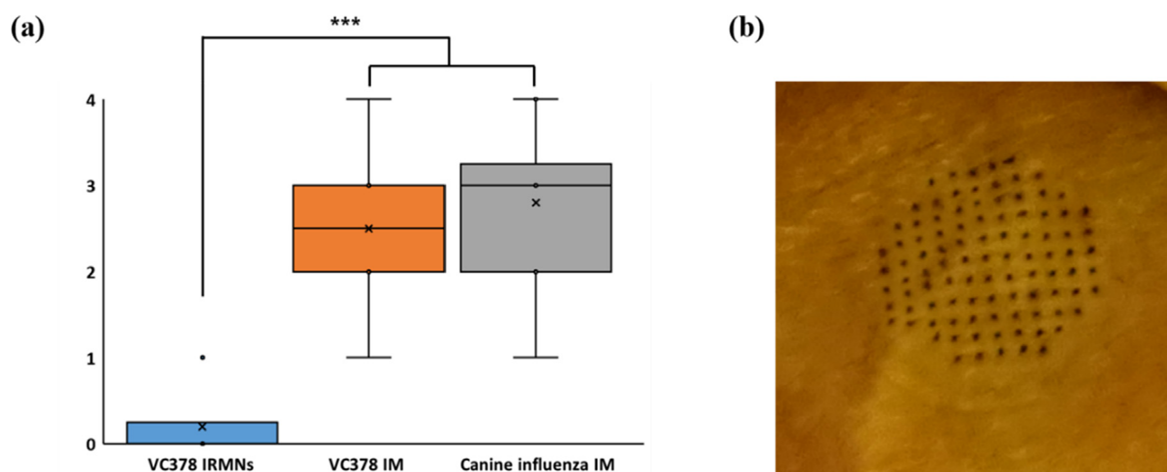

Fig. 8. (a) Canine acute pain score with insertion-responsive microneedles and intramuscular administration according to the Colorado State University Canine Acute Pain scale (0 is comfortable, 1 is slightly unsettled, 2 is uncomfortable, 3 is unsettled and 4 is screaming). Graph shows the mean ± standard deviation values. ***$p < 0.001$ (paired t test; $n = 10$/group). (b) The tips remaining inside a dog's ear after insertion of VC378-coated IRMN for 10 s.

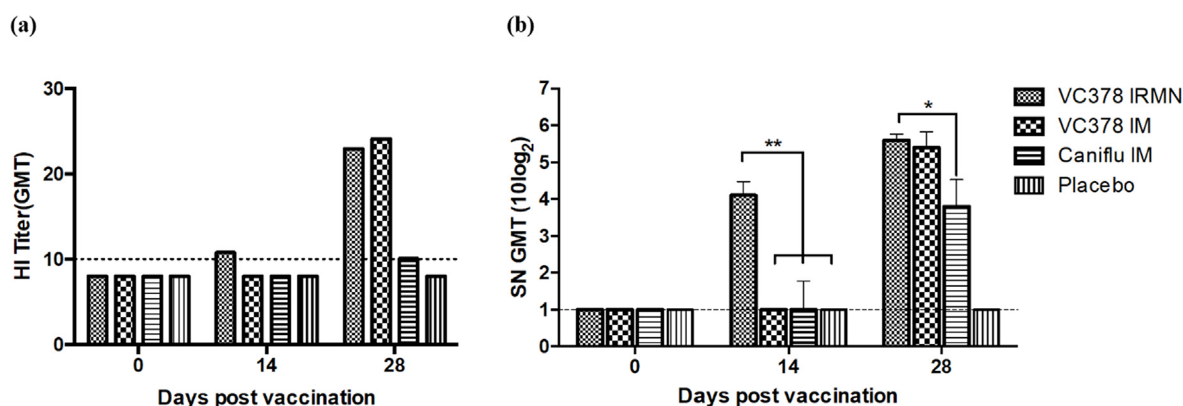

Fig. 9. Serum antibody responses in dogs administered VC378 vaccines i.m. and with IRMN. Groups of dogs were treated with VC378 IRMN, VC378 IM, Caniflu, or mock vaccination twice as described in the Materials and Methods section, and sera were collected 2 and 4 weeks after the first vaccination. Serum antibody responses were evaluated with (a) HI and (b) serum neutralization assays of the VC378 viruses. The detection limit of each test was < 10 HI. Statistical significance of VC378 IRMN compared to each vaccination was determined by a t test (*$p < 0.05$; **$p < 0.005$).

because of the brief insertion time. IRMN can also be administered without removing hair. Thus, the use of IRMN can provide both convenience and compliance for both dogs and their owners.

Vaccine-coated IRMN induced the same level of antibody titer as that induced by IM administration of the vaccine. The comparable titers of the two insertion methods showed that the administration of vaccine with IRMN was successful. Local distribution of the vaccine formulation near the tips of the IRMN reduced the inconsistent delivery of the vaccine. Microneedle tips and bases were separated immediately and successfully by the external force on the microneedles during skin insertion, as shown in the image of the base where no tips remained after separation (Fig. 6C’). However, puncture performance could be lowered if the tips were to be administered to skin covered with long and dense hair. Thus, the ear was a suitable site for vaccine administration using IRMN. The vaccine-coated tips were fully embedded in the skin within 10 s, and complete tip insertion was confirmed. However, insufficient pressure (less than 1 kg of force) on IRMN can cause incomplete insertion.

The use of IRMN is a new delivery method that can successfully deliver the desired dose of vaccine through the ears of hairy dogs. In addition to vaccines, various other drugs can also be delivered to the animals because of improved compliance. Therefore, the use of IRMN is a potential delivery system for animals in the future.

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Conflict of interest statement

BSK and PJH are inventors of patents that have been licensed to companies developing microneedle-based products, and are a shareholder of companies developing microneedle-based products.

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