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Hot spring bathing accelerates wound healing and enhances heat retention effect in guinea pigs

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ABSTRACT. This study aimed to demonstrate the effects of hot springs on wound healing and heat retention by performing comparative experiments with tap water. The hot spring water used in this study was from an alkaline hot spring that was rich in sodium and chloride ions and exhibited high reducibility. Guinea pigs were divided into a hot spring bathing group and a tap water bathing group, and a bathing test was conducted for eight consecutive days. A comparison of the plasma amino acid composition between the two groups after the bathing test revealed differences in the concentrations of several amino acids associated with wound healing. Image analysis demonstrated that wounds made on the abdominal skin of guinea pigs were significantly contracted by hot spring bathing compared to that by tap water bathing, and histopathological findings showed that wound healing was accelerated. In the thermography test, changes in body surface temperature after bathing were investigated in both groups. The heat retention effect was not observed in the tap water bathing group after bathing, whereas it was enhanced in the hot spring bathing group until 30 min after bathing. In conclusion, this study demonstrated that hot spring bathing accelerates wound healing and has a more significant heat retention effect than tap water bathing.

KEYWORDS: heat retention effect, hot spring bathing, wound healing

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Hot springs have been widely used by people for a long time to improve their physical condition. Hot spring therapy does not require chemicals and has few side effects [27, 45]. Therefore, it is advantageous to safely carry out therapy for an extended period without causing any potential health risks. Many legends about the effects of hot springs exist in Japan, and scientific proofs of them have been attempted [20, 21, 71, 82, 88]. Especially for the skin, it has been reported that various therapeutic effects can be expected by hot spring bathing. For example, acidic hot springs are famous for their ability to improve the symptoms of atopic dermatitis [27, 36–38, 80], and the sulfur spring and Dead Sea spa regulate melanin production [13, 58, 61]. Furthermore, the effectiveness of hot-spring bathing for various diseases such as psoriasis and ichthyosis has been reported [16, 24, 35–37, 41, 42, 45]. Alkaline hot springs are supposed to stimulate the skin less than acid hot springs and help smooth the skin; however, this has not been clearly elucidated. The Yuda hot spring, which we use for research, is also a simple alkaline hot spring [26], and the skin-smoothing effect has been handed down empirically for a long time. Therefore, it is believed to accelerate wound healing, and many people visit for this effect. Previously, we conducted a hot spring bathing test using Capybaras [26] and confirmed the improvement in skin properties such as an increase in skin moisture content and the heat retention effect by bathing in Yuda hot spring. However, the test was not under controlled conditions. Thus, it remains unclear whether the Yuda hot spring is superior to simple tap water. Guinea pigs are classified into the same family Caviidae as Capybaras and are known to be closely related species. Preliminary experiments confirmed that guinea pigs can bathe calmly; therefore, we considered that guinea pigs were the most suitable animals for performing more detailed hot spring bathing tests in the laboratory. The purpose of this study is threefold: 1) to clarify the characteristic difference between Yuda hot spring and tap water from two factors: pH and oxidation–reduction potential (ORP); 2) to conduct a comparative analysis of hot spring water and tap water under controlled conditions to examine the wound healing effect; and 3) to verify the heat retention effect of hot springs.

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MATERIALS AND METHODS

Analysis of hot spring water

Component analysis: Yuda hot spring water supplied by the Yuda hot spring distribution cooperative was used in this experiment. The Yuda hot spring is located in Yamaguchi city, Yamaguchi prefecture, Japan. The Yuda hot spring is categorized as a non-volcanic hot spring, and the temperature of its source is as high as 72–76°C. The components were analyzed by the Yamaguchi Prefectural Institute of Public Health and Environment (Yamaguchi, Japan).

pH–ORP relationship: The pH and ORP were measured in tap water and hot spring water. The pH was measured using a pH meter PH-208 (Sato Shoji Co., Ltd., Kawasaki, Japan). The ORP was measured using an SOTA-ORP ORP meter (Sato Shoji Co., Ltd.). An equilibrium diagram between pH and ORP was drawn for tap water and hot spring water according to previous studies [55, 57, 59].

Aging in hot spring water: Tap water and hot spring water were allowed to stand for eight days. The pH and ORP were measured daily for both the water samples.

Wound healing test

Animals: Female albino guinea pigs (SPF, Hartley) were used in this study. Animals were purchased from Kyudo Co., Ltd. (Tosu, Japan) at the age of 3 weeks. A pair of animals was housed in a polysulfone cage (CL-0143, W 355 × D 499 × H 198 mm, CLEA Japan Inc., Tokyo, Japan) with bedding made from recycled pulp (Eco chip, CLEA Japan Inc.). The animal room was maintained at a constant temperature of 24 ± 2°C and relative humidity of 50 ± 5%. The room air was ventilated 10–15 times per hour automatically, and 12 hr light/dark cycle (07:00–19:00) was imposed.

The animals were fed CLEA Guinea Pig Diet CG-7 (CLEA Japan Inc.) and tap water *ad libitum*. After acclimatization, these guinea pigs were used at 8 weeks of age in the wound healing test.

Experimental procedures: Twelve guinea pigs were used for the wound healing test. They were divided into two groups: hot spring bathing group and tap water bathing group. Water temperature was adjusted to 40°C during the bathing test. The test schedule is shown in Fig. 1.

The day before the start of the test (day 0), the abdominal hair coat of the animals was removed using an electric clipper and subsequently shaved with an electric shaver. Under general inhalation anesthesia with isoflurane, split-thickness wounds were made at four sites on the abdominal skin using a sterile punch biopsy (8 mm in diameter, Kai Industries Co., Ltd., Tokyo, Japan).

During the test period (days 1 to 7), the bathing test was conducted consecutively for one week, and the guinea pigs were allowed to bathe for 15 min every day. After the bathing procedure was completed, the wound areas on the abdomen were photographed daily for image analysis. The bedding was changed daily to maintain a clean environment.

On the last day of the test (day 8), the bathing procedure was performed in the same way as in the previous days. Guinea pigs were anesthetized with 4.5% isoflurane and maintained with 3% isoflurane (MSD Animal Health, Tokyo, Japan). Under general inhalation anesthesia, blood samples were collected from the caudal vena cava of guinea pigs using no anticoagulants. Immediately after drawing blood with heparin sodium, the plasma was separated by centrifugation at 1,500 × *g* for 10 min to analyze the amino acid composition. Thirty min after collection of blood samples, sera were separated by centrifugation at 1,500 × *g* for 10 min for biochemical analysis. For hematological samples, blood was collected in tubes containing K2EDTA. Tissue specimens were obtained from all wound sites on the abdominal skin. Other specimens (liver, kidney, spleen, and adrenal gland) were also collected for histopathological examination.

Hematological and serum biochemical examination: In the hematological examinations, the following parameters were examined using an automated cell counter (Microsemi LC-662, HORIBA, Kyoto, Japan): white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (Hgb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), plateletcrit ratio (PCT), and platelet distribution width (PDW). The following parameters were measured during serum biochemical examinations using a blood chemistry analyzer (Fuji DRY-CHEM NX500V, FUJIFILM, Tokyo, Japan): alkaline

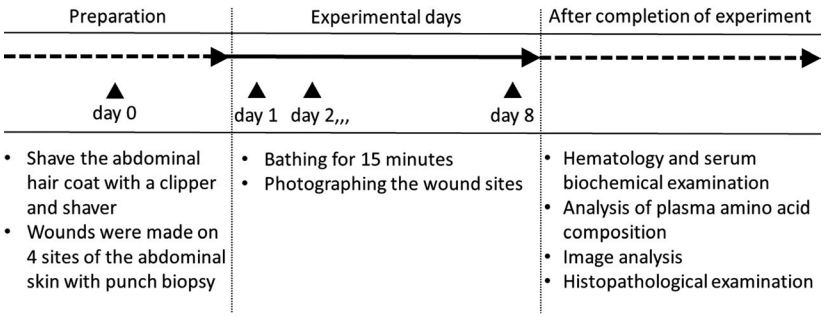

Fig. 1. Experimental schedule of wound healing test. On the day before the start of the test (day 0), the abdominal hair coat was partially shaved, and skin biopsies were performed at four sites with a biopsy punch with a diameter of 8 mm. The bathing test was conducted for one week (days 1 to 8), and the guinea pigs were allowed to bathe for 15 min a day. The inspection items included hematological examination, serum biochemical examination, analysis of plasma amino acid composition, image analysis, and histopathological examination.

phosphatase (ALP), cholinesterase (ChE), aspartate aminotransferase (GOT), alanine aminotransferase (GPT), γ-glutamyl transferase (GGT), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), amylase (AMYL), glucose (GLU), creatine phosphokinase (CK), blood urea nitrogen (BUN), creatinine (CRE), electrolytes (Na, K, Cl, Ca, and Mg), uric acid (UA), total protein (TP), albumin (ALB), globulin (GLOB), total cholesterol (TCHO), triglycerides (TG), total bilirubin (T-bil), and inorganic phosphorus (IP).

Analysis of plasma amino acid composition: Centrifugation was performed to collect plasma from blood samples, and the free amino acid composition in plasma was analyzed using a mass spectrometer. These parameters are listed in Table 3.

Macroscopic observations and image analysis of wound area: Macroscopic changes in the wound area were recorded and calculated using the image-processing software ImageJ® [68, 70]. The wound area is expressed as the mean ± standard deviation (SD) for each group.

Histopathological examination: All tissue specimens obtained by autopsy were fixed in 10% neutral-buffered formalin, and 4 µm tissue sections were prepared. Skin tissue sections were stained with hematoxylin and eosin (HE), van Gieson's (vG) method, and Weigert's (WG) method. The remaining tissue sections were stained with hematoxylin and eosin. In the wound healing test, the severity of epidermal and dermal changes was graded as negligible (−), slight (±), mild (+), moderate (++), and marked (+++).

Skin thermography test

Animals: Twelve guinea pigs were used for skin thermography tests. These guinea pigs were assigned to the following two groups: tap water bathing and hot spring bathing groups. The housing conditions were the same as those used in the wound healing test.

Experimental procedures: The bathing temperature was adjusted to 40°C for both groups. The schedule is summarized in Fig. 2.

On the day before starting the test (day 0), the abdominal hair of each guinea pig was removed using an electric clipper and a shaver.

During the test period (days 1 to 7), thermograms of the abdominal skin were taken daily before seven consecutive baths. Guinea pigs were allowed to bathe for 15 min. Skin surface temperature was recorded on the thermograph immediately after bathing and 10, 20, and 30 min after bathing. After the bathing test, bedding materials were changed every day to maintain a clean-living environment. This process was continued every day for one week.

On the last day of the test (day 8), after bathing and thermography, blood was drawn from the caudal vena cava under general anesthesia with isoflurane. Hematological, serum biochemical, and histopathological examinations verified whether there was a difference in the physiological effects between the tap water bathing group and the hot spring bathing group. Throughout both bathing tests, we examined the stability of the skin surface temperature.

Thermography test: Changes in body surface temperature before and after bathing were recorded using thermography (FLIR E4; FLIR Systems, Inc., Wilsonville, OR, USA). The thermograph was analyzed using image analysis software (FLIR Tools, FLIR Systems, Inc.), and the abdominal skin surface temperatures of the tap water bathing group and the hot spring bathing group were repeatedly determined during the bathing test. Skin temperature is expressed as the mean ± SD.

Statistical analysis

All statistical analyses were performed using Statcel 4 (OMS Publishing Inc., Higashikurume, Japan), and significant differences were assessed using Student's *t*-test and Dunnett's test, which is one of the multiple comparison methods ($P<0.05$, $P<0.01$).

Ethical statement

This experiment was approved by the Institutional Animal Care and Use Committee of Yamaguchi University (Approval No. 409: May 20, 2022), and all experimental procedures using animals followed the Guidelines of Animal Care and Experiments of Yamaguchi University. The animal care and use program at the Advanced Research Center for Laboratory Animal Science in Yamaguchi University has been accredited by AAALAC International since 2018.

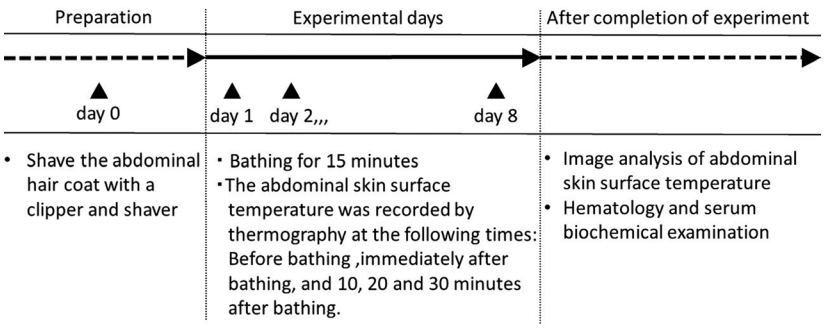

Fig. 2. Experimental schedule of skin thermography test. On day 0, the abdominal hair coat of guinea pigs was partially shaved with a clipper and shaver. From day 1 to day 8, the guinea pigs were allowed to bathe for 15 min per day. The abdominal skin surface temperature was recorded using thermography at the following times: before bathing, immediately after bathing, and 10, 20, and 30 min after bathing. After completing this series of thermography tests, hematological examination, serum biochemical examination, and image analysis of the body surface temperature were conducted.

RESULTS

Analysis of hot spring water

Component analysis: The components of Yuda hot spring are listed in Table 1. According to Table 1, the main cations were sodium ions, the main anions were chloride ions, and sulfur was also present. This analysis revealed that the Yuda hot spring has a simple thermal alkaline quality of water.

pH–ORP relationship: The pH of the fresh state at Day 1 was 7.10 ± 0.08 for tap water and 8.96 ± 0.05 for hot spring water (Fig. 3A). The ORP values on the first day were 397 ± 52.4 mV for tap water and −295 ± 9.69 mV for hot spring water (Fig. 3B). The equilibrium ORPs calculated based on these results are shown in Fig. 3C. The red area indicates that the water sample is in the oxidative region. Conversely, the blue area indicates that the water sample belonged to the reductive region. It was found that the Yuda hot spring water had higher reducibility than tap water.

Aging: The pH on Day 1 of tap water was 7.10 ± 0.08 and that of hot spring water was 8.96 ± 0.05. Until day 3, the pH of both samples converged at 8, and the pH values of both water samples were maintained at an approximately constant pH of 8. The ORP values of tap water remained high (approximately + 400 mV) throughout the study. In contrast, hot spring water showed markedly low ORP levels (−300 mV) on day 1. ORP levels did not increase easily during this experiment. There was a significant difference in the ORP levels between the water samples (P<0.01).

Table 1. Component analysis of Yuda hot spring water

Temperature (°C)	71.20
pH	9.20
Components (mg/L)	
Na^+	212.80
K^+	4.20
Mg^{2+}	0.00
Ca^{2+}	8.50
Sr^{2+}	0.10
Fe^{2+}, Fe^{3+}	0.00
Mn^{2+}	0.00
Al^{3+}	0.02
Zn^{2+}	0.00
Cu_2^+	0.00
H^+	0.00
Li^+	0.20
NH_4^+	0.07
F^-	12.00
Cl^-	288.00
Br^-	0.70
I^-	0.00
HCO_3^{2-}	12.30
CO_3^-	16.90
SO_4^{2-}	22.10
HS^-	1.30
$S_2O_3^{2-}$	0.80
OH^-	0.30
PO_4^{3-}	0.00
BO_2^-	4.80
Non-dissociated component (mg/L)	
H_2SiO_3	90.20
Total dissolved matter (mg/L)	675.20

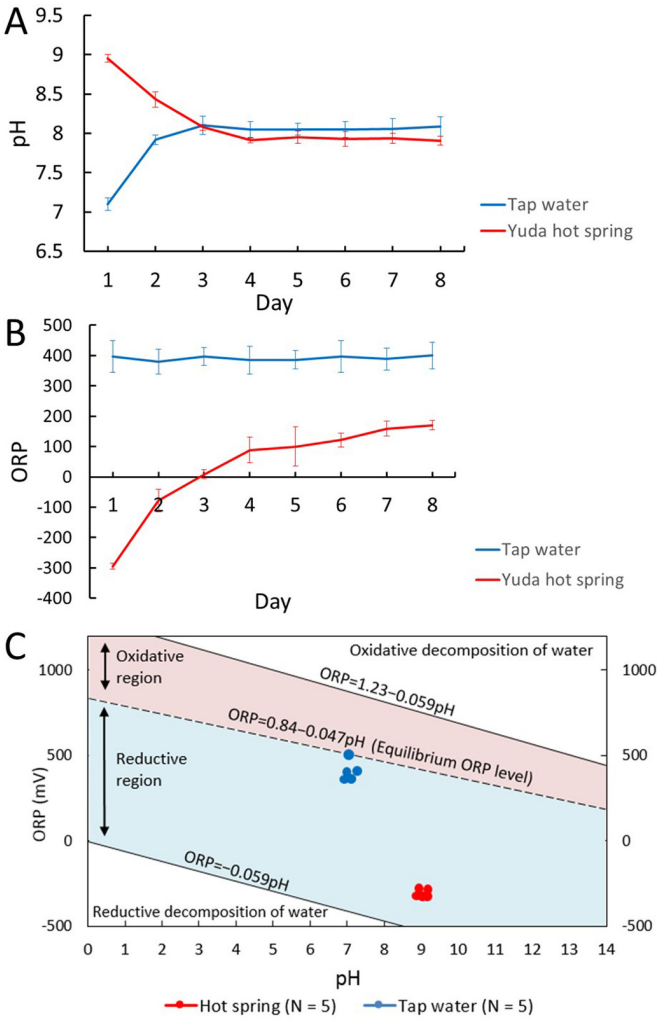

Fig. 3. Changes in water quality over 8 days. (**A**) pH on Day 1: tap water: 7.10 and hot spring water: 8.96. Two days after the start of the experiment, the pH of both samples converged to around 8. (**B**) The ORP value of tap water showed a high value from Day 1 (397 ± 52.4 mV), and no change was observed thereafter. The ORP value of hot spring water on Day 1 was −295 ± 9.69 mV. After Day 2, the ORP value continued to rise gradually, but even on day 8, it was lower than that of tap water. (**C**) Based on pH and ORP value on Day 1, an equilibrium ORP diagram was drawn. It was clarified that the hot spring water had higher reducibility than tap water.

Wound healing test

Hematological and serum biochemical examination: Table 2 shows the results of the hematological and biochemical examinations. There was no difference in the WBCs counts. The levels of erythrocytic parameters (RBCs, Hb, PCV ratio, MCH, and MCHC) were significantly higher in the hot spring bathing group than in the tap water bathing group. The parameters related to platelets (PLTs and PCT) were higher in the tap water group than in the hot spring bathing group. There were a few significant differences in the biochemical findings between the two groups. On day 8 after bathing, GLU concentrations in the hot spring bathing group remained within normal limits, whereas the measurements in the tap water bathing group moderately decreased. CRE levels in the hot spring bathing group remained within the normal range, whereas increased CRE levels were observed in the tap water bathing group.

Analysis of plasma amino acid composition: Table 3 shows a comparison of the compositions of 41 plasma amino acids. Significant differences were observed in 12 amino acids between the two groups. Notably, the hot spring bathing group had significantly higher levels of 11 types of amino acids (CIT, SER, HYP, GLY, THR AABA, ORN, PRO, TPR, VAL, and TYR). Significant differences in aromatic amino acids, branched chain amino acids, and total amino acids were also found based on the results of these items.

Macroscopic observations and image analysis of wound area: Macroscopic findings at the wound sites and the daily transition of the wound area are shown in Fig. 4. The wound area on Day 1 was almost the same in both groups (tap water: 0.48 ± 0.09 cm^2, hot spring: 0.48 ± 0.04 cm^2). From days 2 to 4, no significant difference was observed in the wound areas between the two groups. After day 5, the wound areas significantly began to decrease in the hot spring bathing group (day 5: $P<0.05$, days 6 to day 8: $P<0.01$). On day 8, the wound areas were significantly smaller in the hot spring bathing group (0.024 ± 0.008 cm^2) than in the tap water bathing group (0.059 ± 0.023 cm^2). On days 3, 4, and 5, the wound sites in the tap water group developed a stronger inflammatory reaction than those in the hot spring group (Fig. 4C). From these results, by Day 8, hot spring bathing accelerated the rate of re-epithelialization in the wounds.

Histopathological examination: Histopathological findings of the wound healing test are shown in Fig. 5. The wounds in the tap water bathing group were not sufficiently re-epithelialized compared with those in the hot spring bathing group. Few elastic fibers in the dermis were reconstructed in the tap water bathing group, whereas elastic fibers began to regenerate in the hot spring bathing group. The arrangement of collagen fibers showed a marked difference between the two groups. The arrangement of collagen fibers was disordered and irregular in the tap water bathing group, whereas in the hot spring water bathing group, the collagen fibers were regularly arranged parallel to the epidermis. Histopathological findings were evaluated, as shown in Table 4. No differences in histological findings were observed between the two groups for specimens other than the skin (the kidney, adrenal gland, liver, and spleen).

Skin thermography test

Thermography test: The results of the thermographic tests are shown in Fig. 6. Thermography tests showed that there was a marked difference in the changes in body surface temperature between hot spring bathing and tap water bathing. Comparing the two groups at the same time, there was no significant difference in body surface temperature before and immediately after bathing. However, from 10 min to 30 min later, the hot spring bathing group maintained a significantly higher temperature than the tap water bathing group ($P<0.01$).

Although the body surface temperature of the tap water bathing group increased significantly immediately after bathing, its temperature decreased 10 min after bathing and significantly declined below the body surface temperature before bathing ($P<0.01$). In contrast, notably, the hot spring bathing group maintained a high body surface temperature for 30 min after bathing. Body surface temperature did not return to the initial temperature before hot spring bathing for 30 min under controlled conditions in the laboratory room ($P<0.01$). Hematological and biochemical tests were also performed and were all within the normal range. Histopathological examination revealed no abnormalities.

DISCUSSION

The component analysis of the Yuda hot spring showed that the total amount of dissolved substances was less than 1,000 mg/kg, and it was classified as a simple alkaline hot spring that was relatively rich in sodium and chloride ions. The pH of the Yuda hot spring is higher than the average pH of Japanese hot springs [49], whereas the ORP value is low [1, 59, 83]. The equilibrium ORP demonstrated the high reducibility of the fresh Yuda hot spring compared with that of tap water. Bathing in a hot spring with high reducibility lowers the ORP value of the skin and suppresses oxidation and aging, resulting in improved skin elasticity [56, 60, 62, 64, 65]. We previously reported that bathing in Yuda hot spring improves skin properties, such as skin moisture [26]. It was suggested that the high reducibility of the Yuda hot spring, as revealed in this test, is related to its beneficial effect on the skin, in addition to the properties of an alkaline hot spring that are low in skin stimulation. Fresh hot springs are generally preferred because the quality of hot springs deteriorates over time. This study examined the effects of aging on water quality from the perspective of ORP. The ORP of tap water is high because of the influence of chlorine-based disinfectants [59]. In contrast, the Yuda hot spring maintained relatively high reducibility compared to tap water, even after one week. This series of studies on water quality suggests that fresh Yuda hot spring is less likely to deteriorate and has a positive effect on wound healing.

Hematological examinations of the wound healing test showed significant differences among the seven items. The RBC, HGB, and HCT results for the tap water group were slightly lower than those reported in previous studies [12, 78]. The reason was considered to be stress and loss of appetite due to pain, but the results of the blood tests in the hot spring group were within the reference values. Hot springs have stress-reducing and pain-reducing effects [2, 10, 26, 53, 54, 89, 90], and the results of hematological examination suggested a relationship with these effects.

Table 2. Hematological and serum biochemical examination (Wound healing test)

Analyte	Mean ± SD		*P* value
	Tap water group (N=6)	Hot spring group (N=6)	
WBC (10^9/L)	2.92 ± 1.02	3.33 ± 0.75	0.44
RBC (10^{12}/L)	4.52 ± 0.17	5.24 ± 0.43	0.003
Hb (g/L)	114 ± 4.3	138 ± 11.5	0.0007
PCV ratio	0.37 ± 0.02	0.43 ± 0.04	0.011
MCV (fL)	82.0 ± 1.73	81.1 ± 1.79	0.40
MCH (pg)	25.2 ± 0.69	26.4 ± 0.66	0.013
MCHC (g/L)	308 ± 6.3	325 ± 3.5	0.0001
RDW (%)	12.8 ± 0.63	13.1 ± 0.46	0.37
PLT (10^9/L)	574 ± 65	441 ± 49	0.003
MPV (fL)	5.80 ± 0.28	5.62 ± 0.18	0.20
PCT (%)	0.33 ± 0.05	0.25 ± 0.03	0.005
PDW (%)	12.1 ± 0.54	11.6 ± 0.53	0.10
ALP (U/L)	293.5 ± 67.4	332 ± 46.7	0.28
CHE (U/L)	10.2 ± 0.75	10.8 ± 1.83	0.43
GOT (U/L)	29.5 ± 6.25	62 ± 65.2	0.25
GPT (U/L)	21.2 ± 3.43	27 ± 8.72	0.16
GGT (U/L)	27.2 ± 10.1	24.5 ± 7.06	0.61
LDH (U/L)	118 ± 64.3	157 ± 84.8	0.39
LAP (U/L)	508 ± 19.7	508 ± 63.3	0.995
AMS (U/L)	2,024 ± 400	2,081 ± 407	0.4
GLU (mmol/L)	8.44 ± 0.58	13.2 ± 0.58	0.001
CK (U/L)	268 ± 178	296 ± 245	0.82
BUN (mmol/L)	6.28 ± 0.70	6.46 ± 0.90	0.73
CRE (μmol/L)	40.7 ± 14.1	23.9 ± 1.77	0.02
Na (mmol/L)	130 ± 7.74	129 ± 12.5	0.81
K (mmol/L)	4.12 ± 0.33	4.85 ± 1.50	0.27
Cl (mmol/L)	93.2 ± 5.49	94 ± 9.70	0.89
UA (μmol/L)	87.4 ± 13.7	85.1 ± 29.7	0.88
TP (g/L)	40.5 ± 2.10	42.0 ± 5.90	0.57
ALB (g/L)	18.2 ± 1.20	19.3 ± 4.10	0.52
TCHO (mmol/L)	1.49 ± 0.13	1.08 ± 0.29	0.06
TG (mmol/L)	0.65 ± 0.16	0.63 ± 0.17	0.88
TBIL (mg/dL)	1.71 ± 0	1.71 ± 0	1
Ca (mmol/L)	2.06 ± 0.20	2.28 ± 0.39	0.25
IP (mmol/L)	2.61 ± 0.37	2.61 ± 0.33	0.98
Mg (mmol/L)	1.45 ± 0.21	1.52 ± 0.36	0.67

Table 3. Plasma amino acid composition (Wound healing test)

Analyte (nmol/mL)	Mean ± SD		*P* value
	Tap water group (N=6)	Hot spring group (N=6)	
Arginine (ARG)	57.0 ± 39.6	48.4 ± 43.7	0.73
Glutamine (GLN)	158.9 ± 47.6	186.9 ± 27.1	0.24
Citrulline (CIT)	21.4 ± 9.04	42.1 ± 2.59	0.0003
Anserin (ANS)	2.19 ± 0.74	1.61 ± 0.65	0.18
Serine (SER)	143 ± 23.7	179 ± 18.3	0.01
Asparagine (ASN)	0	0	-
Prolyl hydroxyproline (PHP)	4.63 ± 0.45	4.42 ± 0.58	0.48
4-Hydroxyproline (HYP)	24.7 ± 5.08	31.5 ± 4.20	0.03
3-Methylhistidine (3-MHIS)	2.22 ± 0.21	1.96 ± 0.27	0.08
1-Methylhistidine (1-MHIS)	10.8 ± 0.50	7.76 ± 1.89	0.003
Glycine (GLY)	414 ± 77.2	550 ± 90.2	0.02
Glycyrrhein proline (GPR)	0.25 ± 0.14	0.17 ± 0.13	0.32
Threonine (THR)	102 ± 13.2	145 ± 21.4	0.002
β-Alanine (bALA)	3.01 ± 0.53	2.65 ± 0.68	0.32
Alanine (ALA)	294 ± 65.1	276 ± 44.7	0.58
Sarcosine (SAR)	0.87 ± 0.22	1.12 ± 0.32	0.15
δ-Hydroxylysine (HLY)	0.71 ± 0.29	0.75 ± 0.26	0.81
γ-Aminobutyric acid (GABA)	2.96 ± 0.87	4.16 ± 1.48	0.12
β-aminoisobutyric acid (bAIBA)	0	0.33 ± 0.58	0.2
α-Aminobutyric acid (AABA)	2.62 ± 0.53	3.69 ± 0.79	0.02
Ornithine (ORN)	78.7 ± 13.4	135 ± 36.3	0.005
Carnosine (CAR)	0.09 ± 0.15	0	-
Methionine (MET)	28.9 ± 4.61	34.4 ± 5.30	0.08
Proline (PRO)	102 ± 24.7	130 ± 16.9	0.05
Lysine (LYS)	146 ± 38.7	162 ± 33.1	0.47
Aspartic acid (ASP)	12.74 ± 7.53	17.8 ± 3.77	0.17
Histidine (HIS)	54.2 ± 9.23	51.0 ± 8.94	0.56
Thioproline (TPR)	0.10 ± 0.16	0.45 ± 0.08	0.0006
Valine (VAL)	253 ± 16.6	300 ± 33.8	0.01
Glutamic acid (GLUT)	172 ± 33.1	206 ± 37.1	0.12
Tryptophan (TRP)	47.2 ± 8.51	57.6 ± 11.6	0.11
α-aminoadipic acid (AAA)	3.00 ± 0.69	3.62 ± 0.58	0.12
Phenylalanine (PHE)	61.7 ± 8.13	69.6 ± 9.63	0.15
Leucine (LEU)	146 ± 11.5	158 ± 18.1	0.2
Isoleucine (ILE)	109 ± 10.5	115 ± 11.8	0.38
Aminopimelic acid (APA)	0	0	-
Cystathionine (CTH)	0.94 ± 0.30	0.82 ± 0.10	0.38
Cystine (CYS2)	65.9 ± 12.3	61.0 ± 9.12	0.45
Tyrosine (TYR)	50.6 ± 8.01	66.9 ± 8.25	0.006
Kynurenine (KNU)	2.50 ± 1.28	1.82 ± 0.40	0.24
5-aminolevulinic acid (5-ALA)	0	0	-
Aromatic amino acid	112 ± 15.2	137 ± 17.0	0.03
Branched chain amino acid	507 ± 33.8	572 ± 62.1	0.05
Fischer ratio	4.56 ± 0.39	4.21 ± 0.23	0.08
Total amino acid	2,580 ± 349	3,057 ± 334	0.04

In biochemical tests, all parameters in both groups were within the reference values. The glucose and creatinine levels in the two groups were significantly different, but remained within the reference range.

To the best of our knowledge, this is the first study to comprehensively investigate the plasma amino acid composition in a wound healing test by bathing in a hot spring. Plasma amino acids supply amino acids to each tissue and become constituents of proteins; therefore, an increase in plasma amino acid concentration is thought to be beneficial for wound healing. It has been reported that the supply of amino acids is effective for wound healing [5, 66, 67, 77], whereas amino acid deficiency delays wound healing [6, 8, 87].

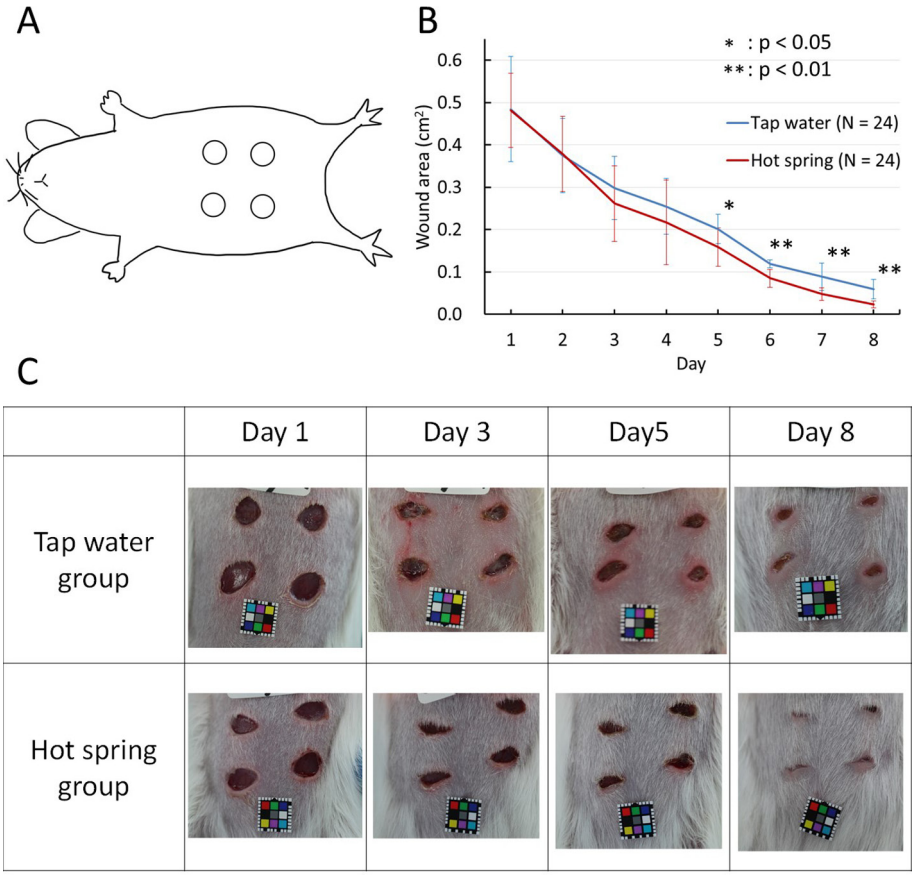

Fig. 4. The results of wound healing test. (**A**) Diagram of four biopsy portions on the abdominal skin in a guinea pig. Split wounds were aseptically made using a biopsy punch with a diameter of 8 mm. (**B**) Comparison of daily changes in wound area. There was no significant difference in the wound area during the first 4 days between the 2 groups. After the 5th day, there was a significant difference in the size of the wound area, and the wound healed more quickly in the hot spring bathing group. (**C**) Examples of the appearance of the wound sites. In the tap water bathing group, the inflammatory reaction was strong on Days 3 and 5. The wound areas on Day 8 appeared smaller in the hot spring bathing group than those in the tap water bathing group as observed by macroscopic findings.

Table 4. Histopathological evaluations

	Tap water	Hot spring
Epidermis		
Reepithelialization	++	+++
Keratohyalin granules	+	+++
Dyskeratosis	±	±
Infiltration of inflammatory cells	+	−
Mast cells	−	−
Dermis		
Granulation	+++	+
Neovascularization	++	++
Infiltration of inflammatory cells	+++	++
Mast cells	−	−
Arrangement of collagen fibers	+	++
Regeneration of elastic fibers	+	++

It is also known that plasma amino acid levels were elevated when wound healing was promoted by amino acid supply [51, 85]. Several studies in humans and animals without wounds have also indicated the possibility that hot spring bathing alters the metabolism of biological materials such as hormones and proteins [3, 18, 25, 86]. In this study, the plasma amino acid composition of the hot spring group showed significantly higher values for some items (CIT, SER, HYP, GLY, THR AABA, ORN, PRO, TPR, VAL, and TYR).

Citrulline contributes to the maintenance of epidermal homeostasis as a natural moisturizing factor (NMF). Natural moisturizing factor is water-soluble small molecule that plays an important role in moisturizing and softening the skin and keeping the stratum corneum healthy. Elevated plasma citrulline levels are presumed beneficial for promoting wound healing. Furthermore, citrulline produces nitric oxide (NO) during the metabolic process, which dilates blood vessels and promotes blood circulation [33, 72, 84]. An improvement in blood flow by NO production is considered to contribute to an increase in body temperature and the heat retention effect.

Serine is the most abundant amino acid in NMF [22, 44], and serine is effective in moisturizing the skin. [31, 50]. Our previous studies have shown that consecutive bathing in hot springs can contribute to skin moisturization [26]. Considering that the plasma serine concentration increased due to consecutive bathing in hot springs, this study suggests that amino acid metabolism is related to the moisturizing effect of hot springs. In addition, serine is associated with improved sleep quality [28]. Some studies have reported that bathing in hot springs improves sleep quality [40]; therefore, it is possible that changes in the plasma concentration of serine are associated with the sleep-improving effects of hot springs.

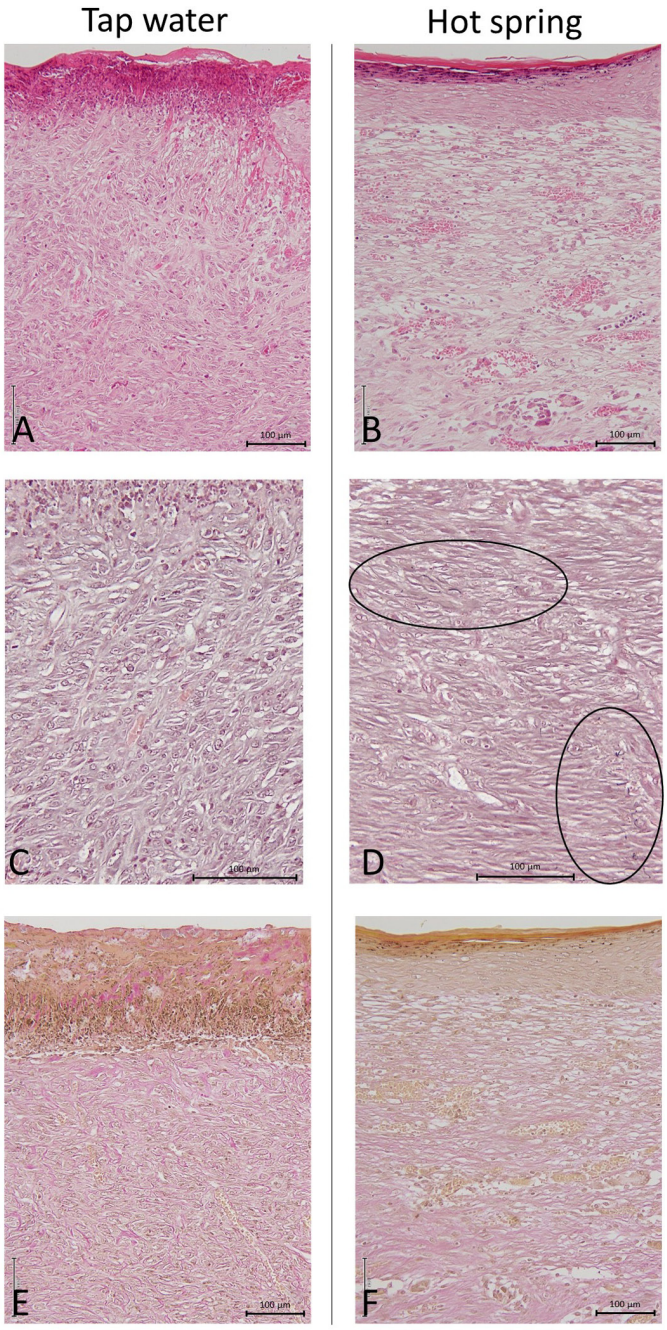

Fig. 5. Comparison of histopathological findings between hot spring group and tap water group in the wound healing test. (**A**) The re-epithelialization of the epidermis was insufficient in the tap water bathing group. HE stain. Bar=100 µm. (**B**) The re-epithelialization of the epidermis was clearly accelerated in the hot spring bathing group. In addition, production of keratohyalin granules was also observed. HE stain. Bar=100 µm. (**C**) The WG method showed that the production of elastic fibers in the dermis layer was rarely seen. Weigert's method. Bar=100 µm. (**D**) In the hot spring bathing group, elastic fibers began to regenerate in the dermis layer. Weigert's method. Bar=100 µm. (**E**) The arrangement of collagen fibers was irregular compared to that of the hot spring bathing group. Van Gieson's method. Bar=100 µm. (**F**) Collagen fibers were regularly arranged parallel to the epidermal layer, which was similar to the original arrangement of collagen fibers. Van Gieson's method. Bar=100 µm.

Hydroxyproline is the primary component of collagen. Collagen is a fibrous protein that constitutes the majority of the dermis, and collagen biosynthesis is an important component of wound healing. Because both collagen synthesis capacity and blood concentration of hydroxyproline decrease with age [46, 48], it is assumed that hydroxyproline in the blood is related to the ability to synthesize collagen. In addition, because hydroxyproline has been reported to be effective in wound healing [14], an increase in the blood concentration of hydroxyproline by bathing in a hot spring is associated with the promotion of collagen metabolism and wound healing.

Glycine occupies one-third of the amino acid sequence that constitutes collagen [63] and is the second most abundant amino acid

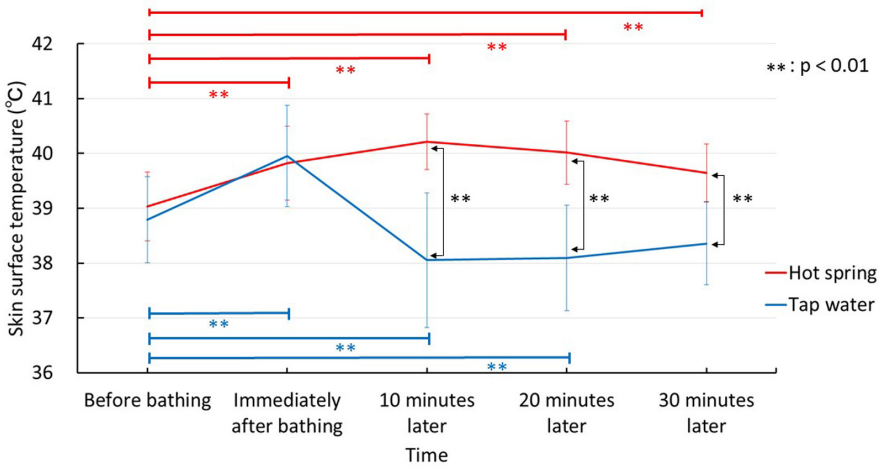

Fig. 6. Comparison of the changes in body surface temperature before and after bathing between tap water bathing and hot spring bathing. When we compared the body surface temperatures in the two groups at the same time point, we found a significant difference at all time points after bathing, and the temperature was significantly higher in the hot spring bathing group (P<0.01). Compared to the temperature before bathing, the hot spring bathing group maintained a significantly higher temperature until 30 min after bathing, whereas the temperature of the tap water bathing group decreased significantly after bathing (P<0.01).

among the amino acids contained in NMF [22]. It is possible that the significant increase in plasma glycine concentration in the hot spring group is associated with the early normalization of collagen fibers and the production of keratohyalin granules.

Threonine is found in the skin as an amino acid constituent of NMF and is a component of essential skin proteins, such as collagen and elastin. Because threonine is an essential amino acid that cannot be synthesized in the body, it was speculated that hot spring bathing did not directly affect this significant difference.

α-Aminobutyric acid is an isomer of aminobutyric acid. Aminobutyric acid has been reported to significantly promote hyaluronic acid production in the epidermis [34]. Hyaluronic acid greatly contributes to improving the moisturizing and elasticity of the skin; therefore, it is likely that aminobutyric acid is related to the factors that cause hot springs to improve the water content and elasticity of the skin.

Plasma ornithine levels in the hot spring bathing group were significantly increased. Ornithine has been reported to be effective in wound healing [9, 73]. Ornithine is a metabolite of arginine, and arginine is also known to be effective in wound healing [15]. These are because both ornithine and arginine have the effect of stimulating the secretion of growth hormone. Growth hormones work effectively in wound healing by promoting protein synthesis [32]. In addition, it has been reported that ornithine is converted to proline, which is necessary for collagen synthesis, and works for wound healing [9]. Therefore, it was suggested that the significant increase in the supply of ornithine is involved in the wound healing effect of the hot springs.

Proline is a precursor of hydroxyproline and an important amino acid that constitutes collagen together with hydroxyproline in the skin [30]. Since oral or intraperitoneal administration of proline promotes wound healing [5, 67], it was suggested that an increase in blood proline concentration due to a hot spring bath promotes local proline biosynthesis and accelerates wound healing. Moreover, as mentioned above, proline is produced from ornithine. Elevated plasma levels of skin-related amino acids, such as ornithine, proline, and hydroxyproline, suggest that wound healing is accelerated.

Thioproline is a derivative of proline but has little effect on the structure of the skin. Although there have been reports on the carcinogenic suppressive effects of thioproline [39, 79], none have focused on the relationship between thioproline and wound healing, and it is unclear whether elevated plasma levels of thioproline have a positive effect on wound healing.

Tyrosine is a precursor to dopamine. Stress reduces dopamine levels, resulting in lethargy and depression. As tyrosine reduces stress [4, 7, 19], it has been suggested that a significant increase in tyrosine by bathing in a hot spring has a psychologically positive effect. Although there have been some reports in humans on the stress-relieving effects of hot springs in the past [54, 89, 90], this study provided new insights into the stress-reducing effects of hot springs in terms of changes in blood amino acid levels.

Plasma concentrations of aromatic and branched-chain amino acids were significantly different between the two groups, but the Fisher ratios were not. The Fisher ratio indicates the balance between aromatic amino acids and branched chain amino acids, and a disturbance in the Fisher ratio indicates abnormal amino acid metabolism in the liver [17, 76]. We could not find data on the normal value of the Fisher ratio of guinea pigs, but the change in plasma amino acid concentration observed in this hot spring bathing test did not show a significant difference in the Fisher ratio; therefore, it can be said that this is a well-balanced change within the normal range.

In the present study, macroscopic findings revealed that the wound size on day 8 contracted significantly, and erythema caused by inflammation was also mild in the hot spring bathing group compared to that in the tap water bathing group. Bathing in tap water is beneficial in that it can contribute to cleansing and promoting blood flow at wound sites. It was speculated that these effects would help accelerate wound healing rather than delay it. However, it is noteworthy that hot springs showed an even higher wound-healing

effect in this study. Histopathological examination revealed that hot spring water accelerated wound healing not only in the epidermis, but also in the dermis. In the epidermis layer, re-epithelialization was clearly advanced in the hot spring bathing group, whereas the structure of the epithelial layer could not be sufficiently confirmed in the tap water bathing group. In the dermis layer, regeneration of elastic fibers and regular arrangement of collagen fibers were observed only in the hot spring group. There are three possible explanations for these differences. The first is the improvement in blood circulation in the wound area due to hot spring bathing. The heat-retention effect of the hot spring demonstrated by this thermography test and our previous study [26] is considered to contribute to the improvement in blood flow by dilating the capillaries in the wound area. Additionally, in rats, bathing in a hot spring raises the skin temperature around the wound site more than hot water does [43]. Hot spring bathing is effective for blood circulation failure in the wound, which is one of the factors that delay wound healing. The second is the anti-inflammatory and bactericidal effects of hot springs. The anti-inflammatory effect of bathing in hot springs and the bactericidal effect of sulfur contained in the Yuda hot spring have been reported in humans [11, 45, 47, 52, 69]. These effects may have contributed to the suppression of wound inflammation and the maintenance of a clean environment. The third factor is the change in plasma amino acid concentration. As mentioned above, some plasma amino acid levels associated with wound healing were observed to increase with hot spring bathing. The involvement of these amino acids in wound healing has been speculated, but further research is required to test this hypothesis. In summary, the wound healing effect of hot springs could be achieved by suppressing inflammation, improving blood flow in the affected area, and supplying abundant amino acids from circulating blood. No abnormal findings were found in tissues other than skin in either group. The animals were not harmed during the one-week hot spring bathing test.

In the thermograph test, the transition of the body surface temperature after bathing was clearly different between the two groups. A similar heat retention effect has been confirmed in the outdoor Yuda hot spring bathing test for large rodents [26]. In this study, it was revealed that the heat retention effect of hot springs is superior to that of tap water under controlled air conditioning. Hot springs containing NaCl are known to have a heat-retention effect [23, 29, 74, 75]. This effect is stated to be caused by these salts covering the body surface with a fine membrane, which prevents the salts from binding to epidermal proteins and dissipating the body's heat [23, 75, 81]. It was considered that sodium and chloride ions, which are abundant in the Yuda hot spring, worked effectively for the heat retention effect. Therefore, a more detailed test system is required to support this hypothesis.

In conclusion, it was demonstrated in guinea pigs that bathing in a hot spring accelerates wound healing and enhances the heat retention effect compared with tap water bathing. In addition, the hot spring bath group had higher plasma concentrations of some amino acids associated with wound healing. These results indicate that the heat retention effect and changes in plasma amino acid concentration caused by bathing in reducible hot springs potentially contribute to accelerated wound healing.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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