

Article

Topical Application of Cha-Koji, Green Tea Leaves Fermented with *Aspergillus Luchuensis* var *Kawachii* Kitahara, Promotes Acute Cutaneous Wound Healing in Mice

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Abstract: “Koji” is one of the most well-known probiotic microorganisms in Japan that contribute to the maintenance of human health. Although the beneficial effects of some probiotics on ulcer healing have been demonstrated, there have been no reports on the wound healing effects of koji to date. In the present study, we investigated the effects of “cha-koji”, green tea leaves fermented with *Aspergillus luchuensis*, on cutaneous wound healing, using a linear incision wound mouse model. Topical application of autoclave-sterilized cha-koji suspension on the dorsal incision wound area healed the wound significantly faster and, notably, with less scarring than did the green tea or the control distilled water treatment. Further in vitro experiments revealed that the accelerated effects of cha-koji could be attributed to its increased anti-bacterial activity, enhanced epidermal cell proliferation and migration, augmented expression of the anti-inflammatory cytokine *transforming growth factor-β1*, reduced expression of inflammatory cytokine *interleukin-6* in macrophages, and decreased endoplasmic reticulum stress. In addition, we conducted a skin sensitizing potential test, which revealed that cha-koji had no adverse effects that posed a sensitizing risk. Thus, cha-koji may have a potent therapeutic effect on cutaneous wound healing, opening up a new avenue for its clinical application as a medical aid.

Keywords: anti-bacterial activity; epidermal cell proliferation and migration; endoplasmic reticulum stress; green tea leaves fermented with *Aspergillus luchuensis* (cha-koji); interleukin-6; skin wound healing; skin sensitizing potential; transforming growth factor-β1



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1. Introduction

The skin represents the body’s most expansive cutaneous tissue and protects the body from environmental stimuli [1]. As a result of its size, the skin is frequently damaged in situations such as accidental trauma and surgical procedures. As damaged skin is at a heightened risk from complications such as infection, it is important to accelerate healing time after the development of a cutaneous wound [2,3]. In the research field of wound healing, researchers have made various therapeutic attempts over the past few decades to accelerate wound healing. However, various challenges still remain to be addressed, such as scar formation, abnormal hyperplasia, and so forth [4]. Because the global market for wound healing applications is reported to exceed more than USD 20 billion [5], the scale of this market may indicate high social demand.

Probiotics have been reported to have various pleiotropic effects on human health [6–10]. Cha-koji comprises green tea leaves fermented with *Aspergillus luchuensis* var *Kawachii* Kitahara and is used as a recently developed probiotic health food [11]. *Aspergillus* species, referred to as “koji” in Japan, have been traditionally used in Japanese fermented food, such as miso and soy sauce. Unlike *Aspergillus* in Europe that is the causative fungus, koji is a safe food (generally regarded as safe, or GRAS), certified by the US Food and

Drug Administration (FDA) [11]. Recently, health damages such as kidney injury have been reported after ingestion of a health food product containing beni-koji in Japan [12,13]. This has been assumed to be due to puberulic acid, a compound produced by blue mold, suggesting contamination at the manufacturing plant as a potential source. The fungus called “koji” is an *Aspergillus* species, but “beni-koji” is a *Monascus* species. During culturing, *Aspergillus* species produce citric acid, which has the ability to inhibit other bacterial growth and the resultant contamination [14], whereas *Monascus* species are unlikely to produce citric acid. This difference might be one of the reasons why beni-koji eventually caused health damage. To date, there has been only one report showing that administration of cha-koji increases regulatory T cell production in both humans and mice [11], but no studies exploring the effect of koji on wound healing have been performed. In a recent study, green tea was reported to have beneficial effects on human health through several mechanisms such as antioxidant, anti-inflammatory, and antimicrobial activity [15–17]. However, the effects of autoclaved green tea on wound healing have not been reported on to date. Considering commercial use, autoclave-sterilized products may be easier to manage and more versatile than raw ones with regard to possible risk of infectious diseases.

In the present study, we investigated the effects of autoclave-sterilized cha-koji on wound healing using a linear incision wound mouse model [18,19]. Topical application of cha-koji suspension on the dorsal incision wound area healed the wound significantly faster and, notably, with less scarring than did the green tea or the control distilled water treatment. Our results demonstrate the possible mechanisms through which cha-koji promotes wound healing as follows: anti-bacterial activity, epidermal proliferation activity, cytokine secretion effects in macrophages, and suppressive effects on endoplasmic reticulum (ER) stress. Furthermore, in the well-known in vitro skin sensitization test, the human cell line activation test (h-CLAT), no skin sensitizing risk was detected [20]. Our results suggest that cha-koji has potent therapeutic potential to cure wounds quickly, safely, and with less scarring. This is the first report on the wound healing activity of cha-koji and the second report on its biological activity.

2. Materials and Methods

2.1. Cell Culture

Mouse keratinocyte cell line PAM212 (provided by Dr. S. Tajima, Keio University, Tokyo, Japan), mouse fibroblast cell line NIH3T3 (provided by Dr. K. Hirokawa, Tokyo Medical and Dental University, Tokyo, Japan), mouse macrophage cell line RAW264.7 (purchased from American Type Culture Collection, Manassas, VA, USA), and human melanoma cell line SK-MEL-37 (provided by Dr. L.J. Old, Memorial Sloan Kettering Cancer Center, New York, NY, USA) were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO₂/95% air. Human monocytic leukemia cell line THP-1 (ATCC-TIB-202, purchased from American Type Culture Collection) was cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO₂/95% air.

2.2. Mice

C57BL/6 female mice, 6–7 weeks of age, were purchased from Sankyo Labo Service Corp. Inc. (Tokyo, Japan). All mice were maintained under pathogen-free conditions and provided with ad libitum access to both water and food. All animal experiments were approved by the President and Institutional Animal Care and Use Committee of Tokyo Medical University (R4-017 approved on 22 March 2022; R5-104 approved on 26 April 2023; R6-016 approved on 14 March 2024) and performed in accordance with institutional, scientific community, and national guidelines for animal experimentation and the Animal Research: Reporting of In Vivo Experiments guidelines.

2.3. Preparation of Cha-Koji

Cha-koji, which comprises green tea leaves fermented with *A. luchuensis* var *kawachii* *kitahara*, and green tea leaves were provided in powder form by the Biogenkoji Research Institute Co., Ltd. (Kagoshima, Japan). Green tea leaves and cha-koji suspensions were prepared by mixing them in distilled water (0.5 g/10.0 mL) and incubation at 4 °C overnight followed by autoclave sterilization (121 °C, 20 min) to inactivate fungi [21]. The resultant suspensions were divided into small aliquots (1.0 mL stock per tube) and stored at −80 °C until use. The suspension was used for wound treatment in in vivo experiments, while for in vitro experiments the supernatant was prepared by quick centrifugation of the suspension for 20 s at 3000 rpm using a centrifuge (MX-150, TOMY SEIKO Co. Ltd., Tokyo, Japan) to just remove the debris, which may have potential to cause any noise on the observation of cells in the in vitro culture experiments.

2.4. Evaluation of Wound Healing in a Linear Incision Wound Mouse Model

For a dorsal skin incision (10.0 mm in length), three mice in each experimental group were prepared; the hair on the dorsal skin of the mice was shaved 1 day before the skin incision was made (Figure 1A). Afterward, the mice were anesthetized with isoflurane (3% for induction and 1–2% for maintenance), and an incision 10.0 mm in length was made perpendicular to the midline of the dorsal skin using sterile scissors. The incision was subsequently coated with gauze soaked in green tea or cha-koji suspension and covered with a transparent film dressing, Tegaderm (3M Corp., St. Paul, MN, USA), to fix the gauze on the wound (Figure 1B). The gauze and Tegaderm were changed daily until day 13, when the wounds of almost all of mice were closed according to the previous report [22]. Photographs of the wounds were taken over time. The wound length in each photograph was evaluated using the software FIJI (an expanded version of ImageJ, version 1.53c; National Institutes of Health, Bethesda, MA, USA) [18,19].

2.5. Anti-Bacterial Activity Test

The anti-bacterial activity of cha-koji was evaluated by measuring the growth rate of *E. coli* (DH5α, Takara Bio Inc., Kusatsu, Shiga, Japan). Thawed DH5α stock (2.0 μL) was added to LB liquid medium containing green tea or cha-koji (0, 0.5, 1, 2, 4, and 5%) and incubated at 37 °C for 8–14 h with gentle shaking. Bacterial growth was analyzed using the turbidity of the LB medium, which was determined by measuring the optical density at 600 nm (OD600) with the GloMax Discovery Microplate Reader (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.6. Cell Proliferation Assay

PAM212 and NIH3T3 cells were seeded (4×10^3 cells/well and 3×10^3 cells/well in 96-well plates, respectively) and stimulated with green tea or cha-koji (0, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0%). After 48 h, cell proliferative activity was determined using the CellTiter-Glo 2.0 Cell Viability Assay (Promega) and measured with the GloMax Discovery Microplate Reader (Promega) according to the manufacturer's instructions.

2.7. Scratch Assay

Mouse keratinocyte PAM212 cells (4×10^5 cells/well) or fibroblast NIH3T3 cells (3×10^5 cells/well) were cultured to semi-confluence in DMEM containing 10% FBS in 24-well plates. The medium was then exchanged with DMEM containing 0.05% green tea or 0.05% cha-koji and 1.0% FBS. A cross-shaped scratch was made in the center of the well using a 10 μL pipette tip, and photographs were taken over time using an optical microscope LEICA DMI1 (Leica Systems Inc., Tokyo, Japan). The remaining wound area size was determined using FIJI, and the ratio relative to the initial wound area on day 0 was calculated [23].

2.8. RT-qPCR

Mouse macrophage RAW264.7 cells were stimulated with 0.3% cha-koji or 0.3% green tea for 24–48 h. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was prepared using oligo (dT) primer and SuperScript VI RT (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantitative PCR was performed using the SYBR Premix Ex Taq II and Thermal Cycler Dice Real Time System according to the manufacturer's instructions (Takara Bio Inc.). *HPRT* was used as the housekeeping gene to normalize mRNA. The relative expression of PCR products was determined by using the $\Delta\Delta C_t$ method to compare the target gene and *HPRT* mRNA expression. The primers used in this study were purchased from Takara Bio Inc. and are listed in Table S1.

2.9. ER Stress

NIH3T3 cells were seeded (4×10^5 cells/well in 24-well plates) and incubated overnight, followed by stimulation with 0.25% green tea or 0.25% cha-koji in the presence of tunicamycin (1.0 $\mu\text{g}/\text{mL}$) for 12 h. RNA was extracted and RT-qPCR analysis was performed to examine the mRNA expression of ER stress-related factors, HSPA5, XBP-1, and CHOP, together with *HPRT*, an internal control. For the cell growth assay, NIH3T3 cells were seeded (4×10^3 cells/well in 96-well plates) and incubated overnight. After stimulation with 0.25% green tea or 0.25% cha-koji for 24 h, photographs of each well were taken, and cell growth activity was determined by measuring the remaining viable cell area relative to the untreated cell area using FIJI.

2.10. h-CLAT Assay

Single-cell suspensions of THP-1 cells were stained with allophycocyanin (APC)-conjugated anti-human CD86 antibody (clone IT2.2). The stained cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and the FlowJo software application (version 10: FlowJo, Ashland, OR, USA). Dead cells were identified using 7-aminoactinomycin D (7-AAD, Sigma–Aldrich, Saint Louis, MO, USA). The flow cytometry gating strategy is shown in Figure 6A. The RFI of CD86 was calculated, and an RFI higher than 150% was considered to be positive for skin sensitizing potential [20].

2.11. Statistical Analyses

Data are expressed as the mean $\pm$ standard deviation (SD) for each group. Statistical analyses were performed using unpaired, one-way or two-way analysis of variance with Tukey's or Dunnett's multiple comparison test to compare more than three groups using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered statistically significant.

3. Results

3.1. Autoclave-Sterilized Cha-Koji Suspension Promotes Cutaneous Wound Healing

The mouse incisional wound is one of the simplest mouse models for skin wound healing [18,19]. We made an incision on the dorsal area of C57BL/6 mice and evaluated the healing effect of daily topical application of gauze soaked in autoclave-sterilized cha-koji, green tea suspension, or distilled water (Figure 1A,B). The healing rate of the mice treated with cha-koji was accelerated compared to the mice treated with green tea or distilled water (Figure 1C,D). From day 6, the wound length of mice treated with cha-koji decreased significantly more rapidly than that of the mice treated with green tea or distilled water, with considerably fewer scars present (Figure 1C). Thus, the above in vivo results indicate that cha-koji has a potent ability to promote cutaneous wound healing.

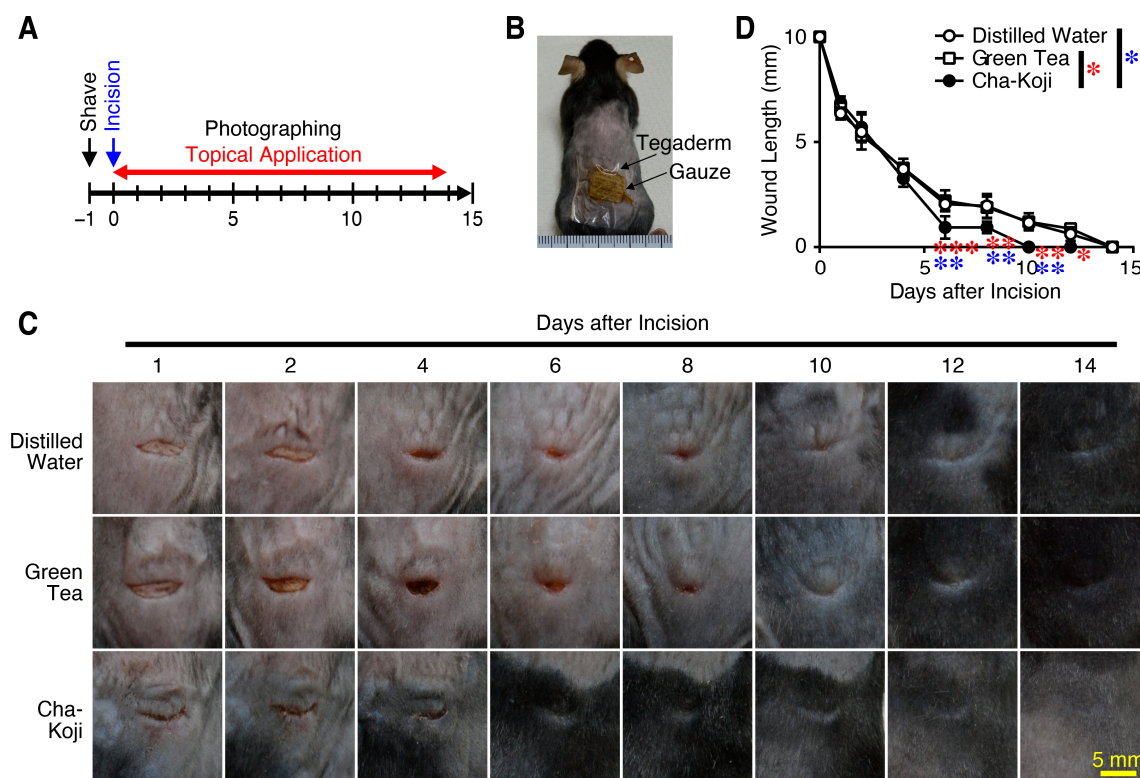


Figure 1. Autoclave-sterilized cha-koji suspension promotes cutaneous wound healing. A cutaneous incision wound was made on the dorsal skin of the mice; coated with gauze soaked in green tea, cha-koji suspension, or distilled water; and covered with a transparent film dressing, Tegaderm, daily until day 13 (A,B). Photographs of the incision were taken over time. Representative photographs of the healing process are shown (C). The length of the incision wound in each photograph was measured using FIJI, and the relative length at each time point to the initial length of 10 mm was calculated (D). Data are shown as the mean $\pm$ SD ($n = 3$) and are representative of five independent experiments. p -values were determined via two-way analysis of variance with Tukey's multiple comparison test. The red and blue asterisks marked on day 6, 8, 10, and 12 in the figure (D) mean that there are significant differences between cha-koji and green tea and between cha-koji and distilled water, respectively. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Scale bar = 5 mm.

3.2. Autoclave-Sterilized Cha-Koji Suspension Shows Anti-Bacterial Activity

Infection is one of the main causes of delayed or discontinued wound healing [3]. To clarify the mechanism underlying the promotive healing effect of cha-koji on cutaneous wound healing, we next examined the anti-bacterial activity of cha-koji using *Escherichia coli* (*E. coli*, DH5 α) as a representative of Gram-negative bacteria. Previous reports have indicated that a list of antibiotic-resistant priority pathogens published by WHO consists mainly of Gram-negative bacteria. Due to their distinctive structure, Gram-negative bacteria are more resistant than Gram-positive bacteria, and major causes of significant increases in morbidity and mortality worldwide [24]. To evaluate the broad anti-bacterial activity of cha-koji, we compared the anti-bacterial activities of autoclave-sterilized green tea and cha-koji against *E. coli*, together with raw, non-autoclaved green tea as a positive control. The turbidity (OD600) of the Luria–Bertani (LB) medium containing *E. coli* gradually increased with time due to bacterial growth; however, increasing concentrations of cha-koji and green tea in the LB medium (0.5, 1.0, 2.0, 4.0, and 5.0%) greatly inhibited turbidity (Figure 2A). Of note, among the treatments, the autoclave-sterilized cha-koji inhibited turbidity, that is, bacterial growth, to the greatest degree (Figure 2B). The above results indicate that cha-koji treatment shows anti-bacterial activity, which should contribute to healing effects on cutaneous wounds.

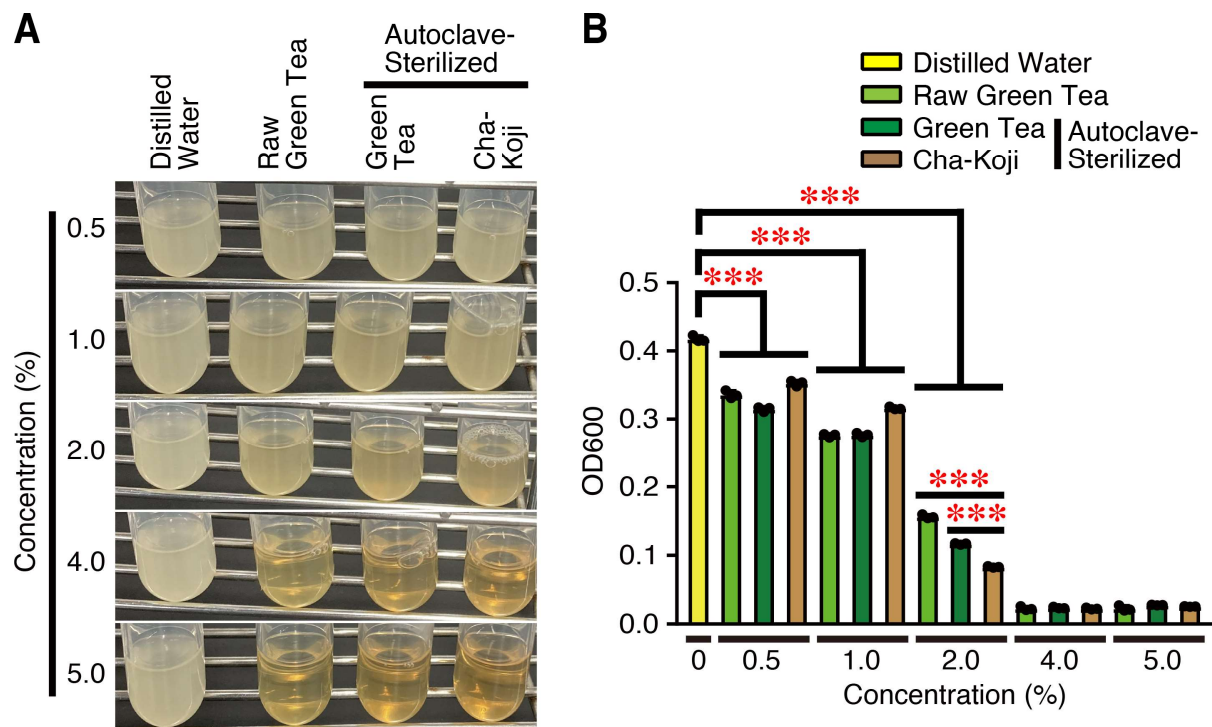


Figure 2. Autoclave-sterilized cha-koji suspension showing anti-bacterial activity. *E. coli* was cultured in LB containing various concentrations of autoclave-sterilized cha-koji, green tea (0.5, 1.0, 2.0, 4.0, and 5.0%) or distilled water for 8 h at 37 °C (A). As a positive control, raw green tea was used. Bacterial growth was examined according to the turbidity of the LB medium, which was determined by measuring optical density at 600 nm (OD600) (B). Data are shown as the mean $\pm$ SD ($n = 3$) and are representative of three independent experiments. p -values were determined via one-way analysis of variance with Tukey's multiple comparison test. *** $p < 0.001$.

3.3. Autoclave-Sterilized Cha-Koji Suspension Promotes Skin Epidermal Cell Proliferation and Migration

Wound closure is induced by epidermal cell proliferation and migration in the remodeling stage of the healing process [25]. To investigate whether cha-koji has any effects on the promotion of wound closure, we examined cell proliferation and migration in mouse keratinocyte PAM212 cells and fibroblast NIH3T3 cells treated with cha-koji using a scratch assay [23]. Because higher concentrations of cha-koji and green tea significantly decreased the proliferation of PAM212 cells and NIH3T3 cells, we first titrated their concentrations and determined the concentrations that do not affect the proliferation of these cells to be 0.05%, which was used for the scratch assay (Figure S1). Cha-koji significantly promoted wound closure in PAM212 cells (Figure 3A,B) but not NIH3T3 cells as compared to green tea or the control distilled water (Figure 3C,D). Conversely, green tea did not promote wound closure in PAM212 and NIH3T3 cells compared to the control distilled water. The above results indicate that cha-koji promotes epidermal cell proliferation and migration to accelerate wound closure in the healing process.

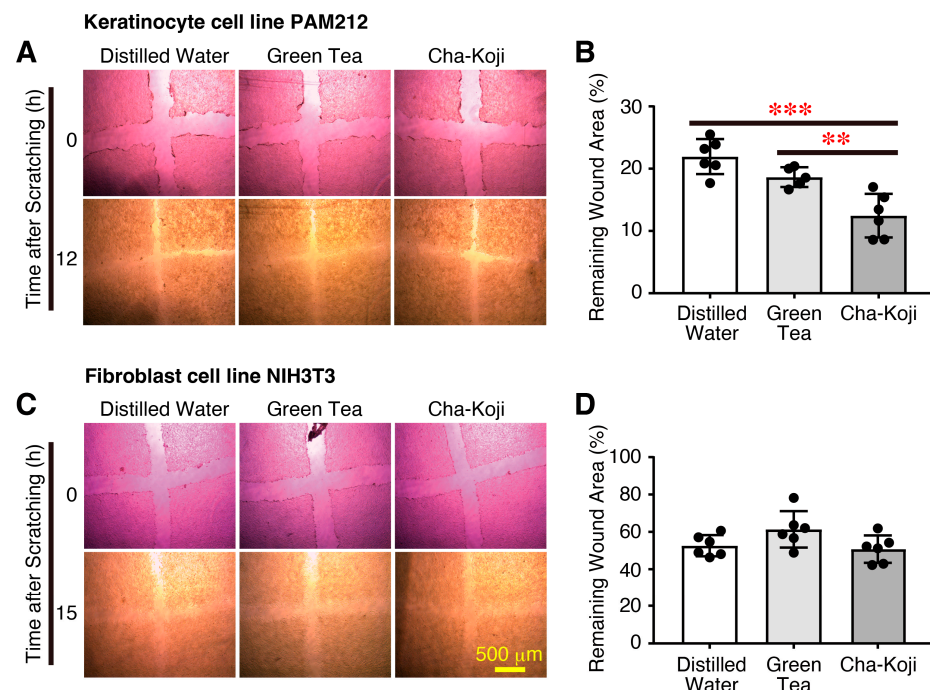


Figure 3. Autoclave-sterilized cha-koji suspension promotes skin epidermal cell proliferation and migration. Keratinocyte PAM212 cells (A,B) or fibroblast NIH3T3 cells (C,D) were seeded in 24-well plates and cultured to semi-confluence in DMEM containing 10% FBS. The medium was then exchanged with DMEM containing 0.05% green tea or 0.05% cha-koji and 1.0% FBS. A cross-shaped scratch was made in the center of the well, and photographs were taken over time. Representative photographs are shown (A,C). The remaining wound area size was determined using FIJI, and the ratio relative to the initial wound area on day 0 was calculated (B,D). Data are shown as the mean $\pm$ SD ($n = 6$) and are representative of four (A,B) and three (C,D) independent experiments. p -values were determined via one-way analysis of variance with Tukey's multiple comparison test. ** $p < 0.01$ and *** $p < 0.001$. Scale bar = 500 μ m.

3.4. Autoclave-Sterilized Cha-Koji Suspension Promotes the Expression of Anti-Inflammatory Cytokine Transforming Growth Factor (TGF)- β 1

It has been previously reported that M2 macrophages contribute to wound healing [4], and anti-inflammatory cytokine TGF- β 1 induces M2-like macrophage polarization to promote wound healing [26]. To explore whether cha-koji contributes to macrophage polarization at the wound site, we performed reverse transcription–quantitative polymerase chain reaction (RT-qPCR) analysis of TGF- β 1 using a mouse macrophage cell line, RAW264.7, stimulated with cha-koji or green tea. Cha-koji and green tea significantly upregulated the mRNA expression of TGF- β 1 compared to distilled water after stimulation for 48 h; this same effect was not seen after stimulation for 24 h, however (Figure 4A,B). In the skin wound healing process, the regenerative stage occurs following the inflammation stage, changing from a pro-inflammatory environment to an anti-inflammatory environment, to promote tissue regeneration [27]. Next, to examine the effect of cha-koji on the state of inflammation, we analyzed the mRNA expression of one of the pro-inflammatory cytokines, interleukin (IL)-6, in RAW264.7 cells stimulated with cha-koji or green tea (Figure 4C,D). Cha-koji but not green tea significantly suppressed the mRNA expression of IL-6 after stimulation for 48 h; however, this same effect was not seen after stimulation for 24 h. The above results suggest that cha-koji contributes to the processes involved in the regenerative stage by promoting conversion from the pro-inflammation stage to the tissue regenerative stage through possible induction of TGF- β 1.

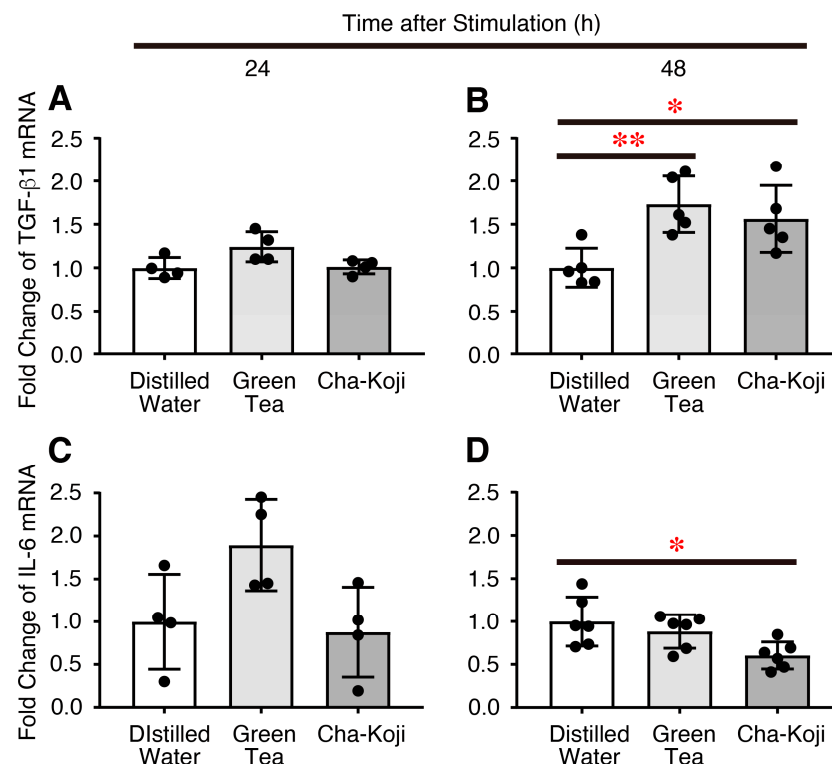


Figure 4. Autoclave-sterilized cha-koji suspension promotes the expression of the anti-inflammatory cytokine TGF-β1. Macrophage RAW264.7 cells were stimulated with 0.3% cha-koji, 0.3% green tea, and distilled water for 24 and 48 h. RT-qPCR was then performed to analyze the mRNA expression of TGF-β1 (A,B) and IL-6 (C,D). HPRT was used as an internal control, and the relative expression of TGF-β1 or IL-6 to HPRT was calculated. Data are shown as the mean ± SD ($n = 4–6$) and are representative of three (A,B) and five (C,D) independent experiments. p -values were determined via one-way analysis of variance with Dunnett’s multiple comparison test. * $p < 0.05$ and ** $p < 0.01$.

3.5. Autoclave-Sterilized Cha-Koji Suspension Suppresses ER Stress

It has been reported that, during the skin wound healing process, ER stress is involved in the differentiation of fibroblasts into myofibroblasts [28] and that it is important to induce skin contraction by myofibroblasts for wound closure [29]. However, strict regulation of this process is required, as excessive wound contraction may result in scar contraction [30]. We next explored whether cha-koji affects ER stress induction at the wound site. After mouse fibroblast NIH3T3 cells were stimulated with a known ER stress inducer, tunicamycin [31], we analyzed well-known ER stress markers such as *heat shock protein family A member 5* (HSPA5), *X-box-binding protein 1* (XBP1), and *C/EBP homologous protein* (CHOP) via RT-qPCR (Figure 5A–C). Cha-koji treatment significantly decreased the mRNA expression of HSPA5 and CHOP; in contrast, green tea only significantly decreased that of HSPA5. Furthermore, to confirm the suppressive effect on ER stress, we analyzed cell growth after tunicamycin treatment (Figure 5D,E). Although tunicamycin treatment reduced cell growth, cha-koji, but not green tea, significantly reversed this process. The above results suggest that cha-koji has suppressive effects on ER stress and resultant cell damage, possibly leading to scarless wound healing.

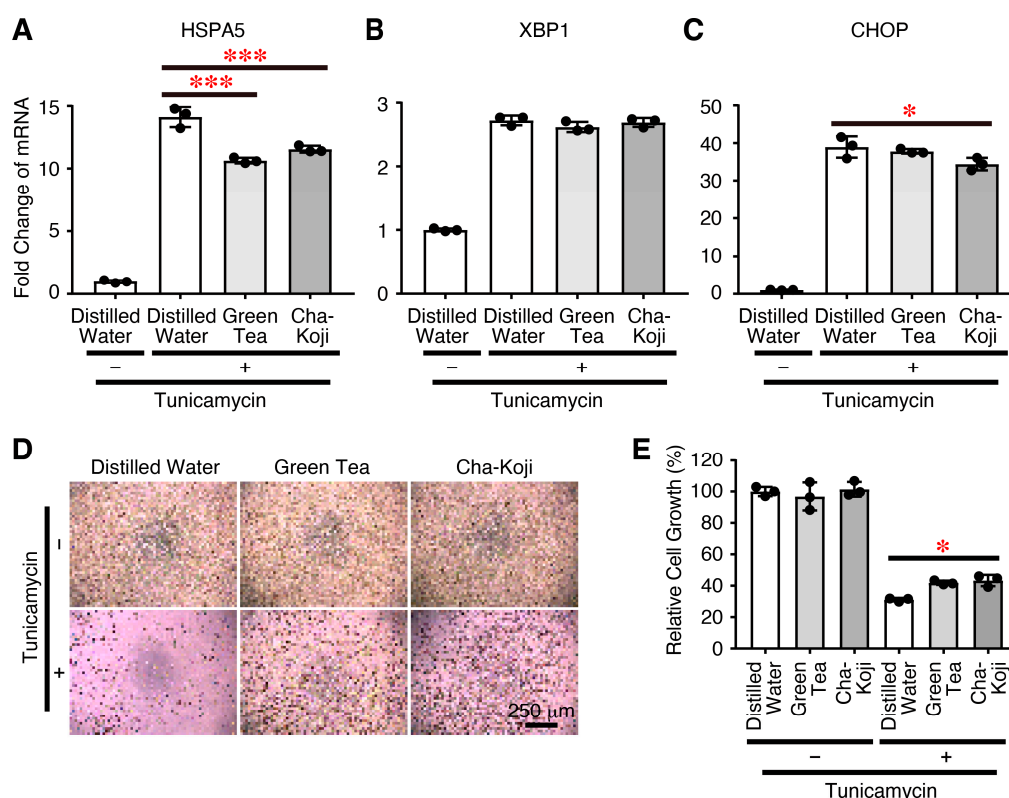


Figure 5. Autoclave-sterilized cha-koji suspension suppresses ER stress. Fibroblast NIH3T3 cells were stimulated with tunicamycin (1.0 μ g/mL) in the presence or absence of 0.25% cha-koji and 0.25% green tea for 12 h. RNA was extracted, and RT-qPCR analysis was performed to examine the mRNA expression of ER stress-related factors, namely, *HSPA5* (A), *XBP1* (B), and *CHOP* (C). *HPRT* was used as an internal control, and its relative expression of *HPRT* was calculated. Data are shown as the mean $\pm$ SD ($n = 3$) and are representative of two independent experiments. p -values were determined via one-way analysis of variance with Dunnett's multiple comparison test. After 48 h, photographs of each well were taken (D), and cell growth activity was determined by measuring the remaining viable cell area relative to the tunicamycin-untreated, distilled water-treated cell area using FIJI (E). Data are shown as the mean $\pm$ SD ($n = 3$) and are representative of three independent experiments. p -values were determined via one-way analysis of variance with Dunnett's multiple comparison test. * $p < 0.05$ and *** $p < 0.001$. Scale bar = 250 μ m.

3.6. Autoclave-Sterilized Cha-Koji Suspension Poses No Potential Skin Sensitizing Risk

In general, when new compounds used for cosmetics or skin treatment ointments are developed, it is necessary to determine whether they have any adverse effects before production, such as skin sensitizing potential. To examine the skin sensitizing potential of cha-koji, we performed an h-CLAT assay [20] using THP-1 cells. The positive control, the conditioned medium of human melanoma SK-MEL-37 cells treated with rhododenol [32,33], greatly upregulated the expression of the relative fluorescent intensity (RFI) of one of the critical costimulatory molecules, the cluster of differentiation (CD)86. In marked contrast, cha-koji and green tea failed to upregulate the RFI of CD86 by more than 150%, which is the critical cut-off value for positive and negative sensitizers [20] (Figure 6A,B). The above results indicate that cha-koji does not pose any potential skin sensitizing risk.

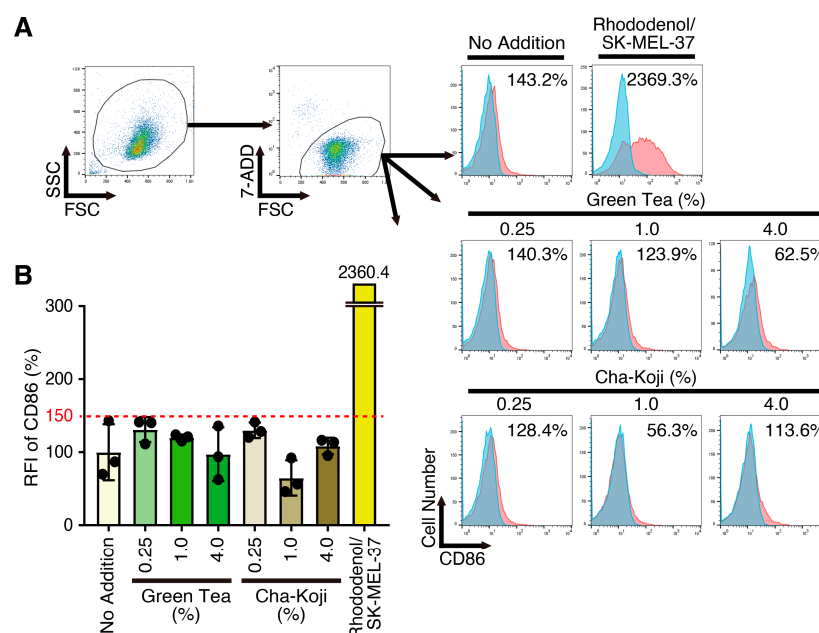


Figure 6. Autoclave-sterilized cha-koji suspension poses no potential skin sensitizing risk. Human monocytic THP-1 cells were stimulated with cha-koji or green tea suspension (0.25, 1.0, and 4.0%) together with the positive control, conditioned medium from rhododenol-treated melanoma SK-MEL-37 cells. After 24 h, CD86 expression in the THP-1 cells was analyzed via flow cytometry using anti-CD86 (red-shaded histogram) or a control antibody (blue-shaded histogram). Conditioned medium from the rhododenol-treated melanoma (SK-MEL-37) was used as a positive control. Representative histograms for the cell surface expression of CD86 are shown (A). The RFI values of each sample were calculated and compared (B). An RFI higher than 150% is considered to be positive for skin sensitizing potential.

4. Discussion

The beneficial effects of koji on wound healing have not been reported to date; in comparison, other bacteria such as lactobacillus species have been demonstrated to promote wound healing [34]. Besides bacteria, several compounds derived from plants or animals, including tetrapeptides from sea cucumber [35], epigallocatechin gallate from green tea [36], and polyphenols from strawberry and blackberry [37], have also been reported to contribute to the promotion of wound healing. Cha-koji, green tea fermented with *Aspergillus luchuensis* var *Kawachii* kitahara, is a recently developed health food in Japan [11]. In the present research, to investigate whether autoclave-sterilized cha-koji has any promotive effect on cutaneous wound healing, we used a linear incision wound mouse model [19]. After making a 10 mm incision on the dorsal skin of the mice, daily topical application with gauze soaked in autoclave-sterilized cha-koji suspension resulted in significantly faster wound healing with less scarring than application of gauze soaked in autoclave-sterilized green tea suspension or distilled water (Figure 1C,D). Our preliminary histological evaluation with hematoxylin and eosin of the skin sections on day 2 suggested that the number of infiltrating inflammatory cells under the scab in cha-koji-treated mice tended to be less than those of distilled water- or green tea-treated mice. These preliminary results may indicate that cha-koji treatment has the potential to accelerate the convergence of inflammation, although further studies are necessary to prove it in the near future.

Cutaneous wound healing is a complex process involving clot formation, inflammation, angiogenesis, cell migration and proliferation, remodeling, and resolution [27]. In the wound area specifically, it has been reported that bacterial infections cause delays in the healing process [27]. Cha-koji may contain green tea ingredients such as catechins, which are widely known to have anti-bacterial activity [38,39]. It has also been reported that some catechins in green tea are still active even after being exposed to high temperatures or

placed in an autoclave [40,41]. Moreover, *Aspergillus luchuensis* has the ability to produce citric acid [42], which is reported to have anti-bacterial activity as well [43]. Therefore, to investigate the possible mechanisms through which cha-koji promotes wound healing, we first focused on its anti-bacterial activity. We performed a bacterial growth assay and found that all raw green tea and autoclave-sterilized green tea and cha-koji display potent anti-bacterial activity (Figure 2A,B). Among them, autoclave-sterilized cha-koji showed the strongest anti-bacterial activity (Figure 2B). Similarly, cha-koji and green tea appeared to display potent inhibitory activity against the proliferation of PAM212 cells and NIH3T3 cells (Figure S1). Indeed, catechins in green tea have been demonstrated to have potent antitumor activity against a variety of cancers such as breast cancer, lung cancer, and stomach cancer [38,39]. It has also been reported that *Aspergillus oryzae* produces a potent anti-cancer compound, deferri-ferrichrysin [44,45]. *Aspergillus luchuensis* used in cha-koji may produce this compound because it is a species closely related to *Aspergillus oryzae*.

We next focused on wound closure using a scratch assay and found that cha-koji but not green tea promoted the proliferation and migration of keratinocyte PAM212 cells (Figure 3A,B); however, this same effect was not noted for fibroblast NIH3T3 cells (Figure 3C,D). Although the specific details of this mechanism are unclear, cha-koji may contain unique compounds that contribute to the proliferation and migration of keratinocytes. Indeed, *Aspergillus oryzae*-fermented peptone has been demonstrated to enhance the proliferation potential of human keratinocytes [46]. Whether a similar peptone may be produced from cha-koji and contribute to the enhanced proliferation and migration of keratinocytes remains unclear. To induce angiogenesis for wound healing, it has been reported that M2 macrophages contribute to the induction of angiogenesis by secreting platelet-derived growth factor and vascular endothelial growth factor [47] and that TGF- β 1 induces M2 macrophage polarization, suppressing a pro-inflammatory phenotype at the wound site [26]. Therefore, after the stimulation of macrophage RAW264.7 cells with cha-koji or green tea, we analyzed the mRNA expression of anti-inflammatory and pro-inflammatory cytokines, TGF- β 1 and IL-6. Cha-koji but not green tea induced the upregulation of TGF- β 1 but the downregulation of IL-6 (Figure 4B,D), possibly resulting in M2 macrophage polarization, subsequent promotion of angiogenesis, and consequent accelerated wound healing [48].

After the incision was made on the mice, the application of cha-koji significantly induced faster wound closure with less scarring than green tea or distilled water treatment (Figure 1C,D). Interestingly, the peripheral wound area of cha-koji-treated mice showed moderate wrinkles compared to that of the other groups (Figure 1C). From day 4, in particular, the differences were evident to such a degree that the wrinkles caused by contraction appeared to be less prominent in the cha-koji-treated mice. It has been reported that contraction is induced by the differentiation of fibroblasts into myofibroblasts around the wound area [49]. Although this contraction is necessary to promote wound closure, excessive contraction can cause scar contracture and fibrosis [30]. Therefore, strict regulation is required for the induction of contraction. Furthermore, the findings of another study indicate that ER stress promotes differentiation into myofibroblasts [28]. From these findings, we speculated that cha-koji treatment would have an effect on the regulation of ER stress, and we examined the effect of cha-koji on ER stress in mouse fibroblast NIH3T3 cells treated with tunicamycin. Cha-koji treatment significantly decreased the expression of the ER stress markers *HSPA5* and *CHOP* compared with green tea and distilled water treatment (Figure 5A–C) and consequently improved the cell growth of tunicamycin-treated NIH3T3 cells (Figure 5D,E). The above results suggest that cha-koji may suppress scar formation by regulating ER stress.

Focusing on the topical application of cha-koji for cutaneous wound healing, we needed to clarify whether cha-koji has any harmful side effects on the skin. We therefore performed the h-CLAT assay to examine the sensitizing potential of cha-koji (Figure 6A). Both cha-koji and green tea at any concentrations ranging from low to high that reduced

cell viability (Figure S1) did not show any sensitizing activity, suggesting that the topical application of cha-koji is likely to be highly safe.

The results presented in this study indicate that autoclave-sterilized cha-koji suspension has potent therapeutic potential to cure wounds quickly, safely, and with less scarring. Although the *in vitro* analyses revealed several mechanisms are possibly involved, one limitation of this study is that the active ingredients for individual mechanisms in the autoclave-sterilized cha-koji suspension remain to be identified using mass spectrometry analysis. Therefore, further studies on the effectiveness and safety of the cha-koji, as well as its active ingredients, are necessary in near future.

5. Conclusions

The results presented in this study indicate that autoclave-sterilized cha-koji induced faster cutaneous wound healing with less scarring in the utilized linear incision wound mouse model. *In vitro* analysis revealed that cha-koji increased anti-bacterial activity, enhanced epidermal cell proliferation and migration, augmented the expression of the anti-inflammatory cytokine *TGF-β1*, reduced the expression of the inflammatory cytokine *IL-6* in macrophages, and decreased the ER stress. Although further studies on the effectiveness and safety of cha-koji are necessary, the present results suggest that cha-koji may have a potent ability to promote cutaneous wound healing without posing any sensitizing potential risk and could therefore be applied to medical aids such as Band-Aids.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/scipharm92030044/s1>. Figure S1: Autoclave-sterilized supernatant of cha-koji or green tea suppresses cell proliferation; Table S1: Primers used in the study.

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