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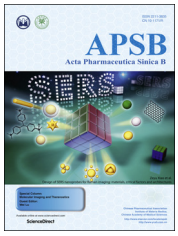

ORIGINAL ARTICLE

Synergistic immunoreaction of acupuncture-like dissolving microneedles containing thymopentin at acupoints in immune-suppressed rats

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transdermal drug delivery;
Acupuncture

Abstract Dissolving microneedles carried drug molecules can effectively penetrate the stratum corneum of skin to improve the transdermal drug delivery. The traditional Chinese medicine acupuncture is based on the needle stimulation at a specific location (acupoint) to generate and transmit biochemical and physiological signals which alter the pathophysiological state of patients. However, the pain associated with conventional acupuncture needles and the requirement of highly trained professionals limit the development of acupuncture in non-Asian countries. The purpose of this study is to investigate whether the dissolving microneedles can be utilized as a self-administered painless replacement for acupuncture and locally released drug molecules can achieve expected therapeutic outcomes. Immunosuppressive rats were treated with acupuncture at Zusanli (ST36) acupoint using microneedles containing thymopentin. The immune functions and psychological mood of the immunosuppressed animals were examined. The proliferation of splenocytes was examined by CCK-8 assay. CD4 and CD8 expression patterns in spleen cells were detected by flow cytometry. The current study showed that use of either microneedles containing thymopentin or conventional acupuncture both resulted in immune cell proliferation, which was confirmed by flow cytometry. Furthermore, either conventional acupuncture or microneedles were able to effectively mitigate the anxiety caused by immune-suppression when applied on the ST36.

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1. Introduction

The immune system plays an important role in protecting against diseases[1]. Immunosuppression can be caused by malignant tumors, infertility, cerebrovascular diseases, AIDS and other conditions[2] in which patient's immune function is weakened as evidenced by the decreased number of immunocompetent cells, abnormal cytokine expression and secretion and increased activities of immunosuppressive factors. As the onset of compromised immune function weakens the body's defenses, some fungi which are usually non-pathogenic for immunocompetent humans can cause severe infections in immunocompromised patients[3]. In addition, immunocompromised patients are often psychologically depressed[4,5].

Acupuncture is one of the important treatments of traditional Chinese medicine in China and some Asian countries and is becoming an alternative treatment in western countries for many diseases which are difficult to cure, including pain, asthma and major depression[6]. A large number of experimental and clinical studies confirmed that acupuncture could bidirectionally (up- and/or down-) regulate the immune system[1]. In the acupuncture treatment, the meridian and acupoints are the basis for effective acupuncture. Acupuncture treatment is actually the stimulation of the meridian and the acupoints. The acupoint injection[7], acupressure[8], electrical stimulation[9] and other a variety of alternatives of acupuncture are known to achieve a good clinical treatment effect.

Zusanli (ST36) is an important acupoint on the meridian of the human body and many treatments involve this point. It is an acupoint located at the posterolateral knee and about 5 cm (in human) under the fibula capitulum, and is related to the stomach, hence being named ST by the World Federation of Acupuncture-Moxibustion Societies[10]. According to the acupuncture meridian chart, immune functions could be regulated by the stomach and spleen which are the conceptual parts of human body and may not be exactly equivalent to anatomical organs defined by modern medicine. To treat and prevent diseases, acupuncture and moxibustion at ST36 are common practices in China and Japan, Koreas and Southeast Asian countries. A large number of studies have confirmed that acupuncture at ST36 can regulate immune functions which are both holistic and bidirectional, *i.e.*, achieving overall improvement of pathophysiological states by either up- or down-regulating patient's immune functions. It has been shown that acupuncture at ST36 can increase serum levels of immunoglobulin, lectins, hemolysin and antibodies, the number of antibody-forming cells and complements. ST36 has been widely used in the treatment of many conditions such as immunocompromised or immunodeficiency patients, and patients with hyperthyroidism disease. It has been shown that either needle stimulation of ST36 or injecting antiviral agents at ST36 can reduce the level of aminotransferase activity associated with hepatitis viruses in hepatitis patients. In addition, hepatitis viral infection leads to elevated levels of serum IgG by increasing total contents of complement C3, C4, B factor and decreasing immune complexes. It was shown that the overly expressed IgG was significantly reduced to a normal level and hepatitis B induced liver damage was repaired following acupuncture at ST36[11]. For older patients acupuncture at the ST36 point could increase their serum levels of IgA, IgG and IgM, thereby enhancing the immune functions to prevent the invasion of pathogens and to speed up recovery[1,12]. The stimulation at ST36 site was found to significantly increase the levels of white blood cells, neutrophils, erythrocytes and thrombocytes caused by conventional chemotherapy in patients with malignant tumors[13]. Therefore, acupuncture at ST36 can be an auxiliary treatment to alleviate side effects caused by chemotherapy and improve the quality of life of cancer patients. However, traditional acupuncture requires hypodermic needles that can cause needle phobia and generate biohazardous wastes. It also requires specifically trained personnel.

A novel microneedle-mediated transdermal drug delivery has received increasing attention in recent years[14]. Microneedle (MN) technology offers a distinctive method of cutaneous drug delivery. It painlessly pierces into the skin to administer drug in a minimally invasive and targeted manner[15]. Another advantage of MN is its potential for self-administration which can considerably increase patients' compliance[16]. Furthermore, MN avoids the first-pass effect, especially valuable for macromolecular biological agents such as proteins and polypeptides. Using MN technology, drug can be delivered through the stratum corneum of the skin in diversified forms such as drug-coated MNs, drug-encapsulated MNs, hollow MNs, or application of the drug on skin prior to being punctured with MNs[14,17–19]. Dissolving microneedle array (DMNA) patches, a new form of MN for transdermal delivery, dissolve in the body fluid as soon as the array is inserted into the skin, and the encapsulated drug is released as the needles dissolve. Moreover, DMNA made from biopolymers are low in cost and have no safety concerns associated with the risk of breakage like silicon and metal needle[20].

Therefore, the purpose of this study is to investigate whether the dissolving microneedles can be utilized as a self-administered painless replacement for acupuncture and locally released drug molecules with expected therapeutic outcomes. A thymic pentapeptide, thymopentin (TP5), was used as a model drug. TP5 loaded DMNAs composed of dissolving polymers were designed to percutaneously deliver TP5, an immune-stimulant, to the ST36 in immunosuppressed rats and the effects on immuno-response and behaviors of the animals were studied.

2. Methods

2.1. *Fabrication of DMNAs to encapsulate TP5*

DMNAs were prepared by a two-step micro-molding process described previously[21]. Briefly, the female mold was first made from polydimethylsiloxane (PDMS, Meryer Co., Ltd., Shanghai, China) by exactly inversely replicating the master structure of a copper-alloy made master mold as shown in Fig. 1. The female mold consisted of 100 (10 × 10) conical microcavities and the dimension of each cavity was 300 μm in base diameter and 800 μm in needle height. One gram of dextran (molecular weight 40,000, Aladdin Co. Ltd., Shanghai, China) was dissolved in 2 mL of deionized water to form a blank needle solution. To prepare TP5 (KaiJie Peptide Co., Ltd., Chengdu, China) loaded DMNAs, a solution containing 1 g dextran in 4 mL deionized water was prepared. TP5 (0.8 g) was dissolved in the solution to form TP5 loaded needle solutions. The base solution was prepared by adding PVP K90 (1.0 g) into 2.7 mL of ethanol and the preparation was left overnight. The blank needle solution or TP5 loaded needle solution prepared above was poured over the female mold, and then centrifuged at 4000 × *g* (Thermo Electron LED GmbH, Osterode, Germany) for 15 min at 4 °C to completely fill microcavities of the mold. Excess solution on the surface of the mold was removed. Next, the base solution was placed on the mold in which microcavities were already filled with the needle solution, then centrifuged at 4000 × *g* for 15 min and dried for 12 h at room

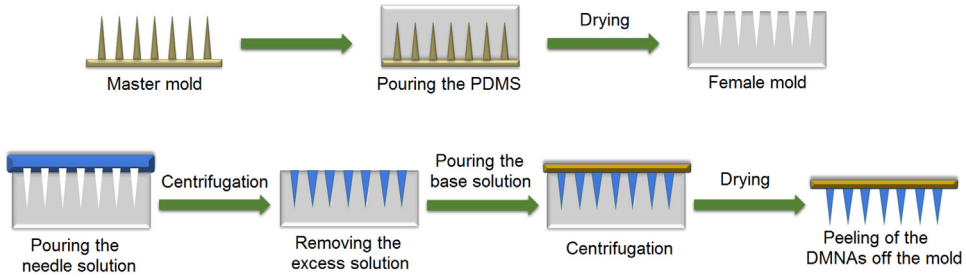

Figure 1 Schematic diagrams of making DMNAs.

temperature. Finally, the blank and TP5-loaded DMNAs were gently peeled off from the mold. The area of the obtained dissolving microneedle array was 153.76 mm^2.

2.2. *Characterization of TP5-loaded DMNAs*

To evaluate suitability for transdermal administration, the properties of TP5-loaded DMNAs, including physical form, mechanical strength, *in vitro* drug release and dissolution, were examined. The physical morphology of TP5-loaded DMNAs were observed by scanning electron microscope (SEM). The needle mechanical strength of TP5-loaded DMNAs was tested by a texture analyzer (TA-XT plus, Stable Micro Systems, Godalming, UK).

Given that each acupuncture procedure lasts about 20 min[22], blank and TP5-loaded DMNAs were manually applied onto depilated rat skin. At different time intervals, the base of DMNAs was removed from the rat and the remaining needles were visually counted under a microscope to determine the dissolution ability. The *in vitro* drug release study was conducted at 37 °C using phosphate buffer solution (pH 5.8) as the medium.

After TP5-loaded DMNA was made, all needles were cut and dissolved completely in deionized water. The amount of TP5 was analyzed by high performance of liquid chromatography (HPLC, Shimadzu Co., Kyoto, Japan) using a reverse phase column (C18, 250 mm × 4.6 mm) under the following conditions: the mobile phase of 50 mmol/L PBS (pH 7.0)/MeOH: 85/15, temperature of 26 °C and flow rate of 1.0 mL/min. Percentage of TP5-loading efficiency was calculated using the quantity of TP5 obtained by HPLC analysis divided by the theoretical quantity based on the total volume of mold microcavities.

2.3. *DMNA therapy in immunosuppressive rats*

All procedures were in accordance with National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals and approved by the Ethical Committee on Animal Experimentation at Sun Yat-Sen University (Guangzhou, China). Eight-week-old male Sprague–Dawley rats purchased from the Sun Yat-Sen University Animal Experiment Center (Guangzhou, China) were housed at room temperature (22 ± 1 °C) at up to 5 rats per cage under specific-pathogen-free conditions, and with free access to food and water.

Forty-eight rats were randomly divided into eight groups with 6 per group: 1) healthy rats (healthy); 2) rats with no treatment (control); 3) rats subject to acupuncture needles at ST36 site (acupuncture); 4) rats subject to blank DMNAs at ST36 site (DMNA); 5) rats subject to TP5-loaded DMNA with lower dose at ST36 site (TP5-L DMNA on acupoint); 6) rats subject to TP5-loaded DMNA with normal dose at ST36 site (TP5-N DMNA on acupoint); 7) rats subject to TP5-L-loaded DMNA on back of the rat (TP5-L DMNA on back); 8) rats subject to TP5-N-loaded DMNA on back of the rat (TP5-N DMMA on back). To create an immuno-suppressed rat model, each rat in Groups 2–8 was injected intraperitoneally with freshly prepared cyclophosphamide solution (1 mg/mL of normal saline), a chemotherapeutic agent, at a dose of 30 mg/kg for 3 days, and rats in Group 1 received same amount of normal saline. Cyclophosphamide is known to cause immunosuppression at higher doses (20–100 mg/kg)[23].

The bilateral (both legs) ST36 acupoints in rats were selected according to well-established criteria[10,24]. The two ST36 acupoints are located at each side of the posterolateral knee and about 5 mm under the fibula capitulum. On the day of experiment, animals were lightly anesthetized for about 20–30 min by inhaling diethyl ether. After shaving off the hair around the ST36 sites on both legs, conventional acupuncture needles and DMNAs containing two different doses of TP5 (TP5-L and TP5-N) were pierced into the ST36 sites for 20 min. Treatments were repeated daily for the next seven days.

2.4. *Open field activity test*

At the end of acupuncture treatment (day 8), rats were subjected to an open field test (OFT), an ethologically based paradigm providing objective measures of exploratory behavior as well as a valid initial screening for anxiety-related behavior in rodents. The apparatus consisted of a white PVC arena (50 cm × 50 cm × 20 cm) divided into 25 squares (10 cm × 10 cm). The 9 central squares were defined as the “center” region. Rat to be tested was placed in a corner square facing the wall. The total number of vertical movement and center crossing were recorded for 3 min. The vertical movement represents upright position using rear paws (both front paws off the ground), and the center crossing number represents total number of squares crossed the center region (all four paws in the next square). The walls and floor of the arena were thoroughly cleaned between experiments.

2.5. *Proliferation of rat spleen cells*

At the completion of OFT study, rats were terminated and spleen was removed, homogenized and centrifuged at 1500 rpm for 5 min. Supernatant was discarded and the spleen cells were washed with red blood cell lysis buffer. Lysis was stopped by washing the spleen cells with complete medium containing 2% fetal bovine serum. The obtained cells were diluted with complete medium. Ninety μL cell suspension (5×10^6/mL for all other groups and 3×10^6/mL for Group 1 rats) was placed in each well of a 96-well microplate. The lymphocyte transformation reaction was initiated by adding 10 μL concanavalin A (ConA, 5 μg/mL,

Sigma–Aldrich Co. LLC) to each well except for the corresponding control in which 10 μL complete medium was added. The blank wells contained 100 μL culture medium. Microplates were kept in an incubator (5% CO_2) at 37 °C for 48 h. Samples were run in triplicate. The reaction was terminated by adding 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) to each well. Microplates were read using a microtiter plate reader at 450 nm.

2.6. *Flow cytometric analysis*

Splenocytes were suspended in 100 μL of RPMI 1640 complete medium. The antibodies used were anti-CD3 FITC (clone 17A2, eBioscience), anti-CD4 RPE (clone W3-25, AbD Serotec) and anti-CD8 Cy5 (clone OX-8, AbD Serotec). Cells (10^7/mL) were labeled with conjugated antibodies for 30 min at 37 °C. After labeling, cells were washed and analyzed. In all experiments, stained cells were acquired with Beckman Coulter flow cytometer and analyzed using Summit 5.2 software.

2.7. *Statistical analysis*

Statistical analysis was performed with Prism 5 for Windows (Graphpad, San Diego, USA). The differences between groups were analyzed by analysis of variance (ANOVA). A P value of 0.05 was considered to be statistically significant.

3. Results

3.1. *Characterization of DMNAs*

It was found that both DMNAs and TP5-loaded DMNAs were perfectly replicated from the PDMA master structure based on the SEM images shown in Fig. 2. As expected both preparations have almost identical cone-shaped pointy needles. To ensure the quality of the preparations, a blank DMNAs and a trypan blue-dyed TP5-loaded DMNAs were prepared and their images under a microscope captured by a camera are shown in Fig. 3. It was found that both formed perfect MN arrays with no obvious missing/damaged needles. To quantify the amount of TP5 incorporated in the MN, HPLC analysis was carried out and results showed that TP5 contents in TP5-loaded DMNAs were 388.6 ± 15.5 μg. It has been reported that 800 μg is a clinically relevant dose of TP5 for the size of rats studied[25,26]. Therefore, adequate dose will be achieved when two patches of TP-DMNA are applied on the bilateral ST36 acupoints in rats.

The strength of DMNAs was examined by a texture analyzer. DMNAs were pressed on a piece of Tibet pig skin with a force applied by a probe. The applied force was increased until maximum resistance was observed. The force at which DMNAs start to break though the stratum corneum was recorded and analyzed by a software to assess the changes in the microneedle geometric characteristics. The pierced skin was stained with trypan blue solution (0.4% *w*/*v*) for 5 min and the excess dye was removed. As shown in Fig. 4, three stages were observed in the mechanical strength test. In the first stage, the DMNA contacted the skin and the base part of the microneedle was compressed with the force raised slowly. Subsequently, the needle part was compressed and the force increased substantially until breaking though the stratum corneum, where the maximum bearable pressure of the DMNA was recorded. Further, the needles inserted into the skin and the force increased sharply. It was found that two types of DMNA could piece into the skin and generated obvious holes. For the skin punctured with the edge, the main failure mode is tensile rupture, and those value has been much higher than the range of skin tensile strength[27].

Successful drug release depended upon the dissolving ability of microneedles. It was found that the presently obtained microneedles were completely dissolved within 30 min (Fig. 5a). The *in vitro* drug release study showed that TP5 could be immediately released from TP5-DMNA. As shown in Fig. 5b, 48.55% of TP5 was released from the microneedles during the first min, and about 90% of drug was released from TP5-DMNA over 15 min.

3.2. *Cell proliferation of rat spleen cells*

ConA is an antigen-independent mitogen and functions as a signal to induce T cells proliferation and activation. The incremental proliferation index (PI) of T lymphocytes was calculated according to: $PI = [(A_d - A_b)/(A_w - A_b)] \times 100\%$, where A_d represents the optical density (OD) values after the treatment, A_w represents the OD values before the treatment, and A_b represents the OD values of the blank wells (only the culture medium, no cells). As shown in Fig. 6, treatment at ST36 by DMNA without immune-stimulant, TP5, failed to increase the PI value significantly. The spleen cells of rats treated with TP5-DMNAs or conventional acupuncture at ST36 showed significant proliferation following ConA treatment. The PI value in immunosuppressive rats increased more than 100% upon treatment by conventional acupuncture at ST36 for 20 min compared to untreated animals. However, no significant difference in PI value was found between the groups of TP5-L and TP5-N.

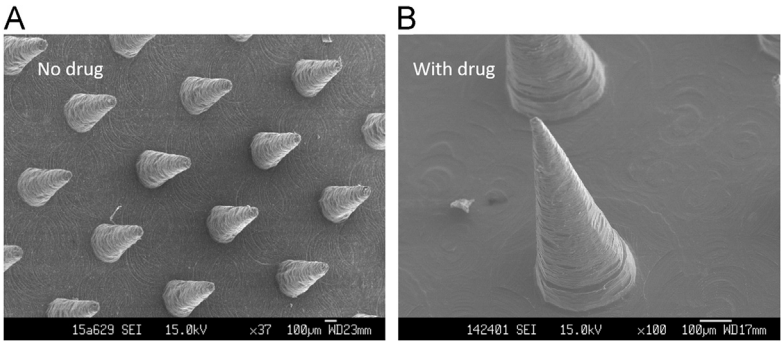

Figure 2 SEM images of DMNAs (A) and TP5-loaded DMNA (B).

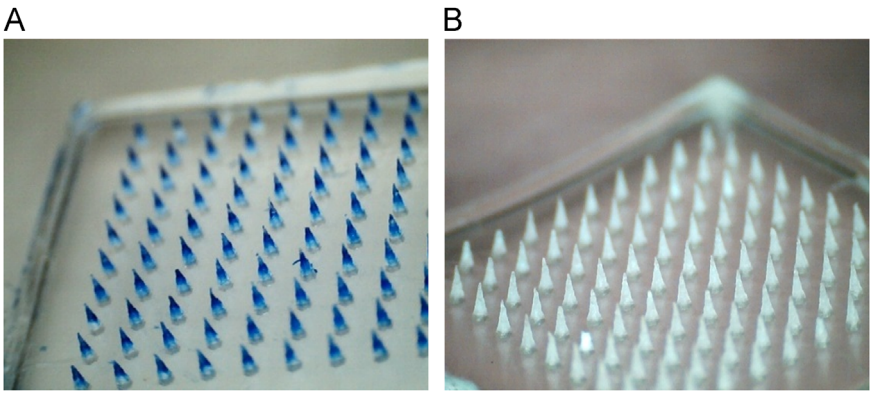

Figure 3 The images of two DMNAs viewed under a microscope equipped with a camera. (A) Blank DMNAs loaded trypan blue dye; (B) DMNAs loaded with TP5.

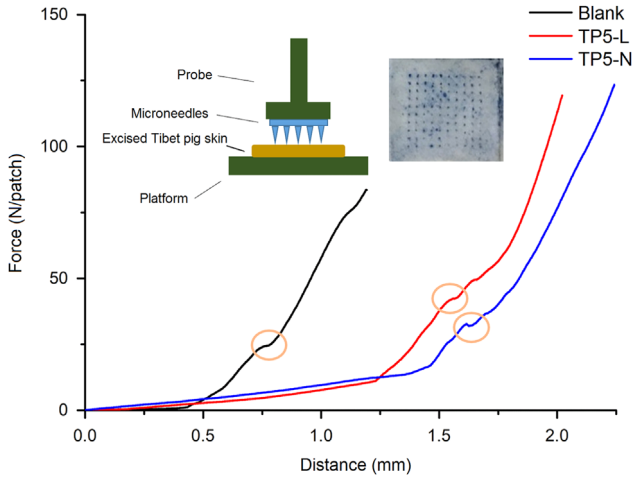

Figure 4 Mechanical strength of blank DMNAs, TP5-L-loaded DMNAs and TP5-N-loaded DMNAs analyzed by a texture analyzer. The pierced skin stained with trypan blue solution was shown in the insert.

3.3. Lymphocyte receptor expression in rats

To assess the possible impact of acupuncture-like therapy on immune responses, the effects on the development of T cells in the spleen was studied. The results showed an increase in lymphocyte counts in rats received either DMNA or conventional acupuncture stimulation in comparison to the control group (Fig. 7). The percentage of CD4 subgroup within the CD3 cells showed a similar trend. The TP5-N on acupoint showed similar results as the conventional acupuncture. Increases in CD4 and CD8 indicate positive cell-mediated immune responses. Fig. 7 shows that the absolute values of CD4 and CD8 were increased in conventional acupuncture and DMNA groups, suggesting the recovery of cell-mediated immune responses in immunosuppressive rats following TP5-DMNA treatments. However, the absolute values between the groups of TP5-L and TP5-N did not show significant difference.

3.4. Effects of DMNA on anxiety-like behavior in rats

Throughout 8 days of treatment, body weight change, fur conditions, piloerection, body temperature, food and water intake, and social aggressive behavior were monitored. Changes in behavior, in particular anxiety-like behavior were assessed using OFT. It was found that both vertical movement and center crossings in healthy rats were similar to the groups receiving ST36 stimulation with either conventional acupuncture or DMNAs, suggesting that the anxiety of those immunosuppressed rats was relieved (Fig. 8). However, DMNA treatment at non-acupoints (back) showed less evidence of such relief.

4. Discussion

Although acupuncture treatment is a common medical practice in China and several Asian countries, some hurdles limit the practice in other countries such as the need for professional training and the pain experienced in patients with low compliance. This study aimed to evaluate a new method to replace conventional acupuncture. The immune responses in an immunosuppressive experimental model were examined by TP5-loaded and blank DMNAs. The results were compared to that of traditional acupuncture. A DMNA patch was designed and fabricated. There are many advantages associated with this novel approach in comparison to conventional acupuncture. DMNAs dissolve in skin's interstitial fluid and there is no sharps waste. There is also no risk of infection which is potentially a problem with acupuncture. Moreover, the cost of polymers used in DMNA is low and production of DMNA is easy. In addition, protein and peptide drugs can be readily incorporated into DMNAs made of water soluble polymers with good biodegradability[27] and transdermal delivery *via* DMNAs. The method used was able to produce a nearly perfect DMNA with good drug loading efficiency (~91%).

The mechanical strength of needles is strong enough to pierce into the skin, and the drug-loaded microneedles displayed even better mechanical strength. According to Euler's buckling formula[28], the most critical factors for the mechanical strength of microneedle were attributed to needle length, tip and base diameter, and materials selected. Since all the microneedles were fabricated from the same mold, only needle materials of blank DMNAs and TP5 containing DMNAs were different. TP5 is a peptide with molecular weight of 679.77 KD, which was expected to improve the elasticity factor of the needle materials and subsequently increase the mechanical strength. The previous study of our group showed that the DMNA had inserted into the skin and created 700 μm deep microholes, revealed by the fluorescein isothiocyanate (FITC) signal[21]. It indicated that the DMNAs do not stimulate the nerves within the dermis without causing pain.

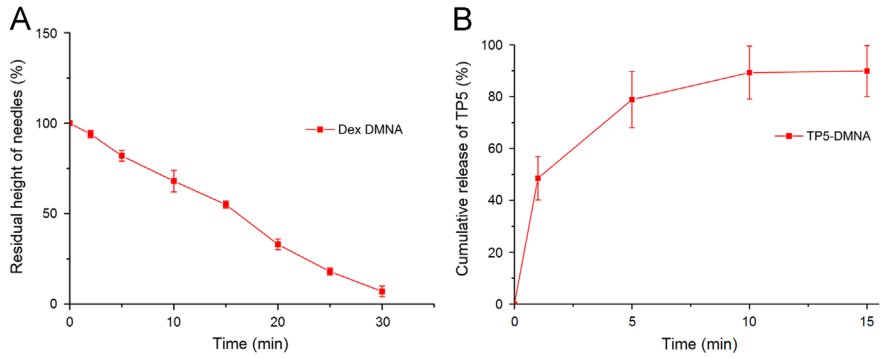

Figure 5 (A) Dissolving time of TP5-DMNA; (B) The cumulative release percentage of TP from TP5-DMNA (mean ± SD, $n = 3$).

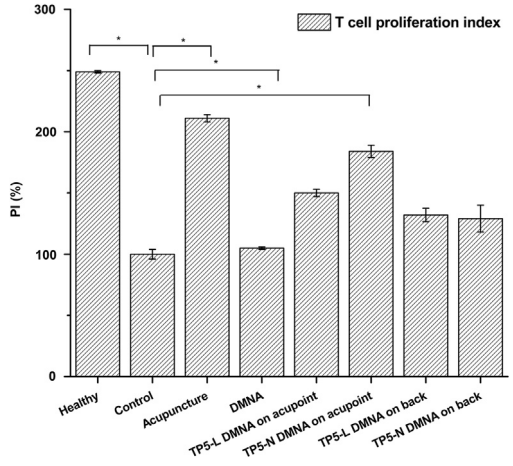

Figure 6 PI values of spleen cells in each group (mean ± SD, $n = 3$).

According to the research conducted by Koh in 1999[29], stimulating the special point on rat could lead the physiological changes, which is similar to human after stimulating the acupoint. The previous publication related to the experimental acupuncture science also provided the connection between the acupoints in a rat and in human[30–32], suggesting the equivalence of the acupoint in rats and human. Therefore, the rats model was used in the present *in vivo* study.

Lymphocyte proliferation plays an important role to restore immune functions in immunosuppressive animals. The incremental PI is a useful parameter to reflect the growth rate of T cells. The higher the PI value is, the faster the cell growth rate is, an indication of improved immune activity. Compared to untreated animals, the lymphocyte proliferation increased to nearly 50% and 80% for TP5-L and TP5-N, respectively. On the other hand, the same treatment at non-acupoint (back of the animal) showed little improvement. These results suggest that in addition to the pharmacological effect of TP5 (immune stimulant), acupuncture stimulation effect of DMNAs applied at the acupoint also played a role, although such an effect was weaker than that of conventional acupuncture. There are two possible reasons for the weaker response, the length of needles and the effective acupuncture time using DMNAs. The length of DMNAs is 800 μm which might be too short to reach the deep center of acupoint to generate the maximum stimulation. After being pierced into the skin, microneedles start to dissolve and disintegrate. Consequently, the effective acupuncture stimulation time is shorter than that of conventional acupuncture needles. Nevertheless, the data presented in this study clearly demonstrated that DMNAs containing TP5 were comparable to conventional acupuncture. Future studies should focus on the development of longer (> 800 μm) microneedles with stronger mechanic strength. To maximize effect, drug can be coated on the surface of the needles or be applied on the skin around acupoints prior to application of DMNA.

T cells are a heterogeneous population of cells. According to cell surface differentiation antigens, glycosylated T cells become activated and can be divided into $CD4^+$ and $CD8^+$ two subgroups. CD4 is mainly expressed in helper T (Th) lymphocyte and is a co-receptor of Th cell receptor recognizing antigen. It binds to the non-polypeptide region of major histocompatibility complex (MHC) class II molecules and participates in the signal transduction of T cell recognition antigens. CD8 T cells recognize endogenous antigen peptide presented by MHC class I molecules and subsequently be activated into $CD8^+$, which is also known as cytotoxic T (Tc) cell. Tc cells play an important role in killing virus-infected and tumor cells. CD4 and CD8 numbers could be significantly reduced by various disease states. It was shown that cyclophosphamide, a commonly used chemotherapeutic agent, could reduce the counts of both CD4 and CD8 by 50%–90% in cancer patients receiving high doses of cyclophosphamide (20–80 mg/kg), resulting immunosuppression[25]. Therefore, cyclophosphamide was used in the present study to create the immunosuppressed rat model.

Flow cytometric studies (Fig. 6) showed that both CD4 and CD8 numbers of immunosuppressive rats treated by conventional acupuncture and DMNAs loaded with TP5 (lower dose and normal dose) at the acupoints (ST36) were increased. The same treatment using DMNA without TP5 did not result in significant increase of CD4 and CD8. It should be noted that the numbers of CD4 and CD8 in rats treated with TP5 loaded DMNAs at non-acupoint (back) also increased to similar levels of those treated at the acupoints. This suggests that TP5 exerted its immune stimulating effect after being delivered transdermal at either the acupoints or non-acupoints. Unlike the PI results, the global indicator of activated T cells ($CD4^+$), the increase of absolute numbers of CD4 and CD8 molecules contributed by microneedle (mechanic not pharmacological) stimulation in the treatments by DMNAs was weak. CD4 and CD8 molecules might not able to be activated. This might be due to the lack of MHC factors, exogenous/endogenous antigen peptides to activate CD4 and CD8. Studies by Wang et al.[33] demonstrated that acupuncture could stimulate the formation of MHC factors. However, the acupuncture effect contributed from DMNAs was relatively weak

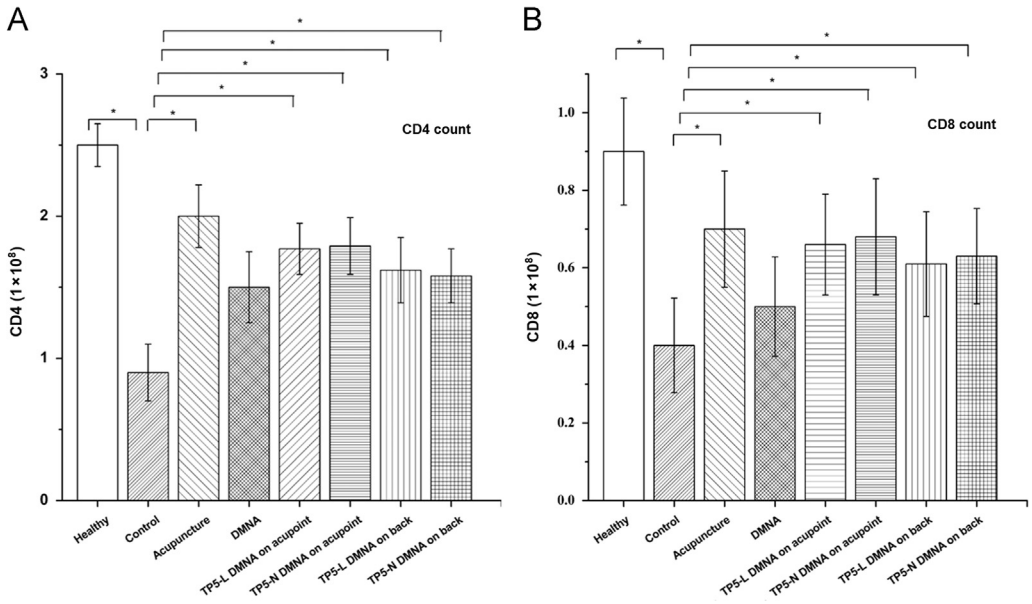

Figure 7 Absolute counts of CD4 (A) and CD8 (B) of rats from different treatment groups obtained by flow cytometry (mean ± SD, $n = 3$). $^{*}P<0.05$ compared to control (no treatment).

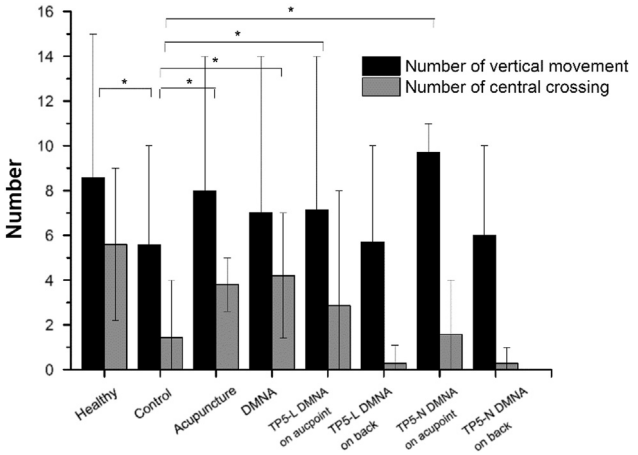

Figure 8 The number of vertical movement and center crossing of rats tested in OFT study (mean ± SD, $n = 3$). $^{*}P<0.05$ compared to the control group.

due to the short needle length and reduced duration of effective acupuncture as discussed earlier. Besides, the acupuncture had been confirmed to exhibit synergistic effect with drugs, which could weaken the importance of drug dose and minimize drug side effects[34]. Therefore, the application of acupuncture with reduced drug dose could still achieve the same treatment effect with normal drug dose.

Acupuncture treatment could alter not only the physiological conditions but also psychological state of immunosuppressive rats. Innes and colleagues found that acupuncture could adjust the pathological conditions caused by the negative psychological mood[35,36]. So, the effect on reducing anxiety was also investigated. The OFT study was used to determine the behavior of immunosuppressed rats. The results showed there were significant differences in the numbers of center crossing and vertical movement (Fig. 8), suggesting that DMNA can induce significant changes in the animal welfare, exploratory activities and/or anxiety like behavior.

The ultimate goal of acupuncture is to restore the body's internal balance and as a means of alleviating disease symptoms[37]. The success of acupuncture treatment has been linked to the hypothalamus that modulates various immune functions. Several studies have demonstrated that acupoint stimulation affected the hypothalamic–pituitary–adrenal axis (HPA axis) resulting in the production of corticotropin-releasing hormone, Adrenocorticotropic hormone and glucocorticoid[38]. These neurotrophic hormones are involved in regulating many of the body's activities, such as the immune system and mood. For example, glucocorticoid is known to promote Th1 cell proliferation and to adjust Th1/Th2 balance[39]. As $CD4^{+}$ cells, Th1 participate in regulating cellular immunity, auxiliary cytotoxic T cell differentiation, cellular immune response, and late-onset hypersensitivity. The mechanism(s) of attenuating psychological distress by acupuncture have to be clarified. One of the possible reasons is that acupoint treatment of anxiety could activate the nervous system by stimulating the different mechanoreceptors found in skin (*e.g.*, Meissner and Pacinian corpuscles, Merkel disc endings, Ruffini and free nerve endings)[40]. Signals could be transmitted along the myelinated nerve fibres to the limbic lobe[41]. Alternatively, the release of soluble messengers such as substance "P" or serotonin may be responsible for mediating the 'relaxing effects' [40–42]. The serotonin and norepinephrine are implicated in the etiology of depression.

5. Conclusions

The current study is a combination of modern pharmaceutical technology and traditional acupuncture treatment. The data presented in this study clearly demonstrate that acupuncture using DMNAs could potentially replace conventional acupuncture. This method may be an alternative therapy for traditional acupuncture in addition to deliver therapeutic agents subcutaneously. DMNA

technology provides a versatile platform to manage diseases. In future, a disease-specific DMNA patch administered onto a specific acupoint could potentially release drug subcutaneously during the stimulation at acupoint by DMNAs.

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