Evaluation of wound healing effects of ginsenoside Rg1 and red ginseng extract in STZ-induced diabetic wound model: an in vivo pilot study

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Authors' contributions

Ji Yun Lim is the first author, performed the experiments, and was responsible for the overall writing and revision of the manuscript. Young Suk Choi, Hye Rim Lee, and Hye Min An are co-authors, analyzed the result data and performed parts of the experiments. Young Koo Lee is a corresponding author, played a role in interpreting the results, and gave final approval.

Abstract

Red ginseng is an immune-enhancing compound that exhibits anti-inflammatory action. The ginsenoside Rg1, an ingredient of red ginseng, has been shown to play an important role in tumor suppression, wound healing, and angiogenesis. This study evaluated the effects of red ginseng extract and Rg1 in a diabetic wound model. Diabetes was induced with streptozotocin (STZ) in 8-week-old male Institute of Cancer Research (ICR) mice weighing 30–35 g. A full-thickness skin defect was treated by applying a dressing every 3 days. The mice were divided into three groups. Group 1 was administered an extract of red ginseng (10 mg/kg/d, $n = 27$, oral) and group 2 was administered Rg1 (10 mg/kg/d, $n = 27$, oral). Group 3 was a control group treated with phosphate-buffered saline (0.3 mL/kg/d, $n = 27$, oral). Red ginseng extract and Rg1 were orally administered to mice daily for 10 days following injury in groups 1 and 2, respectively. Both increased mRNA and protein levels of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 compared to controls. In addition, the wounds of animals in the Rg1 group were significantly smaller between days 7 and 10 ($p < 0.05$). VEGF and TGF-β1 were not expressed in diabetic mice in the control group. Both red ginseng extract and Rg1 promoted the production of VEGF and TGF-β1, which are important in wound healing. Our results for Rg1 suggest its potential to promote diabetic wound healing by stimulating the production or activity of VEGF and TGF- β1 factors involved in the wound healing process.

Keywords

Ginsenoside Rg1, Red ginseng extract, Growth factor, Diabetic wound, Diabetes

Introduction

By the year 2035, it is expected that 592 million people will suffer from diabetes, making it one of the most important medical issues globally [1]. Diabetes is divided into two types: type 1 diabetes (T1D) is characterized by impaired pancreatic β-cell function resulting in insulin deficiency and chronic hyperglycemia while type 2 diabetes (T2D) is characterized by insulin resistance and hyperglycemia [2]. Chronic hyperglycemia causes deterioration of blood vessels and nerves, resulting in cardiovascular diseases and neuropathy [3, 4]. Additional complications, such as diabetic wounds, impair the four stages of wound healing (e.g., hemostasis, inflammation, proliferation and remodeling), delaying the healing process [5]. As a result, chronic wounds in diabetic patients damage wounds and surrounding tissues due to excessive inflammatory reactions, and delay wound healing through delayed expression of growth factors. In serious cases, this results in loss of the affected limb or death [6, 7]. However, a detailed understanding of the delayed healing of diabetic wounds is lacking and current forms of treatment such as oral antidiabetics and insulin injection, have many unwanted side effects, such as hypertension, weight gain, and endothelial cell damage [8, 9]. This has stimulated the search for safe and effective treatments. *Panax ginseng* is one of the oldest traditional herbs and it contains approximately 32 species of ginsenosides [10]. In recent years, ginsenosides have been demonstrated to exhibit many properties, including immune-stimulating activity and anticancer, anti-inflammatory, antiallergic, antihypertensive, and antidiabetic effects [11]. Ginsenosides with antidiabetic effects include Rg1, Rg3, Rg5, Rb1, Rb2, and Rb3, and ginsenosides have been used as an adjuvant for the treatment of diabetes because they exhibit antidiabetic effects [12]. Of the many types of saponins, the ginsenoside Rg1 reduces intestinal glucose uptake by inhibiting the expression of the Na^+/glucose cotransporter 1 (SGLT1) gene

103 responsible for glucose uptake in intestinal epithelial cells [13]. Rg1 has been proposed to
104 reduce oxidative stress and cardiomyocyte death and prevent cardiovascular damage in diabetic
105 mice [14]. Rg1 is also excellent for skin regeneration [15]. Representative factors involved in
106 wound healing include transforming growth factor (TGF)-β, fibroblast growth factor (FGF),
107 and vascular endothelial growth factor (VEGF). VEGF plays an important role in wound
108 healing, and participates in endothelial migration, proliferation, and granulation tissue and
109 blood vessel formation. TGF-β promotes wound healing, fibroblast proliferation, and
110 expression of major components of the extracellular matrix such as fibronectin, collagen I and
111 III, and VEGF [16]. Although wounds heal as the expression of these growth factors gradually
112 increases, the roles of these factors throughout the healing process have not been fully clarified
113 [17]. The use of Rg1 in wound healing and angiogenesis is being actively studied [18,19],
114 although neither its effect nor that of red ginseng on diabetic wounds is known. Therefore, the
115 purpose of this pilot study was to evaluate the effects of red ginseng extract and Rg1 in a
116 streptozotocin (STZ)-induced type 1 diabetic mouse wound model. The main hypothesis of this
117 pilot study was that the expression of VEGF and TGF-β1 in animal models treated with red
118 ginseng extract and Rg1 is important in wound healing.

119

120 **Materials and methods**

121 *Animal model*

122 Male Institute of Cancer Research (ICR) mice aged 6 to 8 weeks (30–35 g) were purchased
123 from Orient BIO Inc. (Seongnam, Korea). All mice were allowed to adjust to the environment
124 for 1 week prior to the experiment. The mice were housed in cages with a 12 h/12 h light/dark
125 cycle at 23 ± 2°C and 50 ± 20% humidity with freely available water and rodent chow (LabDiet

5L79®; Orient BIO Inc.). The experimental design was approved by the Institutional Animal Care and Use Committee of Soonchunhyang University Medical School (SCHBC-Animal-2016–03).

Diabetic wound model using STZ

Diabetes was induced by intraperitoneal (IP) injection of 100 mg/kg STZ (S0130; Sigma-Aldrich, St. Louis, MO, USA**)** in 0.1 M citrate buffer (pH 4.5) to 81 mice following a 6 h fast. After 24 h, blood was collected from the tail vein and blood glucose levels were measured using the Accu-Chek® system (Roche Diagnostics, Mannheim, Germany). Diabetes was defined as a blood glucose level greater than 200 mg/dL after STZ injection. The mice were anesthetized by IP injection of zolazepam/tiletamine (Zoletil 50®; Virbac, Carros, France) and xylazine (Rompun®; Bayer Korea, Seoul, Korea). The hairs on the back of the mice were shaved, the exposed skin was cleansed with 70% ethanol, and a full-thickness skin defect wound of 8 mm in diameter was made using a sterile skin biopsy punch (BP-80F; Kai Industries, Gifu, Japan). The methicillin-resistant *Staphylococcus aureus* (MRSA) standard strain (ATCC®; 43300 MINIPACK™, Manassas, VA, USA) was incubated overnight at 37°C on Mueller – Hinton agar plates. MRSA was diluted in sterilized saline to adjust to McFarland 0.5 standard turbidity and applied to the skin defects. Vaseline (Samhyun Pharmaceutical, Seoul, Korea) was applied to all wounds using a gauze pad to prevent the wound from drying. Then the wounds were covered with Opsite-film (Opsite Flexifix®; Smith & Nephew Medical Ltd., Hull, UK) dressings. The dressings were replaced every 3 days.

Experimental design for red ginseng extract and Rg1 treatment

149 STZ-induced diabetic mice were orally administered 10 mg/kg of red ginseng extract or Rg1
150 once daily for 10 days after induction of injury. Concentration and date were selected according
151 to Yu *et al.* [20]. Red ginseng extract (Korean Red Ginseng Extract Gold®; The Korean
152 Ginseng Research Institute, Daejeon, Korea) and the ginsenoside Rg1 (purity > 98%; 22427–
153 39–0; Abcam, Cambridge, UK) were dissolved in phosphate-buffered saline (PBS). The STZ-
154 induced diabetic mice (n = 81) were randomly divided into three different treatment groups as
155 follows: PBS (0.3 mL/kg/d, n = 27, oral), red ginseng extract (10 mg/kg/d, n = 27, oral), and
156 Rg1 (10 mg/kg/d, n = 27, oral). Treatments were administered orally once daily for 10 days
157 following induction of injury (Figure 1).
158
159 *Measurement of wound area*
160 Images of the skin wound were obtained on days 1, 4, 7, and 10 and the wound size was
161 measured using the UTHSCSA image tool (version 3.0; Microsoft Corporation, Redmond, WA,
162 USA). The wound size on the first day after wound induction was defined as 100% and wound
163 sizes measured on days 4, 7, and 10 are expressed as a percentage of wound size at day 1.
164
165 *Histological analyses of diabetic wound tissue*
166 Wound tissues were compared in the three groups by euthanizing the mice at days 1, 4, 7, and
167 10 after injury. The skin tissues were harvested to include the entire wound, fixed for 12 h in
168 4% paraformaldehyde prepared in PBS (pH 7.4; Santa Cruz Biotechnology, Dallas, TX, USA),
169 washed with PBS at room temperature for 6 h, and processed overnight in an automatic tissue
170 processor. Sections of 4 μm thickness were cut, placed on coated glass slides (5116–20F; Muto
171 Pure Chemicals Co., Ltd., Tokyo, Japan), and deparaffinized before staining with hematoxylin

and eosin (H&E; Gills hematoxylin, 1% eosin; Muto Pure Chemicals Co., Ltd.). Three pathologists blinded to the three groups scored the tissues according to Tables 1 and 2 based on light microscopy (DXM1200; Nikon, Tokyo, Japan) images of the sections at 200× magnification. These images were assessed for granulation tissue area, inflammatory cells, connective tissue, and damage to the dermal layer.

Expression of VEGF and TGF-β1 in diabetic wound tissue by immunohistochemistry

The paraffin-embedded wound tissue blocks were sectioned, placed on slides, and incubated at 60–70°C for 30 min. The sections were deparaffinized in a graded series of xylene and ethanol (100%, 95%, 80%, and 70%) and treated with an endogenous peroxidase blocking agent, which then was diluted with methanol (99%) and a 1.4% hydrogen peroxide solution (FW = 34.01). After 30 min, the slides were washed with 1× Tris-buffered saline (TBS) and sections were blocked with 1.5% normal serum (PK-6102, horse serum, mouse IgG or PK-6101, goat serum rabbit IgG; Vector Laboratories, Burlingame, CA, USA) for 1 h followed by incubation overnight at 4°C with anti-VEGF monoclonal antibody (SC-7269, 1:500 dilution; Santa Cruz Biotechnology) and anti-TGF-β1 polyclonal antibody (ab92486, 1:1000 dilution; Abcam) in blocking buffer. Then the samples were washed with 1× TBS followed by incubation for 1 h at room temperature with a biotinylated secondary antibody (PK-6102, horse serum, mouse IgG or PK-6101, goat serum rabbit IgG; 1:3000 dilution; Vector Laboratories) in blocking buffer, and washed again with 1× TBS. Incubation with the VECTASTAIN Elite avidin-biotin complex (PK-6102 or PK-6101; Vectastain®; Vector Laboratories) was used to increase the target antigen-antibody reaction during a 30 min incubation. Then the sections were stained with the chromogen diaminobenzidine (C02-100; Liquid DAB + Chromogen Kit; Golden

Bridge International Inc., Mukilteo, WA, USA) for 5–6 min followed by hematoxylin for 1 min before they were covered with a coverslip and evaluated using a digital microscope (DXM1200; Nikon) at 200× magnification.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated from wounded skin tissue samples using TRIzol Reagent® (TR118; Molecular Research Center, Cincinnati, OH, USA). mRNA was isolated using chloroform and isopropanol (C2432, I9516; Sigma-Aldrich). After precipitation with isopropanol, the RNA pellet was dissolved in distilled water treated with diethyl pyrocarbonate (DEPC; WR2004; Biosesang, Sungnam, Korea). Total RNA was quantified to 3 μg to react 1 μg/μL random hexamer (C1181; Promega, Madison, WI, USA) at 70°C for 5 min, then incubated at 4°C for 5 min. Each reaction contained 1 μL of 10 mM dNTPs, 1 μmol/L $MgCl_2$, 1 μg/μL RNaseOUT, 1 μg/μL 5×reaction buffer, 1 μL GoScript reverse transcriptase (A5003; Promega), and 7 μL RNase-free double-distilled H2O (N2511; Promega) and was incubated at 25°C for 5 min, followed by incubation at 42°C for 1 h and 70°C for 15 min. The VEGF, TGF-β1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer gene sequences were as follows: VEGF forward primer, 5′- CAG GCT GCT GTA ACG ATG AA -3′; VEGF reverse primer, 5′- AAT GCT TTC TCC GCT CTG A -3′; TGF-β1 forward primer, 5′- ATT CAG CGC TCA CTG CTC TT-3′; TGF-β1 reverse primer, 5′- TTC TCT GTG GAG CTG AAG CA-3′; and GAPDH forward primer, 5′- CCT TAA ACA GGC CCA CTT GA-3′; GAPDH reverse primer, 5′- CCT TCC ACA ATG CCA AAG TT-3′ (Table 1). All primers were ordered from

Macrogen Oligo (Seoul, Korea). PCR was performed using 1 μL cDNA in a mixture with 2× PCR premix (LGT-1212; Lugen Science Co., Seoul, Korea). The conditions for VEGF included initial denaturation at 95°C for 5 min, melting at 95°C for 30 s, annealing at 60°C for 1 min, and elongation at 72°C for 30 s. For TGF-β1 and GAPDH, annealing was performed at 58°C for 30 s. PCR products were visualized using a bioimaging system (C280; Azure Biosystems, Dublin, CA, USA) after electrophoresis on a 1% agarose gel containing Safe Shine Green DNA Staining Solution (GC6051; Biosesang). GAPDH was selected for normalization of specific primer target bands, and density measurements of the target bands were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

The skin tissue was homogenized using complete EDTA free protease inhibitor cocktail (11836170001; Roche Diagnostics GmbH), phosSTOP phosphatase inhibitor (30498800; Roche Diagnostics GmbH), and RIPA buffer (R4200; GenDEPOT, Barker, TX, USA). Dissolved protein was analyzed for protein concentration by micro-scale BCA assay (Micro BCA Protein Assay Kit #23225; Thermo Fisher Scientific Inc., Waltham, MA, USA). Lysates measured at 50 μg were separated using 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (10600023; GE Healthcare Life Sciences, Chalfont, UK). After blocking with 5% skim milk (232100; Becton Dickinson, Franklin Lakes, NJ, USA) at 4°C for 24 h, the membranes were incubated with anti-VEGF monoclonal antibody (SC-7269; 1:500 dilution; Santa Cruz Biotechnology), anti-TGF-β1 polyclonal antibody (ab92486; 1:1000 dilution; Abcam), and β-actin rabbit antibody (4970S; 1:5000 dilution; Cell Signaling, Beverly, MA, USA) at room

239 temperature for 2 h followed by the appropriate secondary antibody (SC-2005; 1:3000 dilution;
240 goat anti-mouse or SC-2030; 1:3000 dilution; goat anti-rabbit; Santa Cruz Biotechnology) for
241 1 h. Bands of the target size were visualized by enhanced chemiluminescence (ECL 2232; GE
242 Healthcare Life Sciences) and a bioimaging system (C280; Azure Biosystems). The band
243 intensities were normalized with β-actin and the density measurements were analyzed with
244 ImageJ software (National Institutes of Health).
245
246 *Statistical analysis*
247 The data were analyzed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the Mann–
248 Whitney U test. All *p*-values less than 0.05 were considered statistically significant.
249
250 **Results**
251 *Wound size measurement*
252 In each group, the wound size decreased over time (Figure 2A). On day 4, there was larger
253 decrease in wound size in the two treatment groups compared to the control group. From days
254 7 to 10, wound size was significantly decreased in the Rg1 group ($p < 0.05$). These results
255 showed that wound contraction was the highest and wound healing was the fastest in the Rg1
256 group (Figure 2B).
257
258 *Histology and immunohistochemistry analyses*
259 The central portion of the wounded tissues was collected to evaluate granulation tissue
260 formation, inflammatory cell infiltration, and growth factor expression. The wound healing

effects of the red ginseng extract and Rg1 groups were evaluated histologically. Samples of all groups exhibited histological patterns of dermal tissue (consisting of three layers of epidermis, dermis, and subcutaneous tissue). H&E and immunohistochemistry (IHC) staining were performed (Figure 3A–C). The degree of granulation and inflammatory cell infiltration was not different in the three groups at day 4. However, at day 10, granulation tissue was formed in the Rg1 group whereas the control group remained unchanged ($p < 0.05$). On the other hand, inflammatory cell infiltration in the Rg1 group was significantly lower at day 10 compared to the other two groups ($p < 0.05$) (Figure 3D, E). Expression of VEGF and TGF-β1 was confirmed by IHC staining; VEGF was mainly present in granulation tissues and TGF-β1 was observed on the wound surface.

Expression of VEGF and TGF-β1 by RT-PCR

We investigated the effects of wound healing on mRNA expression of VEGF and TGF-β1 in a diabetic wound model. GAPDH was used to normalize the expression level of specific target bands (Figure 4A). At day 7, mRNA expression of VEGF and TGF-β1 was significantly increased in the red ginseng extract group ($p < 0.05$) and decreased at day 10. The expression of VEGF in the Rg1 group significantly increased on days 4–10 ($p < 0.05$). Expression of TGF-β1 was observed from day 4 in the red ginseng extract group but gradually increased on days 7–10 in the Rg1 group. In the control group, the expression of VEGF and TGF-β1 was delayed (Figure 4B–C).

Expression of VEGF and TGF-β1 by western blotting

Protein levels of VEGF and TGF-β1 were observed by western blotting; β-actin was used to

284 normalize the expression level of specific target bands (Figure 5A). VEGF expression was
285 significantly increased ($p < 0.05$) in the red ginseng extract and Rg1 groups at days 4–7, but
286 decreased significantly at day 10 in the Rg1 group ($p < 0.05$). The expression of TGF-β1 was
287 significantly higher on days 4–7 in the red ginseng extract group than in the other groups
288 ($p < 0.05$) and decreased on days 7–10. In the control group, VEGF tended to increase over
289 time, but TGF-β1 expression did not (Figure 5B–C).
290

291 **Discussion**

292 This study indicates that administration of red ginseng extract and Rg1 can regulate the healing
293 of diabetic wounds. Red ginseng extract and Rg1 have antidiabetic and wound healing effects
294 on normal wounds [21, 22], but the effects on diabetic wound animal models have not been
295 confirmed. This study showed that administration of red ginseng extract and Rg1 increases the
296 expression of VEGF and TGF-β1, thereby promoting wound healing in an animal model of
297 STZ-induced diabetic wounds (i.e., chronic wounds with delayed healing). Wound healing is
298 generally divided into four stages: hemostasis, inflammation, proliferation, and remodeling.
299 The first stage is hemostasis and clotting [23]. When this process begins, various immune cells
300 such as platelets, neutrophils, and monocytes, as well as growth factors, such as platelet-derived
301 growth factor (PDGF) and TGF-β, are expressed. Inflammatory processes produce neutrophils,
302 macrophages, and inflammatory cytokines following tissue damage [24]. Some growth factors,
303 such as PDGF, epidermal growth factor (EGF), and VEGF, are also expressed; however, in
304 diabetic patients, due to this imbalance of cytokines, would healing does not follow the four
305 general stages [25, 26], resulting in delayed healing. Rg1 not only lowers blood sugar but also
306 increases the expression of factors involved in wound healing, thus promoting wound healing

in mice [27, 28]. Kim *et al.* [29]. reported that RG promotes angiogenesis by stimulating VEGF and improves the proliferation of epidermal cells by upregulating cytokine expression in keratinocytes. These results suggest that RG accelerates the wound-healing process by increasing TGF-β and VEGF expression in the early stages of wound healing [30]. Diabetes is characterized by the overexpression of factors such as nuclear factor-κB as well as pro-inflammatory mediators, cytokines, and nitric oxide (NO), which increase intracellular oxidative stress due to insulin resistance and hyperglycemia, resulting in abnormal cells and inhibition of angiogenesis [31]. In addition, high glucose-derived reactive oxygen species affect the onset of diabetic complications [32]. Recent studies have shown that growth-factor regulatory defects in wounds in both diabetic models and diabetic patients exacerbate wound-healing disorders [33, 34]. Although prophylactic treatment with red ginseng contributes to wound healing by controlling the expression of VEGF and TGF-β1, excessive expression can lead to chronic inflammation and negatively affect wound healing [35, 36, 37]. Therefore, it is important to evaluate the inflammatory response in diabetic wounds and the underlying molecular mechanisms of growth factors. In the present study, we examined whether the administration of red ginseng extract and Rg1 affects expression of VEGF and TGF-β1 in a diabetic wound model. Our results provide evidence of the wound healing effects of red ginseng extract and Rg1 on the diabetic wound model. To determine whether red ginseng extract and Rg1 are effective for wound healing at mRNA and protein levels, we confirmed the expression of VEGF and TGF-β1 by RT-PCR and western blotting. Treatment with Rg1 in a diabetic wound model with delayed wound healing resulted in upregulated mRNA expression level of VEGF and TGF-β1 from the wound-healing inflammation phase to the proliferation phase (4–10 days after the injury) (Figure 4). Treatment with Rg1 also induced protein expression of VEGF and TGF-β1 during the wound-healing inflammation phase (4–7 days

after the injury), resulting in downregulation of expression at day 10 after the wound was fully healed (Figure 5). Other studies have shown that treatment with Rg1 increases angiogenesis and insulin secretion by increasing VEGF expression in the proliferative phase and progressively decreasing VEGF expression over time [38, 39]. In particular, Rg1 treatment reduced wound size compared to the control group (red ginseng extract, Rg1, and control mice were all diabetic). Factors such as TGF-β, PDGF, and interleukin (IL)-1 produced by T cells and macrophages stimulate a variety of growth factors; the expression of VEGF during the inflammatory stage also plays an important role in wound healing [40, 41, 42]. VEGF is an angiogenic cytokine that contributes to the proliferation phase of wound healing by promoting angiogenesis and the formation of fibrous cells, collagen, and granulation tissue around the wound [43, 44]. Many studies have focused on VEGF as a factor promoting angiogenesis and its role in various diseases [45, 46]. In the remodeling phase, VEGF induces cell degranulation, fibroblast proliferation, and migration of existing blood vessels by proteases; fibroblasts are transformed into α-smooth muscle actin bundles similar to smooth muscle cells, resulting in contraction of the wound [47, 48]. Nogami *et al.* [49] reported that VEGF mRNA levels were reduced significantly in diabetic animal models compared to normal models; thus, regulatory defects in VEGF delayed wound healing. Our results also showed that both red ginseng extract and Rg1 were affected by a delayed wound healing due to a lack of VEGF and TGF-β1 expression in untreated diabetic wounds (i.e., in the control group). In our histological analyses, both red ginseng and Rg1 treatment promoted formation of granulation tissue at days 7–10 after injury; wound contraction, reduction of inflammatory cells, and promotion of growth factors were observed during reconstruction at day 10. Treatment of tissues with Rg1 contributes to wound healing by promoting collagen production and VEGF expression [50, 51].Red ginseng positively affects wound healing by modulating VEGF and TGF-β1

355 expression in diabetic wounds. Similarly, our results suggest that treatment with red ginseng
356 may promote diabetic wound healing. In particular, Rg1 by regulates the expression of VEGF
357 and TGF-β1; growth factors were also expressed at the inflammatory stage, promoting
358 proliferation (Figure 3). This is the first report to show that red ginseng extract and Rg1 regulate
359 the expression of VEGF and TGF-β1 to induce healing of diabetic wounds. Expression levels
360 of these factors were confirmed by histological staining, IHC, and molecular biology.

361

362 **Conclusion**

363 Rg1 treatment increases the expression of VEGF and TGF-β1, which are important for wound
364 healing in STZ-induced diabetic wounds. Rg1 can improve diabetic wound healing by
365 stimulating the production or activity of factors related to wound healing.

366

367 **Limitations**

368 Although factors such as STZ can cause T1D, T2D is a complex metabolic disorder due to
369 insulin resistance driven by environmental factors (e.g., obesity, and age). Additionally, animal
370 experiments may not reflect all clinically observed complexities associated with disease [52].
371 Further studies of T2D may provide clinical data to corroborate the results obtained in this
372 study.

373

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Availability of data and materials

All study data used to support our findings are available from the corresponding authors upon request.

Ethics approval and consent to participate: All procedures involving animals were in accordance with the ethical standards of the institution. The experimental design was approved by the Institutional Animal Care and Use Committee of Soonchunhyang University Medical School (SCHBC-Animal-2016–03).

References

1. Dagogo-Jack S. Diabetes mellitus in developing countries and underserved communities: Springer; 2017.
2. Association AD. Diagnosis and classification of diabetes mellitus. Diabetes care. 2014;37(Supplement 1):S81-S90.
3. Di Carli MF, Janisse J, Ager J, Grunberger G. Role of chronic hyperglycemia in the pathogenesis of coronary microvascular dysfunction in diabetes. Journal of the American College of Cardiology. 2003;41(8):1387-93.
4. Juster-Switlyk K, Smith AG. Updates in diabetic peripheral neuropathy. F1000Research. 2016;5.
5. Patel S, Srivastava S, Singh MR, Singh D. Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. Biomedicine & Pharmacotherapy. 2019;112:108615.
6. Chawla A, Chawla R, Jaggi S. Microvasular and macrovascular complications in diabetes mellitus: distinct or continuum? Indian journal of endocrinology and metabolism. 2016;20(4):546.
7. Gill G, Famuyiwa O, Rolfe M, Archibald L. Serious hand sepsis and diabetes mellitus: specific tropical syndrome with western counterparts. Diabetic medicine. 1998;15(10):858-62.
8. Gupta R, Sharma AK, Dobhal M, Sharma M, Gupta R. Antidiabetic and antioxidant potential of β-sitosterol in streptozotocin-induced experimental hyperglycemia. Journal of diabetes. 2011;3(1):29-37.
9. Sebastião I, Candeias E, Santos MS, de Oliveira CR, Moreira PI, Duarte AI. Insulin as a bridge between type 2 diabetes and Alzheimer disease–how anti-diabetics could be a solution for dementia. Frontiers in endocrinology. 2014;5:110.
10. Kim HJ, Kwak I, Kim HJ, Ahn JS, Son YB. A study on the amendment scheme of ginsenoside content standard regulation for red ginseng products in Korea. Journal of Food Hygiene and Safety. 2013;28(1):24-30.
11. Christensen LPJAif, research n. Ginsenosides: chemistry, biosynthesis, analysis, and potential health effects. 2008;55:1-99.

433 12. Bai L, Gao J, Wei F, Zhao J, Wang D, Wei J. Therapeutic potential of ginsenosides
434 as an adjuvant treatment for diabetes. Frontiers in pharmacology. 2018;9:423.
435 13. Wang C-W, Su S-C, Huang S-F, Huang Y-C, Chan F-N, Kuo Y-H, et al. An essential
436 role of cAMP response element binding protein in ginsenoside Rg1-mediated inhibition of
437 Na+/glucose cotransporter 1 gene expression. 2015;88(6):1072-83.
438 14. Yu H-t, Zhen J, Pang B, Gu J-n, Wu S-sJJoZU-SB. Ginsenoside Rg1 ameliorates
439 oxidative stress and myocardial apoptosis in streptozotocin-induced diabetic rats.
440 2015;16(5):344-54.
441 15. Choi J-H, Oh S-K. Studies on the anti-aging action of Korean ginseng. Korean
442 Journal of Food Science and Technology. 1985;17(6):506-15.
443 16. Pakyari M, Farrokhi A, Maharlooei MK, Ghahary AJAiwc. Critical role of
444 transforming growth factor beta in different phases of wound healing. 2013;2(5):215-24.
445 17. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors
446 and cytokines in wound healing. Wound repair and regeneration. 2008;16(5):585-601.
447 18. Yue PYK, Mak NK, Cheng YK, Leung KW, Ng TB, Fan DTP, et al.
448 Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor, angiomodulating and
449 steroid-like activities of ginsenosides. Chinese medicine. 2007;2(1):1-21.
450 19. Savari R, Shafiei M, Galehdari H, Kesmati M. Expression of VEGF and TGF-β
451 genes in skin wound healing process induced using phenytoin in male rats. Jundishapur
452 Journal of Health Sciences. 2019;11(1).
453 20. Yu H, Zhen J, Yang Y, Gu J, Wu S, Liu Q. Ginsenoside Rg1 ameliorates diabetic
454 cardiomyopathy by inhibiting endoplasmic reticulum stress-induced apoptosis in a
455 streptozotocin-induced diabetes rat model. Journal of Cellular and Molecular Medicine.
456 2016;20(4):623-31.
457 21. Kim YS, Cho I-H, Jeong M-J, Jeong S-J, Nah SY, Cho Y-S, et al. Therapeutic effect
458 of total ginseng saponin on skin wound healing. Journal of ginseng research. 2011;35(3):360.
459 22. Mohanan P, Subramaniyam S, Mathiyalagan R, Yang D-C. Molecular signaling of
460 ginsenosides Rb1, Rg1, and Rg3 and their mode of actions. Journal of Ginseng Research.
461 2018;42(2):123-32.
462 23. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clinics in
463 dermatology. 2007;25(1):9-18.

24. Nguyen TT, Mobashery S, Chang M. Roles of matrix metalloproteinases in cutaneous wound healing: InTech; 2016.

25. Lan CC, Liu IH, Fang AH, Wen CH, Wu CS. Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes. British Journal of Dermatology. 2008;159(5):1103-15.

26. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiological reviews. 2003;83(3):835-70.

27. Yang N, Chen P, Tao Z, Zhou N, Gong X, Xu Z, et al. Beneficial effects of ginsenoside-Rg1 on ischemia-induced angiogenesis in diabetic mice. Acta Biochim Biophys Sin. 2012;44(12):999-1005.

28. Kimura Y, Sumiyoshi M, Kawahira K, Sakanaka M. Effects of ginseng saponins isolated from Red Ginseng roots on burn wound healing in mice. British journal of Pharmacology. 2006;148(6):860-70.

29. Kim YG, Sumiyoshi M, Sakanaka M, Kimura Y. Effects of ginseng saponins isolated from red ginseng on ultraviolet B-induced skin aging in hairless mice. European journal of pharmacology. 2009;602(1):148-56.

30. Park K-S, Park D-H. The effect of Korean Red Ginseng on full-thickness skin wound healing in rats. Journal of ginseng research. 2019;43(2):226-35.

31. Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. International journal of vascular medicine. 2012;2012.

32. Panigrahy SK, Bhatt R, Kumar A. Reactive oxygen species: sources, consequences and targeted therapy in type 2 diabetes. Journal of drug targeting. 2017;25(2):93-101.

33. Dogan S, Demirer S, Kepenekci I, Erkek B, Kiziltay A, Hasirci N, et al. Epidermal growth factor-containing wound closure enhances wound healing in non-diabetic and diabetic rats. International wound journal. 2009;6(2):107-15.

34. Blakytny R, Jude E. The molecular biology of chronic wounds and delayed healing in diabetes. Diabetic Medicine. 2006;23(6):594-608.

35. Namgoong S, Lee H, Han SK, Lee HW, Jeong SH, Dhong ES. Effect of Panax ginseng extract on the activity of diabetic fibroblasts in vitro. International wound journal. 2019;16(3):737-45.

36. Yang L, Chan T, Demare J, Iwashina T, Ghahary A, Scott PG, et al. Healing of burn wounds in transgenic mice overexpressing transforming growth factor-β1 in the epidermis. The American journal of pathology. 2001;159(6):2147-57.

37. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. Graefe's archive for clinical and experimental ophthalmology. 2004;242(1):91-101.

38. Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The role of vascular endothelial growth factor in wound healing. Journal of Surgical Research. 2009;153(2):347-58.

39. Goldman R. Growth factors and chronic wound healing: past, present, and future. Advances in skin & wound care. 2004;17(1):24-35.

40. Kondo T, Ohshima T. The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. International journal of legal medicine. 1996;108(5):231-6.

41. Pierce GF, Mustoe TA, Altrock BW, Deuel TF, Thomason A. Role of platelet-derived growth factor in wound healing. Journal of cellular biochemistry. 1991;45(4):319-26.

42. Enoch S, Leaper DJ. Basic science of wound healing. Surgery (Oxford). 2008;26(2):31-7.

43. Grazul-Bilska AT, Johnson ML, Bilski JJ, Redmer DA, Reynolds LP, Abdullah A, et al. Wound healing: the role of growth factors. Drugs Today (Barc). 2003;39(10):787-800.

44. Wilgus TA, DiPietro LA. Complex roles for VEGF in dermal wound healing. The Journal of investigative dermatology. 2012;132(2):493.

45. Khurana R, Simons M, Martin JF, Zachary IC. Role of angiogenesis in cardiovascular disease: a critical appraisal. Circulation. 2005;112(12):1813-24.

46. Pandya NM, Dhalla NS, Santani DD. Angiogenesis—a new target for future therapy. Vascular pharmacology. 2006;44(5):265-74.

47. Ellis S, Lin EJ, Tartar D. Immunology of wound healing. Current dermatology reports. 2018;7(4):350-8.

48. Goldberg MT, Han Y-P, Yan C, Shaw MC, Garner WL. TNF-α suppresses α-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound

healing. Journal of Investigative Dermatology. 2007;127(11):2645-55.

49. Nogami M, Hoshi T, Kinoshita M, Arai T, Takama M, Takahashi I. Vascular endothelial growth factor expression in rat skin incision wound. Medical molecular morphology. 2007;40(2):82-7.

50. Jang K-J, Kim HK, Han MH, Oh YN, Yoon H-M, Chung YH, et al. Anti-inflammatory effects of saponins derived from the roots of Platycodon grandiflorus in lipopolysaccharide-stimulated BV2 microglial cells. International Journal of Molecular Medicine. 2013;31(6):1357-66.

51. Lim A-K, Kim K-S, Park S-J, Hong J-H, Choi H-J, Kim D-I. Healing effects of ginsenoside Rg1 on experimental open wound in rat. Journal of the Korean Society of Food Science and Nutrition. 2010;39(10):1452-8.

52. Chatzigeorgiou A, Halapas A, Kalafatakis K, Kamper E. The use of animal models in the study of diabetes mellitus. In vivo. 2009;23(2):245-58.

Figure 1. Protocol for wound induction.

STZ-induced diabetic mice were orally administered red ginseng extract (10 mg/kg) and Rg1 (10 mg/kg). Wounds were made on day 0 and measured on days 1, 4, and 7. Mice were sacrificed on days 4, 7, and 10. Mice were randomly divided into three groups according to oral-administration treatment. G1: red ginseng extract treatment group (10 mg/kg/day, $n = 27$); G2: Rg1 treatment group (10 mg/kg/day, $n = 27$); G3: control (PBS) group (0.3 mL/kg/day, $n = 27$). STZ: streptozotocin, Rg1: ginsenoside Rg1.

Figure 2. Changes in wound size by date in MRSA-infected diabetic rats.

(A) Images of wound size on different dates. (B) Wound size measurements for each group. At each point, the wound healing rate changed compared to the first day of injury. Data are expressed as a percentage by measuring the wounds of the mice in each group. Data are expressed as mean ± standard deviation ($n = 9$). MRSA: methicillin-resistant *Staphylococcus aureus*.

Figure 3. Histology and IHC analysis.

For histological analysis, the slides corresponding to days 4, 7, and 10 for each group were stained with hematoxylin and eosin (H&E). IHC staining of VEGF and TGF-β1 (important factors for wound healing) was observed. (A) Tissues from mice of each group sacrificed 4 days after injury (H&E stain in the left panels, VEGF antibody IHC stain in the middle panels, and TGF-β1 antibody IHC stain in the right panels). (B) Tissues from mice of each group

576 sacrificed 7 days after injury. (C) Tissues from mice of each group sacrificed 10 days after
577 injury. The original magnifications of 200× and 400× (bottom panel) are displayed. (*) Position
578 of the granulation tissue and wound. (D) The degree of granulation tissue formation and
579 infiltration of inflammatory cells were scored to objectify as H&E stain (Tables 2 and 3). An
580 objective scoring table shows that repeated scoring of three times was performed by blind test.
581 IHC staining shows hematoxylin (blue) and VEGF and TGF-β1 antibody (brown). Mice were
582 divided into three groups (red ginseng extract group, Rg1 group, and the PBS-treated group as
583 a control). *$p < 0.05$ compared to the control group and # $p < 0.05$ compared to the treatment
584 group. E: epidermis, D: dermis, TGF: transforming growth factor, VEGF: vascular endothelial
585 growth factor.
586
587 **Figure 4.** Effects of red ginseng extract and Rg1 on the expression of VEGF and TGF-β1 in
588 diabetic wounds indicated by reverse transcription polymerase chain reaction (RT-PCR)
589 analysis. (A) RT-PCR was performed for the tissue of diabetic mice using VEGF and TGF-β1
590 primers. GAPDH was used to normalize the intensity of the analyzed bands. (B–C) mRNA
591 expression of VEGF and TGF-β1 increased significantly and then decreased during the wound-
592 healing period (4–10 days after wound injury) in the red ginseng extract group; expression of
593 VEGF and TGF-β1 increased gradually in the Rg1 group. Significance was determined using
594 the Mann–Whitney U test. Mice were divided into three groups (red ginseng extract group,
595 Rg1 group, PBS-treated group as a control group). *$p < 0.05$ compared to the control group
596 and # $p < 0.05$ compared to the treatment group. TGF: transforming growth factor, VEGF:
597 vascular endothelial growth factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
598

Figure 5. Effects of red ginseng extract and Rg1 on the expression of VEGF and TGF-β1 in diabetic wounds indicated by western blot analysis. (A) Immunoblotting was performed for diabetic mouse tissue using VEGF and TGF-β1 antibodies. β-actin was used to normalize the intensity of the analyzed bands. (B–C) Protein expression of VEGF and TGF-β1 increased significantly in the red ginseng extract and Rg1 groups during the wound healing period (4–10 days after injury) and then decreased with time. Significance was determined using the Mann–Whitney U test. Mice were divided into three groups (red ginseng extract group, Rg1 group, PBS-treated group as a control group). *$p < 0.05$ compared to the control group and # $p < 0.05$ compared to the treatment group. TGF: transforming growth factor, VEGF: vascular endothelial growth factor, β-actin: beta-actin.

Tables

Table 1. Reverse transcription-polymerase chain reaction primer sequence.

VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. [1)]All primers were designed directly and purchased from Macrogen Corporation (Seoul, Republic of Korea). The primers for VEGF, TGF-β1, and GAPDH used in this experiment are shown in the table and reverse transcription–polymerase chain reaction was performed using each primer.

Target primer[1)]	Oligonucleotide (5' to 3')	Size
VEGF	Forward: CAG GCT GCT GTA ACG ATG AA Reverse: AAT GCT TTC TCC GCT CTG A	221 bp
TGF-β1	Forward: ATT CAG CGC TCA CTG CTC TT Reverse: TTC TCT GTG GAG CTG AAG CA	219 bp
GAPDH	Forward: CCT TAA ACA GGC CCA CTT GA Reverse: CCT TCC ACA ATG CCA AAG TT	201bp

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644

645 **Table 2.** Degree of granulation tissue of formation score.

646 The degree of granulation tissue formation was scored in mouse skin tissue. Hematoxylin and

647 eosin staining were used to evaluate the degree of granulation formation histologically.

648

Judgment element	Standard	Score
Granulation tissue of formation	Not formation	0
	More than 2/3 of defect area	1
	1/3 to 2/3 of defect area	2
	Less than 1/3 of defective area	3

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Table 3. Degree of inflammatory cells infiltration score.

The degree of infiltration of inflammatory cell was scored in mouse skin tissue. Hematoxylin and eosin staining was performed histologically to evaluate the degree of infiltration of inflammatory cell.

Judgment element	Standard	Score
Inflammatory cell of infiltration	No necrosis and inflammation	0
	Not clusters and only exist in cell form	-1
	When small clusters are formed and inflammatory cells are visible	-2
	When large clusters are formed and many inflammatory cells are visible	-3

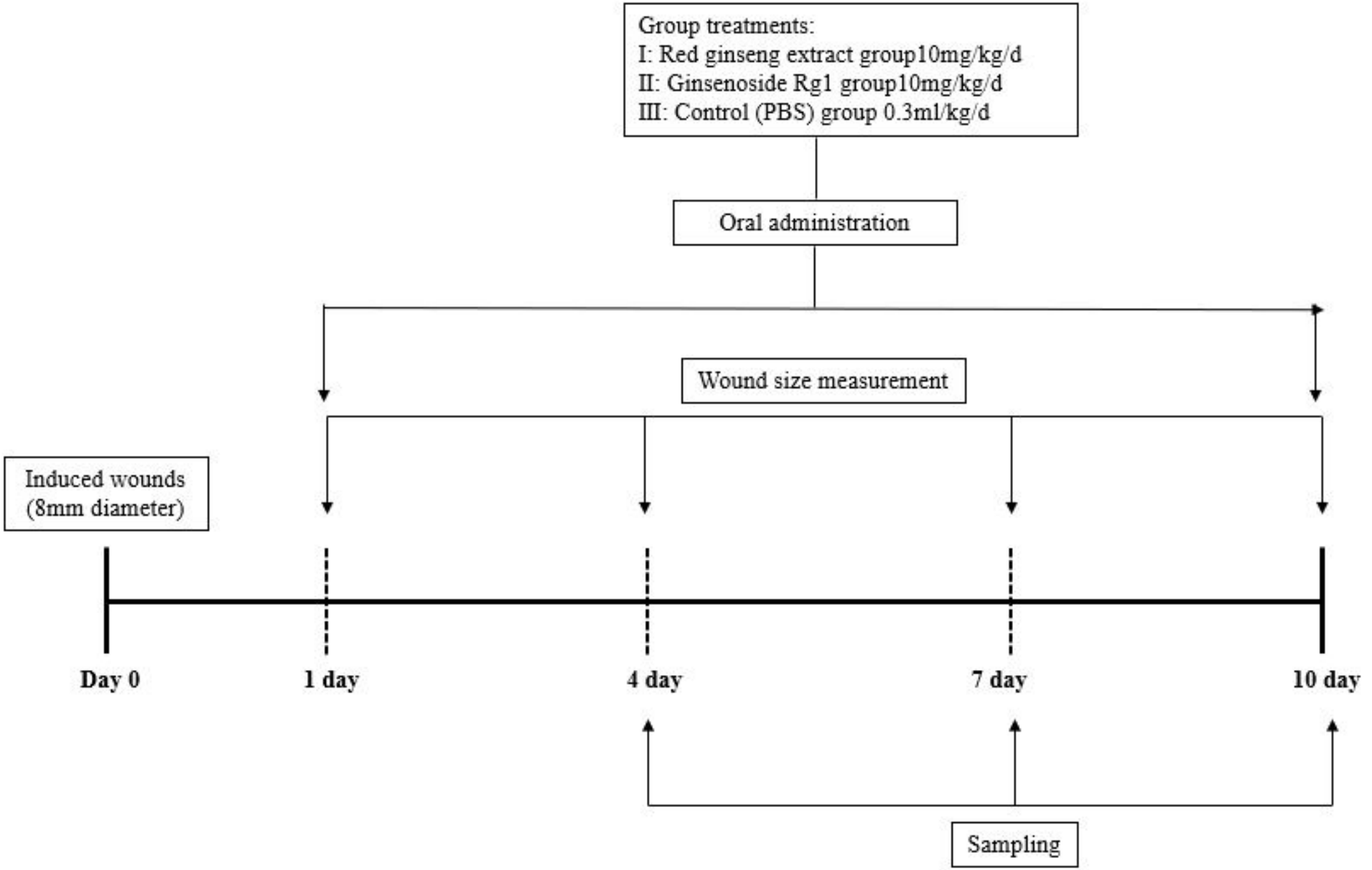
Group treatments:
I: Red ginseng extract group10mg/kg/d
II: Ginsenoside Rg1 group10mg/kg/d
III: Control (PBS) group 0.3ml/kg/d
Oral administration
Wound size measurement
Induced wounds
(8mm diameter)
Day 0
1 day
4 day
7 day
10 day
Sampling

Figure 1

A

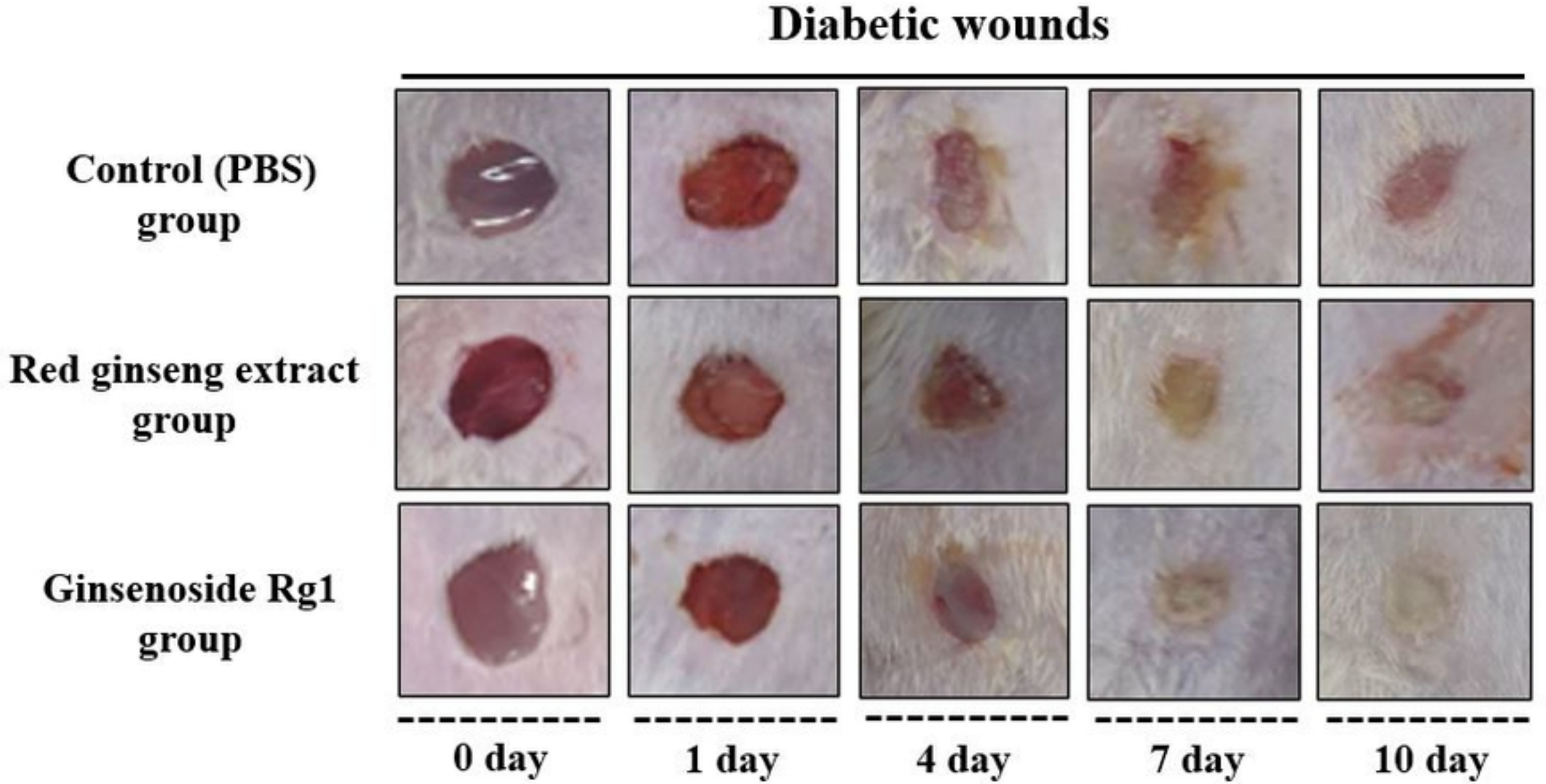
Diabetic wounds
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
0 day
1 day
4 day
7 day
10 day

Figure 2a

B

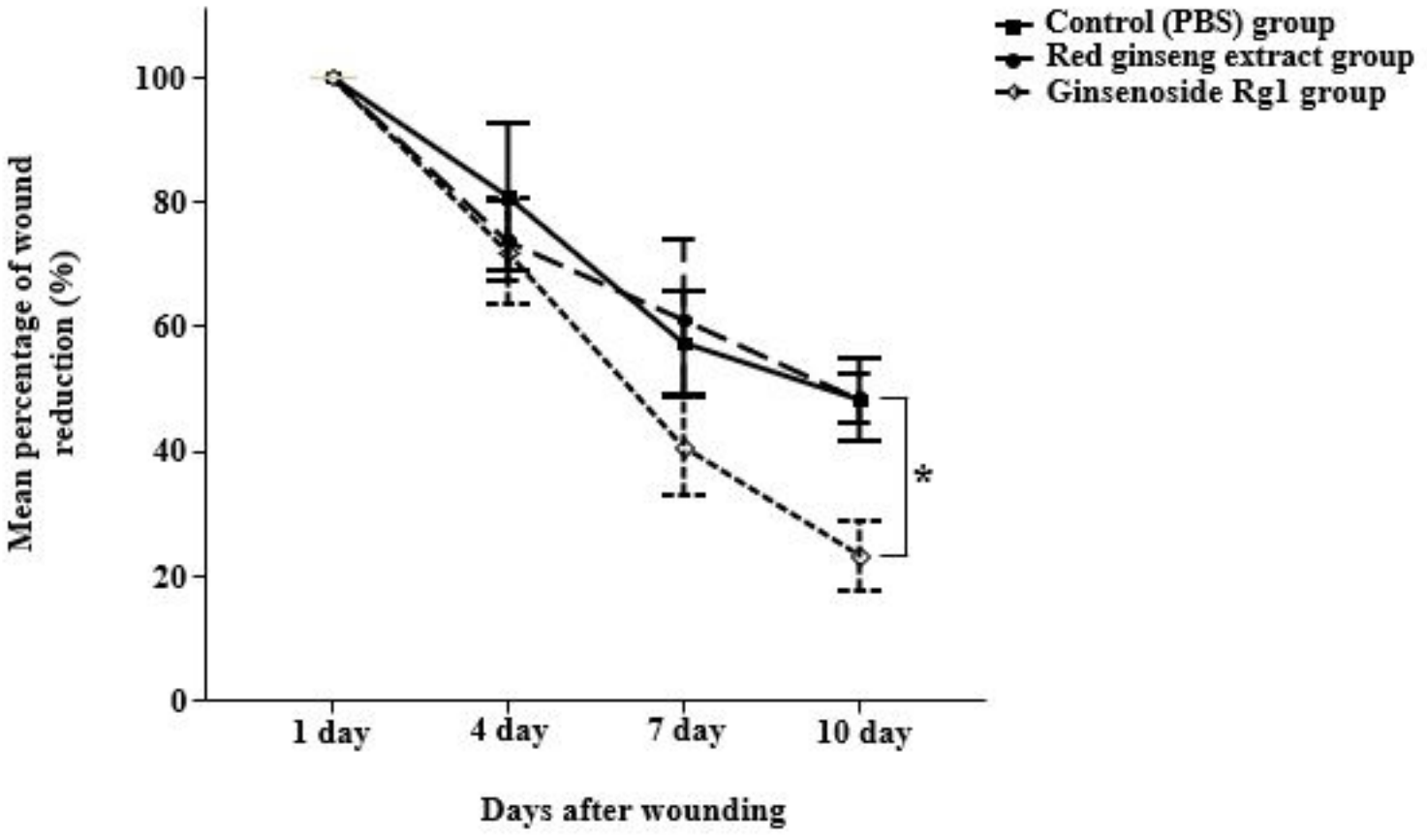
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
Mean percentage of wound reduction (%)
100
80
60
40
20
0
1 day
4 day
7 day
10 day
Days after wounding
*

Figure 2b

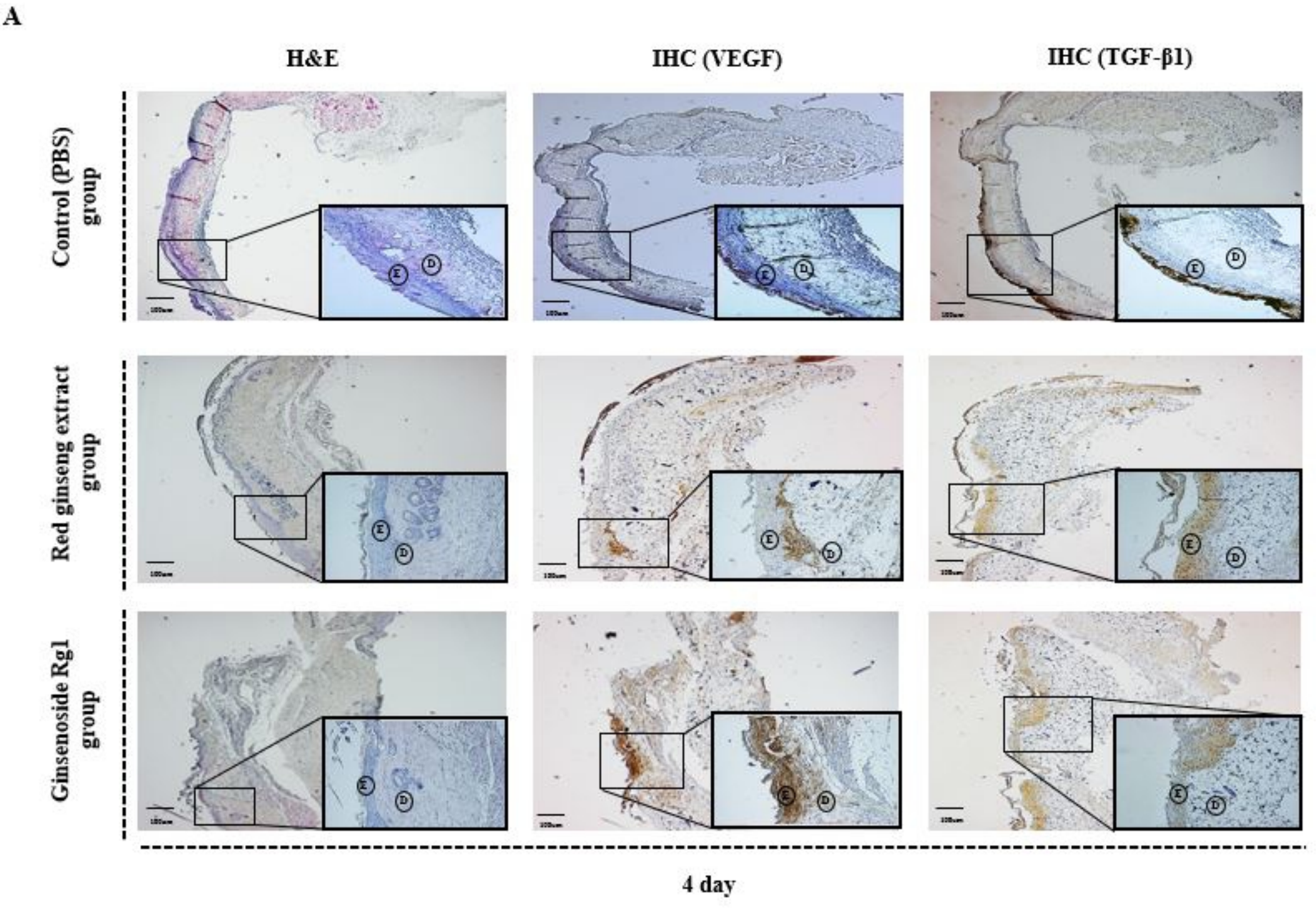
A
H&E
IHC (VEGF)
IHC (TGF-β1)
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
4 day

Figure 3a

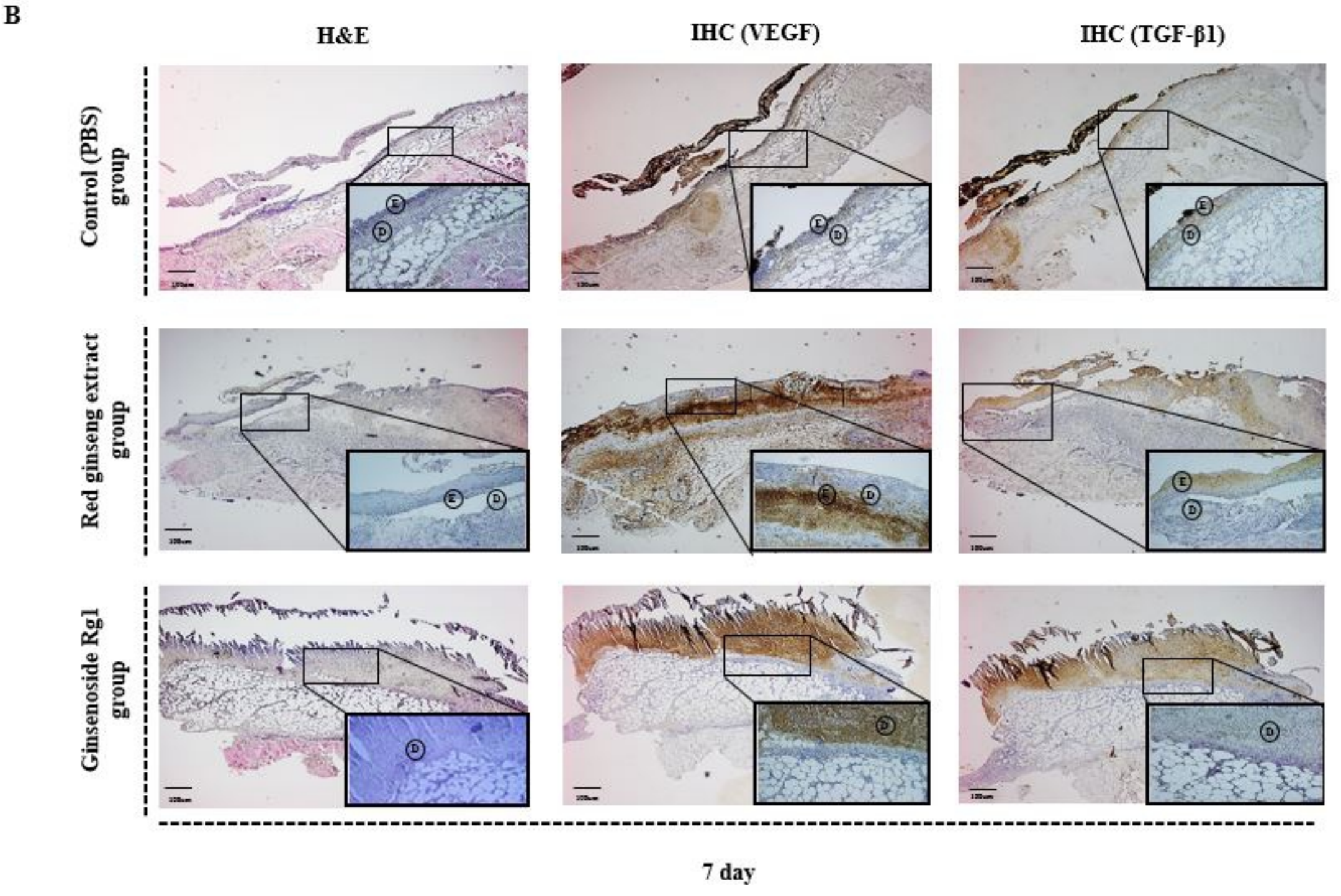
B
H&E
IHC (VEGF)
IHC (TGF-β1)
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
E
D
7 day

Figure 3b

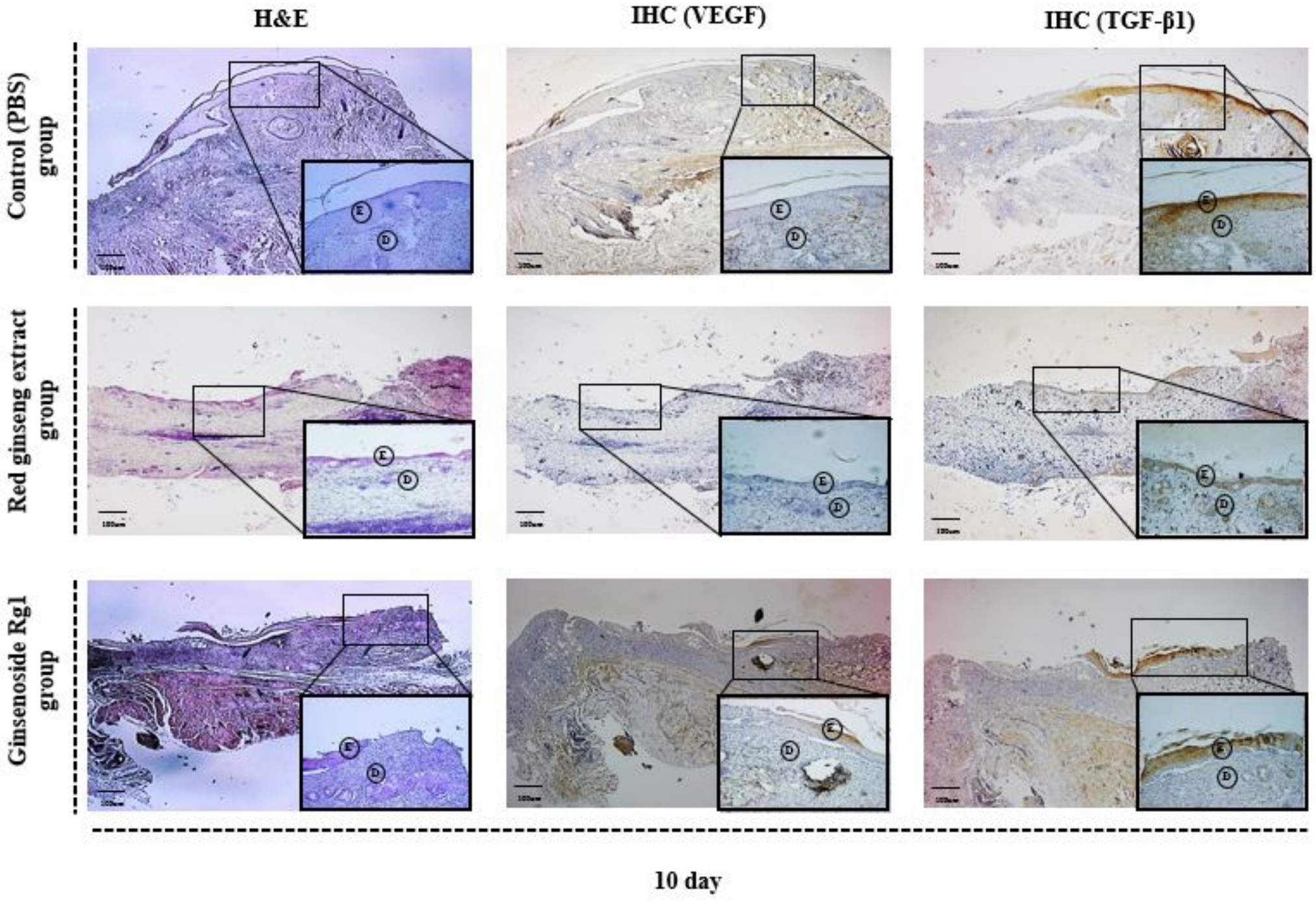
C
H&E
IHC (VEGF)
IHC (TGF-β1)
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
E
D
10 day

Figure 3c

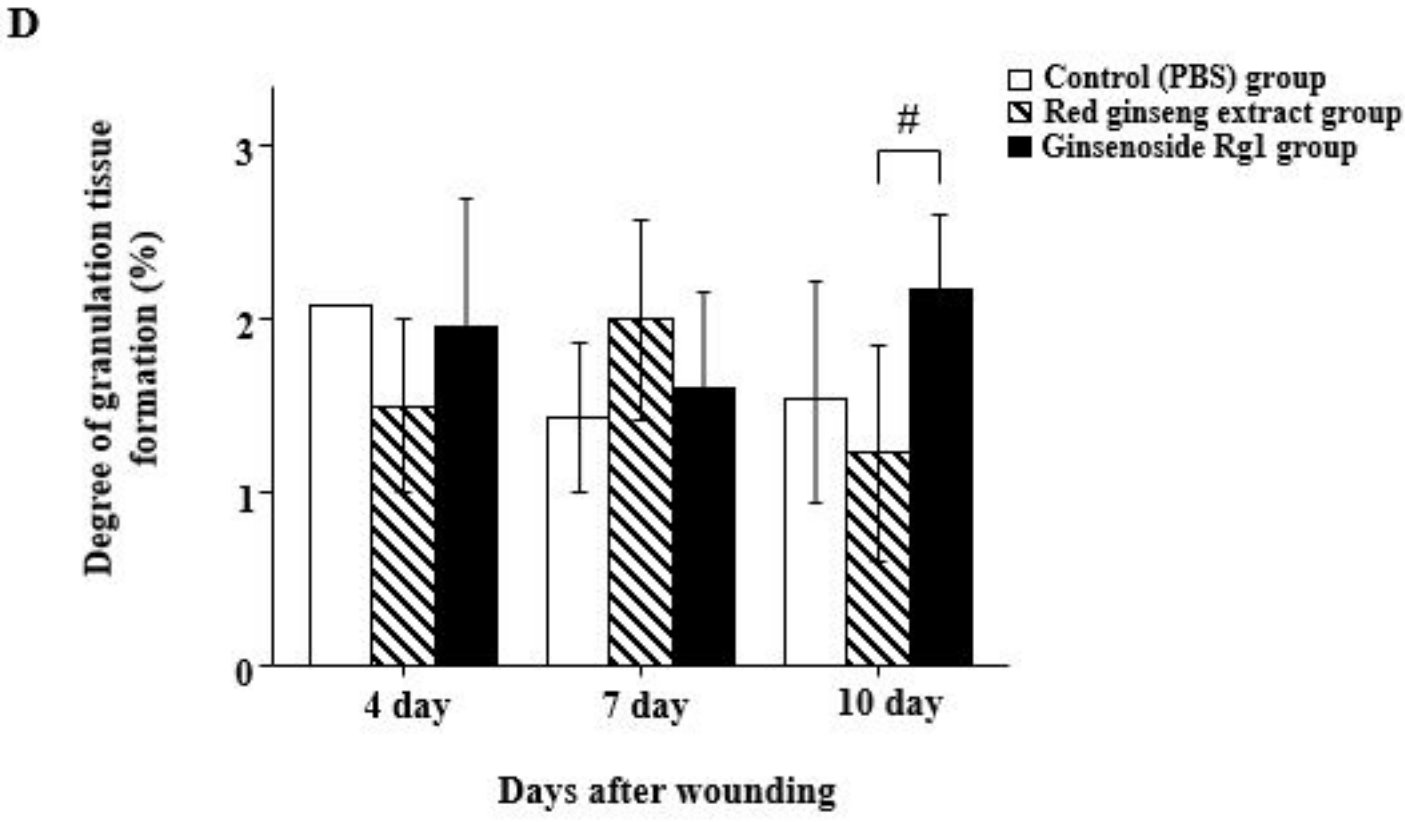
D
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
#
Degree of granulation tissue formation (%)
3
2
1
0
4 day
7 day
10 day
Days after wounding

Figure 3d

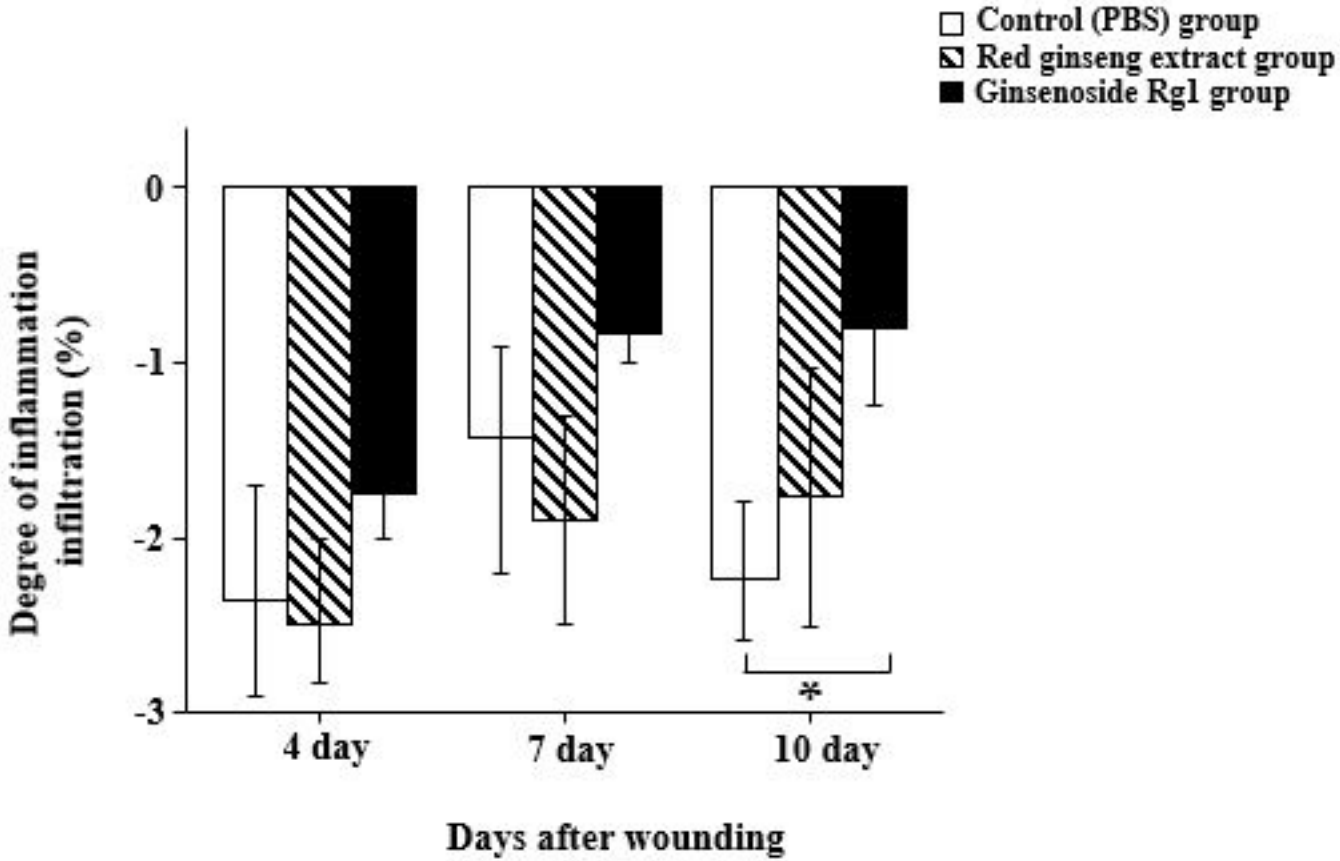
E
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
Degree of inflammation infiltration (%)
0
-1
-2
-3
*
4 day
7 day
10 day
Days after wounding

Figure 3e

A

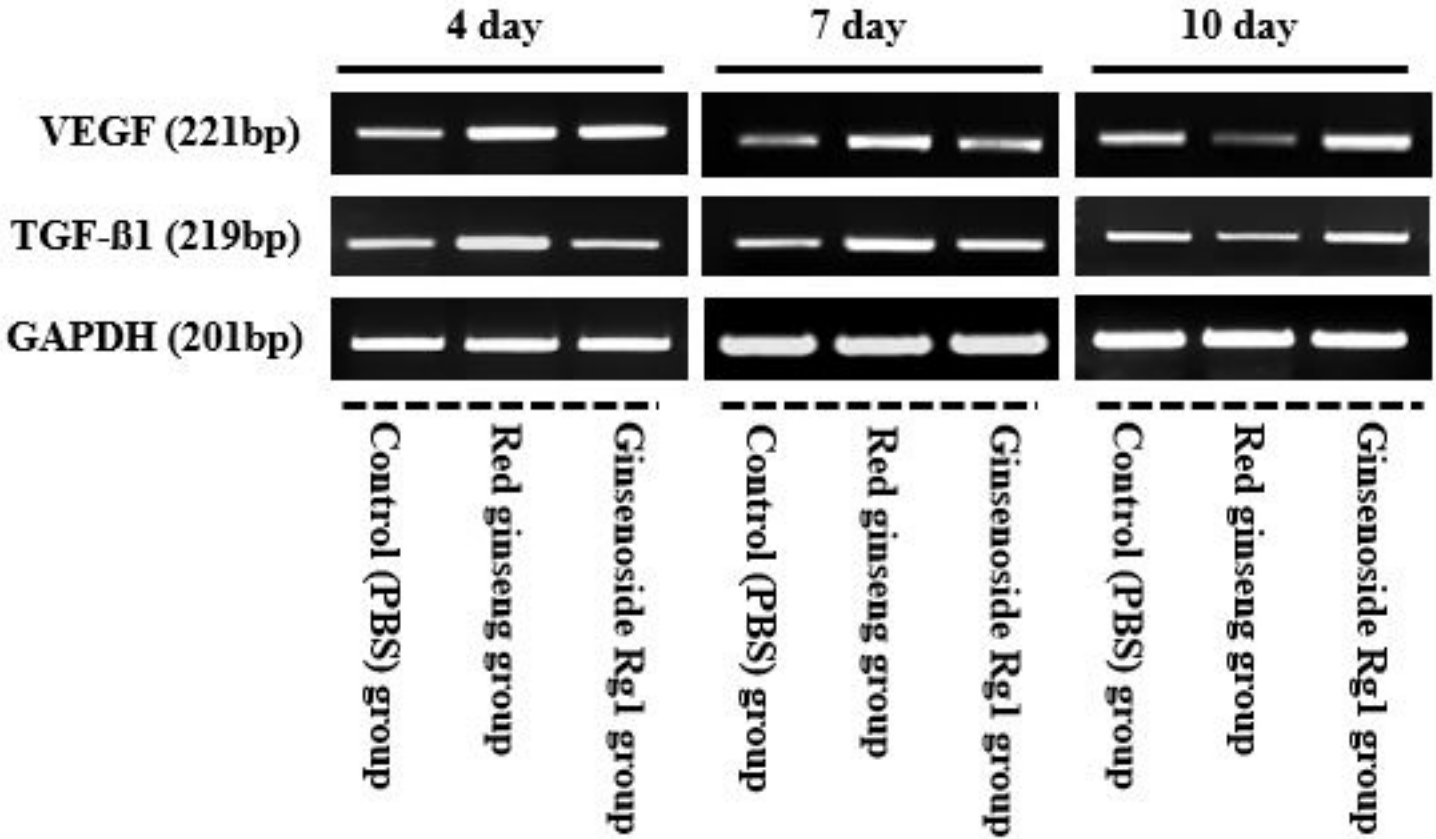
4 day
7 day
10 day
VEGF (221bp)
TGF-ß1 (219bp)
GAPDH (201bp)
Control (PBS) group
Red ginseng group
Ginsenoside Rgl group
Control (PBS) group
Red ginseng group
Ginsenoside Rgl group
Control (PBS) group
Red ginseng group
Ginsenoside Rgl group

Figure 4a

B

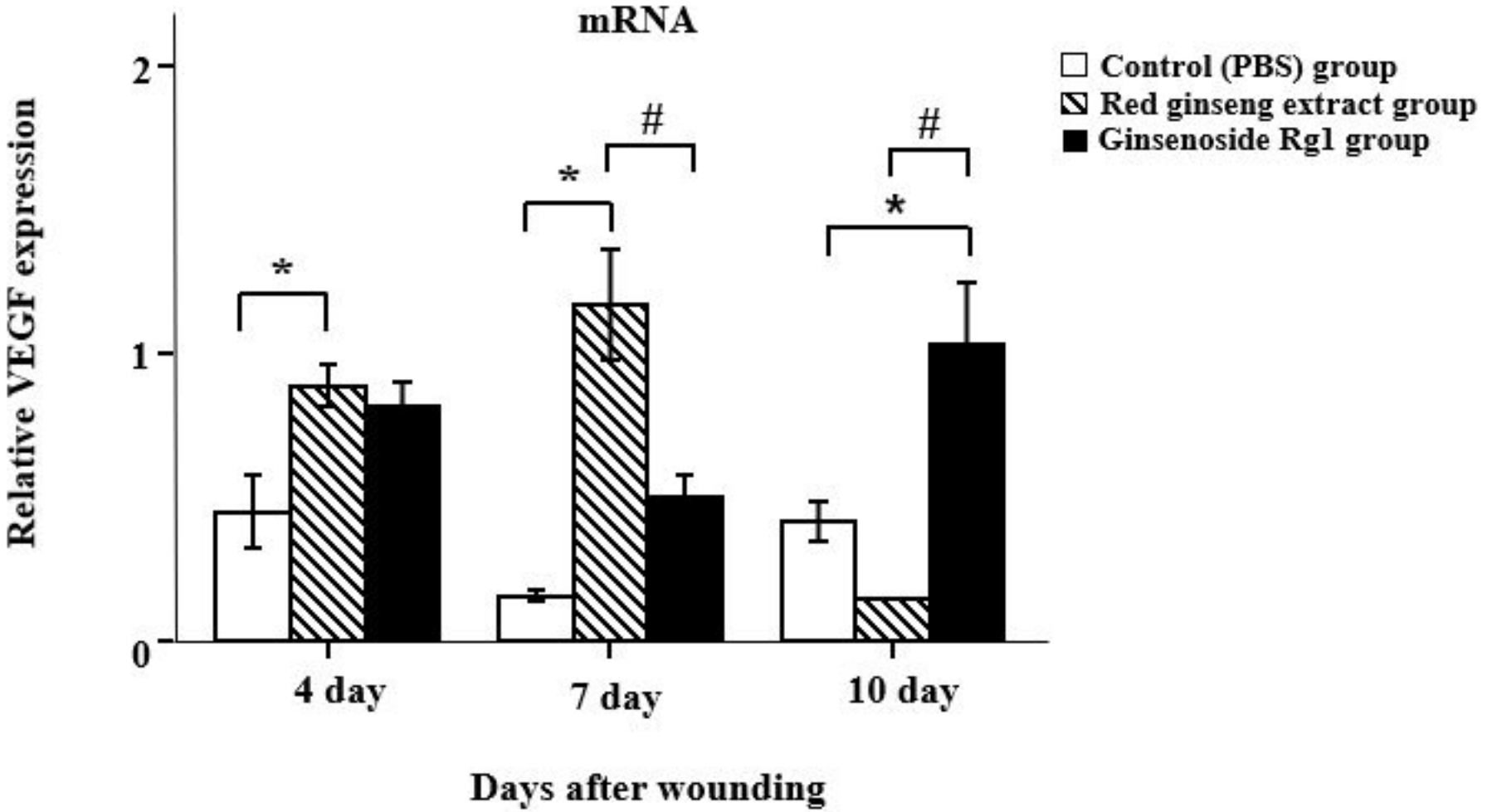
mRNA
Relative VEGF expression
2
1
0
*
*
*
#
#
4 day
7 day
10 day
Days after wounding
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group

Figure 4b

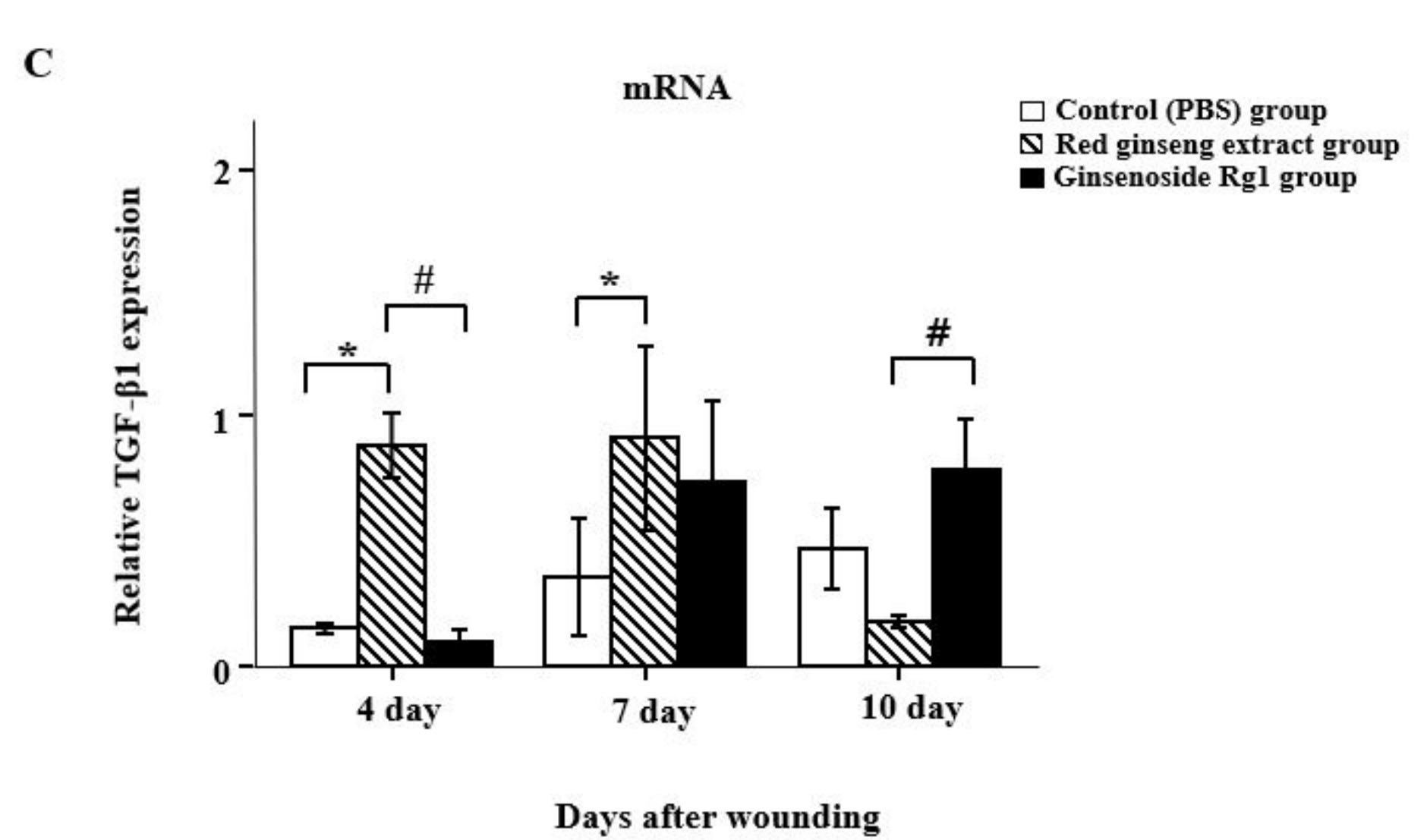
C
mRNA
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
Relative TGF-β1 expression
2
1
0
*
#
*
#
4 day
7 day
10 day
Days after wounding

Figure 4c

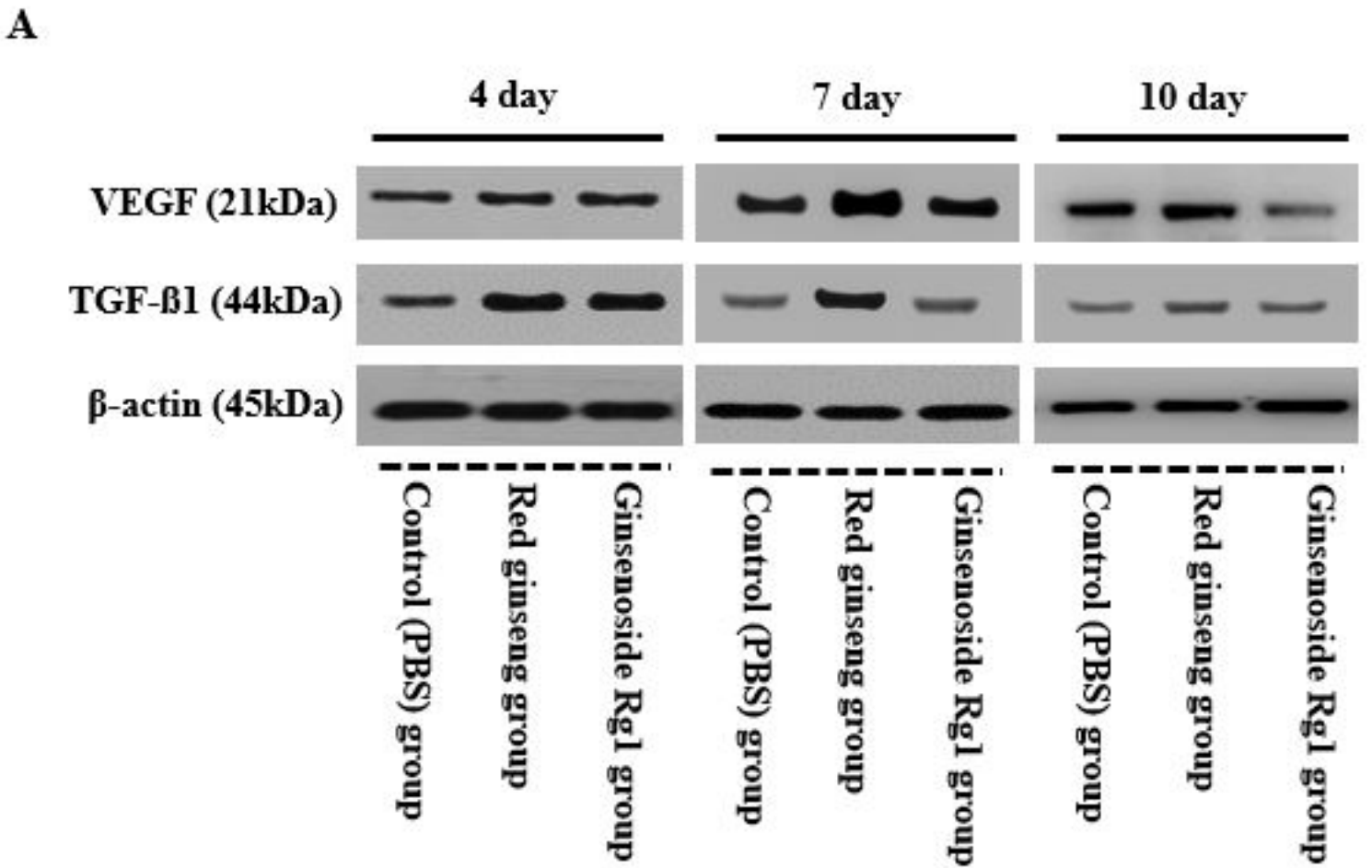
A
4 day
7 day
10 day
VEGF (21kDa)
TGF-ß1 (44kDa)
β-actin (45kDa)
Control (PBS) group
Red ginseng group
Ginsenoside Rg1 group
Control (PBS) group
Red ginseng group
Ginsenoside Rg1 group
Control (PBS) group
Red ginseng group
Ginsenoside Rg1 group

Figure 5a

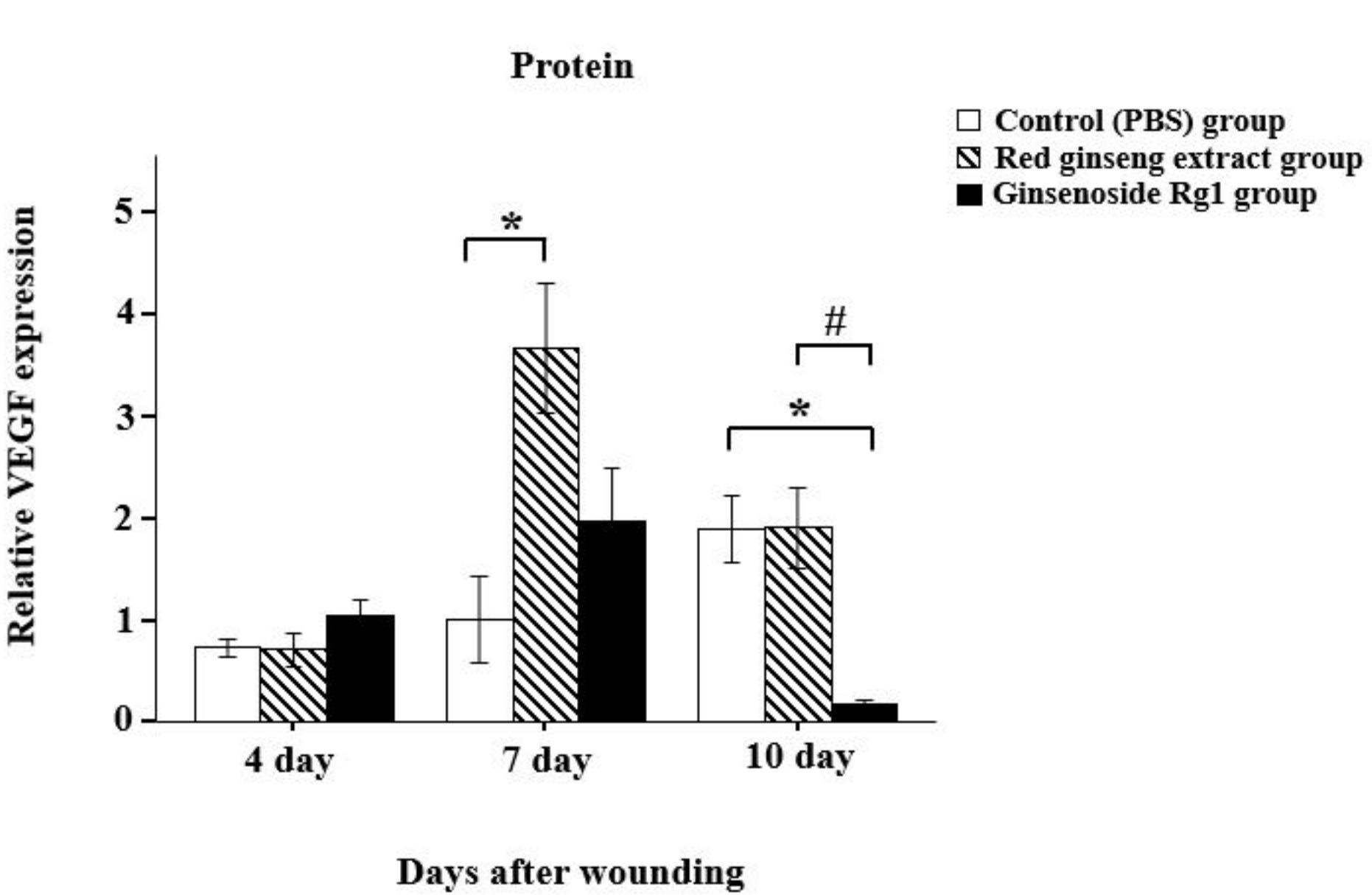
B
Protein
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
Relative VEGF expression
0
1
2
3
4
5
*
#
*
4 day
7 day
10 day
Days after wounding

Figure 5b

C

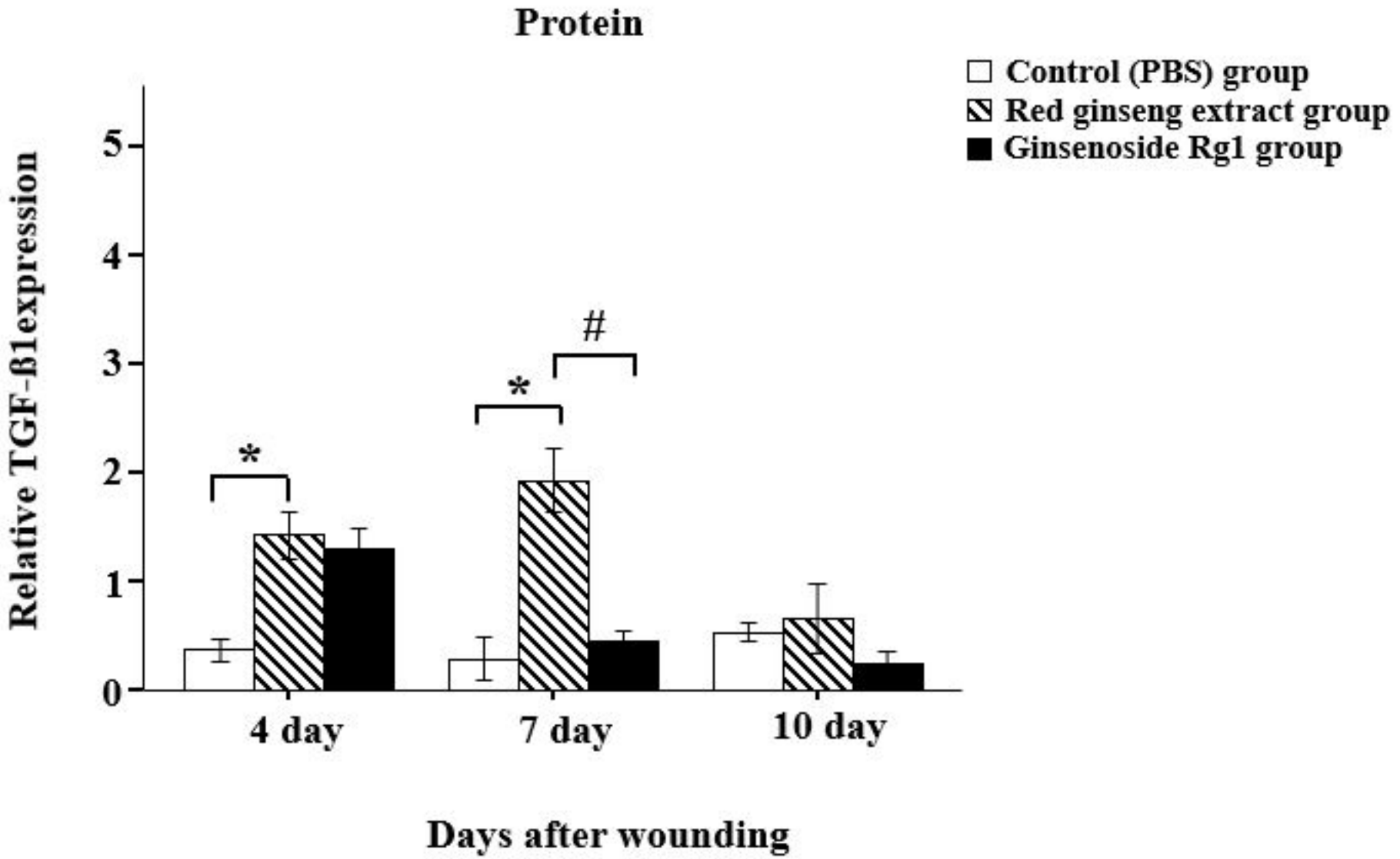
Protein
Control (PBS) group
Red ginseng extract group
Ginsenoside Rg1 group
Relative TGF-ß1expression
0
1
2
3
4
5
*
*
#
4 day
7 day
10 day
Days after wounding

Figure 5c