PROF. HYUNGIL JUNG (Orcid ID : 0000-0002-5716-5426)

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Adenosine-Loaded Dissolving Microneedle Patches to Improve Skin Wrinkles, Dermal Density, Elasticity, and Hydration

G. Kang[1], T. N. T. Tu[2], S. Kim[1], H. Yang[1], M. Jang[1,3], D. Jo[4], J. Rye[4], J. Baek[4] and H. Jung[1, 3, *]

[1]Department of Biotechnology, Yonsei University, Seoul, Korea

[2]Underwood International College, Yonsei University, Seoul, Korea

[3]JUVIC Inc., Seoul, Korea

[4]DERMAPRO LTD., Seoul, Korea

[*]**Correspondence**

Hyungil Jung

[1]Department of Biotechnology, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

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[3]Juvic Inc., Building 102, Yonsei Engineering Research Park, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address hijung@yonsei.ac.kr

Geonwoo Kang

[1]Department of Biotechnology, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address kkw0601@gmail.com

Tu Ngoc Thu Trang

[2]Life Science and Biotechnology, Underwood International College, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address thutrang.tn.h2@gmail.com

Suyong Kim

[1]Department of Biotechnology, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address ksy880521@gmail.com

Huisuk Yang

[1]Department of Biotechnology, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address huisuk@yonsei.ac.kr

Mingyu Jang

[1]Department of Biotechnology, Yonsei University, 50, Yonsei-ro, Seodaemun-gu, Seoul, 03722, Korea

[3]Juvic Inc., Building 102, Yonsei Engineering Research Park, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

Tel. +82-2-2123-2884, Fax +82-2-362-7265, E-mail address god1540@hanmail.net

Da Jung Jo

[4]Dermapro Skin Research Center, 30 Bangbaejoongang-ro, Seocho-gu, Seoul, 06684, Korea

Tel. +82-2-597-5435, Fax +82-2-597-5430, E-mail address dermapro@dermapro.co.kr

Ja Hyun Rye

[4]Dermapro Skin Research Center, 30 Bangbaejoongang-ro, Seocho-gu, Seoul, 06684, Korea

Tel. +82-2-597-5435, Fax +82-2-597-5430, E-mail address dermapro@dermapro.co.kr

Ji Hwoon Baek

[4]Dermapro Skin Research Center, 30 Bangbaejoongang-ro, Seocho-gu, Seoul, 06684, Korea

Tel. +82-2-597-5435, Fax +82-2-597-5430, E-mail address dermapro@dermapro.co.kr

Abstract

OBJECTIVE: Although dissolving microneedle patches have been widely studied in the cosmetics field, no comparisons have been drawn with the topical applications available for routine use. In this study, two wrinkle-improving products, adenosine-loaded dissolving microneedle patches and an adenosine cream, were evaluated for efficacy, with respect to skin wrinkling, dermal density, elasticity, and hydration, and safety in a clinical test on the crow's feet area.

METHODS: Clinical efficacy and safety tests were performed for 10 weeks on 22 female subjects with wrinkles around their eyes. The adenosine-loaded dissolving microneedle patch was applied once every 3 days, in the evening, for 8 weeks to the designated crow's feet area. The adenosine cream was applied two times per day, in the morning and evening, for 8 weeks to the other crow's feet area. Skin wrinkling, dermal density, elasticity, and hydration were measured by using PRIMOS® premium, Dermascan® C, Cutometer® MPA580, and Corneometer® CM 825, respectively. In addition, subjective skin irritation was evaluated by self-observation, and objective skin irritation was assessed through expert interviews.

RESULTS: The adenosine-loaded dissolving microneedle patches had a similar or better efficacy than the adenosine cream. Both groups showed statistically significant efficacy for almost all parameters ($P < 0.05$). The dissolving microneedle patches had a long-lasting effect on the average wrinkle depth ($P < 0.05$), only showed efficacy in dermal density ($P <$

0.05), had an early improving effect on elasticity ($P < 0.05$), and demonstrated better hydration efficacy ($P < 0.001$). No adverse effects were observed in either group during the test period.

CONCLUSIONS: In the clinical efficacy test of four skin-improvement parameters, adenosine-loaded dissolving microneedle patches showed the same or better effect than the adenosine cream, although the weekly adenosine dose was 140 times lower. The dissolving microneedle patches caused no adverse reactions. These adenosine-loaded dissolving microneedle patches are expected to be safe, effective, and novel cosmetics for skin improvement.

Keywords

Dissolving microneedle, Wrinkle improvement, Safety testing, Skin physiology/structure, Delivery/vectorisation/penetration

Introduction

Dissolving microneedles (DMNs), micro-scale needles with a biodegradable polymer matrix, have been developed for transdermal drug delivery [1-3]. DMNs physically penetrate the skin, after which their biodegradable polymer matrix is dissolved by bodily fluids, which results in the release of the encapsulated drugs within the skin. Typically, DMNs are constructed using safe and biocompatible materials, such as hyaluronic acid, carboxymethyl cellulose, and maltose; they completely dissolve in the skin with no remaining biohazardous waste products after use [4-6]. Owing to these advantages, DMNs have been widely

investigated for medical and cosmetic purposes, such as the treatment of diabetes, dementia or anemia, skin vaccinations [7-10], and skin whitening or wrinkle improvement [11-14].

In the clinical applications of DMNs in cosmetics, the delivery of the active compounds across the stratum corneum, the outermost skin barrier, is of critical importance [15]. DMNs have been developed to deliver many active compounds, such as retinyl retinoate, ascorbic acid, 4-n-butylresorcinol, and epidermal growth factor, for wrinkle improvement and skin depigmentation [11-14]. Usually, the efficacy of these DMNs has been analyzed in comparison to a control sample of blank DMNs (i.e., without the active compound) [12-14], and thus reveals the effects of the active ingredients carried by DMNs. In cases where the effect of the active compound is reduced because of activity loss during encapsulation into the microneedles, or the delivery efficacy is low, it is likely no significant difference in efficacy will be found between active compound-loaded DMNs and blank DMNs.

The activity of the encapsulated active compound depends on several environmental factors, such as temperature, pH, and interaction with the polymer backbone during microneedle fabrication [16-18]. In previous clinical trials, most of the active compounds were encapsulated into DMNs via droplet-born air blowing, which is sensitive to external environmental factors [19]. For the delivery of retinyl retinoate, ascorbic acid, and 4-n-butylresorcinol via DMNs, cosmetic ingredient-loaded DMNs demonstrated statistically significant differences in the improvement of wrinkles and depigmentation [11, 13, 14]. However, in epidermal growth factor-loaded DMNs, there was no significant difference in wrinkle improvement between loaded and blank DMNs because of the loss of activity of EGF, which is sensitive to the air blowing and temperatures used during DMN fabrication [20]. It is important to minimize the activity loss during DMN fabrication to maximize the effect of DMNs in clinical applications.

Although DMNs have been developed to deliver active compound by overcoming the stratum corneum, hyaluronic acid, the backbone of DMN which is a booster of collagen production, was delivered (21). Because this improves wrinkles, a comparison between the active compound encapsulated in DMNs and the blank DMNs masks the improvement effect generated by hyaluronic acid. Therefore, it is difficult to estimate the complete efficacy of the DMNs, which is the combination of the effects of the active compound delivered by the DMNs and hyaluronic acid, the backbone matrix. As DMNs have been introduced to overcome the current topical applications of active compounds, a comparison of the complete efficacy of DMNs should be made with the commercially available topical applications on the market. However, to the best of our knowledge, there have been no studies comparing efficacy of active compound-loaded DMNs with topical creams. Thus, we added commercially available topical cosmetic cream as an additional comparison group to show how DMNs overcome the limitations of topical application and exhibit combinative effects of active compound and hyaluronic acid.

In this study, we compared adenosine-loaded DMNs (Ad-DMN) with an adenosine cream (Ad-Cream), which is known to improve crow's feet wrinkles [22]. We fabricated Ad-DMNs by using centrifugal lithography [23], which exerts less stress on the drug-loaded DMNs, minimizing the activity loss than other DMN fabricating methods [24]. After the *in vitro* skin penetration test of Ad-DMN patches, improvement in skin wrinkling, dermal density, elasticity, and hydration effect in human crow's feet was evaluated in a randomized clinical efficacy and safety of adenosine application in the DMN group and the cream group. The trials showed that the DMN group had the same or better efficacy than the cream group with respect to all factors and that neither treatment caused side effects to the skin. Consequently, the Ad-DMN patches are expected to improve skin wrinkling, dermal density, elasticity, and hydration, without sensitization and irritation problems.

Materials and methods

Adenosine-loaded DMN patch and cream fabrication

To fabricate the Ad-DMN patches, an adenosine-mixed polymer solution was prepared by dissolving 0.42% (w/w) of adenosine (Bojing Chemical, Shanghai, China) and 39.23% (w/w) of 30 kDa hyaluronic acid (HA) (Soliance, Pomacle, France) in distilled water. HA was selected for the backbone of the Ad-DMN patches because it has the optimal viscosity for DMN fabrication, biodegradability, biocompatibility, and high water solubility. This prepared solution was homogenized and degassed by using a vacuum mixer (ARV-310, Thinky, Tokyo, Japan) and dispensed with a 7 × 7 array by using a precision dispensing robot (SHOTmini 200Sx, Musashi Engineering, Inc., Tokyo, Japan) on a polyurethane film, which was placed on a sticky hydrocolloid patch. The dispensed polymer solution droplets were fabricated into the DMNs by centrifugal lithography to produce a 7 × 7 Ad-DMN array on a 10-mm square polyurethane film. In each Ad-DMN patch, there were a total of 49 DMNs, and the mass of DMNs were measured as 1,276.19 μg. The expected adenosine content per patch was calculated to be 5.36 μg from the weight of DMN and the adenosine loading rate. For sterilization of Ad-DMN patches, E-beam irradiation was conducted by Seoul Radiology Services (Seoul, Korea) using 8-kW high-energy linear accelerator (MB10-8/635, Mevex Corp., ON, Canada) at 25 kGy.

The Ad-Cream for the clinical test, which contained 0.04% (w/w) adenosine, was kindly provided by Cosnine (Gimpo, Korea). A single dose of the Ad-Cream comprised 250 mg, which contained approximately 100 μg of adenosine.

DMN morphology and penetration of Ad-DMN patches into pig cadaver skin

To validate the morphological properties of microneedles, the Ad-DMN patches were visualized by using an optical microscope. After a uniform cone-shaped array was confirmed, each pair of microneedle patches was packed in a plastic case. To evaluate the skin-penetration capability of Ad-DMN, separate 5 × 5 Ad-DMN patches were made by same fabrication method, and applied to 1-mm-thick hairless pig cadaver skin (Cronex, Hwaseong, Korea) using thumb force for 15 min. After the DMN patch was detached from the pig cadaver, the skin was stained with 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min to visualize the DMN penetration area. After the excess dye was washed with distilled water, the Ad-DMN patch array and the stained pig cadaver skin were observed by using an optical microscope.

Skin wrinkle measurement through three-dimensional imaging

Eye wrinkles were analyzed by using PRIMOS® premium (GFMesstechnik GmbH, Berlin, Germany), a non-contact device that uses Digital Micro Mirror Devices technology. The images were captured at each evaluation time point and analyzed at the same site by using a matching function. The changes in the skin wrinkles were analyzed by using a wrinkle analysis parameter. In this test, the wrinkles of the left and right eye tails were measured at the following time points: before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped.

Dermal density measurement using ultrasonic imaging

Dermal density was measured by using Dermascan® C (Cortex Technology, Hadsund, Denmark), a diagnostic ultrasound device that images the echoes reflected from inside the human body. The degree of echo is determined by the sum of the number and the size of the reflected sound waves among tissues with different densities. Using this principle, a two-dimensional image can be obtained, in which the moisture content of epidermis and dermis is expressed in color. In this test, the dermal density was analyzed before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped.

Skin elasticity measurement using the absorption method

Skin elasticity was measured by using Cutometer® MPA580 (Courage+Khazaka Electronic, GmbH, Kern, Germany), which graphically and numerically displays the elasticity of the skin by adsorbing the skin at a constant negative pressure of 450 mbar for 2.0 s and stopping for 2.0 s, three times in succession. In this test, the R2 parameter (gross elasticity; as the value approaches 1, the elasticity is improved) was measured before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped.

Skin hydration measurement using capacitance

Skin hydration was measured by using a Corneometer® CM 825 (Courage+Khazaka Electronic, GmbH, Kern, Germany), which measures the moisture content in the stratum corneum. The epidermal stratum corneum has a high resistance to electricity and this resistance is reduced when an alternative current wave is applied. The capacitance changes depending on the moisture content of the skin; the higher the capacitance, the higher the

moisture content. In this test, the skin moisture was measured three times and the average value was analyzed before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped.

Clinical efficacy test of adenosine-loaded DMNs patch and cream

To measure the clinical efficacy of Ad-DMNs and the Ad-Cream, 22 healthy female subjects (average age 48.27 ± 4.27 years) with crow's feet (wrinkles around the eyes) were selected by using subject selection criteria (Data S1). The subjects were prohibited from using functional cosmetics or medicines other than the test product. To perform a blind test, the Ad-DMNs and the Ad-Cream were grouped into samples A and B, respectively. Sample A was applied once every 3 days, in the evening, for 8 weeks, because frequent microneedle application may cause erythema and cannot provide enough time for skin regeneration. After cleansing, the subjects trimmed their skin with the facial toner and then applied sample A to the designated crow's feet area (right or left) by their thumb force. Approximately 250 mg of sample B was applied two times per day, in the morning and the evening, for 8 weeks based on the general usage of topical cosmetics. After cleansing, in the final step of skincare, the subjects gently applied sample B to the designated crow's feet area (right or left). Because the purpose of this study was to compare microneedle and traditional topical application, each microneedle and cream was applied based on their optimized method, and thus the amount of adenosine was different. Skin wrinkle parameters, such as elasticity, hydration, and dermal density, were evaluated before and after using the products at each time point (after 4 and 8 weeks of use and 2 weeks after the test).

Skin sensitization and irritation test

After using the product, subjective skin irritation was assessed by self-observation of the tester and objective skin irritation was evaluated through expert interviews, based on the degree of skin adverse reactions including itching, prickling, burning, erythema, edema, and scale.

Statistical analysis

The statistical significance of all data was computed by using the SPSS® Package Program (IBM, New York, NY, USA). Normality was verified by the Shapiro–Wilks test and kurtosis and skewness, with pre-homogeneity verified by a paired t-test. In parametric cases, the repeated-measures analysis of variance was used to consider the interdependence (data interchange) in repeated data from the same subjects for three or more time points and the paired t test was used for two time points. In non-parametric cases, the Wilcoxon signed rank test with Bonferroni correction was used. In the comparisons between the groups at each point, repeated-measures analysis of variance was used when the homogeneity was satisfied, and analysis of covariance was used when the homogeneity was not satisfied.

Results and Discussion

Morphology analysis and skin penetration test of the Ad-DMN patch

Prior to *in vitro* analysis and the clinical safety and efficacy tests, the morphology of the DMN array was analyzed. The 7 × 7 Ad-DMN array with a constant shape and length (Fig. 1a) and a single DMN with a perfectly symmetrical cone shape were observed by using an optical microscope (Fig. 1b). The average length and tip diameter of the DMN was 256.7 ± 22.6 μm and 29.6 ± 3.1 μm, respectively. Two Ad-DMN patches were packed into one plastic case for convenient use in the clinical test and applied twice per week (Fig. 1c)

To confirm the skin penetration ability of the prepared Ad-DMN patch, the DMN patch was applied to the pig cadaver skin for 15 min and then detached. The dissolution of the DMN array (Fig. 1d) and a single DMN applied to the pig cadaver skin were observed by using an optical microscope (Fig. 1e). No DMNs were observed after the 15 min application on pig cadaver skin. To confirm this dissolution was caused by the bodily fluids in the pig cadaver skin, the skin was stained with trypan blue solution to visualize the penetration area of the DMNs after the patch was detached. After the solution was washed, complete microchannel formation stained with trypan blue solution was observed in white dotted circles (Fig. 1f). This implied that the Ad-DMNs had sufficiently strong mechanical force to physically penetrate the pig skin, and the penetrating DMN structure was fully dissolved by bodily fluids after 15 min. Because the skin insertion depth of microneedle was approximately 92% of the 280 μm height DMN, the insertion depth of Ad-DMN is expected to be similar [25] and microchannel caused by skin insertion of DMNs can be useful for the delivery of adenosine contained in the Ad-DMN. [26].

Clinical test: Improvements of skin wrinkling and dermal density

Clinical efficiency was determined through the application of Ad-DMNs once every 3 days and the Ad-Cream two times per day for 8 weeks. The measurements were recorded after 4 and 8 weeks of application and 2 weeks after the product application was stopped.

The wrinkle depth was assessed with a standard optical image and 3D image as shown in Fig. 2a. The standard optical image, real skin photographs, showed an improvement in skin wrinkling over time in the DMN and adenosine cream groups. In addition, the improvement in the wrinkles was more evident in the 3D image. As shown in the wrinkled area in the dotted circle, a gradual reduction was observed in wrinkle shape after 4 weeks and 8 weeks of application, with further reduction observed at 2 weeks after the use of the products was stopped, compared with the wrinkle depth before use of product in both the DMN and cream groups. Although skin image analysis demonstrated wrinkle-improving effects in both groups, the DMN group appeared to have better efficacy. To quantitatively confirm the wrinkle reduction in the DMN and cream groups and the efficacy difference between the two groups, which were confirmed by using optical and 3D image analysis, the change in the average depth of wrinkles was measured.

The reduction in wrinkles in both group was quantitatively analyzed by using a 3D skin-measuring instrument. The average depth of wrinkles was found to decrease in both the DMN group and the cream group. The average wrinkle depth improvement in the DMN group was 0.40%, 3.69%, and 4.43% after 4 and 8 weeks of use and 2 weeks after the use of the product was stopped, respectively; in contrast, the improvements in the cream group were 3.67%, 4.01%, and 3.38%, respectively. These results indicate that adenosine can be delivered into the skin through both delivery methods and it exerted a wrinkle-improving effect (Fig. 2b). The DMN group showed less improvement in the average wrinkle depth after

4 weeks of use than the cream group; however, at 2 weeks after the product application was stopped, the improvement was remarkable. Thus, Ad-DMNs had a longer-lasting effect on wrinkle reduction than the Ad-Cream. The improvement after 4 weeks of use in both group and at 2 weeks after the product application was stopped in the DMN group were statistically significant when compared with the values before use ($p < 0.05$).

In addition, the dermal density ultrasonography image showed that the dermis becomes dense when Ad-DMNs or the Ad-Cream was applied for more than 4 weeks (Fig. 3a). The sparse green dots, which represent the dermis in the white dotted circle, became denser as the product was used for 4 weeks and 8 weeks and there was no significant difference between groups by image analysis.

Although the quantitative analysis of the dermal density showed increases in both group, the improvement in the DMN group was statistically more significant (Fig. 3b). The degree of improvement in DMN group was 0.41%, 0.71%, and 0.00% after 4 and 8 weeks of use and 2 weeks after the product application was stopped, respectively; in the cream group, the values were 0.26%, 0.26%, and 0.06%, respectively. The DMN group showed more improvement in dermal density after 4 and 8 weeks of use than the cream group. The degree of improvement of dermal density in the DMN group was statistically significant when compared with the values before product use ($p < 0.05$).

Clinical test: improvement of skin elasticity and hydration

The skin elasticity showed similar improvement tendencies in both groups (Fig. 4); a significant improvement was observed after 8 weeks of product application compared with before application ($p < 0.001$). The skin elasticity improvement in the DMN group was

0.82%, 1.56% and 0.24% after 4 and 8 weeks of use and 2 weeks after the product application was stopped, respectively; in the cream group, the values were 0.37%, 1.33%, and 0.40%, respectively. In the DMN group, greater improvement was observed after 4 of weeks use than in the cream group.

The trends in the improvement of skin hydration values were also similar in both groups (Fig. 5). The skin hydration increment percentage in the DMN group was 1.38%, 2.87%, and 0.09% after 4 and 8 weeks of use and 2 weeks after the product application was stopped, respectively; in the cream group, the values were 1.24%, 2.02%, and -0.09%, respectively in cream group. These results showed that the Ad-DMNs were more effective in improving skin hydration than the Ad-Cream. The skin hydration value was even decreased at 2 weeks after the product application was stopped, which is an indication that sustained efficacy of the Ad-Cream was inferior to that of Ad-DMNs. The improvement after 4 and 8 weeks of use in both groups was significant compared with the values before use ($p < 0.05$).

If the hydration of the skin is increased in both the epidermal and dermal layers, more dermal interstitial fluid is gathered, which loosens skin fibers and makes the skin less viscous and more fluid-like; hence, skin elasticity is positively correlated with skin hydration [27]. The improvement in skin elasticity and hydration showed that Ad-DMNs exerted better skin-improvement effects than the Ad-Cream. The explanation of the comparatively superior efficacy of Ad-DMN, despite the low adenosine content, was attributed to the special properties of the microneedles that were not found in cream formulations, as described above.

Skin sensitization and irritation test

No adverse events on the skin were observed in any subjects during the study period in either group (Data S2). Both products are considered safe for use as cosmetics.

Comparison of skin-improving effects of Ad-DMNs and the Ad-Cream

Although the apparent effects of both Ad-DMNs and Ad-Cream on wrinkles and dermal density were similar in clinical efficiency tests, Ad-DMNs exerted longer-lasting wrinkle improvement efficiency and greater dermal density enhancement than the Ad-Cream. The adenosine content in the Ad-DMNs and the Ad-Cream was different. The DMN group received 10.72 μg of weekly adenosine dose, whereas the cream group received 1400 μg, an increase of approximately 140 times. Despite the lower quantity of active ingredient, the skin-improving effects of Ad-DMNs were better than those of the Ad-Cream.

DMNs had better drug delivery efficacy than cream formulations because DMNs physically penetrate the skin barrier and directly release the encapsulated drug inside the skin, whereas the cream formulations are applied on skin and only a small amount of encapsulated drug is delivered owing to the barrier presented by the stratum corneum [28]. Moreover, the DMNs have inherent skin-improvement effects through the induction of micro-needling, which leads to a natural wound healing effect on the skin after the activation of collagen production and the improvement of various factors, such as wrinkle and dermal density [21]. In the DMN fabrication process, HA was used in the structure of DMN. HA is reported to promote collagen synthesis and partially restore dermal matrix components, thereby relieving wrinkles and improving the conditions of the skin [29].

The purpose of this study is to compare DMN with traditional topical applications, thus the cream was applied on the general usage of topical cosmetics and microneedle was applied with the optimized method without side effects. Even though the statistical significance between Ad-DMN and Ad-Cream was not prominent, considering adenosine content of Ad-DMN is 140 times lower than Ad-Cream, those results demonstrate the scientific merit of this study.

Conclusion

In this study, we compared the efficacy of DMNs with the application of topical cosmetics at a clinically relevant dose. In a 10-week clinical test, we found that the DMN group had the same or better skin improvement effects, with respect to skin wrinkling, dermal density, elasticity, and hydration, although the applied dose of adenosine was 140 lower than that of the cream group. We also found that the DMNs did not cause any adverse events on the skin. Therefore, adenosine-loaded DMN patches may be a novel cosmetic product with good safety and wrinkle-improvement effects.

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Supporting Information

Data S1. Clinical trial subject selection criteria, exclusion criteria and guideline.

Data S2. Skin adverse reactions of adenosine-loaded dissolving microneedle patch and cream.

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Figure legends

Figure 1 (a) Image of the Ad-DMN array. The central part of the hydrocolloid patch is coated with a polyurethane film, on which the DMN array is placed. Scale bar: 1 mm. (b) Image of a single DMN in the patch. Scale bar: 200 μm. (c) Image of adenosine-loaded DMN patches in the plastic case. Scale bar: 10 mm. (d) Dissolved DMN array after 15 min application on pig cadaver skin. Scale bar: 1 mm. (e) Close-up image of dissolved DMN. Scale bar: 200 μm. (f) After the application of DMNs for 15 min, the pig cadaver skin was stained with trypan blue solution. White dotted circles indicate the penetration area of DMN Scale bar: 1 mm.

Figure 2 (a) Images of the wrinkles of subjects. The standard optical image and 3D image

showed changes in skin wrinkling. Wrinkles gradually disappeared after 8 weeks of application and 2 weeks after the product application was stopped in both the dissolving microneedle (DMN) group and the cream group. (b) The change in average depth of wrinkles in crow's feet after the application of adenosine-loaded dissolving microneedles (DMNs) and the adenosine cream. The application was continued for 8 weeks, with measurements recorded before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped. Both products showed a significant improvement in the average depth of wrinkles (mean ± SEM; $*p < 0.05$ compared with values before use).

Figure 3 (a) Images of the dermal density of subjects. Ultrasonography images showing changes in the dermal density of subjects. The density of the dermal cells gradually increased after 8 weeks of application and 2 weeks after the product application was stopped in both the DMN group and the cream group. (b) The change of dermal density of the crow's feet after the application of adenosine-loaded dissolving microneedles (DMNs) and adenosine cream. The application was conducted for 8 weeks, with measurements collected before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped. Both products caused a significant improvement in dermal density. (mean ± SEM; $*p < 0.05$ compared with the values before use).

Figure 4 Change of skin elasticity on crow's feet after the application of adenosine-loaded dissolving microneedles (DMNs) and adenosine cream. The application was conducted for 8 weeks, with measurements collected before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped. Both products showed a significant improvement in skin elasticity. (mean ± SEM; $*p < 0.05$, $***p < 0.001$ compared with the values before use).

Figure 5 The change in skin hydration of crow's feet after the application of adenosine-loaded dissolving microneedles (DMN) and adenosine cream. The application was conducted for 8 weeks, with measurements taken before use, after 4 and 8 weeks of use, and 2 weeks after the product application was stopped. Both products resulted in a significant improvement in skin hydration (mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, compared with the values before use).