International Journal of Pharmaceutics 392 (2010) 127–133

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Transdermal delivery of insulin using microneedle rollers in vivo

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ARTICLE INFO

Article history:
Received 4 January 2010
Received in revised form 6 March 2010
Accepted 18 March 2010
Available online 25 March 2010

Keywords:
Transdermal drug delivery
Microneedle roller
Transepidermal water loss
Insulin
Diabetes

ABSTRACT

This study characterizes skin perforation by commercially available microneedle rollers and evaluates the efficacy of transdermal delivery of insulin to diabetic rats. Three different needle lengths, 250, 500 and 1000 μm, were used in this work. Creation and resealing of the skin holes that were produced by the needles were observed by Evan's blue (EB) staining and transepidermal water loss (TEWL) measurements. EB clearly showed that microchannels were formed in the skin and that the pores created by the longest microneedle (1000 μm) persisted no longer than 8 h, while the hypodermic injury was still observed 24 h later. TEWL significantly increased after the application of the needles and then decreased with time, which explains the recovery of skin barrier function and agrees well with EB results. The extent of permeation was demonstrated by insulin delivery in vivo. The rapid reduction of blood glucose levels in 1 h was caused by the increased permeability of the skin to insulin after applying microneedle rollers. The reduced decrease after 1 h is closely associated with hole recovery. In conclusion, microneedle rollers with 500-μm or shorter lengths are safe and useful in transdermal delivery of insulin in vivo.

1. Introduction

Despite the advantages of drug delivery through the skin, such as extreme convenience, good patient compliance, prolonged therapy, avoidance of the liver's first-pass metabolism and degradation in the gastrointestinal tract, transdermal drug delivery is only used with a small subset of drugs. This is because the stratum corneum (SC), which is the outermost layer of the skin, constitutes the major barrier (Naik et al., 2000; Prausnitz and Langer, 2008). In recent years, microneedle technology as proposed by Henry et al. (1998) has been developed as an advanced technique for penetration of large molecular weight and hydrophilic compounds into the skin. Four different types of microneedle designs such as poke-and-patch (Martanto et al., 2004; Verbaan et al., 2007; Li et al., 2009), coat-and-poke (Cormier et al., 2004; Widera et al., 2006), poke-and-release (Pearton et al., 2005; Ito et al., 2006), and poke-and-flow (Martanto et al., 2006a; Nordquist et al., 2007) have been developed and investigated in vitro and in animals, even in humans (Bal et al., 2008; Gill et al., 2008; Haq et al., 2009; Van Damme et al., 2009).

Poke-and-patch provides a simple method to enable delivery of hydrophilic drugs and macromolecules from a transdermal patch (Prausnitz, 2004). Solid microneedle arrays can be pressed onto the skin to create microscopic holes; drugs from a patch or topical formulation can then be administered. From a review of the literature, getting a flat microneedle array to pierce the skin using a manual application is far from easy (Yang and Zahn, 2004; Teo et al., 2006). With the help of the electric applicator, a short needle length was able to pierce the mouse's skin (Ding et al., 2009a). Most of the studies which have demonstrated in vivo delivery of drugs using microneedle arrays have utilized some sort of high-velocity impact applicator or longer needle lengths to aid the penetration of microneedle arrays into the skin (Verbaan et al., 2008; Ding et al., 2009b). However, in some vitro experiments, these microneedle arrays can increase the skin's permeability when applied manually with shorter needle lengths. These findings are not in agreement with those obtained from in vivo experiments (Teo et al., 2005; Kolli and Banga, 2008). This result is probably due to the "bed-of-nails" effect and the thick subcutaneous fat and muscle layers in vivo, which render microneedle penetration more difficult in vivo (Martanto et al., 2006a,b).

To circumvent the above difficulties, we used another microneedle denoted the microneedle roller, which is commercially available for cosmetic purposes. The microchannels in the skin formed by rolling the microneedle roller are similar to those created by microneedle arrays; the former is technologically simplified and easier to handle. The needles of the rollers are made from stainless steel with good biocompatibility and torsional properties (Disegi and Eschbach, 2000). An in vitro evaluation of skin penetration by the microneedle roller had been reported (Badran et al., 2009). However, there is little information available regarding their in vivo

Abbreviations: EB, Evan's blue; TEWL, transepidermal water loss; SC, stratum corneum; SEM, scanning electron microscopy.

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doi:10.1016/j.ijpharm.2010.03.041

Fig. 1. A photograph of microneedle rollers with different needle lengths.

character and efficacy. In this work, we conduct systematic studies in vivo, including EB staining, TEWL, and transdermal drug delivery. The response over time of the treated skin to microneedle injury as assessed by EB staining and TEWL provides information which is highly significant in determining drug safety and drug delivery time. Insulin was chosen as a model drug for macromolecule tests due to the ease of analyzing its pharmacodynamic action, as well as the substantial barrier to transdermal permeation by passive diffusion. Diabetes is one of the leading lethal diseases in China and around the world and is often treated by hypodermic injection of insulin, which creates problems such as pain when insulin delivery is performed by those who are inexperienced with the technique. These factors can result in reduced patient compliance. So injection is generally not an ideal method for the administration of these drugs, especially in the context of long-term treatment. Transdermal delivery of insulin by microneedle rollers is convenient and easy to handle and would overcome these limitations. Three different needle lengths were used to evaluate the efficiency of skin perforation and the degree of insulin penetration enhancement, with a special focus on the method used to select a microneedle roller that was found to be safe and effective in the context of manual application. It is noteworthy that drug delivery by microneedle rollers is also suitable for other macromolecules.

2. Materials and methods

2.1. Materials

Recombinant human insulin was purchased from Dongbao Enterprise Group Co. Ltd. (JiLin, China). Streptozotocin (STZ), sodium pentobarbital and Evan's blue (EB) were purchased from Sigma–Aldrich Chemical Company (Shanghai, China). All other materials used in this study were obtained from Chemical Reagent Company (Beijing, China) and were of analytical grade.

Male Sprague–Dawley rats were procured from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The microneedle rollers (ZGTS^{TM}) were bought from JianLi Trade Ltd. (GuangDong, China). Three different models of ZGTS^{TM} with needle lengths of 250, 500 and 1000 μm were used. A photographic picture of the microneedle rollers is shown in Fig. 1. Each microneedle roller contains 192 very fine medical grade stainless steel tiny needles in eight rows in a cylindrical assembly (the diameter and the length of the cylinder are 2 cm). There is a handle for operation.

2.2. Methods

2.2.1. Characterization of the microneedle rollers

Fig. 2 shows microscope images (taken with an Olympus BH3-WHP6) of microneedle rollers of three different needle lengths. The lengths of needles shown in Fig. 2a–c are about 250, 500, and 1000 μm, respectively. The specific diameters at the top and base of the needles are: 10 and 100 μm for the 250-μm needles; 15 and 140 μm for the 500-μm needles; 20 and 250 μm for the 1000-μm needles.

2.2.2. EB staining studies for microchannel visualization

The male rats were first anesthetized with intraperitoneal injections of 60 mg/kg sodium pentobarbital. The hair on their abdomen was clipped using an animal hair clipper, and the rest of the hair roots were removed using a depilatory cream. The skins were closely examined after 24 h to ensure their intactness in case of any destruction during handling. Then microneedle rollers with different needle lengths were rolled in perpendicular lines over the skin surface, ten times each. Considering the individual differences among rats, three microneedle rollers and a 25-G hypodermic needle were applied to four locations on the abdomen of the same rat. Only a single puncture is necessary when using hypodermic needles.

To visualize the microchannels that formed in the skin, 1 mL of EB (1% solution) was applied to the treated skin surface. The EB was left on the treated skin for 5 min before excess EB was removed using water. Then the rats were sacrificed and the full-thickness skins were carefully removed using a scalpel and surgical scissors. The excess subcutaneous fat and connective tissue were also removed. To determine when the wound had healed, the skins were obtained at 0, 1, 2, 3, 5, 8 and 24 h after the application of the needles and examined under the biological microscope (Olympus MIX41) with a digital camera (COOLPIX 4500, Nikon Co., China). Scanning electron microscopy (SEM)

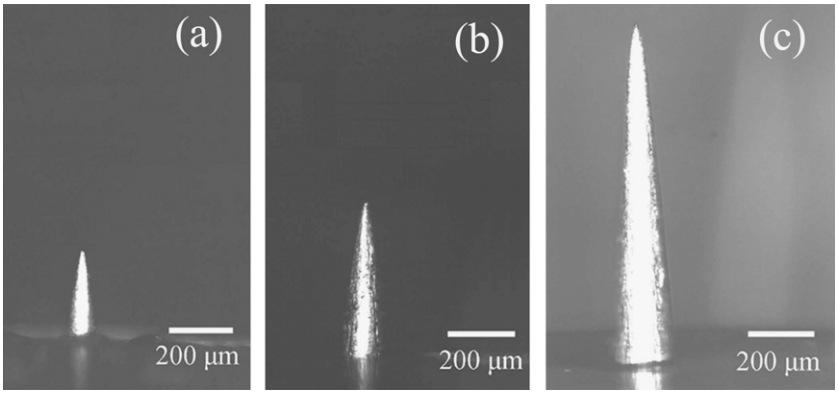

Fig. 2. A photograph of microneedle rollers with three different lengths: 250, 500 and 1000 μm (a–c).

images of skin treated with a 500-μm microneedle roller were also obtained.

Animal handling was performed according to Good Laboratory Practice (GLP). The protocol for animal experiments was approved by the Institutional Animal Ethical Committee of Beijing, China, and studies were carried out in accordance with principles of laboratory animal care and approved protocols.

2.2.3. Transepidermal water loss (TEWL)

Frequently used in the cosmetics and dermatology industries, transepidermal water loss (TEWL) measurement is a standardized method to determine changes in skin barrier properties. The high TEWL value corresponds to damaged skin while low TEWL value is correlated to less damaged skin or healthy skin. In our study, TEWL was measured to determine the extent to which skin barrier function was disrupted following application of the microneedle rollers and the 25-G hypodermic needle. The rats were acclimatized to ambient room temperature and relative humidity, which were maintained at 22 ± 2 °C and 45 ± 5%, respectively for 30 min. Then rats were anesthetized with intraperitoneal injections of 60 mg/kg sodium pentobarbital. Every rat had five prescribed circles located on the abdomen where the hairs had been removed 24 h previously. The skins were treated with 250-, 500- and 1000-μm microneedle rollers as well as the hypodermic needle, using the same method as in the staining studies. A negative control without any treatment was also prepared. Note that two basic TEWL detections were performed 0 and 30 min before the needle treatments. If the two detected values were not significantly different, treatment was initiated. Immediately after piercing the skin, TEWL was measured by a Tewameter (MPA 9, Courage + Khazaka, Cologne, Germany). The measurements were taken by carefully resting the TEWL probe horizontally on the treated site, with the probe head vertical and perpendicular to the skin. The values represent the mean TEWL readings for the 20 s before the measurements were stopped. If there were any uncharacteristic spikes during this period, a more representative 20 s reading was used. TEWL readings were recorded 1, 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 min after the application of microneedle rollers and the 25-G hypodermic needle.

2.2.4. Diabetic animal model

Male Sprague–Dawley rats used in this study were acclimatized in the animal facility for one week before the experiment. They were housed at 22 ± 2 °C and 50–70% relative humidity. The diabetic model was induced in rats (200 ± 20 g) with streptozotocin (50 mg/kg in citrate buffer, pH 4.0) administered by intraperitoneal injection. Rats were fasted overnight, but had free access to water prior to this experiment. Rats were deemed to be diabetic when their baseline glucose levels exceeded 16.7 mM (300 mg/dl) after 48 h. Blood glucose levels were measured with a glucose assay kit on a Blood Analyzer (ONETOUCH® Ultra™, China). Blood glucose levels were allowed to stabilize for one week.

2.2.5. In vivo absorption studies

The rats with blood glucose levels between 20 and 30 mM were selected for study and were divided in groups containing six rats each. The abdomen skin surface where the hairs were removed 24 h previously was washed using cotton soaked with 75% ethanol, after the Sprague–Dawley rats were anesthetized with intraperitoneal injections of 60 mg/kg sodium pentobarbital. Before application of the microneedle rollers, the blood glucose values were detected to establish baseline glucose levels. Next, microneedle rollers which had been disinfected with 75% ethanol were rolled in two perpendicular lines over the skin surface, ten times each. A 2.5 cm^2 × 0.5 cm cotton piece was then placed on the treated skin and 2 mL insulin solution which was prepared by dissolving the recombinant human insulin in phosphate buffered saline (pH 7.4) was added to form the insulin patch. It was covered with a thin plastic film to prevent any leakage of the insulin solution. Blood samples were withdrawn from the tail vein at 0, 1, 2, 3, 4, 5 and 6 h after the beginning of the experiments and blood glucose levels were measured. After the 3 h insulin delivery period, the insulin patches were removed from the skin, which was then cleaned with water. Blood glucose measurements were continued every 1 h for another 3 h. The drug transport studies were performed with the following pretreatments:

a. Variation in the lengths of microneedle rollers: 250, 500 and 1000 μm. The concentration of the insulin solution was 200 IU/mL. The results of the experiment dictated the microneedle roller selected for the following experiment.
b. Variation in the routes of administration: transdermal insulin delivery using microneedle roller (300 IU/mL) vs. subcutaneous injection using hypodermic needle (5 IU/kg). The latter also served as a positive control group.
c. Variation in the concentrations of the insulin: 50, 100, and 300 IU/mL.

As a negative control experiment, the same protocol was followed, except no microneedle rollers were applied to the skin, and the insulin solution was placed passively on the skin for 3 h. Finally, in order to observe the changes in blood glucose levels with time, a time-controlled group was added.

2.2.6. Statistical analysis of results

Statistical analysis of differences between treatments was performed using unpaired Student's *t*-test and data, expressed as mean ± S.D. A level of significance of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. EB staining studies for microchannel visualization

Monitoring the presence of skin punctures provides a primary indication of skin healing followed the application of microneedle rollers and hypodermic needles. Fig. 3 shows that microchannels were formed in the skin after these needle applications. There was a positive correlation between pore size and microneedle size. The same result was also obtained with regard to scab healing time; the values obtained were approximately 2, 3 and 8 h for the 250-, 500- and 1000-μm microneedle rollers, respectively. However, a larger pore mark was observed when a 25-G hypodermic needle was applied to the skin. In addition, bleed and erythema, which did not appear in skin treated with microneedle rollers, were clearly observed. EB staining showed that the hypodermic injury was still visible at 24 h. The dermal side of the skin was also examined at 0 h. No blue staining could be seen after application of 250- and 500-μm microneedle rollers, while the 1000-μm microneedle roller and the hypodermic needle could be observed as shown in Fig. 3.

The pores created by the 500-μm long microneedle could be visualized in more detail by SEM. Pore size was about 70 μm, which is in between the sizes of the top and base of the microneedle (Fig. 4).

3.2. TEWL

As shown in Fig. 5a, the TEWL values were measured immediately before and after treatment with 250-, 500-, and 1000-μm microneedle rollers or the hypodermic needle. TEWL values increased significantly ($P < 0.05$) as compared to baseline for all

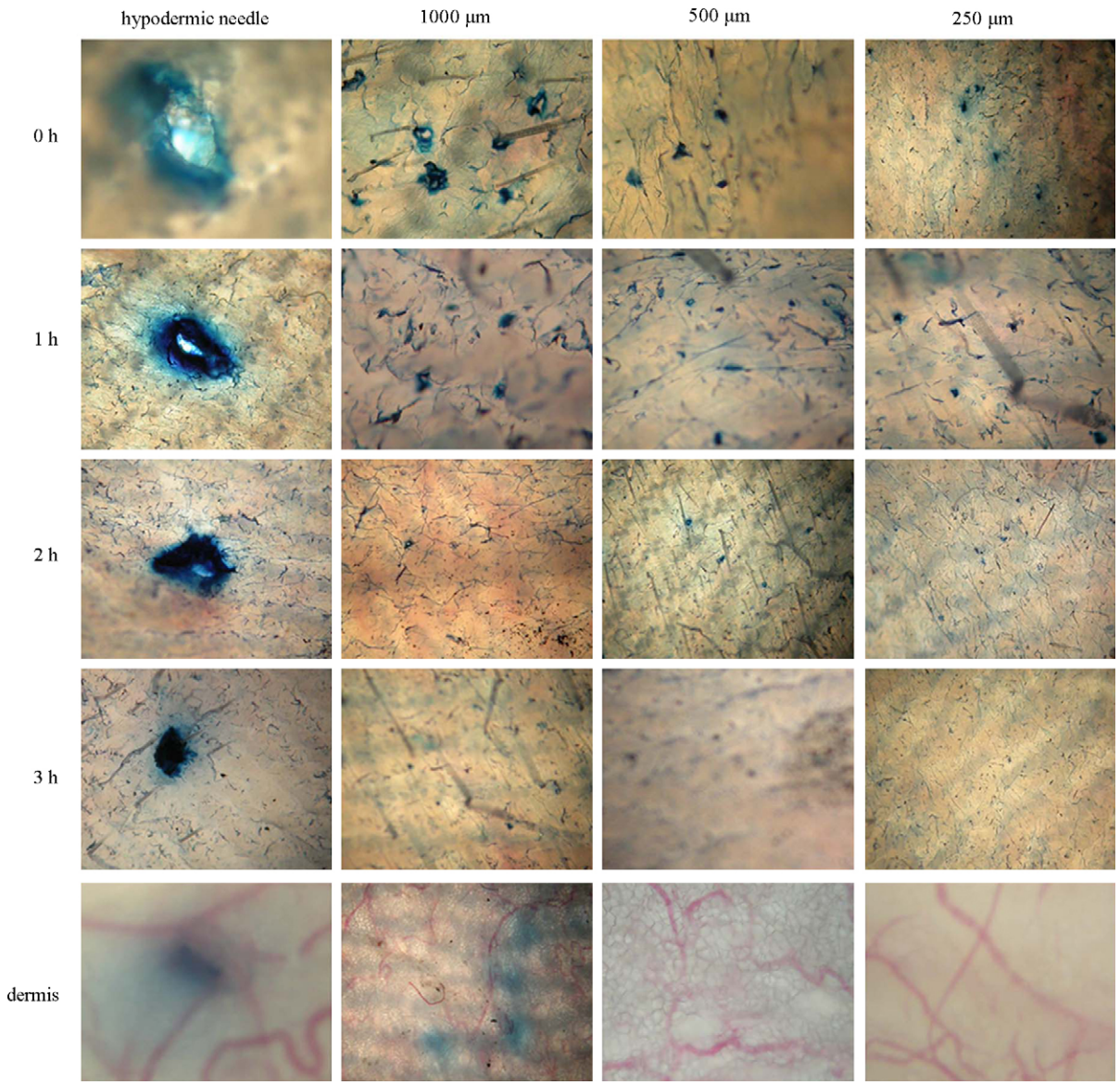

Fig. 3. Rat skin surface after the application of microneedle rollers and 25-G hypodermic needles in vivo, followed by topical staining with EB dye, were observed over time by light microscopy. Pictures were taken from the SC and the dermal sides at 0 h.

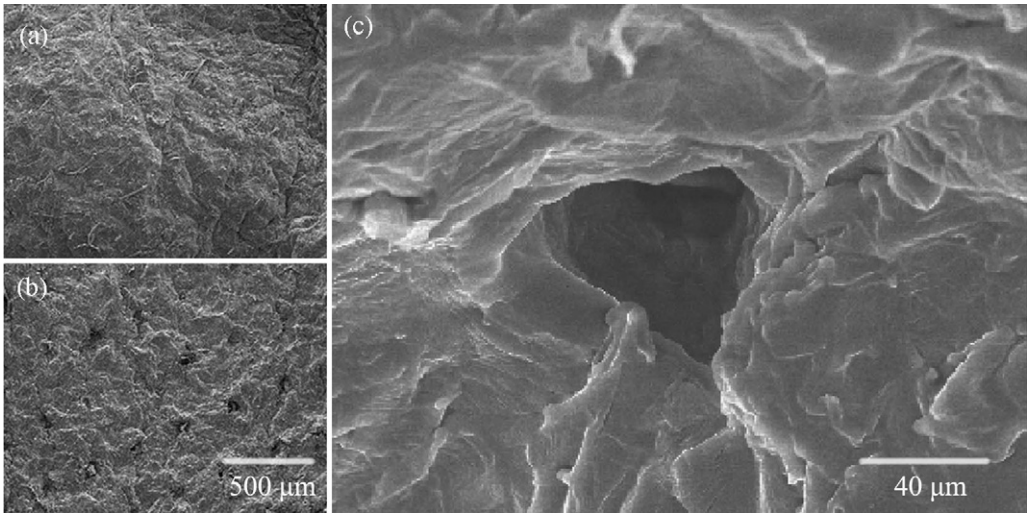

Fig. 4. Scanning electron micrograph images of the surface morphology of intact rat skin (a) and skin treated with a 500-μm microneedle roller (b and c).

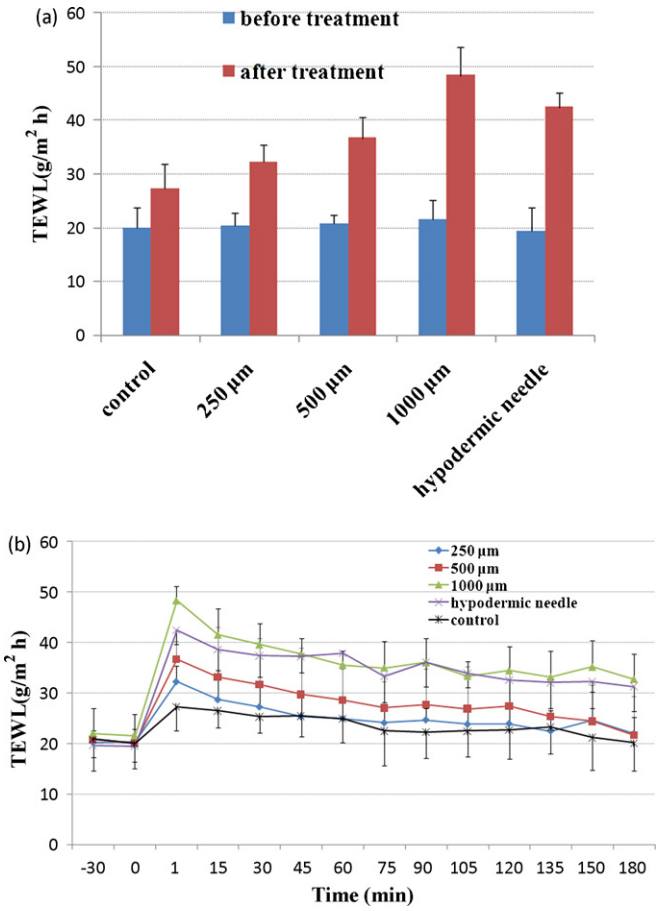

Fig. 5. TEWL was detected by the application of the needles on rat skin in vivo: (a) TEWL detected before and after the application of the needles and (b) TEWL before and after application of different needles and the dependence on time. The microneedle rollers and the 25-G hypodermic needle were applied at $t = 1$ min. Data are presented as average values ± S.D.; $n = 6$.

groups. Fig. 5a also showed a positive correlation between TEWL values and the lengths of the needles. The TEWL values increased 1.57%, 1.62%, and 2.25% for the 250-, 500-, and 1000-μm microneedle rollers, respectively. TEWL values increased 1.36% and 2.18% for the control group and the hypodermic needle, respectively.

The TEWL values were obtained at different intervals over a period of 3 h after treatment with the microneedle rollers and the hypodermic needle shown in Fig. 5b. For all the needles, the TEWL values reached the maximal peak after piercing and decreased continuously with time. The 250- and 500-μm treatment TEWL values recovered to baseline within 3 h, with no significant difference ($P > 0.05$) as compared to control, while the 1000-μm and hypodermic needle treatment groups did not return to baseline values within the time frame of the experiment.

3.3. Effect of the length of microneedle rollers in vivo

As shown in Fig. 6, the various groups displayed reduced blood glucose levels. The blood glucose levels at various times were normalized against the initial blood glucose levels. For all three microneedle roller groups, blood glucose levels decreased remarkably in 1 h and continuously throughout the 3 h insulin delivery period. Blood glucose level then increased slowly after the insulin was removed. With increasing needle length, slight differences in blood glucose values were observed. It was also observed that after removing the insulin patch, the rate of the elevation in blood glucose level was inversely proportional to the length of the

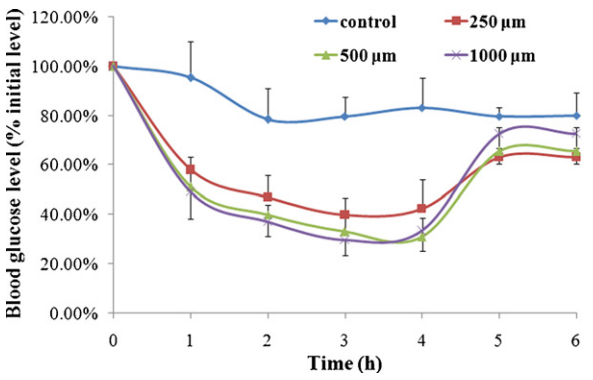

Fig. 6. The effects of microneedle length on changes in blood glucose level in diabetic rats. Data are presented as average values ± S.D.; $n = 6$.

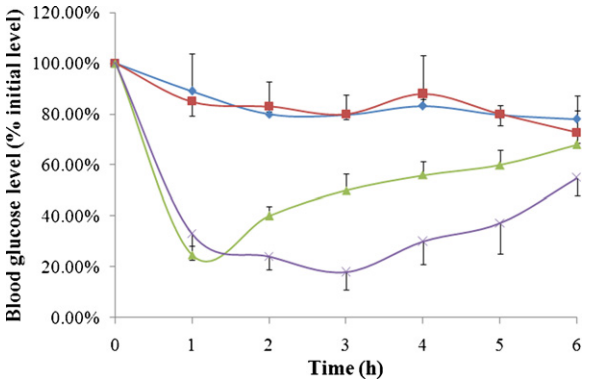

Fig. 7. Changes in blood glucose level in rats as determined using 500-μm microneedle rollers (×), subcutaneous hypodermic injections of insulin (▲), passive delivery across untreated skin (■) and time control (♦). Data are presented as average values ± S.D.; $n = 6$.

microneedle: the 250-μm microneedle roller treatment resulted in the slowest recovery and the 1000-μm microneedle facilitated the most rapid recovery. Based on previous and present results, the 500-μm microneedle roller was chosen for the following experiments.

3.4. Effect of the route of administration

As shown in Fig. 7, the group in which insulin was applied to the rat's skin without microneedle rollers was not significantly different ($P > 0.05$) as compared with the time-controlled group, confirming the well-known inefficiency of passive insulin absorption via the transdermal route. The subcutaneous injection of insulin resulted in a rapid decrease in blood glucose; the blood glucose level at 1 h was about 25% of its initial value. For the 500-μm group, the minimum glucose level was about 18% of the initial value at 3 h, which is significantly different from the negative control rats ($P < 0.05$). In comparison to the positive control group receiving subcutaneous injections with a hypodermic needle after 3 h, the changes in blood glucose levels induced by microneedle roller are greater ($P < 0.05$), but there was no significant difference during the initial 3 h. This indicates that microneedle rollers could increase skin permeability during long-term treatment to deliver physiologically relevant amounts of pharmacologically active insulin.

3.5. Effect of insulin patch concentration

Fig. 8 shows the effect of varying insulin solution concentration, where higher insulin concentration (300 IU/mL) reduced blood glucose levels to a greater extent than lower insulin concentra-

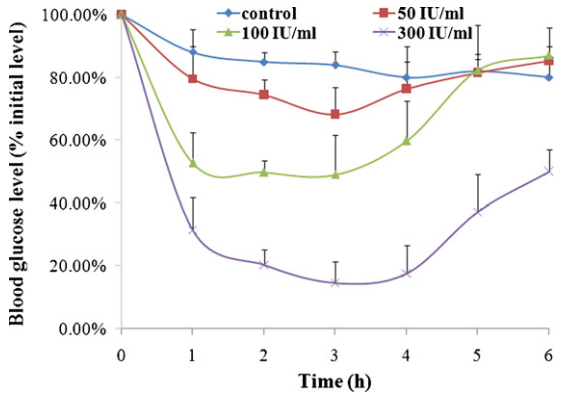

Fig. 8. The effects of insulin concentration on changes in blood glucose level in diabetic rats following application of the 500-μm microneedle roller. Data are presented as average values ± S.D.; $n = 6$.

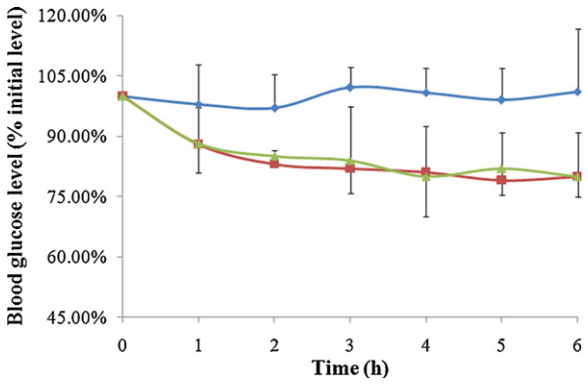

Fig. 9. Changes in blood glucose level in rats. Anesthetic diabetic animals (■), diabetic animals but not anesthetic animals (▲) and normoglycemic rats (♦). Data are presented as average values ± S.D.; $n = 6$.

tion (50 IU/mL) ($P < 0.05$), which is consistent with the expected dose–response relationship.

4. Discussion

In this study, microneedle rollers commonly used for cosmetic purposes have been utilized to disrupt the barrier posed by the skin. The ability of microneedle rollers to produce pores and the related ability of holes in the skin to reseal was determined by EB staining and measurement of TEWL in vivo. Furthermore, the efficiency of drug transdermal delivery through the microchannels produced by the microneedle roller was demonstrated by in vivo studies of insulin. We systematically compared microneedle rollers with three different needle lengths which would affect skin perforation. The cylindrical assembly of the microneedle rollers used in this study has the advantage of lower forces used to pierce the skin, because rolling the device over the skin surface is all that is necessary.

First, the presence of blue staining confirmed the successful interruption of the SC barrier by treatment with microneedle rollers. The resealing time for the microneedle rollers was much less than that observed for the hypodermic needle. This implies that the microneedle would involve less microbial penetration than puncture using a normal hypodermic needle (Donnelly et al., 2009). The pores created by the application of microneedle rollers were smaller than the base of the microneedle, probably due to partial skin recoil after microneedle removal, as well as partial piercing of the skin with the microneedle. After needle application, the 1000-μm roller might have reached the nerves in the dermis, which would cause pain and damage small capillaries.

TEWL analysis further demonstrated the compromised intactness of the SC followed application of the microneedle rollers and the hypodermic needle. Maximal TEWL values were observed after application of the needles in all groups including the control group. It is well known that TEWL is an extremely sensitive measurement of skin permeability and is likely to be affected by many factors (Roskos and Guy, 1989). In this experiment, the elevated TEWL values observed in the control group may be due to irritation of the rats by the needles. So, rigorous precautions must be taken during measurement.

TEWL data were consistent with the results obtained through staining experiments. The increases in TEWL values were observed immediately after needle application due to the pores that had formed; decreases in these values can be attributed to the recovery of holes with time. The results also demonstrated that the TEWL values obtained by using the 1000-μm microneedle roller were similar to those obtained with the hypodermic needle but were larger than those obtained after use of the 250- and 500-μm microneedle rollers ($P < 0.05$). The TEWL values correlated directly with total pore area. This result indicated that the total pore area of skin treated with 1000-μm microneedle rollers was similar to that of skin treated with hypodermic needles. Notably, the 250- and 500-μm microneedle rollers yielded a smaller disrupted area.

Finally, the ability to deliver insulin through the microchannel in vivo was also verified. As determined through the application of microneedles with different needle lengths as well as varying concentrations of insulin solution, we found that blood glucose level was a function of time. Over time, blood glucose levels decreased in both control groups, one subjected to passive intradermal administration of insulin solution and the other to anesthesia without any treatment. The results could be explained by the following two interpretations. First, anesthesia may influence blood glucose levels in rats. Second, the physiological conditions of the diabetic animals are not stable, such that fluid losses are twice as high as those of normoglycemic animals. This factor could in turn affect blood glucose levels. The following three groups of experiments were designed to verify the assumptions outlined above: one group of normoglycemic animals, one group of diabetic animals that were not placed under anesthesia and one group of diabetic animals placed under anesthesia. Fig. 9 shows that blood glucose levels decreased in both of the diabetic groups but there were only small fluctuations in normoglycemic animals. Therefore the second interpretation outlined above appears to be appropriate and reasonable.

The results showed that blood levels decreased with increasing needle length. However, slight differences in blood glucose levels after treatment with different needle lengths were observed. This may be because the SC layer has a phospholipid bilayer structure while the model drug (insulin) is hydrophilic. These factors may have induced limited entry of the drug into the microchannel. Also, deeper skin that has fat and subcutaneous tissue may also hamper entrance of the macromolecular drug, but not to the stratum corneum. However, the inverse relationship between increases in blood glucose and length of the microneedle could be explained by the higher amount of residual insulin in the SC treated by the 250-μm microneedle roller after removal of the patch. Badran et al. (2009) delivered the drug in vitro through pores created by the microneedle rollers. These authors also found that the shortest needle length (150 μm) was most effective for drug delivery in the SC.

5. Conclusions

In this study, we have shown that microneedle rollers with different needle lengths can be used to improve transdermal drug delivery. The ability of microneedles to create transport pathways

and the extent of hole resealing were successfully assessed by staining and TEWL data. Such data have rarely been obtained through in vivo studies on animals. The enhanced transdermal delivery of insulin was also demonstrated in vivo by effective control of blood glucose level. Taken together, our results demonstrate that the 250- and 500-μm microneedle rollers can pierce the skin during manual application and are the most promising tools for in vivo delivery of biologically active proteins such as insulin. This delivery method is easy to handle, applicable to many other vaccines and holds great potential for future applications.

Acknowledgement

This work was supported by the National Natural Science Fund (grant number is 60976086).

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