Original Research

Gamma-tocopherol supplementation ameliorated hyper-inflammatory response during the early cutaneous wound healing in alloxan-induced diabetic mice

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Impact statement

Gamma tocopherol has shown ameliorative effect on diabetic wound healing by regulation of inflammation, oxidative stress, and apoptosis demonstrated by nuclear factor kappa B, nuclear factor (erythroid derived 2)-like 2, and sirtuin-1.

Abstract

Delayed wound healing is one of the major diabetic complications. During wound healing process, the early inflammatory stage is important for better prognosis. One of antioxidant nutrient, gamma-tocopherol (GT) is considered to regulate inflammatory conditions. This study investigated the effect of GT supplementation on mechanism associated with inflammation, oxidative stress, and apoptosis during early cutaneous wound healing in diabetic mice. Diabetes was induced by alloxan injection in ICR mice. All mice were divided into three groups: non-diabetic control mice (CON), diabetic control mice (DMC), and diabetic mice supplemented with GT (GT). After two weeks of GT supplementation, excisional wounds were made by biopsy punches (4 mm). Diabetic mice showed increases in fasting blood glucose (FBG) level, hyper-inflammatory response, oxidative stress, and delayed wound closure rate compared to non-diabetic mice. However, GT supplementation reduced FBG level and accelerated wound closure rate by regulation of inflammatory response-related proteins such as nuclear factor kappa B, interleukin-1β, tumor necrosis factor-α, and c-reactive protein, and oxidative stress-related markers including nuclear factor (erythroid derived 2)-like 2, NAD(P)H dehydrogenase quinone1, heme oxygenase-1, manganese superoxide dismutase, catalase and glutathione peroxidase and apoptosis-related markers such as sirtuin-1, peroxisome proliferator-activated receptor gamma coactivator 1-α, and p53 in diabetic mice. Taken together, GT would be a potential therapeutic to prevent diabetes-induced delayed wound healing by regulation of inflammatory response, apoptosis, and oxidative stress.

Keywords: Gamma-tocopherol, diabetes, cutaneous wound healing, inflammation, oxidative stress

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Introduction

Diabetes mellitus (DM) is becoming increasingly prevalent worldwide. In particular, diabetic complications contribute to the mortality of diabetic patients. Among major complications, diabetic neuropathy also known as a diabetic foot ulcer or diabetic wound is highly prevalent.[1] As many as 15% of diabetic patients will progress foot ulcer or wounds, and 3% will need lower limb amputation.[2]

It is well known that oxidative stress and inflammation are related to diabetic complications including diabetic wounds as well as diabetes. Hyperglycemia-induced formation of reactive oxygen species (ROS) is able to cause the dysfunctions of antioxidant defense systems besides beta-cell death, to induce insulin resistance and alter many biological pathways in diabetes.[3] Moreover, exacerbated oxidative stress in DM is associated with systemic inflammation in diabetic wounds.[4]

Normal wound healing process consists of three stages that overlap in space and time: inflammation, proliferation, and remodeling.[5] The initial event of the inflammatory stage during wound healing is the infiltration of inflammatory cells such as neutrophils and macrophages into the wound site to attack contaminating bacteria and to phagocytose cellular debris resulting in the production of ROS. Proliferation including angiogenesis and granulation tissue formation start in the next stage of wound healing.[6] During the last remodeling stage, collagen is realigned along tension line and cells.[7]

Among the three stages, the inflammatory stage is considered the most important step that regulates entire healing process, because it is involved in the production of

various growth factors and cytokines that coordinate movements of various cells necessary for repair.[6] In particular, there are both major sources and targets of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), which are produced by activated neutrophils and macrophages. TNF-α and IL-1β promote recruitments of more inflammatory cells and activation of nuclear factor kappa B (NFκB).[5,8] NFκB is an essential transcription factor in the inflammatory response for cell survival, differentiation, proliferation, and apoptosis in DM.[9] A previous study showed that NFκB activation in hepatic tissue was significantly elevated in diabetes.[10] However, another study showed that NFκB activation was significantly reduced in cutaneous wounds in high fat diet-induced obese mice.[11] The discrepancy may be due to decreased functions of inflammatory cells during cutaneous wound healing in DM.

A previous study has shown that NFκB activation is regulated by sirtuin-1 (SIRT-1). SIRT-1, an NAD+-dependent protein deacetylase, plays important roles in modulating inflammation, metabolism, stress resistance, DNA repair, and cell survival by deacetylation of transcription factors, enzymes, and proteins.[12] SIRT-1 is shown to be an upstream of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is sufficient to reduce NAD(P)H oxidase expression and to inhibit pro-inflammatory cytokine production.[13,14] SIRT-1 is shown to suppress NFκB activation through deacetylation of the p65 subunit of NFκB.[15] Furthermore, SIRT-1 interacts with p53, which plays an important role in regulation of cell cycle progression and the activation of DNA repair.[16] p53 has protective effects of apoptosis and DNA damage.[17] A previous study has shown that SIRT-1 expression is significantly decreased in DM.[18]

Moreover, NFκB also generally works with other transcription factors such as nuclear factor (erythroid derived 2)-like 2 (Nrf2).[19] Under oxidative stress conditions including DM, Nrf2 modulates its downstream antioxidant defense enzymes such as superoxide dismutase (SOD), heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone 1 (NQO1), catalase, and glutathione peroxidase (GPx), which remove excessive ROS.[20,21] In a previous study, perilesional skin tissues showed higher Nrf2 activation in diabetic patients.[22] Taken together, inflammation, apoptosis, and oxidative stress demonstrated by NFκB, SIRT-1, and Nrf-2 play important roles in DM.

Vitamin E, a lipid-soluble antioxidant nutrient, is well known to have various beneficial effects on prevention of metabolic disorders such as obesity, cancer, diabetes, and cardiovascular disease (CVD).[23–26] Whereas alpha(α)-tocopherol (AT) is known as a biologically active nutrient with anti-inflammatory and antioxidant properties in humans monocytes[27,28] gamma-tocopherol (GT) is a major form of dietary vitamin E, which is abundant in plant seeds including flaxseed, corn, and soybean oils.[29] GT is well absorbed and accumulated in specific human tissues including intestine as efficiently as AT.[29] GT is more effective to trap electrophils than is AT.[30] GT metabolite, 2,7,8-trimethyl-2-(β-carboxuethyl)-6-hydroxychroman (γ-CEHC), significantly inhibited cyclooxygenase-2 activity in lipopolysaccharide-activated macrophages and IL-1β-treated epithelial cells although γ-CEHC was easily excreted.[30–32] Previous studies suggested that GT has a potential role in regulation of oxidative stress and inflammation at low dose (33 mg/kg body weight) in murine models.[30,31] Because of its strong nucleophilic properties, GT may be a more potent pro-apoptotic agent than AT in trapping ROS in colon cancer.[15] Moreover, GT partially protects insulin-secreting cells against functional inhibition by nitric oxide. The protective effect of GT may be of importance for the development of the impaired insulin secretion characterizing diabetes.[33]

However, there are limited studies focusing on effects of GT on cutaneous wound healing in diabetes. This study investigates that GT supplementation ameliorates oxidative stress, inflammation, and apoptosis by interactions among SIRT-1, NFκB, and Nrf2 during the early stage of cutaneous wound healing in alloxan-induced diabetic mice.

Methods

Animals

Four-week-old male ICR mice (Nara Bio, Gyeonggi-do, South Korea) were used in all experiments. They were lodged in individual cages and access to food and water *ad libitum*. Mice were housed with controlled temperature and humidity ($22 \pm 3^{\circ}C$ and $55 \pm 5\%$) under 12 h light–dark cycle. After one-week acclimation, diabetes was induced by one-time intraperitoneal injection of 150 mg/kg alloxan monohydrate (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.9% NaCl solution. Non-diabetic control mice were injected with only 0.9% NaCl solution. Fasting blood glucose (FBG) was measured from the mouse tail vein using the One-Touch blood glucose meter (LifeScan Inc., Milpitas, USA) at one-week after injection. Once FBG levels reached above 250 mg/dL, mice were considered as diabetes and used for the study. All experiments were approved by Kyung Hee University for the care and use of laboratory animals (KHUASP(SE)-14-007) and were performed in accordance with the guideline.

Experimental design

All mice were fed a modified AIN-76A rodent diet (Research Diets, New Brunswick, NJ, USA). Mice were divided into three groups ($n = 24$ in each group): (1) CON: non-diabetic mice were administered with tocopherol-stripped corn oil (MP Biomedicals Inc, Solon, OH, USA), (2) DMC: diabetic mice were administered with a tocopherol-stripped corn oil, and (3) GT: diabetic mice were administered with 35 mg/kg GT (Acros organics, Geel, Belgium) dissolved in a tocopherol-stripped corn oil. Dietary treatment was administered by oral gavage five times per week for two weeks. During the experiment period, body weights, food consumption, and FBG levels were measured once a week. Mice were fasted 8 h after the last treatment for sacrifice and anesthetized by isoflurane (Ilsung Pharmaceutical, Seoul, South Korea). Blood, wound sites were collected at baseline (0 h), 24, and 72 h after wounding. The endpoint was considered as the time

when wound was close in the CON group after wounding. Plasma was collected by cardiac puncture in heparin (Sigma-Aldrich, St. Louis, MO., USA) and isolated by centrifugation at 850 *g* for 15 min at 4°C. The hepatic tissue was washed in saline and frozen immediately in liquid nitrogen. All the samples were stored at −80°C until they were analyzed.

Plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

Plasma GOT and GPT levels were measured using Reitman–Frankel method (Bio-Clinical System, Gyeonggi-do, South Korea).

Wound biopsy and wound closure rate

Each mouse was anesthetized with isoflurane and the back of the mouse was shaved using an electronic clipper and sterilized using an alcohol swab. The back skin was then nipped and bent, and the full-thickness excisional wounds were made on the bent skin using a sterile biopsy punch (4 mm diameter, Kai Medical, Gifu city, Japan). Four wounds in an individual mouse were photographed every day using a digital camera with a standard equivalent to the initial wound area placed beside the wound site. Wound closure was quantified by Canvas 11 software (Deneba, Miami, FL, USA). Wound closure rate was defined as the ratio of the wound size to the initial wound size.

Western blot analysis

One hundred milligram of skin tissue was homogenized in a lysis buffer (containing Trizma base, 150 mM $NaCl_2$, pH7.5, 10% NP40, 10% Na-deoxycholate, 100 mM EDTA 10% SDS) with a mixture of protease inhibitors (1:200 v/v, Sigma-Aldrich) and centrifuged at 14,000 rpm for 30 min at 4°C. Protein supernatant was used for western blot analysis. For the preparation of nuclear fraction, skin tissues were homogenized in buffer A containing 0.6% Nonidet P40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM PMSF, leupeptin, pepstatin, and aprotinin. After the homogenization procedure, each sample was centrifuged at 5000 rpm and 4°C for 30 s. The supernatant was incubated for 5 min on ice and centrifuged at 5000 rpm for 5 min. The nuclear pellet was suspended in buffer B containing 25% glycerol, 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, benzamidine, leupeptin, pepstatin, and aprotinin followed by incubation on ice for 20 min. The supernatant was used to determine nuclear protein concentration. Protein concentration was quantified using the Bradford assay and equal amounts of protein were separated with SDS-PAGE and then transferred to the PVDF membrane (Millipore, Billerica, MA, USA).

After blocking in a blocking buffer containing 3% BSA, the membrane was incubated overnight at 4°C with a primary antibody: NFκB (cell signaling technology, Inc., Danvers, MA, 1:500), SIRT-1 (Abcam, Cambridge, MA, USA, 1:2000), Nrf2 (Abcam, Cambridge, MA, USA, 1:1000), NQO1 (cell signaling technology, Inc., Danvers, MA, USA 1:1000), HO-1 (Stressgen, Victoria, Canada, 1:1000), GPx (Abcam, Cambridge, MA, USA 1:1000), catalase (Cell Signaling Technology, Inc., Danvers, MA, USA 1:1000), manganese superoxide dismutase (MnSOD) (SOD2) (Santa Cruz Biotechnology, CA, USA, 1:5000), TNF-α (Santa Cruz Biotechnology, CA, USA, 1:200), C-reactive protein (CRP; Abcam, Cambridge, MA, USA 1:500), PGC-1α (Santa Cruz Biotechnology, CA, USA, 1:2000), p53 (Cell Signaling Technology, Inc., Danvers, MA, USA 1:1000), 4-hydroxynonenal (4-HNE; R&D systems, Minneapolis, MN, USA, 1 μg/mL), β-actin (Santa Cruz Biotechnology, CA, USA, 1:1000), or Lamin B1 (Abcam, Cambridge, MA, USA 1:8000). Then, the blots were washed four times for 10 min with PBS-T. Thereafter, the membranes were incubated with the secondary antibodies for 1 h and washed with PBS-T. Signals were then developed using the ECL luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then recorded and quantified with the Syngene G box (Syngene, Cambridge, UK, USA).

Statistical analysis

All data were presented as means ± SEM. The significance of differences was analyzed by one-way ANOVA followed by Duncan's multiple range test. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0K for Windows) and Graph Pad Prism (version 5.0).

Results

Effects of GT supplementation on body weight and food intake in alloxan-induced diabetic mice

At one week after alloxan injection, diabetic mice significantly had lower body weight than CON group. However, GT supplementation did not affect body weight change in diabetic mice throughout the experimental periods. There were no significant differences of body weights among diabetic mice regardless of FBG levels. Food intake was significantly higher in the DM group than the CON group. However, GT supplementation did not change food intake in diabetic mice (Table 1).

Effect of GT supplementation on FBG and plasma GOT and GPT in alloxan-induced diabetic mice

In all diabetic groups, FBG levels were significantly higher than in the CON group. After two weeks GT treatment, the GT group showed significantly lower FBG levels compared to the DMC group (Table 1). Plasma GOT and GPT activities were measured as biomarkers of liver injury. GOT level was significantly higher in the DMC group than in the CON group, and the GT group showed significantly lower GOT level compared to the DMC group (Table 1). However, there was no significant difference in GPT levels among all mice groups.

Table 1 Effects of GT supplementation on body weights, food intake, fasting blood glucose, plasma aspartate transaminase (AST), and alanine transaminase (ALT) in alloxan-induced diabetic mice

	CON	DMC	GT
Body weight (g)			
Before treatment	33.66 ± 0.45a	28.45 ± 0.52b	28.57 ± 0.50b
After treatment	34.80 ± 0.44a	26.84 ± 0.69b	26.08 ± 0.81b
Food intake	5.37 ± 0.11a	8.24 ± 0.36b	7.25 ± 0.34b
Fasting blood glucose (mg/dL)	116.67 ± 3.75a	466.50 ± 16.62c	393.25 ± 20.13b
GPT (nM)	67.36 ± 11.64a	139.70 ± 19.34b	95.77 ± 18.46a
GPT (nM)	9.22 ± 0.91	11.69 ± 1.86	8.04 ± 1.05

CON: non-diabetic control mice; DMC: diabetic control mice; GT: diabetic mice supplemented with GT (GT: gamma-tocopherol).
Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$).

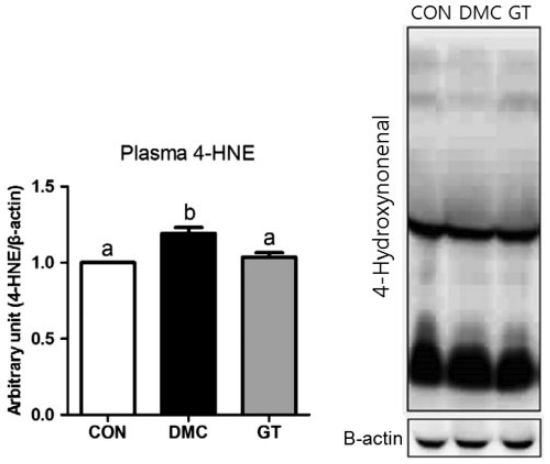

Figure 1 Effect of GT supplementation on plasma 4-HNE in alloxan-induced diabetic mice. Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$). 4-HNE: 4-hydroxynoneal; GT: gamma-tocopherol

Effect of GT supplementation on plasma 4-hydroxynoneal (4-HNE) level in alloxan-induced diabetic mice

Plasma 4-HNE was used as a systemic oxidative stress marker (Figure 1). Plasma level of 4-HNE showed significantly higher in the DMC group than that of the CON group. However, GT supplementation lowered plasma 4-HNE levels in DM mice.

Effect of GT supplementation on wound closure rate during cutaneous wound healing in alloxan-induced diabetic mice

Three days (D3) after wounding, the wound closure rate in the CON group was significantly slower than that in the DMC group, and this tendency lasted the end of wound closure (D13: END). The wound closure rate of the GT group was faster significantly since day 4 (D4) compared to that of the DMC group, until the endpoint (D13: END) of wound closure. Wound closure rates and representative wound images were presented in Figure 2(a) and (b), respectively.

Effect of GT supplementation on inflammatory markers in diabetic cutaneous wounds

The protein levels of inflammatory markers including NFκB, IL-1β, TNF-α, and CRP were measured by western blots in the wounded skin. We examined whether GT supplementation reduced the protein levels of inflammatory markers in hyperglycemia-induced diabetic condition. As shown in Figure 3(a), the protein level of nuclear NFκB was significantly higher in the DMC group than that of the CON group at baseline and 72 h after wounding. However, the level of NFκB in the GT group was significantly lower than that of the DMC group at 24 and 72 h after wounding. The protein levels of IL-1β and TNF-α, target genes of NFκB activation, in the DMC group were significantly higher compared to that of the CON group at 24 and 72 h after wounding (Figure 3(b) and (c)). However, the protein levels of IL-1β and TNF-α in the GT group, regardless of FBG levels, were lower in contrast to the DMC group. The protein level CRP in the DMC group was significantly higher compared to that of the CON group at 24 and 72 h. However, the GT group did not show a difference in the protein level of CRP compared to the DMC group (Figure 3(d)).

Effect of GT supplementation on protein levels of SIRT-1 and apoptosis-related markers in diabetic cutaneous wounds

The protein level of SIRT-1 in the DMC group was significantly lower than that of the CON group at all time points. The GT group showed a significantly higher level of SIRT-1 than that of the DMC group at baseline and 24 h (Figure 4(a)). The protein levels of nuclear PGC-1α (Figure 4(b)) and p53 (Figure 4(c)) were significantly lower in the DMC group compared to that of the CON group except the level of nuclear p53 at 24 h. However, the GT group showed significantly higher levels of nuclear PGC-1α and p53 than the DMC group at all the time points.

Effect of GT supplementation on oxidative stress-related markers in diabetic cutaneous wounds

The level of 4-HNE in cutaneous wound was used as a marker for lipid peroxidation (Figure 5(a)). The protein level of 4-HNE was significantly higher in the DMC

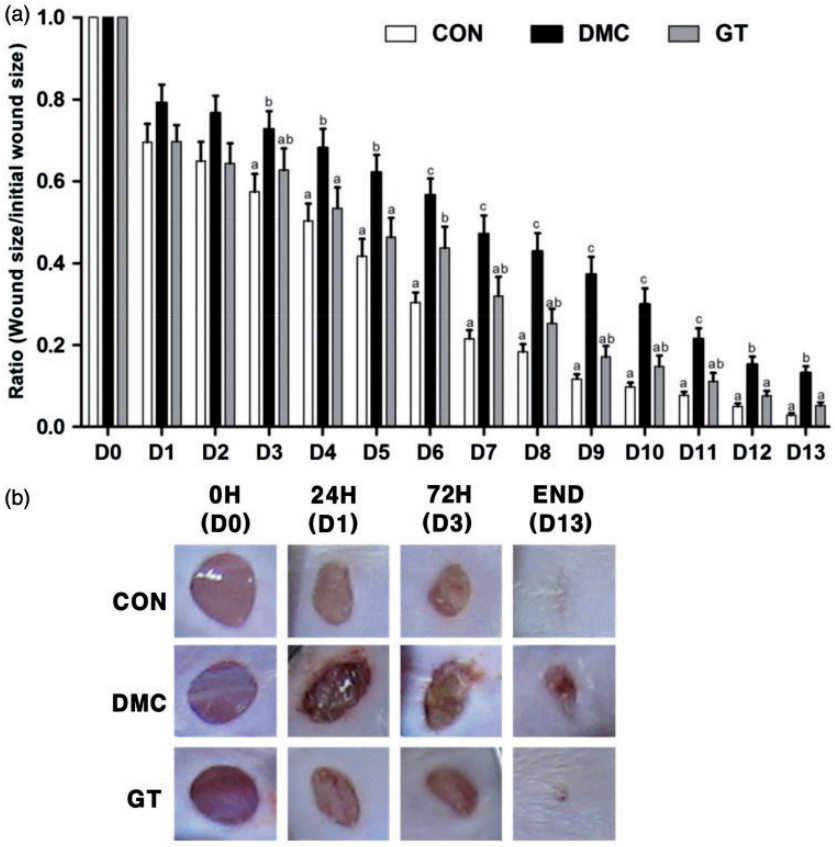

Figure 2 Effects of GT supplementation on wound closure rate (a) and representative wound images (b) during cutaneous wound healing in alloxan-induced diabetic mice. Representative wound (b) on 0 h (D0), 24 h (D1), 72 h (D3), and end (D13) after wounding. Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$). GT: gamma-tocopherol. (A color version of this figure is available in the online journal.)

group than that of the CON group at 24 and 72 h. In addition, the GT group showed significantly lower 4-HNE level in diabetic cutaneous wounds compared to DMC at 72 h. However, there were no significant differences in 4-HNE level in diabetic cutaneous wounds among all groups at baseline.

The protein levels of oxidative stress markers including nuclear Nrf2, cytosolic NQO1, HO-1, GPx, MnSOD, and catalase were significantly higher in the DMC group at 24 h compared to the CON group. The protein level of nuclear Nrf2 was significantly higher in the DMC group compared to that of the CON group at all time points. The protein level of the nuclear Nrf2 in the GT group was significantly lower compared to that of the DMC group at 24 and 72 h (Figure 5(b)). The protein levels of HO-1 and NQO1, representative target genes in Nrf2 pathway, were significantly higher in the DMC group compared to those of the CON group at 24 and 72 h. The GT group showed significantly lower protein levels of HO-1 and NQO1 compared to those of the DMC group (Figure 5(c) and (d)). Moreover, the protein level of NQO1 in the GT group at 72 h was significantly lower than that of the DMC group. The protein levels of GPx, MnSOD, and catalase at 24 and 72 h in the DMC group were significantly higher than those of the CON group (Figure 5(e) to (g)). Protein levels of GPx and MnSOD at 24 h showed significantly lower in the GT group compared to the DMC group (Figure 5(e) and (f)). However, the level of catalase in the GT group at 24 h was not significantly different from that of the DMC group (Figure 5(g)).

Discussion

The inflammatory stage is the most important step in the entire wound healing process because the proper healing process requires recruitment of inflammatory cells and secretion of various mediators in the early inflammatory stage.[34]

In the current study, we investigated that GT supplementation ameliorated delayed wound healing through improvement of inflammation, oxidative stress, and apoptosis, in particular, in the early inflammatory stage of cutaneous wound healing in alloxan-induced diabetic mice. GT is a more potent anti-inflammatory substance compared to AT.[35] Although GT has a beneficial effect on both oxidative stress and inflammation,[26] there is no obvious fundamental mechanism, in which GT improves protective roles in diabetic delayed wound healing. Our results demonstrated that GT supplementation down-regulated hyperglycemia, attenuated lipid peroxidation, and accelerated cutaneous wound healing in diabetes. By extension, our data confirmed that GT supplementation is effective in attenuation of hyper-inflammation, oxidative stress, and apoptosis

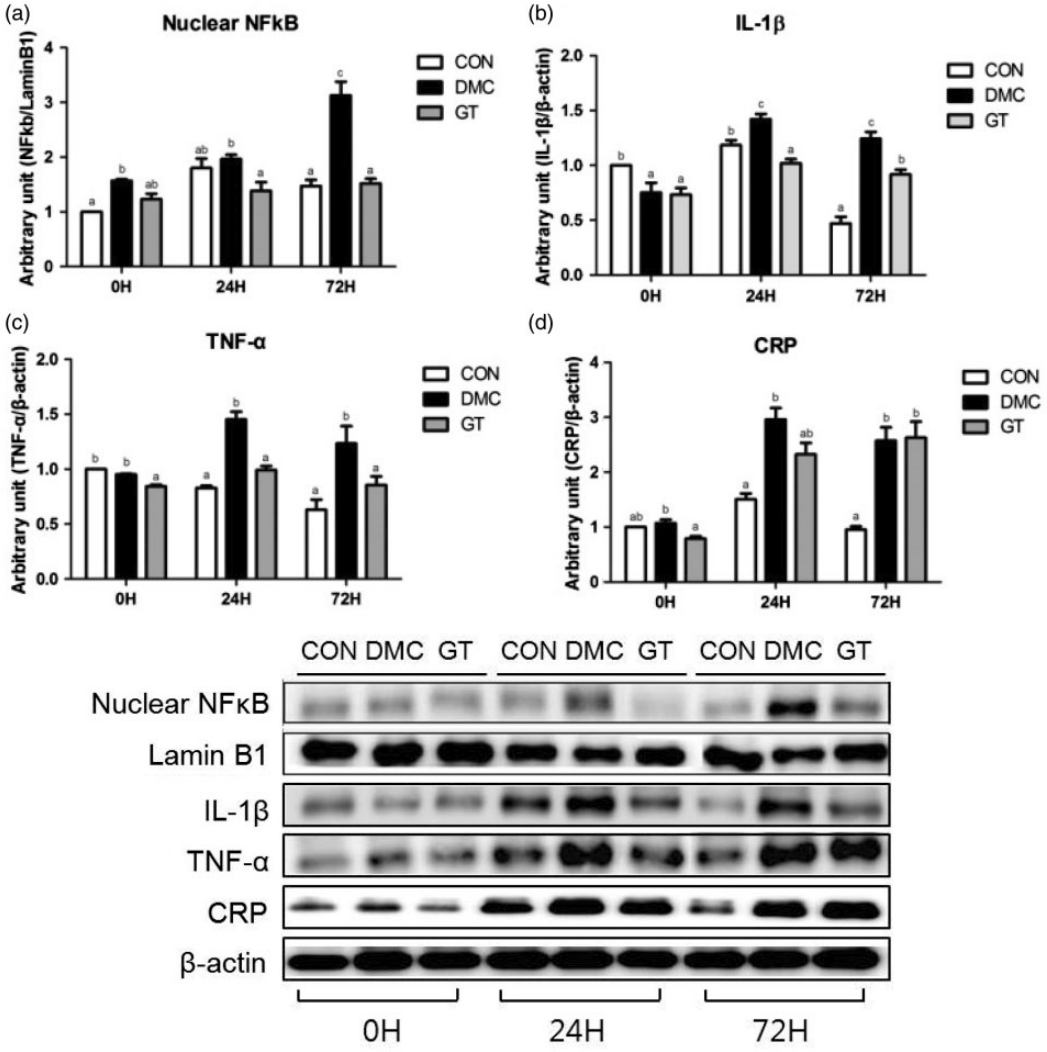

Figure 3 Effect of GT supplementation on cutaneous protein levels of inflammatory markers: nuclear NFκB (a), IL-1β (b), TNF-α (c), CRP (d) in alloxan-induce diabetic mice. The cutaneous protein measured by western blot. There were pooled from five mice per group. A representative image is shown in the below panel. The bands were normalized to the band levels of β-actin (cytosol) or Lamin B1 (nucleus). Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$). CRP: C-reactive protein; GT: gamma-tocopherol; IL-1β: interleukin-1 beta; NFκB: nuclear factor kappa B; TNF-α: tumor necrosis factor alpha

demonstrated by NFκB, Nrf2, and SIRT-1 during the early stage of cutaneous wound healing in DM.

The present study found that GT supplementation has the ability to reduce FBG without insulin treatment in DM mice. Our previous study reported that GT ameliorated FBG level in diabetic nephropathy.[36] Therefore, GT has a potential in attenuating hyperglycemia which is a common symptom in DM with reduction of hepatic damage demonstrated by GOT level.

A previous study showed increases in oxidative stress demonstrated by lipid peroxidation in DM. Hyperglycemia can induce production of prostaglandin E(2), which plays a key role in inflammatory diseases such as neurodegenerative and CVDs.[31,37] Oxidative stress is the basis of the pathogenesis of delayed wound healing in DM. We measured lipid peroxidation demonstrated by 4-HNE, a valid biomarker of oxidative stress in blood and tissues.[38,39] Our data showed that GT supplementation significantly attenuated lipid peroxidation. The results suggest that GT suppresses hyperglycemia-induced overproduction of ROS induced by DM. During the early inflammatory stage of wound healing, innate immune cells including neutrophils and macrophages produce large amount of ROS, which are of necessity to protect the organism against invading bacteria.[40] If ROS are produced in excessive amounts or the detoxification of ROS is insufficient, oxidative stress occurs. Nrf2, a redox-sensitive transcription factor, modulates its downstream antioxidant defense enzymes such as SOD, HO-1, NQO1, catalase, and GPx.[20,21] HO-1 and superoxide production are increased in the cell response against oxidative damage.[21,41]

Moreover, the present study demonstrated that GT supplementation, regardless of FBG levels, lowered the expression of NQO1 levels, which is a representative target gene in Nrf2 pathway, compared to the DMC group. NQO1 expression is induced by exposure to radiation and internal stress such as a streptozotocin-induced diabetic mice and cultured human mesangial cells.[21] External and internal

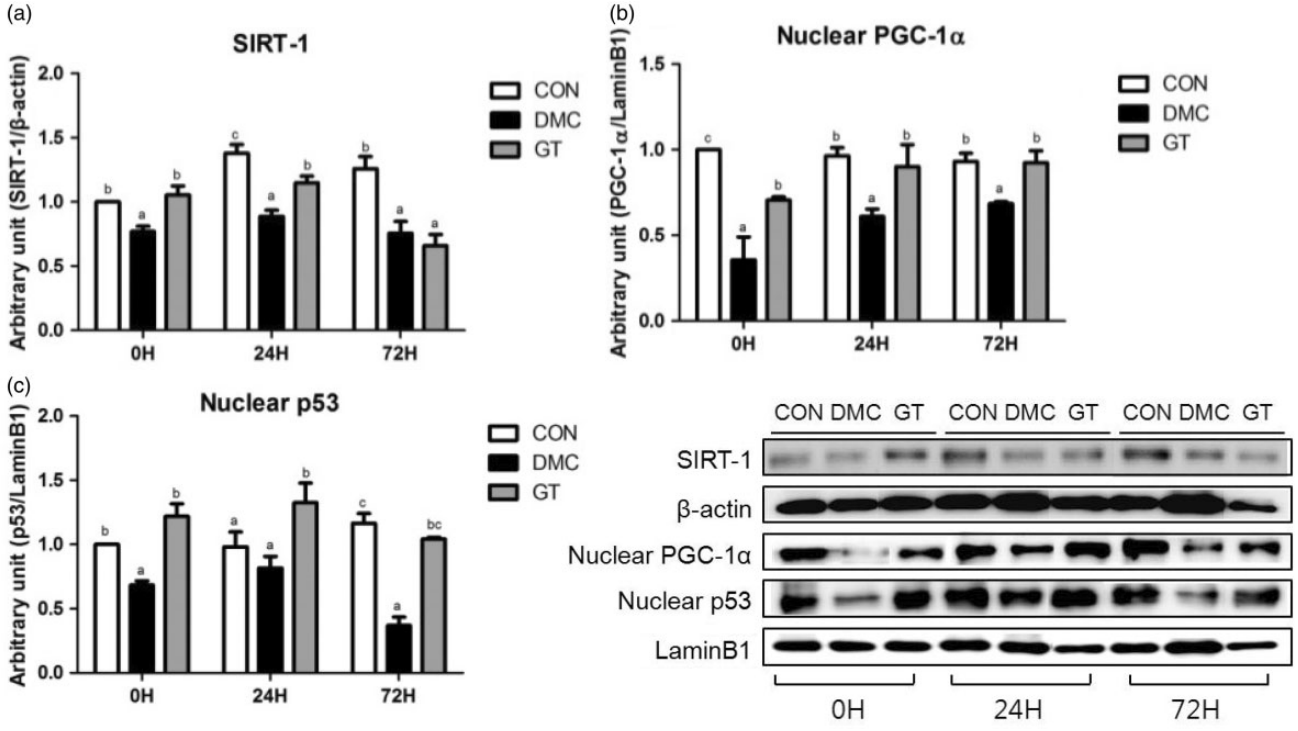

Figure 4 Effect of GT supplementation on cutaneous protein levels of SIRT-1 and apoptosis-related markers: SIRT-1 (a), nuclear PGC1-α (b), and nuclear p53 (c) in alloxan-induced diabetes mice. The cutaneous protein measured by Western blot. There were pooled from five mice per group. A representative image is shown in the below panel. The bands were normalized to the band levels of β-actin (cytosol) or Lamin B1 (nucleus). Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$). GT: gamma-tocopherol; PGC1-α: proliferator-activated receptor gamma coactivator 1-alpha; SIRT-1: sirtuin-1

stimuli such as chemicals and stresses induce NQO1, which is mediated solely through the Keap1/Nrf2/ARE.[20,21] Nrf2 is consequently stabilized and accumulated in the nucleus upon which it binds to the AREs and initiates expression of cytoprotective genes including NQO1.[42] Our result suggests that amelioration of NQO1 by GT supplementation can be a beneficial mechanism for Nrf2 downstream oxidative-related enzymes in diabetic wounds.

The protein levels of GPx and catalase were significantly increased in the DMC group compared to the CON group. Both GPx and catalase involve in the conversion of H_2O_2 into H_2O and O_2. GPx and catalase levels have known to be associated with an increase in oxidative stress in DM.[20,21] Collectively, GT is considered as a useful substance which alleviates oxidative stress in DM. At molecular level, our data demonstrated the protein levels of oxidative stress markers including nuclear Nrf2 and cytosolic HO-1, NQO1, GPx, catalase, and MnSOD in cutaneous wounds were higher in the DMC group compared to those in the CON group. Moreover, plasma 4-HNE level in the DMC group was higher than that in the CON group. The results suggest that diabetic mice suffer from excessive oxidative stress even in cutaneous tissue. Moreover, GT shows a potential benefit in attenuating oxidative stress which leads to progression of chronic inflammation in DM. The results are similar to other studies which showed that palm vitamin E or alpha-tocopherol increased antioxidant enzyme activities and enhanced wound repair in diabetic mice although they were used at relatively high dose (200 mg/kg).[43,44]

Another major target of ROS is the inflammatory-mediated transcription factor called NFκB.[45–47] During the inflammatory stage, wounded tissue secretes growth factors and cytokines for initiation of healing process. Neutrophils phagocytize cell debris and bacteria and gradually replace monocytes with macrophages.[17] Macrophages phagocytize microorganisms, fibrin, neutrophils, and fragments of the extracellular matrix and secrete biomolecules for effective wound repair.[21] The released molecules by macrophages proceed to the next stages of the wound in order to provide the basis for formation of the extracellular matrix.[18] In our result, delayed wound closure started on D3 and continued by D13 (endpoint) in diabetic mice. These results reflect the delayed wound repair in diabetes. NFκB is composed of a family of inducible transcription factors that serve as essential regulators of the host immune and inflammatory response.[48] The facilitation of wound healing by the antioxidant supplement is likely achieved by the inactivation of NFκB and a reduction in TNF-α expression levels.[49–51] We observed a similar effect of GT on down-regulation of inflammatory mediators (NFκB, TNF-α, etc.) by reducing ROS.[52] Previous studies showed that the protein level of NFκB was higher in DM than that of non-diabetics.[53] Moreover, inhibition of NFκB activation during resolution of inflammation prevented apoptosis in inflammatory diseases.[54] Induction of CRP is caused by a variety

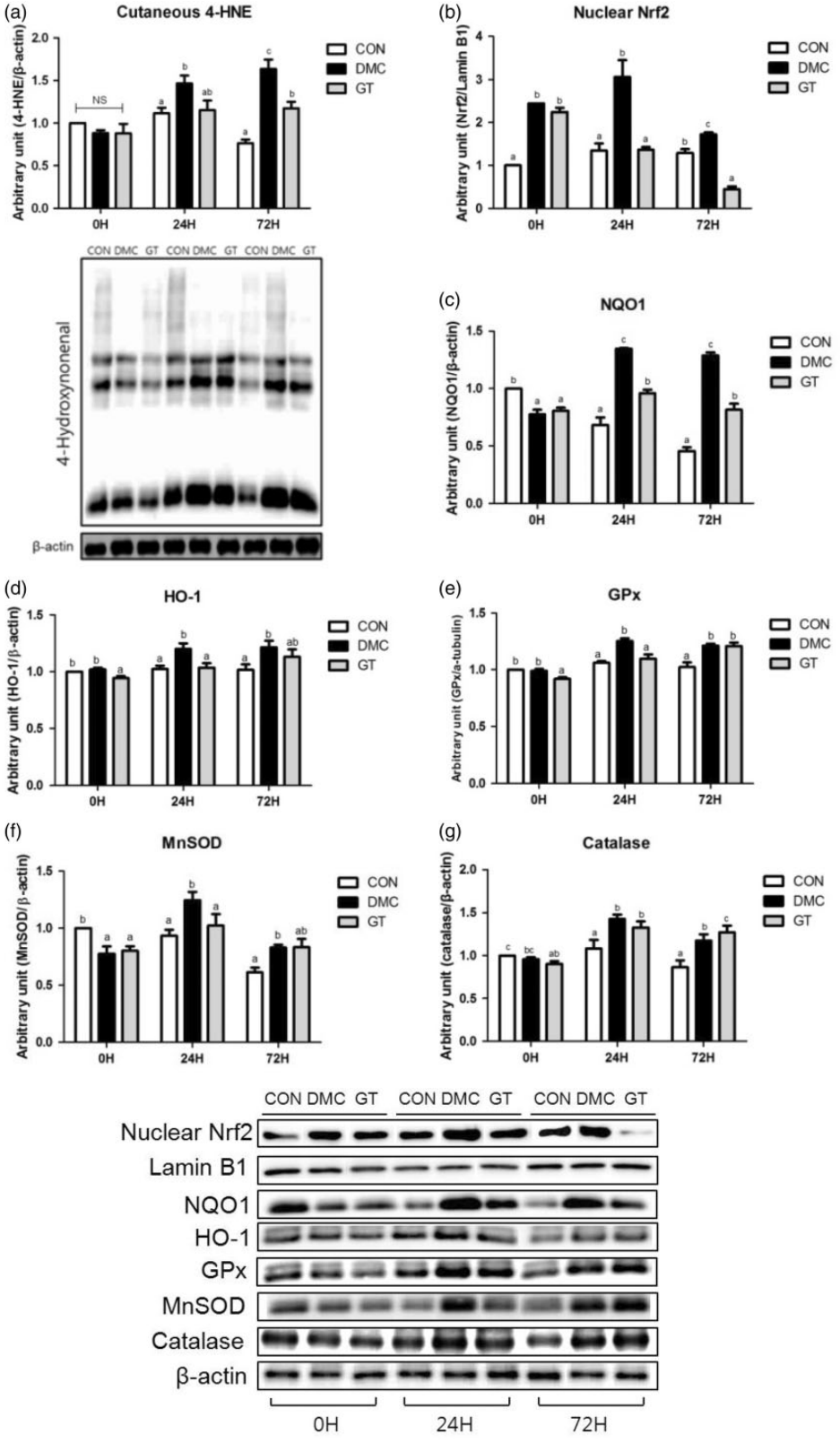

Figure 5 Effect of GT supplementation on cutaneous protein levels of 4-hydroxynonenal (4-HNE) (a) and oxidative stress-related markers: Nrf2 (b) and NQO1 (c), HO-1 (d), GPx (e), MnSOD (f), and catalase (g) in alloxan-induced diabetes mice. The cutaneous protein measured by western blot. There were pooled from five mice per group. A representative image is shown in the below panel. The bands were normalized to the band levels of β-actin (cytosol) or Lamin B1 (nucleus). Values are means ± SEM (n = 6). Mean values with different letters were significantly different ($P < 0.05$). GPx: glutathione peroxidase; GT: gamma-tocopherol; HO-1: heme oxygenase-1; MnSOD: manganese superoxide dismutase; NQO1: NAD(P)H dehydrogenase quinone 1; Nrf2: nuclear factor (erythroid derived 2)-like 2

of pro-inflammatory cytokines such as IL-1β and IL-6.[55] An increase in CRP has been found in pathological conditions such as diabetes and its complications.[56] Similarly, our data showed an increase in CRP in the DMC group along with an increase in IL-1β. In a prior study, AT (50 and 100 μmol/L) significantly decreased productions of IL-1 and IL-6 released from human monocytes.[28] This result showed that pro-inflammatory cytokines such as TNF-α and IL-1β were increased during early wound healing process in diabetes. The increases in pro-inflammatory cytokines including IL-1β and TNF-α activate NFκB which stimulates gene expressions of TNF-α and IL-1β through amplification loop.[5,8] In the current study, GT supplementation attenuated the levels of IL-1β and TNF-α as well as NFκB in the DMC group, thereby moderating the consequence of inflammation-mediated diabetic cutaneous damage.

SIRT-1 associated with improving insulin sensitivity inhibits NFκB transcription and sensitizes cells to TNF-α-induced apoptosis.[57,58] A previous study reported an increase in NFκB activity along with a decrease in SIRT-1 activity in the diabetic heart.[59] Another study showed that NFκB activation was significantly elevated in diabetic liver.[10] Some studies suggest that an increase in SIRT-1 has a therapeutic benefit under condition of excessive ROS production by amelioration of oxidative stress in cell types and organs.[60,61] A previous study suggests an increased activity of NFκB along with decreased SIRT-1 activity in the diabetic heart.[59] SIRT-1 regulates various processes that alter cell response to genotoxicity including the detoxification of ROS by up-regulation of sensitivity of cells to apoptosis.[62] The current study showed that the GT supplementation decreased the protein level of SIRT-1 in the DMC group. The result suggests that GT supplementation ameliorates DM-induced hyper-inflammatory response with suppression of NFκB activation by SIRT-1.

Furthermore, SIRT-1 also interacts with p53 which has effects of antiapoptosis and protection from DNA damage.[17,63] Upon DNA damage or other stresses, p53 induces a cell cycle arrest to allow cell repair and survival or apoptosis to discard the damaged cell.[16] The present study showed the protein level of p53 is lower in the DMC group contrary to that in the CON group. GT supplementation increased the levels of p53 in the DMC group. Therefore, the GT group could discard the damaged cells and repair the cells after wounding. Moreover, SIRT-1 modulates mitochondrial function and metabolic homoeostasis through PGC-1α regulation.[64] PGC-1α, markedly upregulates GLUT4 (glucose transporter 4) expression and glucose transport activity. The effect of PGC-1α on gene expression of GLUT4 reflected the increased ability of myocytes to transport glucose, suggesting that the SIRT-1-regulated activation of PGC-1α influences insulin sensitization.[65]

This study showed that the increase in SIRT-1 induced nuclear PGC-1α and modulated p53 by GT supplementation in diabetic cutaneous wounds. Therefore, GT supplementation has beneficial effects on SIRT-1-related apoptosis during cutaneous wound healing in DM. Furthermore, we suggest that activated SIRT-1 by GT supplementation leads to suppression of NFκB and consequently, down-regulates expression of inflammatory genes including TNFα, IL-1β, and CRP. Collectively, the results of this study suggest that GT supplementation ameliorated the early inflammatory response during cutaneous wound healing in diabetes. Taken together, regulation of inflammatory response demonstrated by NFκB- and SIRT-1-associated molecular mechanism would be important in amelioration of DM-induced delayed wound healing and GT has beneficial effects on attenuating DM-induced delayed wound healing. However, chemical-induced diabetic models such as alloxan or streptozotocin-induced model showed quiet different mechanism to develop diabetes compared to genetic models or diet-induced models. In spite of the differences between type 1 and type 2 diabetes, diabetic complications such as delayed wound healing and kidney damage were caused by hyperglycemia which involves in oxidative stress and hyper-inflammation. Our laboratory has shown many similarities in inflammatory response and oxidative stress with tissue-specific manner between high fat diet-induced model and a chemical-induced model in previous studies.[66,67] However, we need further investigations to identify similarities and differences in different diabetic models.

Conclusions

The present study demonstrates that GT supplementation has beneficial effects on delayed wound healing in diabetes. Moreover, this study suggests that GT supplementation ameliorates the early inflammatory response during cutaneous wound healing by attenuating oxidative stress, hyper-inflammation, and apoptosis during the early inflammatory stage of cutaneous wound healing in DM mice. Consequently, this study concludes that GT could be a potential nutraceutical, which may be beneficial in patients with diabetes and its complications, in particular, diabetic delayed wound healing.

Authors' contribution: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; JS performed the experiments, analyzed the data, and wrote the manuscript; SJY revised the manuscript; YL designed the study, analyzed the data, and wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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