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Abstract: Thymopentin (TP5) is commonly used in the treatment for autoimmune diseases, with a short plasma half-life (30 s) and a long treatment period (7 days to 6 months). It is usually administrated by syringe injection, resulting in compromised patient compliance. Dissolving microneedle array (DMNA) offers a superior approach for transdermal delivery of biological macromolecules, as it allows painless penetration through the stratum corneum and generates minimal biohazardous waste after dissolving in the skin. Despite recent advances in DMNA as a novel approach for transdermal drug delivery, problem of insufficient mechanical strength remains to be solved. In this study, TP5-loaded DMNA (TP5-DMNA) was uniquely developed using a modified two-step molding technology. The higher mechanical strength was furnished by employing bovine serum albumin (BSA) as a co-material to fabricate the needles. The obtained TP5-DMNA containing BSA displayed better skin penetration and higher drug loading efficiency than that without BSA. The *in vivo* pharmacodynamics study demonstrated that TP5-DMNA had comparative effect on immunomodulation to intravenous injection of TP5, in terms of ameliorating the CD4+/CD8+ ratio, SOD activity and MDA value to the basal level. Only mild irritation was observed at the site of administration. These results suggest that the novel TP5-DMNA utilizing BSA provides an alternative approach for convenient and safe transdermal delivery of TP5, which is a promising administration strategy for future clinical application.

Keywords: Thymopentin; dissolving microneedle array; bovine serum albumin; mechanical strength; immunomodulation.

1. Introduction

Currently, there emerged an increasing interest in the immunotherapy with proteins and polypeptides, due to their promising therapeutic efficiency. Hypodermic injection is the most common way for the delivery of these therapeutic agents, because of their unstable structure and high molecular weight [1]. However, frequent injection can lead to pain, infection and biohazardous waste, which compromise the patient compliance.

Thymopentin (TP5), a synthetic water-soluble pentapeptide (Arg-Lys-Asp-Val-Tyr), is an immunomodulating agent consisting of the 32^{nd} to 36^{th} residues of the 49 amino acid thymopoietin [2]. TP5 shows promise in promoting the differentiation of thymocytes and affecting the function of mature T-cells. Clinically, TP5 is used for treating autoimmune diseases [3] such as cancer, infections, acquired immunodeficiency syndrome [4], rheumatoid arthritis [5, 6], as well as primary and secondary immune deficiency. The commercially available product of TP5 is in the form of lyophilized powder for intramuscular or intravenous (*i.v.*) injection. And the patients have to receive frequent injections due to the long treatment period (7 days to 6 months) but very short half-life of TP5 in plasma (about 30 s) [7], which greatly reduces the patient compliance. Ideal formulation of TP5 should be readily administrated without pain, maximizing the patients' compliance and improving therapeutic effect. Previous studies about TP5 were mainly focused on the development of sustained-release delivery systems for TP5 by hypodermic injection,

such as microsphere, nanoparticle, liposome, and hydrogel, without considering pain and potential burst influence [8-10].

Recently, microneedles have been proposed as an alternative administration route for immunotherapeutic proteins and polypeptides. Microneedles (< 1 mm) can create temporary conduits in an invasion-minimized manner [11, 12]. Being directly and readily utilized in skin without pain, microneedle arrays can be self-administrated with good patient compliance [13, 14]. Dissolving microneedle array (DMNA) fabricated from biocompatible and biodegradable polymers can gradually dissolve inside the skin without generating any biohazardous waste [15, 16]. Moreover, the application of DMNA can effectively prolong the shelf-life of proteins/polypeptides, and lower the cost of cold chain processes compared with the conventional solution counterparts [17]. Therefore, DMNA can be used as potential vehicles for transdermal delivery of TP5 as a novel treatment for immunodeficiency syndrome [18, 19].

Various methods has been used to fabricate DMNA, including micromolding, high-temperature molding [20, 21], UV photo-polymerization curing [22] and aqueous solution casting [23]. The problems involved in these methods are complicated steps, and potential impairment to the stability of biomacromolecular drugs caused by the radiation, polymerizing reagent or elevated temperature utilized in these processes [13, 24]. To overcome these problems, a two-step molding technology (Fig. 1) was utilized for fabricating DMNA. However, drug concentration gradient between the needles and the base causing the drug diffusion from the needle to the base results in lower

drug-encapsulation efficiency in the needle. In our previous study, the evaporation of base solvent was found to be a key factor determining needle loading proportion (NDP), and improved NDP (90%) was achieved by replacing slow-evaporating water with highly volatile ethanol as the base solvent [25]. However, the mechanical strength of the obtained microneedles was still insufficient for porcine skin penetration.

The aim of this study was to deliver TP5 via novel transdermal route using DMNA (TP5-DMNA) with robust mechanical strength. To achieve higher mechanical strength for easier and better skin penetration of needle, the preparation of DMNA was optimised by employing BSA as a co-material. The mechanical strength of drug-free DMNA was evaluated, and the insertion capability of the TP5-DMNA in porcine cadaver skin was investigated. The immunomodulatory efficacy of this novel delivery system was confirmed via pharmacodynamic study in the induced immunosuppressed Sprague-Dawley (SD) rat model. A histological study of the administration site was also carried out to estimate the biocompatibility of TP5-DMNA.

Please insert Fig. 1 near here.

2. Materials and methods

2.1. Materials and experimental animals

Thymopentin (purity > 99.5%) was purchased from Chengdu Kaijie Biopharm Co., Ltd. (Chengdu, China). Superoxide dismutase kit and maleic dialdehyde (MDA) kit were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Bovine serum albumin, dextran (DEX) 40 and polyvinyl pyrrolidone (PVP K90) were obtained from MBCHEM Co. (New Jersey, USA), Aladdin Industrial Co. (Shanghai, China) and BASF Co. (Ludwigshafen, Germany), respectively. Polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit) was purchased from Dow Corning Co. (Midland, Michigan, USA). All other chemicals and solvents were of reagent grade and used without further purification.

Female SD rats weighted approximately 200 g were obtained from the Experimental Animal Center of Sun Yat-Sen University (Guangdong, China) and maintained under standard conditions with 12 h light/dark cycle. All the procedures used in the animal study were approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (Approval No. IACUC-DD-16-0908) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Fabrication of molds

A master mold made from brass containing 100 (10×10) microneedles with 300 μm in base diameter, 800 μm in height and 900 μm tip-to-tip space was manufactured using micromilling technique according to the exact design drawing using computer aided design (CAD). PDMS female molds were made by pouring a defined amount of PDMS solution over the brass master mold and allowing the polymer to cure at 80°C for 2 h. The master array was subsequently removed to yield the PDMS mold, which

was then repeatedly used to make dissolving microneedle arrays.

2.3. Preparation of DMNA

Different amounts of BSA as co-material were utilized for the fabrication of the drug-free DMNA. To prepare the blank needle solution, different ratios of DEX 40 and BSA (DEX solution without BSA (water : DEX = 1.00 : 0.25, v/w), and DEX solution with BSA (water : DEX : BSA = 1.00 : 0.25 : 0.03/0.05/0.075/0.1, v/w/w, respectively)) were dissolved in deionized (DI) water and kept overnight for swelling. As for the base solution, a defined amount of PVP K90 was added to ethanol (PVP K90 : ethanol = 1 : 2.5, w/v), which was also allowed to swell overnight. The DMNA was fabricated using a modified two-step molding process (Fig. 1) according to the previous report [25]. Basically, the needle solutions were poured over the PDMS mold in the first step, which was subjected to centrifugation in a swinging bucket rotor (Thermo Electron LED GmbH, Osterode, Germany) at 4000 rpm for 10 min at 4°C to completely fill the microcavities of the female mold. Then the excess needle solution was completely removed by a suitable sized spatula. For the second step, the base solution was casted on the mold and centrifuged at 3000 rpm for 5 min, and subsequently dried in an oven desiccator for 24 h at room temperature before the DMNA was peeled off from the female mold.

For the preparation of TP5-loaded DMNA, the mixture of fixed TP5 to blank needle solution ratios (0.10 : 1, 0.05 : 1, and 0.01 : 1, w/v) were allowed to dissolve

overnight at 4°C. The base solution and the preparation process were just as those of the drug-free DMNA. The obtained DMNA was gently peeled off and examined by a field emission scanning electron microscope (SEM, JSM-6330F, Jeol, Tokyo, Japan).

2.4. Mechanical strength assessment of the drug-free DMNA

Mechanical strength assessments were performed using a texture analyser (TA-XT Plus, Stable Micro Systems, London, UK). Briefly, a piece of DMNA was adhered to the probe of the texture analyser with the microneedles facing down the stainless flat. The probe was programmed to move down perpendicular to the flat at a defined speed until mechanical fracture of the DMNA occurred. The force during the whole testing process was recorded.

2.5. *In vitro* insertion assessment of the TP5-DMNA

To assess the skin insertion capability of DMNA, the prepared TP5-DMNA was inserted into full thickness porcine cadaver skin with a force of approximately 15 N using a homemade applicator and removed after 5 min. Then the treated skin surfaces were stained with trypan blue solution (0.3%, w/v). After 5 min of incubation, the solution was washed off three times completely with PBS. Ultimately, pictures of the treated sites were taken using a digital camera (Canon, Japan).

2.6. Water content of the TP5-DMNA

The water content of various TP5-DMNA was measured using a moisture analyzer (MJ33, Mettler Toledo, Switzerland). Briefly, 8 patches of TP5-DMNA (about 0.55 g) of various formulations were placed on the empty sample pan and then the drying and measuring process automatically began, ended with water content recorded. The test was repeated in triplicate.

2.7. Drug distribution in the TP5-DMNA and stability test of the encapsulated TP5

The needle tips and the base portions of DMNA were splitted using a scalpel blade and then dissolved separately in DI water. The concentration of TP5 in the samples was measured at 275 nm by RP-HPLC (LC-20AT binary pump, SIL-20A standard autosampler, SPD-20A UV detector, Shimadzu, Kyoto, Japan) with a RP C18 column (4.6 × 250 mm, 5 μm, Phenomenex, Torrance, USA) using a mobile phase consisting of acetonitrile and 0.3% trifluoroacetic acid (12 : 88, v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL and the column working temperature was kept at 30 °C. Needle drug loading proportion (NDP) was calculated by the following formula:

$$NDP(\%) = \frac{M_{\mathrm{n}}}{M_{\mathrm{n}}+M_{\mathrm{b}}} \times 100\% \quad (1)$$

where M_{n} and M_{b} presented the drug loading in the needle and base portions, respectively.

The stability of TP5 structure exposed to ethanol and BSA during fabrication process was determined using RT-HPLC [26]. TP5-DMNA was dissolved in 4 mL of DI water at 4 ℃ to release the encapsulated drug completely. Aqueous solutions of untreated TP5, BSA, DEX and physical mixture of TP5, BSA and DEX were employed for comparison. All of the samples were evaluated by RP-HPLC with the same validated method. The changes of the retention time and the pattern on the HPLC curves were evaluated.

2.8. Establishment of immunosuppression model and administration

Forty-two normal female SD rats (weight = ~200 g) were randomly divided into six groups (seven rats for each group). As the method described by Jin *et al.* [27], rats in Group 1 received intraperitoneal injection of physiological saline (35 mL/kg) once daily in the first 3 days and tail vain injection of physiological saline (1 mL/kg) in the following 7 days as the control group. Rats from Group 2 to Group 6 received intraperitoneal injection of 1 mg/mL cyclophosphamide solution (35 mg/kg) once daily for three consecutive days. In the following 7 days, rats in Group 2 were administrated by tail vain injection of physiological saline (1 mL/kg) once daily as the immunosuppression control group; rats in Group 3 were administrated with TP5 solution (100 μg/kg) once daily and Group 4/Group 5/Group 6 were given with different doses of TP5-DMNA on the skin of the inner ear once daily. Blood samples were obtained from peritoneal venous and spleen and thymus were collected 24 h after

the last administration. The organ index was calculated by the following formula:

$$\text{Organ index} = \frac{W_O}{W} \quad (2)$$

where W_O represented the weight of spleen or thymus (mg), and W represented the weight of the rat (g).

Please insert Table 1 here.

2.9. Measurement of the Lymphocyte subsets

The blood samples were processed in the following way: 100 μL of anticoagulant blood was added to a test tube containing pre-added fluorescent antibodies (FITC : CD3/PE : CD4 or FITC : CD3/PE : CD8), vortexed for 30 s, followed by incubation for 20 min at room temperature in the dark. Then 1 : 10 dilution of red cell lysis buffer was added for incubation at room temperature for another 10 min. After red blood cells were lysed completely, the cell sediments were collected by centrifugation at 2000 rpm for 5 min, washed twice with PBS, and subsequently resuspended in 0.5 mL of PBS. After staining at 0 ^{o}C for 4 h, the T-lymphocyte subsets were measured by a flow cytometry (Beckman-Cou FACS CantoTMII flow cytometer, USA). Twenty-thousand events were collected for each sample.

2.10. SOD activity and MDA assay

The superoxide dismutase (SOD) activity in plasma was tested according to the SOD kit instruction. For each assay, 100 μL of plasma sample was used and all assay

validations met the requirements of the kit instruction. The absorbance of the tested solutions was tested at 450 nm using a spectrophotometer (752-type, Shanghai, China). Plasma maleic dialdehyde (MDA) level was measured according to the MDA kit instruction. The absorbance of tested samples was read at 532 nm using the spectrophotometer (Molecular Devices, California, USA). For each assay, a volume of 100 μL plasma sample was used. MDA value was expressed as nano-moles per millilitre of plasma.

2.11. Histological study

Following the applications of the drug-free DMNA and TP5-DMNA 2, SD rats were euthanized at designated time points and ear skins were collected for histological analysis. Skin samples of DMNA-administrated sites containing both dermis and epidermis were fixed in 10% paraformaldehyde for 24 h, embedded by paraffin, stained with hematoxylin and eosin (H&E), and then observed by light microscopy.

2.12. Statistical analysis

Statistical analysis was carried out using SPSS (version 22.0, International Business Machines Corporation, New York, Armonk). For pharmaceutics study data, one-way analysis of variance (ANOVA) was used to determine the significance among groups, and subsequently post-hoc tests with the Bonferroni correction were used for comparison between individual groups. For the pharmacodynamics study data,

Kruskal-Wallis test was used for statistical analysis and post-hoc Dunn's multiple comparison tests were used for comparison between individual groups. p values < 0.05 were considered significant.

3. Results

3.1. Mechanical strength of the drug-free DMNA

Drug-free DMNA containing different amount of BSA was fabricated to evaluate the mechanical strength. As shown in Fig. 2, the DMNA experienced four stages during the moving down process. The DMNA contacted the flat and the base part was compressed in the first stage, while the force raised slowly. Subsequently, the needle part was compressed and the force increased substantially until reaching its zenith, where the peak force was recorded and considered as the maximum bearable pressure of the DMNA. Further, the needles bent and the force decrease slowly. Finally the needles broke off with the continued moving down of the probe and the force rapidly declined to zero. It was found that higher amount of BSA was favourable to improve the mechanical strength of the DMNA. Therefore, the formulation with the largest amount of BSA (water : DEX : BSA = 1.00 : 0.25 : 0.10) was adopted for further study for the most robust mechanical strength.

Please insert Fig. 2 here.

3.2 Characterization of the TP5-DMNA

The stratum corneum (SC, 15-25 μm at more likely application sites) is the main barrier to be break through by the DMNA to achieve drug delivery, thus the DMNA should be long enough and have favourable mechanical strength capable for penetrating through SC [28-29]. In this study, a series of TP5-DMNA were produced using identical PDMS female mold by the modified two-step molding process (Table 2). The SEM images of magnified individual needles showed the manufactured microneedles were cones of 800 μm in height without any cavities (Fig. 3A-F), indicating that the loaded drug concentration and the utilization of ethanol as the base solvent had little effect on the needles' morphology. Fig. 3G-H displayed the overall images of TP5-DMNA 2 with or without BSA. The needles were 10×10 with 300 μm in base diameter, 800 μm in height and 900 μm in tip-to-tip space, uniformly distributing on the PVP K90 substrate, suggesting the successful fabrication of the TP5-DMNA with consistent geometric dimensions.

Please insert Fig. 3 here.

3.3. *In vitro* skin insertion of the TP5-DMNA

The drug-loaded DMNA must have sufficient mechanical strength to effectively overcome the SC barrier for successful transdermal drug delivery. In this study, TP5-DMNA was inserted into the porcine skin and removed after 5 min. The pierced skin was stained with a tissue marking dye (trypan blue solution). The representative

photographs of the stained porcine skin surface were shown in Fig. 4. The 10×10 array of blue spots in correspondence to the microneedles penetration sites indicated that the TP5-DMNA containing BSA could completely create conduits and release the loaded drug into the skin (Fig. 4D-F). In contrast, Fig. 4A-C showed no obvious blue spots remaining in the skin, suggesting the insufficient skin penetration of DMNA without BSA.

Please insert Fig. 4 here.

3.4. Water content of the TP5-DMNA

As displayed in Table 2, the water content of all the formulations was recorded less than 5%, which was beneficial for the long-term storage of TP5-DMNA.

Please insert Table 2 here.

3.5. Drug loading amount and drug distribution in different formulations of TP5-DMNA

The ethanol was used as a base solvent in the modified two-step molding technology for DMNA preparation. The drug loading amounts and NDP in different formulations were shown in Table 3 and Fig. 5, respectively. And as shown in Fig. 5, the NDP of all the formulations using ethanol was over 55%, overtaking that using DI water as a base solvent [25]. The NDP of the formulations containing BSA was recorded approximately 33% higher at 10% concentration of TP5 when compared with

DMNA without BSA ($p < 0.001$). However, no significant difference in NDP was found between the formulations with and without BSA containing low concentrations of TP5 (5% and 1%).

Please insert Table 3 here.

Please insert Fig. 5 here.

3.6. Stability of the encapsulated TP5

Since a series of processes and highly volatile solvent were included in the DMNA preparation, it was necessary to determine whether the preparation procedures as well as the addition of BSA would affect the unstable molecular structure of TP5. The TP5 released from the TP5-DMNA was evaluated by RP-HPLC. Fig. 6 showed the chromatograms of untreated TP5 solution, DEX solution, BSA solution, aqueous solution of physical mixture of TP5, DEX and BSA, and TP5 encapsulated in DMNA. No remarkable change was observed in the retention time and the characteristic absorption band of TP5 in various samples, without any artifactual bands existing in the chromatograms except for solvent signal, indicating that the preparation process of the TP5-DMNA and the addition of BSA were non-destructive to the fragile biomolecules.

Please insert Fig. 6 here.

3.7. Measurement of peripheral blood T-lymphocyte subsets

In immunosuppression models, the CD3+CD4+ and CD3+CD8+ T-lymphocyte subset values in blood are usually found abnormal with altered CD4+/CD8+ ratio. Therefore, the lymphocyte subsets in immunosuppression rats after 7 days' treatment of TP5 were determined by a flow cytometry to evaluate the immunomodulatory efficiency. As revealed in Table 4, in the peripheral blood of immunosuppression rats (Group 2), the T-lymphocyte percentage of CD3+CD8+ was significantly lower than that in the normal group (Group 1) ($p < 0.01$). And the percentage of CD3+CD4+ slightly decreased, whilst a significant increase in CD4+/CD8+ ratio was observed ($p < 0.001$), indicating that the successful establishment of the immunosuppression models. After 7 days of treatment with TP5-DMNA 1/2 (Group 4/5), the CD3+CD4+, CD3+CD8+ value were increased obviously and the CD4+/CD8+ ratio was reversed close to the normal level, showing significant difference compared with those of Group 2 (immunosuppression group), demonstrating that the achievement of desired therapeutic effects. Meanwhile, Group 3 (TP5-injection) and Group 6 (TP5-DMNA3) realized similar regulatory levels for CD3+CD4+, CD3+CD8+ and CD4+/CD8+. It was indicated that TP5-DMNA performed similarly with intravenous injection, while better immunomodulation could be achieved with higher dose of TP5.

Please insert Table 4 here.

3.8. SOD activity and MDA content

SOD activity and MDA level, which reflect the anti-aging function of living body, are often used to assess the body responses to oxide free radical. As described in Fig. 7, both SOD activity and MDA level in immunosuppression rats after treatment of p hysiological saline and various TP5 formulations for 7 days differed from each other. Compared with the normal group (Group 1) treated with physiological saline, the SOD activity of immunosuppression group (Group 2) decreased to 77.98 ± 3.78 U/mL while the MDA level increased to 6.30 ± 0. 39 nmol/mL, indicating that the establishment of immunosuppression models ($p < 0.05$ for the SOD activity). An obvious increase in SOD value occurred after administration of different TP5 formulations. In particular, the SOD value of TP5-DMNA1/2 (Group 4-5) returned to normal level ($p < 0.001$ compared to Group 2). Simutaneously, the corresponding MDA level obviously decreased after treatment with TP5 solution or TP5-DMNA. These results suggested that TP5-DMNA was helpful for improving SOD activity and reduction of MDA value, resulting in the elimination of free radicals in immunosuppression rats.

Please insert Fig. 7 here.

3.9. Weight changes of the immune organs

Since cyclophosphamide could cause weight reduction of immune organs in the establishment of immunosuppression model, the thymus index and spleen index were utilized as indicators of health status of typical immune organs. As shown in Fig. 8, the

thymus index and spleen index of the immunosuppression group (Group 2) significantly decreased compared with those of the normal group (Group 1) ($p < 0.01$ for thymus index and $p < 0.001$ for spleen index). Intravenous injection of TP5 solution could adjust the immune organ indexes close to normal level, and the indexes of rats in several TP5-DMNA groups also recovered from the immunosuppressive intervention.

Please insert Fig. 8 here.

3.10. Histological study of the administration sites

A histological study of administration site was carried out to estimate the biocompatibility of the drug-free DMNA and the TP5-DMNA using BSA as a co-material of the needles. As shown in Fig. 9, layers including stratum corneum, epidermal and dermal as well as cartilage and outer side of the ear could clearly be observed.The thickness of stratum corneum and epidermal were not changed after the administration of free-DMNA or TP5-DMNA 2. Only mild edemas appeared in the drug-free DMNA group (< 25%/HPF) (Fig. 9 A), while milder edemas (5-10%/HPF) and slight local lymphocytic infiltration (5-10% /HPF) were detected in TP5-DMNA 2 group (Fig. 9 B). The inflammatory reaction disappeared in one day for both groups (Fig. 9 E-F). It was indicated from the pathological study of that the insertion of the DMNAs into the ear skin induced very slight mechanical damage and caused weak inflammatory reaction, which could recover completely in one day. And the addition

of BSA as mechanical strength regulator and TP5 as therapeutic agent didn't cause any extra inflammation.

Please insert Fig. 9 here.

4. Discussion

Therapeutic biomolecules such as proteins and polypeptides have been extensively utilized in the treatment of many diseases due to their promising efficiency. Dissolving microneedles are being developed to provide a potential alternative to the traditional injection of proteins and polypeptides, with convenient and painless administration. Herein, TP5 was encapsulated in dissolving microneedles using a modified two-step molding technology for the first time.

Although DEX was previously utilized in microneedles fabrication, the mechanical strength of DMNA fabricated from DEX-BSA composition was found higher than the former one (Fig. 2). And TP5-DMNA made up of DEX could not create enough microconduits in the skin compared with TP5-DMNA made up of DEX-BSA composition (Fig. 4). Both of the evaluations suggested that the addition of BSA as a co-material could significantly enhance the mechanical strength of the microneedles for better skin penetration. As a slender and high aspect ratio geometricized device, DMNA fabricated without BSA is more likely to buckle, which is the major cause for mechanical failure [30]. According to Euler's buckling formula

for cylindrical column, the critical buckling load of microneedle (*F*) can be depicted as follows:

$$F = \frac{E\pi^3 d^4}{64(kL)^2} \quad (3)$$

where *E* is the elasticity factor of a material described by Young's modulus, *d* represents the area moment of inertia which is dependent on geometry, *k* is the column effective factor that is determined by the boundary condition and *L* is the unsupported length of the column. Since all of the microneedles were fabricated from the same mold, only *E* values of DEX and DEX/BSA microneedles were different. Due to the *E* value limit of DEX solution, the addition of another material was expected to change the *E* value of the microneedle materials [30]. BSA is a serum albumin protein with molecular weight of 66.5 KD and has a complex three dimensional structure, which can effectively improve the *E* value and thus increases the *F* value higher than the insertion force.

The therapeutic efficacy of DMNA is realized by the effective drug delivery through needles. Therefore, needle drug proportion (NDP) is an important index to evaluate the drug distribution in the microneedles. It was shown in Fig. 5 that higher NDP was obtained in the condition of high drug concentration with the addition of BSA, which might be attributed to the restricted drug migration caused by the higher viscosity of the DEX/BSA solution.

In this study, ethanol was used as the solvent of microneedle base to enhance the drug distribution proportion in needle due to its high evaporation rate [25]. However,

the effects of ethanol diffusion on protein or polypeptide characteristics remained unexplored. Herein, RP-HPLC was employed to determine the change in the structure of TP5 encapsulated in the DMNA, compared with that of untreated TP5 and physical mixture of TP5, DEX and BSA. No significant difference in the chromatograms of different TP5 samples was found (Fig. 6). That may be contributed to the rapid evaporation of ethanol, significantly reducing the exposure time, and meanwhile there was no interaction occurring between BSA and TP5.

T lymphocyte subsets ratio (CD4+/CD8+) was employed in this study to evaluate the immune function. The human peripheral blood T lymphocytes can be divided into four subsets according to the cell surface phenotype and function, i.e., inducer-helper T cell (Th), cytotoxic tell (Tc), delayer type hypersensitivity T cells (Td), and suppressor T cell (Ts) [31]. T lymphocytes with CD4+ and CD8+ markers are Th and Tc cells, respectively. When the rats receive immunosuppressive agent cyclophosphamide, the CD4+/CD8+ ratio in the peripheral blood increases due to the sensitivity difference of T-lymphocyte subsets. As shown in Table 4, the CD4+/CD8+ ratio displayed a remarkable increase in immunosuppression rats compared with that of normal rats. After 7-day treatment with various TP5 formulations, both TP5 solution injected through tail vein and TP5-DMNA with different doses of TP5 were effective for improving the decreased CD4+ and CD8+ count and subsequently reversed the abnormal CD4+/CD8+ ratio to the normal value. When compared with the study conducted by Jing Wang *et al.* [32], in which TP5 was administrated intranasally with

various enhancers, the CD4+/CD8+ ratio in the present study was within the reasonable range. The low dose TP5-DMNA (TP5-DMNA 3) showed an equivalent effect of immunoregulation with intravenous injection in 7 days and higher dose of TP5 delivered by DMNA (TP5-DMNA 1/2) could realize better therapeutic efficiency. The results indicated that TP5-DMNA was a potential clinical dosage form for effective immunoregulation which could avoid the disadvantages of needle injection such as pain and possible infection.

Both the activity of SOD and the concentration of MDA in peripheral blood reflect the anti-aging function of the organism. SOD provides important defense against the toxicity of superoxide radical by catalyzing the superoxide radical into hydrogen peroxide and elemental oxygen. The MDA concentration is a biomarker that indicates the level of lipid peroxidation. In immunosuppression rats, the SOD activity decreased remarkably while the MDA value increased significantly, which was consistent with the reported literature [33]. As shown in Fig. 7, the SOD activity of TP5-DMNA 3 appeared to be similar with that of the injection solution of TP5, while those of the middle and high dose groups presented notably higher activity. The change of SOD activity seemed more sensitive than that of the organ indexes and CD4+/CD8+ ratio, hence it was suggested to be used as the elementary pharmacological index of TP5.

As revealed in previous studies [34-36], the skin irritation caused by solid mircroneedles and hollow microneedles through accession of transepidermal waterloss

(TEWL) or blood flow was proved to be transient and mild. In this study, the irritation caused by TP5-DMNA was evaluated by the histological section patterns. Both drug-free DMNA and TP5-DMNA induced slight inflammation reaction at different degrees after administration, possibly explained by the exogeneity of BSA and TP5 included in the formulation. Compared with the drug-free DMNA, a little inflammatory cell infiltration was caused by TP5-DMNA, which might be helpful for recruiting immunocyte to the site of irritation and initiating an immune response. Moreover, the lymphocytic infiltration was gradually vanished and the skin recovered completely in one day. Therefore, the histological study demonstrated that TP5-DMNA was biocompatible and capable of dissolving within the skin.

5. Conclusion

In this study, novel DMNA containing TP5 was developed by a modified two-step molding technology using BSA as a mechanical strength regulator. The obtained TP5-DMNA was 10×10 microneedles with 300 μm in base diameter, 800 μm in height, and 900 μm tip-to-tip space uniformly distributing on the PVP K90 base. The mechanical strength of TP5-DMNA was significantly improved by the addition of BSA without reducing the NDP. The HPLC chromatograms demonstrated the structure of TP5 remained integrate after encapsulation in the DMNA. Furthermore, *in vivo* pharmacodynamics study revealed that the TP5-DMNA resulted in comparative immunomodulatory efficiency to intravenous injection of TP5. Additionally, the mild

irritation observed in the pathological slice of the administrated sites confirmed good biocompatibility of the TP5-DMNA. In conclusion, the proposed TP5-DMNA is a promising alternative to syringe injection in future therapy, because of its safety, convenience and equivalent therapeutic efficiency.

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Declaration of interest

The authors stated that there was no conflict of interest in the preparation of this paper.

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Figure captions:

Fig. 1. Fabrication process of dissolving microneedle array by a modified two-step molding technology.

Fig. 2. Mechanical strength of the drug-free DMNA.

Fig. 3. SEM images of microneedles: (A-C) Individual needle of TP5-DMNA 1/2/3 with BSA, respectively; (D-F) Individual needle of TP5-DMNA 1/2/3 without BSA, respectively; (G-H) Overall array of TP5-DMNA 2 with/without BSA, respectively.

Fig. 4. Skin insertion experiment of TP5-DMNA: (A-C) TP5-DMNA 1/2/3 without BSA, respectively; (D-E) TP5-DMNA 1/2/3 with BSA, respectively.

Fig. 5. Needle drug loading proportion of the TP5-DMNA (n = 5).

Fig. 6. Chromatograms of different samples.

Fig. 7. SOD activity and MDA value of normal rats or immunosuppression rats administrated with various TP5 formulations for 7 days (n = 7). *p < 0.05 vs Group 1 (normal group); &p < 0.05 vs Group 2 (immunosuppression group); &&p < 0.01 vs Group 2; &&&p < 0.001 vs Group 2.

Fig. 8. Thymus index and spleen index of normal rats or immunosuppression rats administrated with various TP5 formulations for 7 days (n = 7). *p < 0.05 vs Group 1 (normal group); **p < 0.01 vs Group 1; ***p < 0.001 vs Group 1; &p < 0.05 vs Group 2 (immunosuppression group); &&p < 0.01 vs Group 2.

Fig. 9. Histological examination of H&E staining ears of SD rats (A) 0 min, (C) 90 min, and (E) 1 day after administration of the drug-free DMNA; and (B) 0 min, (D) 90 min and (F) 1 day after administration of TP5-DMNA 2.

Table captions:

Table 1. Treatments of different groups of rats (n = 7).

Table 2. Water content of the TP5-DMNA (n = 3).

Table 3. Formulations of the TP5-DMNA (n = 5).

Table 4. The values of T-lymphocytes subsets in blood samples of rats in different groups (n = 7).

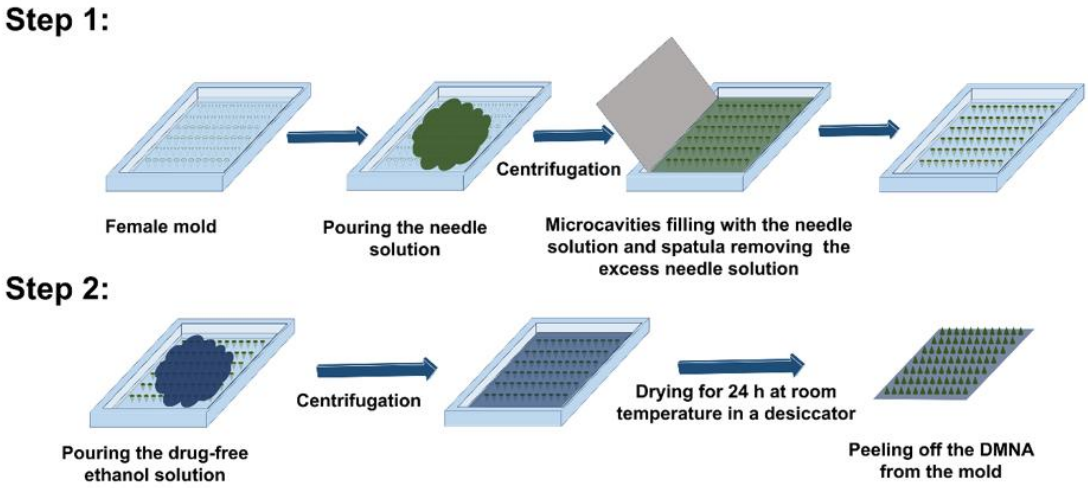
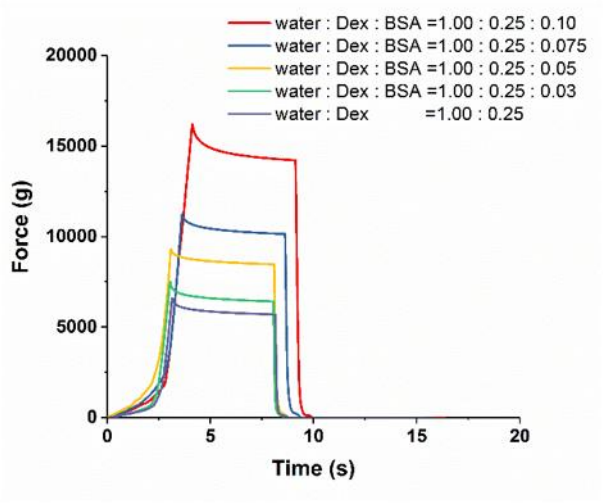


Fig. 1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water : Dex : BSA =1.00 : 0.25 : 0.075
water : Dex : BSA =1.00 : 0.25 : 0.05
water : Dex : BSA =1.00 : 0.25 : 0.03
water : Dex =1.00 : 0.25
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15000
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15
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Fig. 2

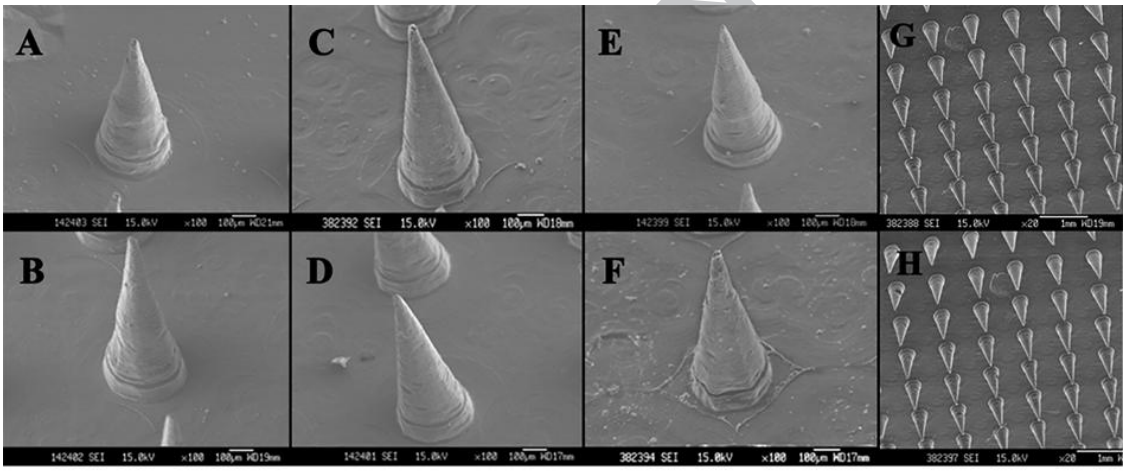
A
C
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Fig. 3

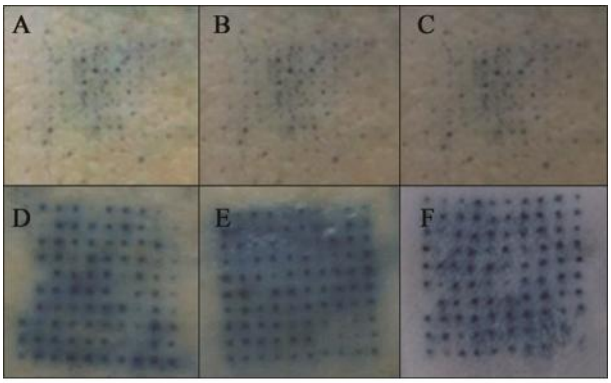

Fig. 4

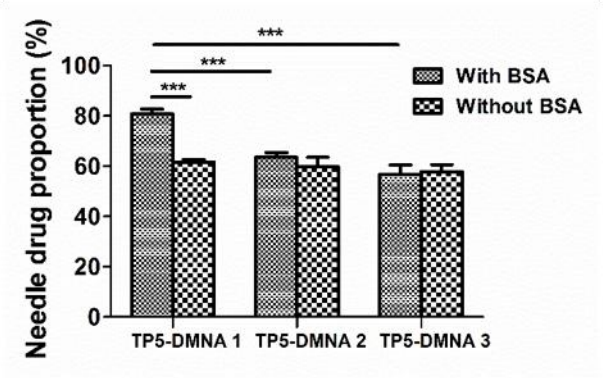

Fig. 5

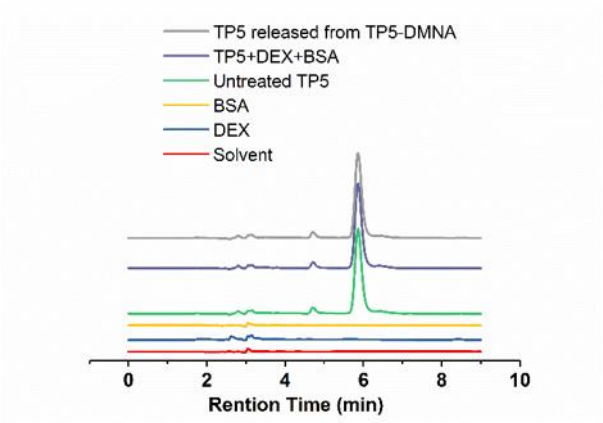

Fig. 6

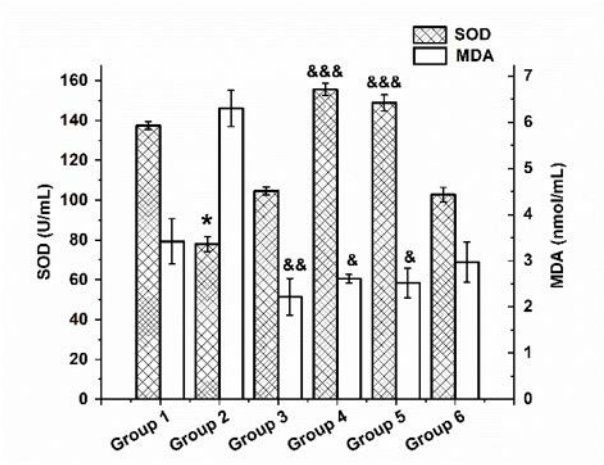

Fig. 7

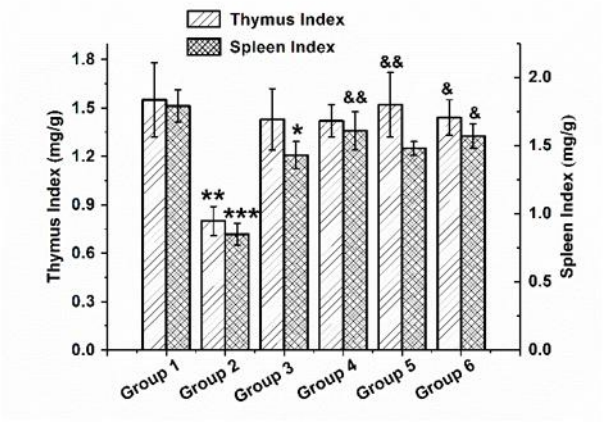

Fig. 8

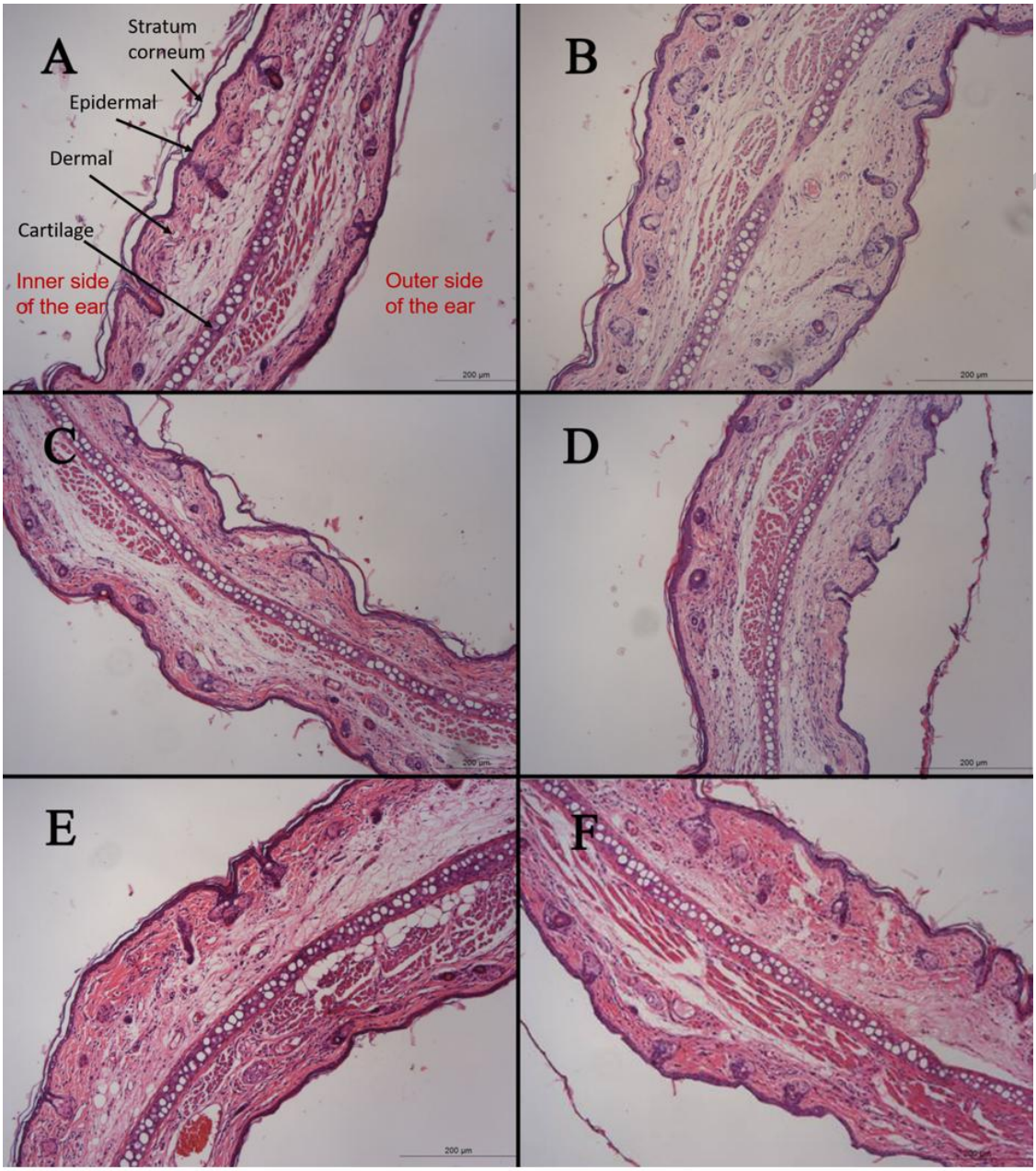
A
Stratum corneum
Epidermal
Dermal
Cartilage
Inner side of the ear
Outer side of the ear
B
C
D
E
F

Fig. 9

Table 1. Treatments of different groups of rats (n = 7).

Group	Annotation	Administration of TP5
1	Normal group	Physiological saline (1 mL/kg), *i.v.*
2	Immunosuppression group	Physiological saline (1 mL/kg), *i.v.*
3	TP5-treated groups	TP5 solution (100 μg/kg), *i.v.*
4		TP5-DMNA 1 (850 μg/kg)
5		TP5-DMNA 2 (300 μg/kg)
6		TP5-DMNA 3 (100 μg/kg)

Table 2. Water content of the TP5-DMNA (n = 3).

Formulation	Water content (%)
TP5-DMNA 1	4.85 ± 0.21
TP5-DMNA 2	3.89 ± 0.23
TP5-DMNA 3	4.07 ± 0.07

Table 3. Formulations of the TP5-DMNA (n = 5).

Formulation	Composition (water : DEX : BSA: TP5)	Drug loading amount (μg)

TP5-DMNA 1	1 : 0.25 : 0.10 : 0.10	170.56 ± 15.14
TP5-DMNA 2	1 : 0.25 : 0.10 : 0.05	60.32 ± 22.12
TP5-DMNA 3	1 : 0.25 : 0.10 : 0.01	21.68 ± 0.51

Table 4. The values of T-lymphocytes subsets in blood samples of rats in different groups (n = 7).

Group	CD3+CD4+ (%)	CD3+CD8+ (%)	CD4+/CD8+
1	44.23 ± 2.30	38.45 ± 1.74	1.16 ± 0.06
2	39.46 ± 1.08	19.68 ± 1.15 **	2.01 ± 0.12***
3	42.18 ± 1.03	33.68 ± 0.91	1.25 ± 0.04
4	49.85 ± 3.26 $^{\&\&\&}$	40.85 ± 1.75$^{\&\&\&}$	1.22 ± 0.12 $^{\&}$
5	48.34 ± 2.00 $^{\&\&\&}$	40.02 ± 0.79$^{\&\&\&}$	1.20 ± 0.06 $^{\&}$
6	41.28 ± 1.32	34.47 ± 1.32	1.19 ± 0.04 $^{\&}$

**p < 0.01 vs Group 1 (normal group); ***p < 0.001 vs Group 1; $^{\&}p$ < 0.05 vs Group 2 (immunosuppression group); $^{\&\&\&}p$ < 0.001 vs Group 2.

Highlights:

- Novel dissolving microneedle array encapsulating thymopentin (TP5-DMNA) was originally developed using a modified two-step molding technology for immunomodulation.
- BSA was used as co-material to fabricate the microneedles for higher mechanical strength.
- The obtained TP5-DMNA showed equivalent immunomodulation efficiency compared with intravenous injection.
- Unlike commonly used intramuscular or intravenous injection, TP5-DMNA can be self-administrated and deliver TP5 in a manner of minimal pain, ultimately resulting in good patient compliance.

Graphical abstract