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Skin collagen reproduction increased by ascorbic acid derivative iontophoresis by frequent-reversal bipolar electric stimulation

YUSUKE HORI, RYUJI AKIMOTO, AKIKO HORI, KATSUHIKO KATO, DAISUKE CHINO, SHOHEI MATSUMOTO, SHOHEI KAMIYA, and YASUO WATANABE, *Tes Holdings Co., Ltd, Tokyo 111-0032 (Y.H); Homer Ion Laboratory Co., Ltd, Tokyo 150-0045 (R.A., S.K.); Gene Expression and Regulation, Institute of Medical Science, University of Tokyo 108-8639 (A.H.); Department of Genetical Education, School of Veterinary Medicine, University of Azabu, Kanagawa 229-8501 (K.K.); Department of Pharmacology and Pharmacotherapy, School of Medicinal Pharmacy, Nihon Pharmaceutical University, Saitama 362-0806 (D.C., Y.W.); and Department of Anesthesiology, Tokyo Medical University, Tokyo 160-0023 (S.M.), Japan.*

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Synopsis

The effect of the iontophoresis of ascorbic acid (Vitamin C; VC) derivative with frequent-reversal bipolar electric stimulation on the production of collagen in rat skin was evaluated in terms of hydroxyproline content through high-performance liquid chromatography. First, a control group was not given electrical stimulation and four groups were stimulated with a unipolar pulse for 0.5–10 min every day for one week. The hydroxyproline level in the skin was increased depending on the length of the stimulation. Second, a control group was not given any electrical stimulation, and three groups were treated with (a) VC solution without any stimulation, (b) a bipolar pulse for 10 min with saline, or (c) a bipolar pulse for 5 min with the VC solution. Significant increases were found in all the stimulation groups, although these treated with the VC solution without any stimulation did not have any effects compared to the control. Thus, in order to increase the hydroxyproline levels in skin, a VC must be delivered with bipolar stimulation as a method of iontophoresis. These results suggest that our newly developed electric stimulation is effective at increasing skin collagen content, and that bipolar stimulation is more effective on the iontophoresis of not only VC but also some medicines such as low- and high-molecular drugs directed to the target organ (7).

INTRODUCTION

Collagen is a major constituent of the dermis, and is therefore involved in many facets of skin disease as well as the recovery process. Collagen also plays an important role in maintaining the moisture content and the elasticity of skin, although collagen is altered and

damaged by aging and/or ultraviolet rays (1,2). Generally, in order to avoid such damage and changes, attempts have been made to regenerate collagen in skin by stimulation with either a laser or far-infrared rays (3). However, these treatments induce erythema or redness of the skin for a few weeks to several months (4,5).

Meanwhile, it has been already reported that ascorbic acid (vitamin C; VC) promotes the synthesis of collagen. Furthermore, in the field of esthetics, VC derivatives, which have a high affinity for skin cells, have been used to regenerate skin collagen because they are highly effective and safe, as shown in previously reported papers (6,7). However, treatment with VC takes a long time to obtain effects because of its weak activity. Recently, a method of treatment using VC derivatives with a weak electric current has been developed for improving the absorption of VC derivatives by the skin, so-called iontophoresis, although satisfying effects have yet to be obtained (7,8). Recently, we developed a frequent-reversal electric stimulation machine, and in preliminary human studies, the effects of the VC derivative iontophoresis with this machine were much more satisfying than those of the VC derivative alone, although the actual increased amount of skin collagen could not be detected (Watanabe, Hori, Oh-I, Urushibata, Akiyama, and Kamiya; unpublished data, 2008). In addition, frequent-reversal electric stimulation is much more harmless and can be used as sequential stimulation for a much longer period than frequent unipolar stimulation.

The main purpose of this study is to prove the effects of a newly developed electric stimulation, "Gunpatsu pulse®" (Application number 2006-048423; filing date, May 26, 2006; registration number 5026198, Japanese Patent Office) on the regeneration of collagen by measuring the hydroxyproline content of the skin using high-performance liquid chromatography (HPLC) with a fluorogenic agent, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) (9). Furthermore, we compared the effects of unipolar Gunpatsu pulse stimulation and bipolar Gunpatsu pulse stimulation either with or without VC derivative (aminopropy1 ascorbyl phosphate) on the increase of rat skin hydroxyproline content within the most effective and shortest duration (one week).

MATERIALS AND METHODS

ANIMALS AND GUNPATSU PULSE STIMULATION

Healthy seven-week-old male Sprague-Dawley rats (SPF) (Charles River Japan Inc., Yokohama, Japan) were used in this experiment. These rats were housed in wire-mesh-bottom cages in an animal room with controlled temperature, humidity, and lighting, with food and water available *ad libitum.* At breeding, plain tap water was supplied. All rats were anesthetized with pentobarbital-Na (45 mg/kg, i.p., Abbot Laboratories, IL, U.S.A) until unconscious and removed of hair on the abdominal and dorsal skin before the experiment, since the electrode for the Gunpatsu pulse was tied around the abdominal and dorsal skin. Before any stimulation, circular dorsal skin samples 8 mm in diameter from the right of the midline as a control were stored at −80°C until analyzed. Electric stimulation (4800 Hz, 5 V; duty: 50% and 1.2 mA) was then applied for one week, and more than one week later, dorsal skin samples from the left of the midline were stored as above (9). This paper is focused on the evaluation of the several effects of Gunpatsu pulse stimulation on the increases in rat skin collagen using the following two experiments:

Experiment 1: Electric stimulation with unipolar Gunpatsu pulse (Figure 1) (time course). A total of 50 rats were randomly divided into five groups and housed individually. The control group (10 rats/group) did not receive any electric stimulation. The other four groups (10 rats/group) received the unipolar Gunpatsu pulse stimulation (see Figure 1) for 0.5, 2, 5, and 10 min every day for one week.

Experiment 2: Electric stimulation with bipolar Gunpatsu pulse (combined with a VC solution). A total of 40 rats were randomly classified into four groups and were housed individually. The control group (10 rats/group) was treated with a saline solution without any electric stimulation. The other three groups (10 rats/group) were treated with (a) the VC derivative (aminopropyl ascorbyl phosphate, pH 7.4) solution without any Gunpatsu pulse stimulation, (b) bipolar Gunpatsu pulse stimulation (see Figure 1) for 10 min with saline, or (c) bipolar Gunpatsu pulse stimulation for 5 min with the VC solution. Gunpatsu pulse stimulation was performed at 4800 Hz and 5 V every day for one week.

MEASUREMENT OF THE AMOUNT OF COLLAGEN IN SKIN

Most methods for determining collagen levels are based on measurements of the quantities of proline and/or hydroxyproline, the principal amino acid components of collagen. Recently, we developed a simple and highly sensitive method of measuring the amount of hydroxyproline in skin by using isocratic HPLC with NBD-F (9).

REAGENTS

NBD-F was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The standards for HPLC were hydroxyproline (Wako Pure Chemical Industries Ltd.) and a standard amino acid mixture, Type AN-2 (Wako Pure Chemical Industries Ltd.). The Wako standard amino acid solution is an equimolar mixture of Asp, Cys, Glu, Ser, Gly,

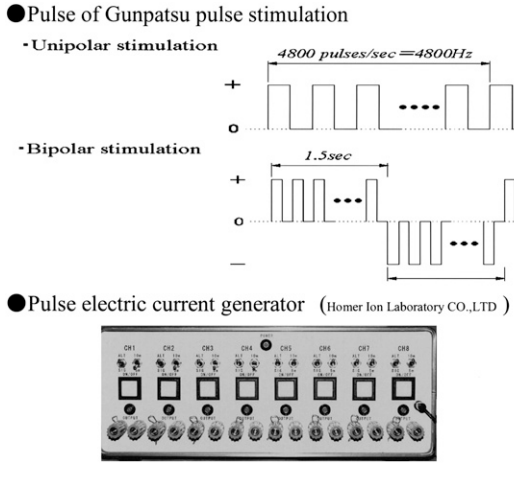

Figure 1. Typical pulse pattern of either unipolar or bipolar stimulation of Gunpatsu pulse. Unipolar stimulation shows the only positive site of 4800 Hz, and the bipolar stimulation shows the both positive and negative sites at each 1.5 sec. Electric stimulation (4800 Hz, 5 V, duty: 50% and 1.2 mA) was obtained by the new device (see photograph).

His, Arg, Thr, Ala, Tyr, Val, Met, Ile, Leu, Phe, Lys, Pro, and hydroxyproline. All other chemicals were of HPLC grade. Hydrochloric acid (HCl), acetonitrile, potassium dihydrogenphosphate, sodium tetra borate decahydrate, and boric acid were purchased from Wako Pure Chemical Industries Ltd. Ethylenediamine tetra acetic acid disodium salt dehydrates (EDTA-2Na) was purchased from Pharmacia Biotech AB (Uppsala, Sweden).

HYDROLYSIS OF PROTEIN AND DERIVATIVE

Tissue samples were hydrolyzed for 22 h at 108°C in evacuated flame-sealed Pyrex tubes with 400 µl of 6 N HCl, to which 1% phenol was added. The hydrolysates were dried *in vacuo.* For the HPLC analysis, the dry residue of each hydrolysate was re-suspended in 500 µl of 50 mM borate buffer containing 20 mM EDTA-2Na (pH 8.0). The resuspended samples were diluted to 1/2000 with a 50-mM borate buffer containing 20 mM EDTA-2Na and 7.5 µM of L-homoserine (pH 8.0). Next, 30 µl of either the hydrolyzed sample or a mixed amino acid standard solution diluted to 1/100 with a 50-mM borate buffer containing 20 mM EDTA-2Na (pH 8.0) was poured into a 500-µl conical tube. Then 10 µl of NBD-F (10 mM in acetonitrile, freshly prepared) was added to this solution and the tube was capped and covered with aluminum foil. The vessel was heated to 60°C for 1 min, and after it had cooled in ice water, 40 µl of 0.05 M HCl was added to the reaction mixture. Finally, 10 µl of the solution was injected into the column.

HPLC CONDITIONS

Briefly, an EP-300 (Eicom Corp., Kyoto, Japan) pump equipped with an L-7200 auto sampler (Hitachi, Tokyo, Japan) was employed. A guard column (200 mm × 3.9 mm) and a main column of SC-5ODS (200 mm × 4 mm, 10 µm; Eicom Corp., Kyoto, Japan) were used. All solvents were filtered and degassed prior to use. The flow rate was 0.8 ml/min, and the column temperature was kept at 30°C using a column oven. A Hitachi L-7485 spectrofluorometer equipped with a 12-µl flow cell was used with an excitation wavelength of 470 nm and emission at 540 nm. A phosphate buffer (pH 5.0) containing 8% acetonitrile was used as the mobile phase.

STATISTICAL ANALYSES

Results are presented as the mean ± SE. All results were analyzed with Dunnett's multiple comparison test of the Pearson correlation after an analysis of variance.

RESULTS

During this experiment, there was no significant difference between the groups in body weight or quantity of water ingested. No adverse effects were observed in any of the rats (data not shown). All results, particularly for the analysis of hydroxyproline content, obtained by HPLC are clearly documented in Figure 2. The retention times of hydroxyproline and Pro were about 8 and 34 min, respectively. L-homoserine, as an internal standard, was eluted for about 12 min.

Experiment 1: Changes in hydroxyproline levels in the skin and increase in body weight. In the control group (Figure 3; control: the electrode for Gunpatsu pulse was tied around the

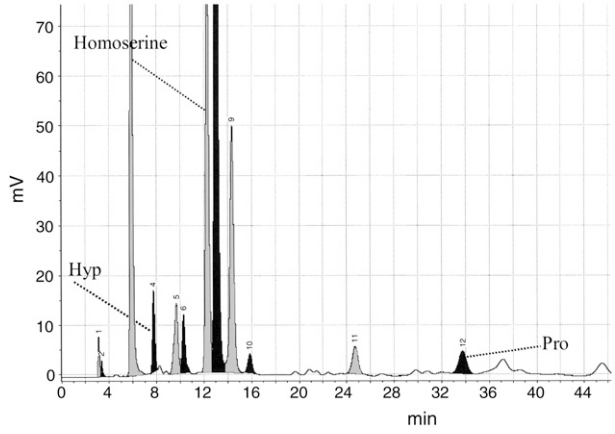

Figure 2. Chromatogram of derivatized rat skin sample (control group) analyzed by isocratic HPLC with the NBD-F method. Homoserine is the internal standard amino acid. Hydroxyproline was eluted at about 8 min.

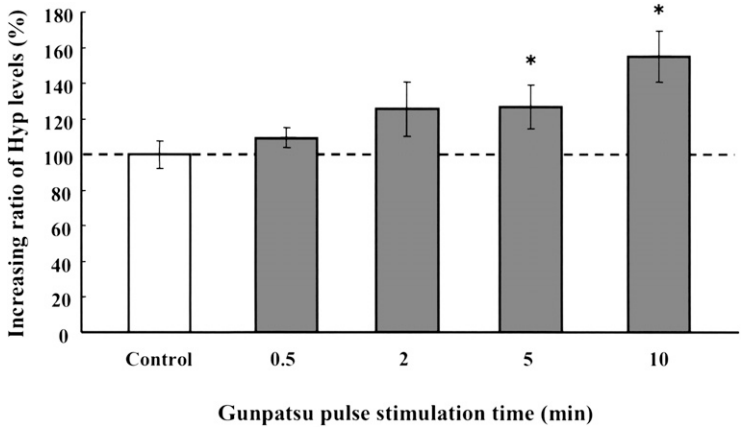

Figure 3. Changes in hydroxyproline levels in skin stimulated by unipolar Gunpatsu pulse stimulation. Each column shows the mean ± SE of 10 animals. The dotted line expresses the value of 100% of the control group. *Significant difference from the control group. *$p < 0.05$.

abdominal and dorsal skin but with no Gunpatsu pulse stimulation), hydroxyproline levels in the skin were 102.6 ± 9.3 nmol/mg (10 rats/group). These levels were increased depending on the length of stimulation with a unipolar Gunpatsu pulse, with significant increases when the stimulation lasted longer than 5 min (Figure 3). In addition, the increases in body weight seen in the Gunpatsu pulse groups were likely to be less than those seen in the control group. More than 10 min of Gunpatsu pulse stimulation seems to prevent a gain in body weight (Figure 4).

Experiment 2: Changes in hydroxyproline levels in skin stimulated by iontophoresis using Gunpatsu pulse. In the control group (Figure 5; saline + non-Gunpatsu pulse: the electrode for Gunpatsu pulse was tied around the abdominal and dorsal skin but with no Gunpatsu pulse stimulation), the hydroxyproline levels in rat skin were 102.8 ± 11.9 nmol/mg (10 rats/group). Significant increases ($p < 0.05$) were found in two Gunpatsu pulse-stimulated groups (Figure 5). In the bipolar Gunpatsu pulse 5-min + VC group, a significant increase

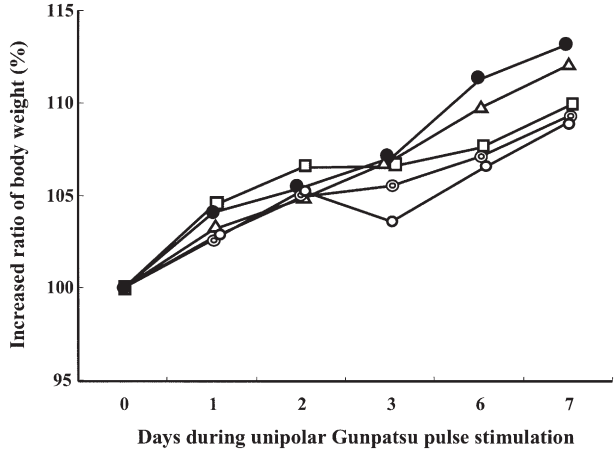

Figure 4. Changes in rat body weight during unipolar Gunpatsu pulse stimulation. Data show the mean ± SE of 10 animals: ●, control group; △, 0.5-min group; □, 2-min group; ◎, 5-min group; ○, 10-min group.

($p < 0.05$) in the concentration of hydroxyproline in skin was detected compared to the control group (Figure 5). Also, a significant increase ($p < 0.05$) was seen in the group that received bipolar Gunpatsu pulse stimulation for 10 min with saline (no VC derivative), although treatment with VC derivative without any Gunpatsu pulse stimulation did not have any significant effect compared to the control group. Thus, in order to increase the amount of hydroxyproline in skin, the VC derivative must be administered with bipolar Gunpatsu pulse stimulation through iontophoresis.

From the results of Experiments 1 and 2, it is clear that the greatest increase in hydroxyproline levels occurred in the group treated with unipolar Gunpatsu pulse stimulation for 10 min (164 ± 20% increase), followed by the group treated with bipolar Gunpatsu pulse

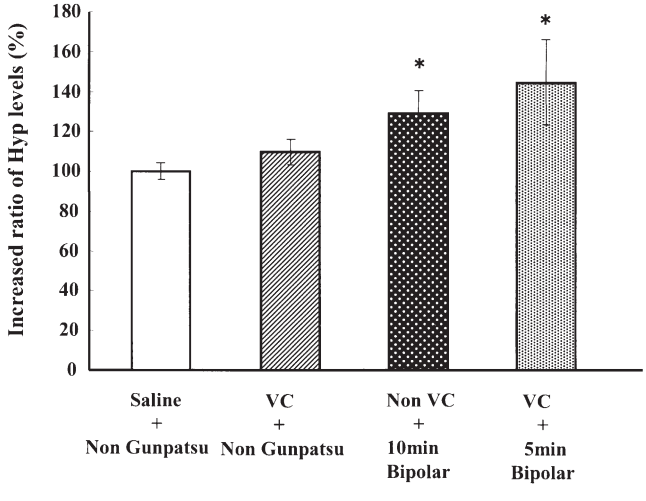

Figure 5. Changes in hydroxyproline levels in skin stimulated by iontophoresis using Gunpatsu pulse stimulation. Data show the mean ± SE of 10 animals. *Significant difference from the control group. *$p < 0.05$.

stimulation for 5 min with VC (144 ± 21% increase), and then the group treated with bipolar Gunpatsu pulse stimulation for 10 min without VC treatment (129 ± 11%), although the values did not differ significantly between the three groups. Gunpatsu pulse stimulation with either a unipolar or bipolar pulse is effective at increasing skin hydroxyproline levels and can also be useful for enhancing the iontophoresis of VC. In general, however, a longer electric stimulation can have adverse effects, such as a suppression of body weight gain, as shown in Figure 4. The decrease in body weight can be seen in the 10-min Gunpatsu pulse group alone, and the correlation between the duration of treatment and the changes in body weight cannot be seen in other groups. Additionally, a bipolar Gunpatsu pulse for 5 min showed no disturbance of growth and a more effective increase in hydroxyproline levels compared to those of a 10-min Gunpatsu pulse. Therefore, the iontophoresis of VC using a bipolar Gunpatsu pulse (5 min) is the best way to produce skin collagen.

DISCUSSION

In this paper, we evaluated the usefulness of the iontophoresis of a VC derivative for regenerating collagen in skin by using a newly developed method of electrical stimulation, Gunpatsu pulse, with either unipolar or bipolar pulses. To measure levels of collagen in skin, we used a newly developed, highly sensitive HPLC with NBD-F for detecting the quantity of hydroxyproline. This method clearly showed the changes in hydroxyproline levels in rat skin following Gunpatsu pulse stimulation and/or treatment with the VC derivative.

Two approaches to the regeneration of collagen, a laser/far-infrared ray technique and the iontophoresis of VC derivatives, are widely used in the field of cosmetic dermatology and esthetic training, although they have weak effects and can cause skin damage (10–15). Thus, the techniques of producing collagen in skin require some improvements (5,15).

As shown in Figure 3, daily treatment for 5 or 10 min with unipolar Gunpatsu pulse stimulation promoted the production of collagen, although stimulation for longer than 10 min may suppress body weight gain. Furthermore, as shown in Figure 5, the iontophoresis of the VC derivative was completely achieved with 5 min of bipolar Gunpatsu pulse stimulation without any severe damage (e.g., burn and body weight gain) to rat skin. These results suggest that collagen can be regenerated by the iontophoresis of a VC derivative potentiated by a short bipolar Gunpatsu pulse stimulation without any adverse effects.

Gunpatsu pulse stimulation (4800 Hz pulse) is patented, since it differs from ordinary pulse stimulation, and also can be reversed as unipolar or bipolar (see Figure 1). As our preliminary data, between 1200 and 4800 Hz, the increase in the VC-derivative iontophoresis was seen in a frequency-dependent manner. Between 4800 Hz and 50,000 Hz, such increases were not shown to be significant. Furthermore, Gunpatsu pulse stimulation did not induce any significant damage in the skin.

Attempts to increase the amount of collagen in skin have mostly used pulses for stimulation. For instance, pulses of UV rays and infrared rays are more than 10,000 Hz, whereas pulses widely used in iontophoresis are between 1 and 500 Hz (3–8). Gunpatsu pulse stimulation uses moderate pulses of 4800 Hz.

In this experiment, we demonstrated that Gunpatsu pulse is neither weak nor strong, requires less stimulus to regenerate the collagen in skin, and can be used for the iontophoresis of VC derivatives. Additionally, bipolar Gunpatsu pulse stimulation is useful for

the iontophoresis of VC derivatives and some medicines directed to not only the skin but other tissues, since the different polarities between skin/target organs and VC derivatives/medicines can be regulated by bipolar stimulation.

CONCLUSION

Gunpatsu pulse was effective at increasing the amount of collagen in skin with either unipolar or bipolar stimulation for less than 10 min. Furthermore, in terms of the iontophoresis of VC derivatives, bipolar Gunpatsu pulse stimulation for 5 min was most effective. These results suggest that our methods can be used to increase the amount of collagen in skin, particularly in the case of iontophoresis of substances to the target organ, where bipolar stimulation was more effective. Furthermore, Gunpatsu pulse stimulation has much less of an adverse effect compared to ordinary pulse stimulation. This method can be developed as a novel and safe treatment for the application of medicines. This is the first report that substantiates that the amount of collagen in skin is increased by electric stimulation, Gunpatsu pulse.

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