Mechanistic studies on transcutaneous vaccine delivery : microneedles, nanoparticles and adjuvants

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Chapter 3

In vivo assessment of safety of microneedles in human skin

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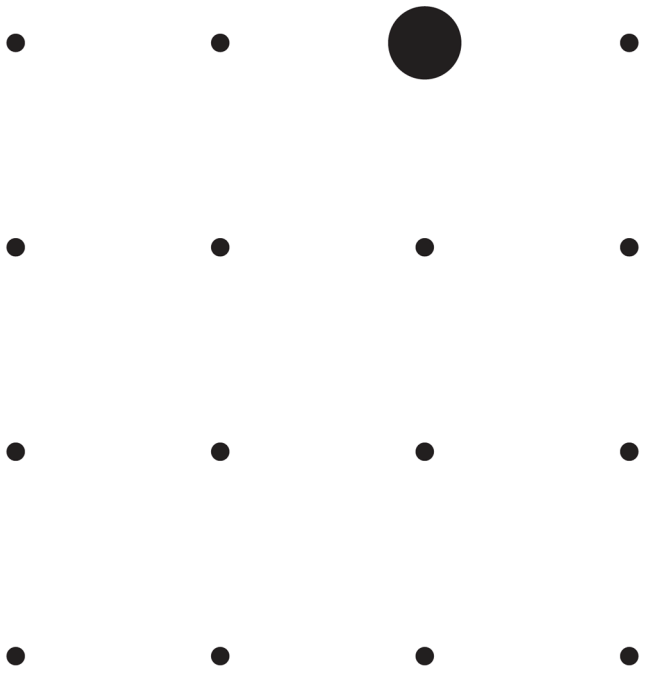

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Abstract

Microneedle arrays are promising devices for the delivery of drugs and vaccines into or through the skin. However, little is known about the safety of the microneedles. In this study we obtained insight in the ability of microneedles to disrupt the skin barrier, which was evaluated by transepidermal water loss (TEWL). We also determined the safety in terms of skin irritation (skin redness and blood flow) and pain sensation. We applied microneedle arrays varying in length and shape on the ventral forearms of 18 human volunteers. An effect of needle length was observed, as TEWL and redness values after treatment with solid microneedle arrays of 400 µm were significantly increased compared to 200 µm. The blood flow showed a similar trend. Needle design also had an effect. Assembled microneedle arrays induced higher TEWL values than the solid microneedle arrays, while resulting in less skin irritation. However, for all microneedles the irritation was minimal and lasted less than 2 hours. In conclusion, the microneedle arrays used in this study are able to overcome the barrier function of the skin in human volunteers, are painless and cause only minimal irritation. This opens the opportunity for dermal and transdermal delivery of drugs and vaccines.

Introduction

Even though the skin is an attractive site for drug delivery, the stratum corneum, the upper part of the epidermis, poses a barrier to the transport of most compounds. In recent years a large number of methods have been developed to increase the permeation across this skin barrier. Among these methods are chemical enhancement such as the use of penetration enhancers and novel formulations and physical enhancement, such as iontophoresis and electroporation [1-5]. Recently microneedles have gained much attention, as they can create little holes in the stratum corneum. Microneedles can be fabricated from a large number of different materials, such as silicon, glass, metal and polymers, and differ in length and in shape [6-8]. The microneedles are excellent candidates for transdermal and dermal delivery. One of the most attractive applications of the microneedle arrays is to use them for transcutaneous vaccination. Microneedle studies are often focused on the fabrication of microneedle arrays. Studies on the enhanced delivery across the skin [9-15] and the increase in immune response generated [16-18] are in progress. An important question that needs to be resolved is whether these microneedles induce skin irritation [15, 17, 19].

Skin irritation is a reversible inflammatory reaction that can lead to erythema and oedema [20, 21]. Many chemical substances act as skin irritants and the mechanism of this process is not completely understood, but the production of cytokines by epidermal cells is deemed important. Keratinocytes, which comprise 95% of the epidermal cells, are the major source of cytokines. Activated Langerhans cells also secrete cytokines, but to a lesser extent [22]. In response to barrier disruption, keratinocytes produce a variety of cytokines of which interleukin-1α (IL-1α) is the most important one. Preformed and active IL-1α is already present in resting keratinocytes and after it has been released, it stimulates further release of more IL-1α and other cytokines such as IL-8, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α) [23-25]. This cytokine cascade leads to dermal vasodilatation and cellular infiltration in the epidermis, which directs the restoration of the skin barrier function [26, 27]. Physical barrier disruption by tape stripping or UV radiation is also known to result in release of IL-1α and the resulting inflammation reaction [25, 28, 29]. It may therefore be possible that microneedles also induce an inflammatory reaction.

There are many non-invasive biophysical techniques to assess skin irritation and barrier disruption, such as transepidermal water loss (TEWL), skin colour, laser Doppler flowmetry, capacitance, reflectance spectroscopy, ultrasound and visual scoring [30-35]. In this study the safety and barrier disruption caused by microneedle arrays was investigated in healthy subjects. Erythema was evaluated by skin colour assessment and by laser Doppler imaging (LDI). LDI is an optical technique that measures the movement of red blood cells. Light

from a laser beam is directed onto the skin. Moving red blood cells scatter the laser light in a different way than static tissue resulting in a frequency shift. This shift is photodetected and processed to provide a blood flow value [36, 37]. The barrier function was investigated by measuring the TEWL [38]. After treatment with different types of microneedle arrays the TEWL, LDI, redness and painscore were assessed on regular intervals during 2 hours. The length of the microneedles as well as the shape of the tip of the microneedles varied.

Materials and methods

Volunteers

Eighteen non-smoking healthy volunteers (9 men and 9 women), aged between 21 and 30 years (mean ± SD, 25 ± 3), with no pre-existing skin conditions participated in the study. They were asked not to apply any cosmetic formulations on the ventral forearm during seven days before the study and to refrain from coffee and tea on the day of the study. The study was approved by the Medical Ethical Committee from the Leiden University Medical Centre.

Microneedles

Two different types of microneedle arrays were used. Solid metal microneedle arrays (figure 1a and b) with a length of 200, 300 or 400 µm (200S, 300S and 400S) were obtained from Transferium (Almelo, The Netherlands). These needles are made from stainless steel wire with a diameter of 200 µm and are die-cut to a tangential shape. The needles were placed in a 4 by 4 pattern in a polyetheretherketone mould (diameter 9 mm) with a pitch of 1.25 mm. Assembled hollow metal microneedle arrays (figure 1c and d) with a length of 300 and 550 µm (300A, 550A) were obtained from Philips (Philips Research Europe, Eindhoven, The Netherlands). These needles were manufactured from commercially available 30G hypodermic needles and have a diameter of 300 µm [14]. These needles were positioned in a 4x4 pattern in a polyetheretherketone mould with a pitch of 1.25 mm, similarly to the solid microneedle arrays.

To precisely tailor the insertion speed of the microneedle array into the skin to 3 m/s a custom made electrical applicator was used (Fine Mechanical Department, Leiden University). An array of microneedles was positioned at the end of the applicator and held in place by a metal holder. A Perspex cover protects this metal holder. The device contains a coil through which on demand current passes, which results in a magnetic driving force that launches a metal rod out of the coil, moving the attached microneedle array.

Experimental procedure

The study was conducted at 23˚C in a temperature controlled room. The subjects acclimatised in this room for 30 minutes prior to the start of the study. Three circular areas were marked on the left ventral forearm and five on the right ventral forearm of each subject. The circular areas were located at approximately the same position on each forearm. However, to ensure that these areas were not located on a vein, which would interfere with the blood flow measurements, the subcutaneous blood flow was imaged before treatment with the microneedle arrays with a laser Doppler imager (LDI) (MoorLDLS, Moor Instruments, Devon, UK). The distance between the LDI measuring head and the skin was set to 15 cm and the images were analysed by calculating the mean blood flow over an area of 0.64 cm^2, corresponding to the size of the mould of the microneedle array. The values are expressed as perfusion units (PU).

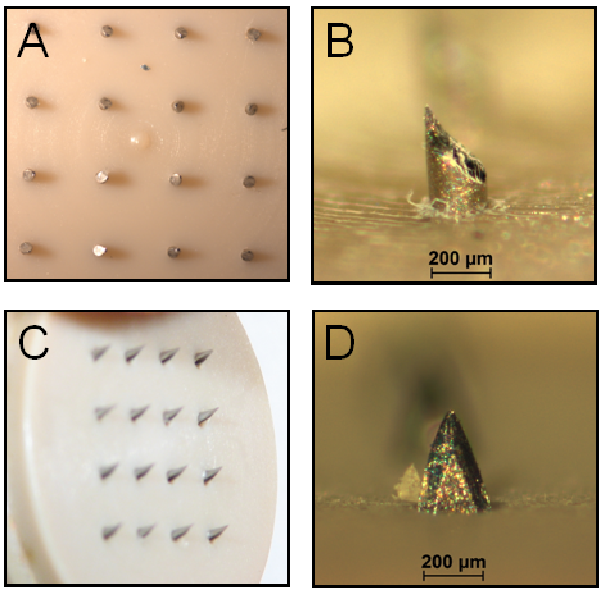

Figure 1. The microneedle arrays used in this study are i) solid metal microneedles in a 4x4 array (300S, a) and a higher magnification of a single microneedle (b) and ii) assembled hollow metal microneedles in a 4x4 array (300A, c) and a higher magnification of a single microneedle (d).

In order to compare the effect of increasing microneedle length, 200S, 300S and 400S microneedle arrays were applied on the left ventral forearm of 18 volunteers in a randomised manner. This experiment was always performed in the morning, between 10 AM and 12 PM. On the right ventral forearm of 15 volunteers five microneedle treatments were carried out to compare single application of the 300S microneedles to the following treatments: i) twofold application of the 300S microneedles, ii) application of 300A and iii) application with 550A, which served as a positive control and iv) application of an empty mould which served as a negative control. All positions were randomised in comparison to the 300S to correct for differences between the application sites. This experiment was always performed in the afternoon, between 1 PM and 3 PM.

Before applying the microneedle arrays, baseline values were recorded for the barrier function (TEWL), the subcutaneous blood flow and the skin colour. The TEWL was measured with a Tewameter TM210 (Courage+Khazaka, Köln, Germany). After placing the probe on the skin, the TEWL values were recorded for a period of 1 min after which an average reading during this time interval was calculated. The values are expressed in g h^{-1} m^{-2}. The skin colour was measured using a Minolta CR-300 chromameter (Minolta Ltd, Milton Keynes, UK). The chromameter was calibrated against a colour standard before measuring each subject, according to the method defined by the manufacturer. The probe of the apparatus was placed gently onto the skin and the colour was measured on the a* scale, the red-green Commission Internationale de l'Éclairage (CIE) axis [39]. The treated areas were also visually inspected for skin damage. The measurements were performed directly after application (0 min) and repeated after 15, 30, 45, 60, 90 and 120 minutes. The subjects were also asked to rate the pain of application on a 1-10 scale directly after the treatment.

Statistical analysis

Statistical analysis was performed with Prism 4 for Windows (GraphPad, San Diego, U.S.A). Data of TEWL, redness and LDI are presented as mean ± SEM (n = 18 for left ventral forearm and n = 15 for right ventral forearm). Because the data for the pain scoring did not show a normal distribution, a box-and-whiskers plot was used to present these data. A repeated measurement analysis of variance (ANOVA) was combined with a Bonferroni multiple comparison post test.

Results

Barrier function

The TEWL values after treatment with the 200S, 300S and 400S are provided in figure 2a. Prior to treatment, TEWL values were around 9.5 g h^{-1} m^{-2}. The 200S treatment did not result in increased TEWL values and 15 minutes after piercing the TEWL values only decreased and reached values that were below the initial baseline values. After piercing with the 300S, TEWL values increased immediately and declined after 15 minutes reaching baseline values after 30 minutes. The pattern of the TEWL values obtained after treatment with the 400S was similar to that obtained with the 300S, but the effect lasted 15 minutes longer. Treatment with the microneedle arrays showed a trend that longer microneedles result in a higher increase in TEWL values. Only a significant difference in response was observed between the 400S and 200S (table 1A). In figure 2b the increase in TEWL after treatment with microneedles of different shapes, positive control (550A), negative control

(550A) and twofold application is provided. In this study all treatments were compared to the treatment with the 300S microneedle arrays. For almost all microneedle arrays the TEWL values increased and reached a peak directly after application. After the first time point at 0 min the TEWL decreased very slowly, but did not return to the baseline value within the time frame of the experiment. The 300A was the only treatment that reached its maximum TEWL values not directly after piercing, but 15 minutes later. TEWL did not increase after treatment with the control. As shown in table 1B**,** treatment with the 300S did not increase the TEWL to a significantly higher level than after the control treatment. The highest TEWL values (maximum of 11.8 g h^{-1} m^{-2}) were obtained with the 550A ($p<0.001$ in comparison to the 300S). Furthermore, the 300A resulted in a significant higher increase in TEWL than the solid microneedle array of the same length ($p<0.001$) and piercing twice with the 300S microneedle array increased the TEWL significantly compared to a single 300S microneedle treatment ($p<0.001$).

Pain

In figure 3a and b box-and-whisker plots of the pain scores as reported by the volunteers are shown. The pain scores of all treatments are similar and very low. No significant differences in pain caused by microneedles of different length or shape were found. The median value of all microneedle arrays was 1, except for the 550A were the median was 2. This array also had the highest maximum pain score of 6. Even though the scores after microneedle treatment and control did not differ significantly, the latter did have the smallest interquartile range.

Table 1. Pairwise comparison of TEWL values (g h^{-1} m^{-2}) between microneedle arrays of different length (A) and type (B).

	400S vs 300S	**400S vs 200S**	**300S vs 200S**	
A) TEWL				
Mean difference	0.548	1.04	0.495	
95 % CI	0.149 to 0.947	0.644 to 1.44	0.0962 to 0.894	
p value	p< 0.01	p< 0.001	p> 0.05	
B) TEWL	**300S vs 300A**	**300S vs 2x 300S**	**300S vs control**	**300S vs 550A**
Mean difference	-0.884	-0.844	0.458	-1.95
95 % CI	-1.33 to -0.435	-1.29 to -0.395	0.00961 to 0.907	-2.40 to -1.51
p value	p< 0.001	p< 0.001	p< 0.05	p< 0.001

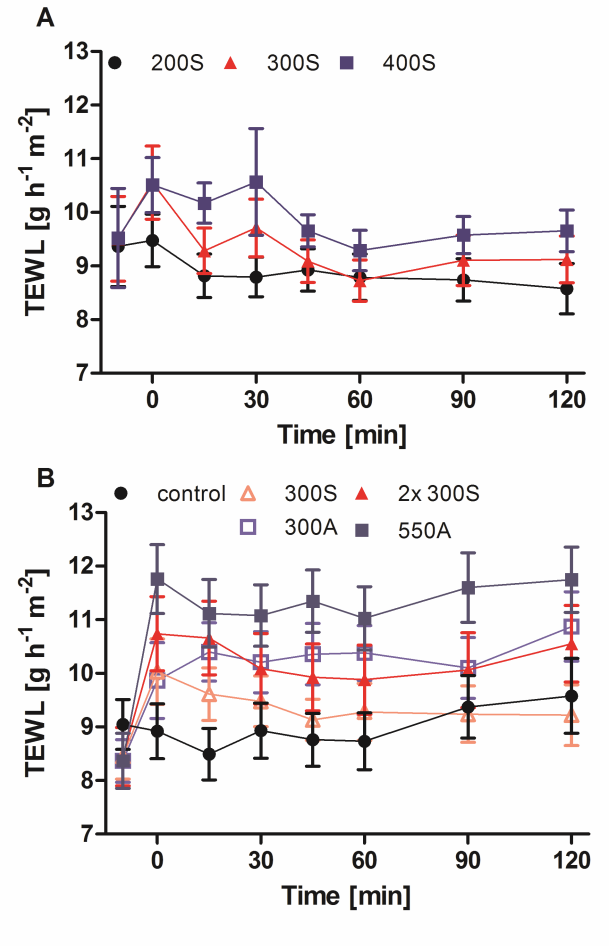

Figure 2. TEWL values before and after applying different microneedle arrays (t=0). (A) Solid metal microneedle arrays of 200, 300 and 400 µm needle length. (B) Solid metal microneedle arrays of 300 µm in comparison to different types of microneedle arrays. Data is presented as average values ± SEM of 18 (A) or 15 (B) volunteers.

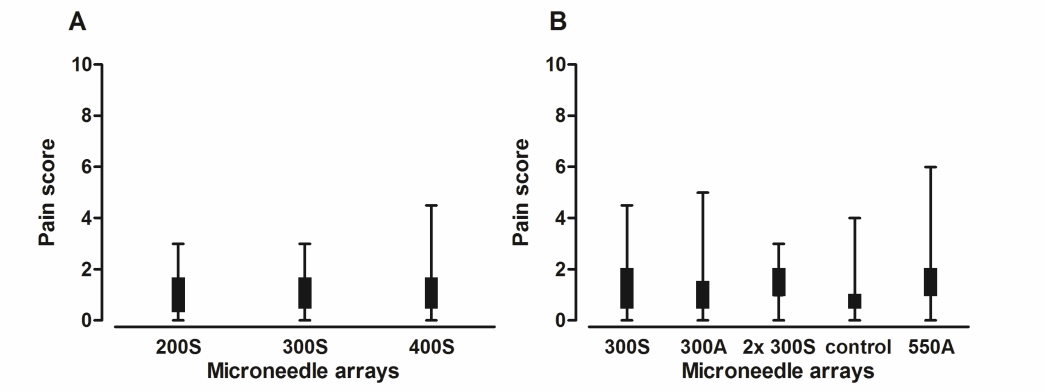

Figure 3. Box-and-whisker plots of the pain scores after treatment with different microneedle arrays. (A) Solid metal microneedle arrays of 200, 300 and 400 µm needle length. n = 18. (B) Solid metal microneedle arrays of 300 µm in comparison to different types of microneedle arrays. n = 15.

Skin Irritation

As a determinant of the degree of irritation the redness of the skin and the blood flow was examined. Figure 4a shows the change in redness (Δa) for the solid microneedle arrays of different length. After application of each microneedle array an increase in Δa was observed. After 15 min the Δa values were maximal and reached values of 1.8 absorption units (AU) for the 300S and 400S and of 1.4 AU for the 200S could be detected. From this time on the Δa values decreased and reached baseline values for the 200S after 60 minutes and for the 300S and 400S after 90 minutes. As shown in table 2A treatment with the 400S resulted in significant higher Δa values than treatment with the 200S ($P<0.001$). In figure 4b the Δa after treatment with the 300S was compared to different types of microneedle arrays. Treatment with the empty mould resulted in maximum values directly after application and almost immediately afterwards the baseline values were reached. For all microneedle arrays, the Δa values were maximal 15 minutes after application, and remained elevated for at least 90 minutes. Treatment with the 550A and the 300S resulted in Δa values that were still higher after 2 hours than before treatment. As shown in table 2B, treatment with the 300S resulted in an increase that was significantly higher than after the control treatment ($P<0.001$). After treatment with the 550A, similar Δa levels were reached as with the 300S, while significantly lower values compared to the 300S were found after treatment with the 300A ($P<0.01$), even though after treatment with the 300A very small spots of blood redness were observed in the skin. Piercing with the 550A also resulted in small blood spots in the skin. Single and twofold piercing with the 300S microneedle array did not result in significant differences in Δa values.

Monitoring changes in subcutaneous blood flow using the LDI was another way to assess skin irritation. In figure 5 examples of pictures and perfusion images of skin reactions after 5 different applications of microneedle arrays are shown. The figure shows scans of the same skin area before treatment and at different time points after treatment. The change in blood flow compared to the baseline values after application of the 200S, 300S and 400S was derived from the blood flow images and is shown in figure 6a. Immediately after treatment the blood flow increased, but reduced to baseline values within 45 minutes. However, no significant differences in blood flow were found after treatment with 400S, 300S and 200S microneedles (table 3A). As shown in figure 6b pressing an empty mould against the skin resulted in a slight increase the subcutaneous blood flow, as an increase of 25 PU could be observed, but after 30 minutes the baseline value was reached again. Applying the microneedle arrays resulted in an immediate increase in blood flow followed by a rapid decrease. The 300S resulted in a significantly higher increase in blood flow than after treatment with the control ($p<0.001$). The blood flow returned to baseline values within 60 minutes for all microneedle arrays except the 550A, which values remained

elevated for at least 2 hours. Treatment with the solid microneedle arrays resulted in a trend of a more pronounced blood flow increase than after applying the assembled microneedles (table 3B). Twofold and single piercing of 300S microneedle arrays did not result in significant differences in blood flow.

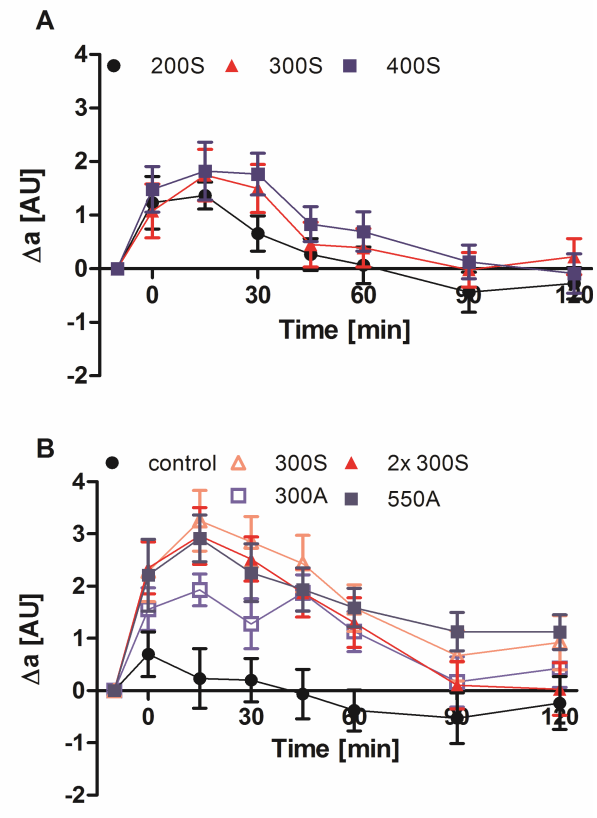

Figure 4. The change in redness (Δa) at different time points after the application of microneedle arrays (t=0) in comparison to the redness before application. (A) Solid metal microneedle arrays of 200, 300 and 400 µm needle length. (B) Solid metal microneedle arrays of 300 µm in comparison to different types of microneedle arrays. values. Data is presented as average values ± SEM of 18 (A) or 15 (B) volunteers.

Table 2. Pairwise comparison of induced redness between microneedle arrays of different length (A) and type (B).

A) Δa	**400S vs 300S**	**400S vs 200S**	**300S vs 200S**	
Mean difference	0.181	0.537	0.356	
95 % CI	-0.112 to 0.481	0.237 to 0.837	0.0559 to 0.656	
p value	p> 0.05	p< 0.001	p< 0.05	
B) Δa	**300S vs 300A**	**300S vs 2x 300S**	**300S vs control**	**300S vs 550A**
Mean difference	0.804	0.408	2.01	0.119
95 % CI	0.244 to 1.36	-0.152 to 0.967	1.45 to 2.57	-0.441 to 0.679
p value	p < 0.01	p > 0.05	p < 0.001	p > 0.05

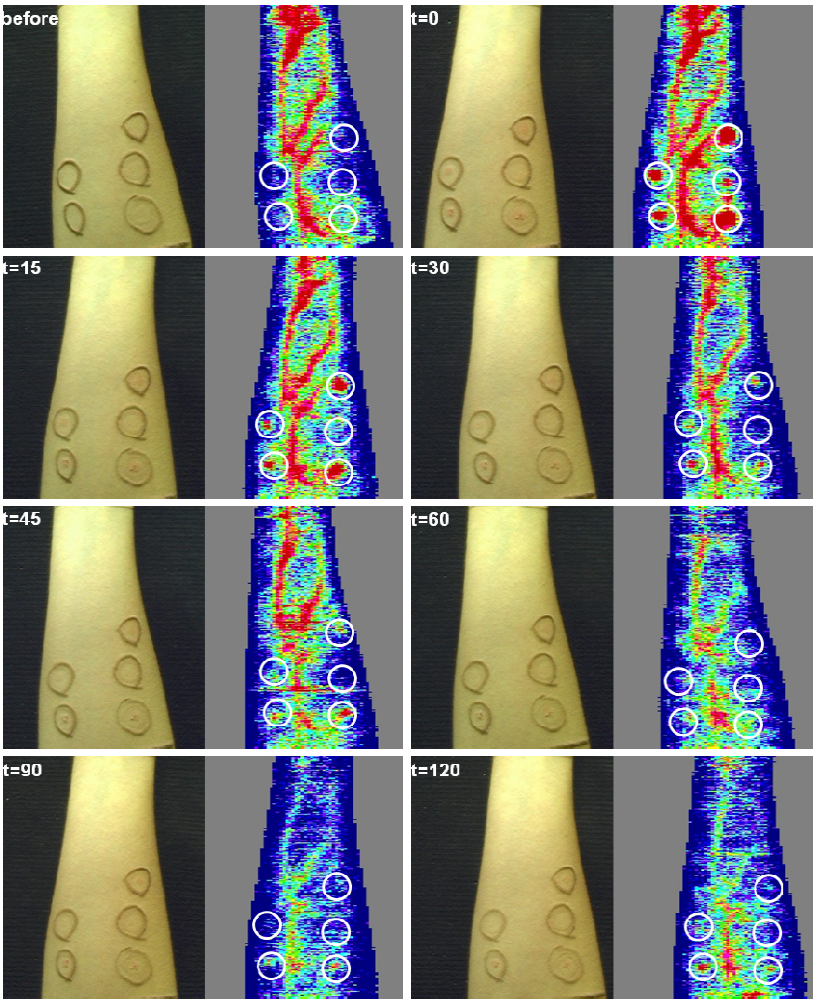

Figure 5. Laser Doppler pictures and perfusion images of a forearm of a volunteer. The figure shows scans of the same skin area before treatment and at different time intervals after treatment.

Table 3. Pairwise comparison of the increase in blood flow (PU) between microneedle arrays of different length (A) and type (B).

Δblood flow	**400S vs 300S**	**400S vs 200S**	**300S vs 200S**	
Mean difference	19.25	18.82	-0.432	
95 % CI	-0.177 to 38.68	-0.608 to 38.25	-19.86 to 19.00	
p value	p> 0.05	p> 0.05	p> 0.05	
Δblood flow	**300S vs 300A**	**300S vs 2x 300S**	**300S vs control**	**300S vs 550A**
Mean difference	15.79	3.823	40.57	-12.6
95 % CI	-9.646 to 41.22	-21.61 to 29.26	15.14 to 66.01	-38.04 to 12.83
p value	p > 0.05	p > 0.05	p < 0.001	p > 0.05

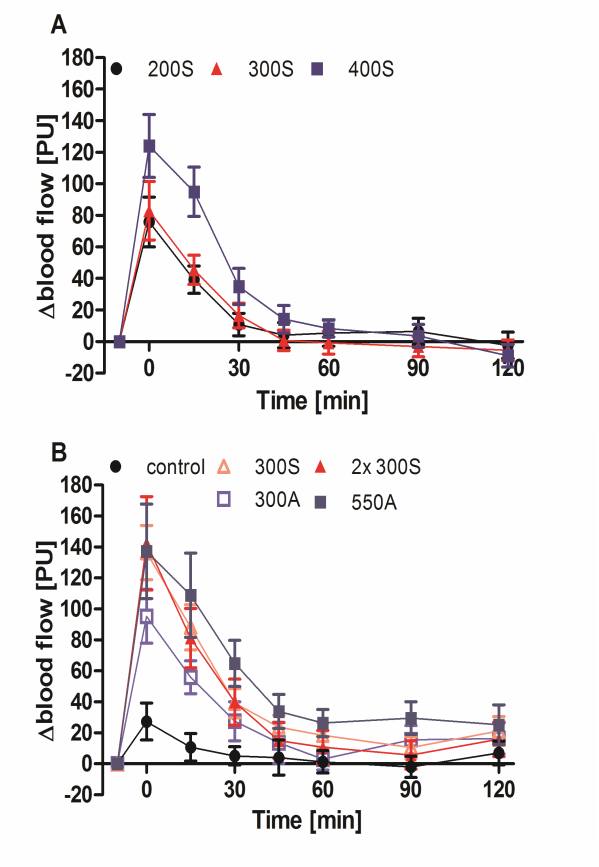

Figure 6. The change in blood flow at different time points after the application of microneedle arrays (t=0) in comparison to the blood flow before application. (A) Solid metal microneedle arrays of 200, 300 and 400 µm needle length. (B) Solid metal microneedle arrays of 300 µm in comparison to different types of microneedle arrays. Data is presented as average values ± SEM of 18 (A) or 15 (B) volunteers.

Discussion

The aim of this study was to obtain insight in the ability of microneedles to disrupt the barrier of the skin and to determine the safety of microneedle treatment in terms of skin irritation and pain sensation. For this purpose we used microneedles varying in microneedle length, diameter and shape. In one study we investigated the effect of increasing microneedle length and in another study single application of the 300S microneedles was compared to treatment with microneedles of different shape and to twofold application.

First, the influence of the microneedles arrays on the barrier function was assessed. For microneedle arrays with the same shape, only treatment with 400S resulted in a significant difference in TEWL in comparison to 200S. Treatment with the 300S was also compared to treatment with microneedle arrays with a differently shaped tip. We found a significant difference between the 300S and the 300A and 550A, indicating that needle shape is an important parameter for barrier disruption. The 300S did not increase the TEWL significantly compared to the control treatment. However, in *in vitro* studies we did show that these needles could pierce human skin by visualising the conduits [40]. The 300S microneedle arrays were used in the study focusing on needle length and in the study

focusing on needle shape. Slight differences in TEWL were observed in both studies. In the microneedle length study, the elevated TEWL values lasted 30 minutes, while in the microneedle shape study the TEWL values remained elevated and increased again after 90 minutes. It is possible that this was caused by circadian variations. The microneedle shape study was performed between 1 and 3 PM. Le Fur et al. showed that TEWL values on the forearm reach a peak at 8 am and at 4 pm and a minimum at noontime [41, 42].

An important reason to develop microneedles for dermal vaccination is to decrease the pain and discomfort that the current delivery of vaccines by injection causes. Several recent studies indicate that approximately 20% of the children suffered serious distress from vaccinations [43]. For this reason we also assessed the pain that treatment with our microneedle arrays might induce. We demonstrated that treatment with microneedle arrays varying in microneedle length, diameter and shape did not cause pain to most of the volunteers. This is in agreement with results from Kaushik et al., who showed that the pain sensation caused by microneedle arrays containing 400 microneedles with a length of 150 µm did not differ significantly from a smooth surface [19]. The pain score of the microneedle arrays do have a larger interquartile range than the control. However, pain scoring is a subjective matter and two volunteers did perceive all microneedle arrays as uncomfortable.

To assess the safety of the microneedles, the irritation that these needles might induce was measured both with a chromameter and a LDI. Both methods measure erythema, which is one of the fundamental markers of inflammation [23]. However, a chromameter measures only the superficial redness, while a LDI measures the blood flow much deeper in the skin. The exact penetration depth of the laser depends on pigmentation, but on average the image is reflecting the blood flow until a skin depth of 1 mm [44]. Because the vasodilatation response caused by the inflammation reaction in the dermis is faster than the redness response on the surface of the skin, the blood flow reached its maximum value directly after treatment with the microneedles, while the maximum Δa was measured 15 minutes after microneedle treatment. The results of both methods correlate excellently. For microneedle arrays of the same needle type, an increase in length results in an increase in Δa. Although treatment with microneedles of varying length did not result in significant blood flow differences, a similar trend was observed. When focusing on the microneedles of different shapes, treatment with the 300S induced clearly more irritation than the control treatment and the 300A microneedles, while between the 300S and 550A no significant differences in skin irritation were observed. Taking the Δa and blood flow data together, the assembled microneedle arrays result in less skin irritation than the solid ones. The effects observed are in agreement with data from Sivamani et al., who observed a higher maximum blood flow after microneedle application of methyl nicotinate compared with topical application [45].

Which microneedle arrays are most suitable to use for transdermal delivery and dermal vaccination purposes? The assembled microneedle arrays have the advantage that they disrupt the stratum corneum barrier to a higher extent, while they induce slightly less irritation. The most likely explanation for the difference in irritation and TEWL between the two needle types is the sharpness of the tips. The solid metal microneedles are 200 µm thick at the base and the tapered shaft of the needles has a length of approximately 280 µm. The slope of the angle is therefore 45°. The assembled hollow metal microneedles are made from 30G needles, which are 300 µm thick at the base, but the tapered shaft of the needles has a length of approximately 1.2 mm. The angle is therefore more acute, resulting in a very sharp tip. For this reason, they can make deeper incisions into the skin, as is suggested by the presence of small blood spots on the skin surface after application of these microneedles. On the other hand, piercing with the solid microneedle arrays appears to form a larger cut and therefore causes slightly more skin damage and irritation. This could mean that the assembled microneedles increase penetration of drugs, without unwanted side effects caused by irritation. However, previous *in vitro* transport studies across human skin performed by Verbaan et al. showed that pre-treatment with the solid microneedle arrays resulted in significant higher fluxes of cascade blue than pre-treatment with the assembled microneedle arrays [40]. Chilcott et al. also postulated that there is no correlation between increased TEWL levels and increased transdermal transport [46]. Further transport studies have to be performed to confirm the significant difference between solid and assembled microneedles *in vivo*. In case of dermal vaccination, the irritation caused by the solid microneedle arrays could be an advantage. It was shown that mechanical barrier disruption induces cytokine release and in that way initiates an inflammatory reaction [28, 29]. The Langerhans cells that are recruited to the site of irritation can take up antigens and consequently initiate an immune response. In this way the irritation caused by the microneedle arrays could function as an enhancer. Langerhans cells are located in the lower epidermis [47], that is approximately 150 µm thick [48]. Verbaan et al. postulated that microneedle arrays do not pierce the skin with their full length, because they have to overcome the bulk elastic tissue compression of the skin [14]. It is therefore advisable to use microneedles that are longer than 150 µm. From this study can be concluded that the minimal length should be 300 µm, because shorter needles did not pierce the skin.

To further evaluate the irritation data, we performed a pilot study in which we compared the TEWL, redness and blood flow values to those directly after tape stripping. We chose for tape stripping as this has been used for many years and is reported to be non-invasive [32]. After 10 tape strips the TEWL reached values of 15 g h^{-1} m^{-2} and remained at that value for at least 2 hours. This was higher than after treatment with the 550S. This larger increase in barrier disruption was accompanied by a higher degree of irritation. The Δa and

increase in blood flow after 10 tape strips were 4 AU and 160 AU respectively, which is higher than the Δa of 2 AU and the increase in blood flow of 140 AU reached after application of the 550S. The effect of tape stripping on the blood flow was short lasting, similar to the effect of the microneedle arrays. Only after removing 30 tape strips the blood flow appeared to remain elevated for 2 hours. The Δa after removing 10, 20 or 30 tape strips lasted longer than after application of the microneedle arrays, probably because with tape stripping the stratum corneum is removed and more superficial damage is done. Previously, Li et al. studied the effects of iontophoresis on TEWL and skin redness [49]. They found comparable redness values (Δa of 4 AU) to the values we obtained after microneedle application, but the redness persisted for a longer time. In this study also increased TEWL values were observed, but it is difficult to compare these values to the values obtained in our study, as the skin was hydrated with buffer solution for the duration of the iontophoresis and this also causes an increase in TEWL.

In conclusion, this study has shown that application of solid and assembled metal microneedle arrays with a length of up to 550 µm can be used to overcome the barrier function of the skin. Furthermore, human volunteers perceived their application as painless. Finally, it causes only minimal irritation in comparison to for instance tape stripping, which is accepted to be non-invasive. The shape and the length of the microneedle arrays have an influence on the degree of irritation, but for all microneedle arrays the irritation is short lasting.

References

1. Kanikkannan N, Kandimalla K, Lamba SS, and Singh M, *Structure-activity relationship of chemical penetration enhancers in transdermal drug delivery.* Curr Med Chem, 2000. **7**(6): p. 593-608.
2. Kalia YN, Naik A, Garrison J, and Guy RH, *Iontophoretic drug delivery.* Adv Drug Deliver Rev, 2004. **56**(5): p. 619-658.
3. Akimoto T and Nagase Y, *Novel transdermal drug penetration enhancer: synthesis and enhancing effect of alkyldisiloxane compounds containing glucopyranosyl group.* J Control Release, 2003. **88**(2): p. 243-252.
4. Prausnitz MR, Mitragotri S, and Langer R, *Current status and future potential of transdermal drug delivery.* Nat Rev Drug Discov, 2004. **3**(2): p. 115.
5. Denet AR, Vanbever R, and Preat V, *Skin electroporation for transdermal and topical delivery.* Adv Drug Deliver Rev, 2004. **56**(5): p. 659-674.
6. Stoeber B and Liepmann D, *Arrays of hollow out-of-plane microneedles for drug delivery.* J Microelectromech S, 2005. **14**(3): p. 472-479.
7. Parker ER, Rao MP, Turner KL, Meinhart CD, and MacDonald NC, *Bulk micromachined titanium microneedles.* J Microelectromech S, 2007. **16**(2): p. 289-295.
8. Park JH, Yoon YK, Choi SO, Prausnitz MR, and Allen MG, *Tapered conical polymer microneedles fabricated using an integrated lens technique for transdermal drug delivery.* IEEE T Bio-Med Eng, 2007. **54**(5): p. 903-913.
9. Henry S, McAllister DV, Allen MG, and Prausnitz MR, *Microfabricated microneedles: A novel approach to transdermal drug delivery.* J Pharm Sci, 1998. **87**(8): p. 922-925.
10. Park JH, Allen MG, and Prausnitz MR, *Biodegradable polymer microneedles: Fabrication, mechanics and transdermal drug delivery.* J Control Release, 2005. **104**(1): p. 51-66.
11. Teo MAL, Shearwood C, Ng KC, Lu J, and Moochhala S, *In vitro and in vivo characterization of MEMS microneedles.* Biomed Microdevices, 2005. **7**(1): p. 47-52.
12. Wu XM, Todo H, and Sugibayashi K, *Effects of pretreatment of needle puncture and sandpaper abrasion on the in vitro skin permeation of fluorescein isothiocyanate (FITC)-dextran.* Int J Pharm, 2006. **316**(1-2): p. 102-108.
13. Gill HS and Prausnitz MR, *Coated microneedles for transdermal delivery.* J Control Release, 2007. **117**(2): p. 227-237.
14. Verbaan FJ, Bal SM, van den Berg DJ, Groenink WHH, Verpoorten H, Luttge R, and Bouwstra JA, *Assembled microneedle arrays enhance the transport of compounds varying over a large range of molecular weight across human dermatomed skin.* J Control Release, 2007. **117**(2): p. 238-245.
15. Wermeling DP, Banks SL, Huclson DA, Gill HS, Glupta J, Prausnitz MR, and Stinchcom AL, *Microneedles permit transdermal delivery of a skin-impermeant medication to humans.* P Nat Acad Sci USA, 2008. **105**(6): p. 2058-2063.
16. Widera G, Johnson J, Kim L, Libiran L, Nyam K, Daddona PE, and Cormier M, *Effect of delivery parameters on immunization to ovalbumin following intracutaneous administration by a coated microneedle array patch system.* Vaccine, 2006. **24**(10): p. 1653-1664.
17. Mikszta JA, Alarcon JB, Brittingham JM, Sutter DE, Pettis RJ, and Harvey NG, *Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery.* Nat Med, 2002. **8**(4): p. 415-419.
18. Matriano JA, Cormier M, Johnson J, Young WA, Buttery M, Nyam K, and Daddona PE, *Macroflux (R) microprojection array patch technology: A new and efficient approach for intracutaneous immunization.* Pharm Res, 2002. **19**(1): p. 63-70.
19. Kaushik S, Hord AH, Denson DD, McAllister DV, Smitra S, Allen MG, and Prausnitz MR, *Lack of pain associated with microfabricated microneedles.* Anesth Analg, 2001. **92**(2): p. 502-504.
20. Corsini E and Galli CL, *Cytokines and irritant contact dermatitis.* Toxicol Lett, 1998. **103**: p. 277-282.
21. Chew A and Maibach HI, *Irritant Dermatitis*. 2006, Berlin: Springer.

22. Williams IR and Kupper TS, *Immunity at the surface: Homeostatic mechanisms of the skin immune system.* Life Sci, 1996. **58**(18): p. 1485-1507.
23. Corsini E and Galli CL, *Epidermal cytokines in experimental contact dermatitis.* Toxicology, 2000. **142**(3): p. 203-211.
24. Kock A, Schwarz T, Kirnbauer R, Urbanski A, Perry P, Ansel JC, and Luger TA, *Human keratinocytes are a source for Tumor-Necrosis-Factor-alpha - Evidence for synthesis and release upon stimulation with endotoxin or ultraviolet-light.* J Exp Med, 1990. **172**(6): p. 1609-1614.
25. Wood LC, Jackson SM, Elias PM, Grunfeld C, and Feingold KR, *Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice.* J Clin Invest, 1992. **90**(2): p. 482-487.
26. De Jongh CM, Verberk MM, Withagen CET, Jacobs JJL, Rustemeyer T, and Kezic S, *Stratum corneum cytokines and skin irritation response to sodium lauryl sulfate.* Contact Dermatitis, 2006. **54**(6): p. 325-333.
27. Bauer D, Grebe R, and Ehrlacher A, *A new method to model change in cutaneous blood flow due to mechanical skin irritation: Part I: Comparison between experimental and numerical data.* J Theor Biol, 2006. **238**(3): p. 575.
28. Fluhr JW, Akengin A, Bornkessel A, Fuchs S, Praessler J, Norgauer J, Grieshaber R, Kleesz P, and Elsner P, *Additive impairment of the barrier function by mechanical irritation, occlusion and sodium lauryl sulphate in vivo.* Brit J Dermatol, 2005. **153**(1): p. 125-131.
29. Schwarz T and Luger TA, *Effect of uv irradiation on epidermal-cell cytokine production.* J Photoch Photobio B, 1989. **4**(1): p. 1-13.
30. Lee JY, Effendy I, and Maibach HI, *Acute irritant contact dermatitis: recovery time in man.* Contact Dermatitis, 1997. **36**(6): p. 285-290.
31. Agner T and Serup J, *Sodium lauryl sulfate for irritant patch testing - a dose-response study using bioengineering methods for determination of skin irritation.* J Invest Dermatol, 1990. **95**(5): p. 543-547.
32. Fluhr JW, Kuss O, Diepgen T, Lazzerini S, Pelosi A, Gloor M, and Berardesca E, *Testing for irritation with a multifactorial approach: comparison of eight non-invasive measuring techniques on five different irritation types.* Brit J Dermatol, 2001. **145**(5): p. 696-703.
33. Andersen PH and Maibach HI, *Skin irritation in man - a comparative bioengineering study using improved reflectance spectroscopy.* Contact Dermatitis, 1995. **33**(5): p. 315-322.
34. Berardesca E, Elsner P, and Maibach HI, *Bioengeneering of the skin: Cutaneous blood flow and erythema*. 1994, Boca Raton, FL: CRC Press.
35. Berardesca E, Elsner P, Wilhelm KP, and Maibach HI, *Bioengineering the skin: Methods and instrumentations*. 1995, Boca Raton, FL: CRC Press.
36. Holloway GA and Watkins DW, *Laser Doppler measurement of cutaneous blood-flow.* J Invest Dermatol, 1977. **69**(3): p. 306-309.
37. Fullerton A, Stucker M, Wilhelm KP, Wardell K, Anderson C, Fischer T, Nilsson GE, and Serup J, *Guidelines for visualization of cutaneous blood flow by laser Doppler perfusion imaging - A report from the Standardization Group of the European Society of Contact Dermatitis based upon the HIRELADO European community project.* Contact Dermatitis, 2002. **46**(3): p. 129-140.
38. Pinnagoda J, Tupker RA, Agner T, and Serup J, *Guidelines for transepidermal water-loss (Tewl) measurement - A report from the Standardization-Group-of-the European-Society-of-contact-dermatitis.* Contact Dermatitis, 1990. **22**(3): p. 164-178.
39. Fullerton A, Fischer T, Lahti A, Wilhelm KP, Takiwaki H, and Serup J, *Guidelines for measurement of skin colour and erythema - A report from the standardization group of the European society of contact dermatitis.* Contact Dermatitis, 1996. **35**(1): p. 1-10.
40. Verbaan FJ, Bal SM, van den Berg DJ, Dijksman JA, van Hecke M, Verpoorten H, van den Berg A, Luttge R, and Bouwstra JA, *Improved piercing of microneedle arrays in dermatomed human skin by an impact insertion method.* J Control Release, 2008. **128**(1): p. 80-88.
41. Le Fur I, Reinberg A, Lopez S, Morizot F, Mechkouri M, and Tschachler E, *Analysis of circadian and ultradian rhythms of skin surface properties of face and forearm of healthy women.* J Invest Dermatol, 2001. **117**(3): p. 718-724.

42. Yosipovitch G, Xiong GL, Haus E, Sackett-Lundeen L, Ashkenazi I, and Maibach HI, *Time-dependent variations of the skin barrier function in humans: Transepidermal water loss, stratum corneum hydration, skin surface pH, and skin temperature.* J Invest Dermatol, 1998. **110**(1): p. 20-23.
43. Jacobson RM, Swan A, Adegbenro A, Ludington SL, Wollan PC, and Poland GA, *Making vaccines more acceptable - methods to prevent and minimize pain and other common adverse events associated with vaccines.* Vaccine, 2001. **19**(17-19): p. 2418-2427.
44. Larsson M, Steenbergen W, and Stromberg T, *Influence of optical properties and fiber separation on laser Doppler flowmetry.* J Biomed Opt, 2002. **7**(2): p. 236-243.
45. Sivamani RK, Stoeber B, Wu GC, Zhai HB, Liepmann D, and Maibach H, *Clinical microneedle injection of methyl nicotinate: stratum corneum penetration.* Skin Res Technol, 2005. **11**(2): p. 152-156.
46. Chilcott RP, Dalton CH, Emmanuel AJ, Allen CE, and Bradley ST, *Transepidermal water loss does not correlate with skin barrier function in vitro.* J Invest Dermatol, 2002. **118**(5): p. 871-875.
47. Romani N, Holzmann S, Tripp CH, Koch F, and Stoitzner P, *Langerhans cells - dendritic cells of the epidermis.* APMIS, 2003. **111**(7-8): p. 725-740.
48. Kanitakis J, *Anatomy, histology and immunohistochemistry of normal human skin.* Eur J Dermatol, 2002. **12**(4): p. 390-400.
49. Li GL, Van Steeg TJ, Putter H, Van Der Spek J, Pavel S, Danhof M, and Bouwstra JA, *Cutaneous side-effects of transdermal iontophoresis with and without surfactant pretreatment: a single-blinded, randomized controlled trial.* Brit J Dermatol, 2005. **153**(2): p. 404-412.