

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

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50 **Authors' contributions**

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

55

57 **Abstract**

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

76

77 **Keywords**

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

79

80 **Introduction**

81 By the year 2035, it is expected that 592 million people will suffer from diabetes, making it
82 one of the most important medical issues globally [1]. Diabetes is divided into two types: type
83 1 diabetes (T1D) is characterized by impaired pancreatic β -cell function resulting in insulin
84 deficiency and chronic hyperglycemia while type 2 diabetes (T2D) is characterized by insulin
85 resistance and hyperglycemia [2]. Chronic hyperglycemia causes deterioration of blood vessels
86 and nerves, resulting in cardiovascular diseases and neuropathy [3, 4]. Additional
87 complications, such as diabetic wounds, impair the four stages of wound healing (e.g.,
88 hemostasis, inflammation, proliferation and remodeling), delaying the healing process [5]. As
89 a result, chronic wounds in diabetic patients damage wounds and surrounding tissues due to
90 excessive inflammatory reactions, and delay wound healing through delayed expression of
91 growth factors. In serious cases, this results in loss of the affected limb or death [6, 7]. However,
92 a detailed understanding of the delayed healing of diabetic wounds is lacking and current forms
93 of treatment such as oral antidiabetics and insulin injection, have many unwanted side effects,
94 such as hypertension, weight gain, and endothelial cell damage [8, 9]. This has stimulated the
95 search for safe and effective treatments. *Panax ginseng* is one of the oldest traditional herbs
96 and it contains approximately 32 species of ginsenosides [10]. In recent years, ginsenosides
97 have been demonstrated to exhibit many properties, including immune-stimulating activity and
98 anticancer, anti-inflammatory, antiallergic, antihypertensive, and antidiabetic effects [11].
99 Ginsenosides with antidiabetic effects include Rg1, Rg3, Rg5, Rb1, Rb2, and Rb3, and
100 ginsenosides have been used as an adjuvant for the treatment of diabetes because they exhibit
101 antidiabetic effects [12]. Of the many types of saponins, the ginsenoside Rg1 reduces intestinal
102 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- β 1 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 $23 \pm 2^\circ\text{C}$ and $50 \pm 20\%$ humidity with freely available water and rodent chow (LabDiet

126 5L79[®]; Orient BIO Inc.). The experimental design was approved by the Institutional Animal
127 Care and Use Committee of Soonchunhyang University Medical School (SCHBC-Animal-
128 2016–03).

129

130 *Diabetic wound model using STZ*

131 Diabetes was induced by intraperitoneal (IP) injection of 100 mg/kg STZ (S0130; Sigma-
132 Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) to 81 mice following a 6 h fast.
133 After 24 h, blood was collected from the tail vein and blood glucose levels were measured
134 using the Accu-Chek[®] system (Roche Diagnostics, Mannheim, Germany). Diabetes was
135 defined as a blood glucose level greater than 200 mg/dL after STZ injection. The mice were
136 anesthetized by IP injection of zolazepam/tiletamine (Zoletil 50[®]; Virbac, Carros, France) and
137 xylazine (Rompun[®]; Bayer Korea, Seoul, Korea). The hairs on the back of the mice were
138 shaved, the exposed skin was cleansed with 70% ethanol, and a full-thickness skin defect
139 wound of 8 mm in diameter was made using a sterile skin biopsy punch (BP-80F; Kai
140 Industries, Gifu, Japan). The methicillin-resistant *Staphylococcus aureus* (MRSA) standard
141 strain (ATCC[®]; 43300 MINIPACK[™], Manassas, VA, USA) was incubated overnight at 37°C
142 on Mueller – Hinton agar plates. MRSA was diluted in sterilized saline to adjust to McFarland
143 0.5 standard turbidity and applied to the skin defects. Vaseline (Samhyun Pharmaceutical,
144 Seoul, Korea) was applied to all wounds using a gauze pad to prevent the wound from drying.
145 Then the wounds were covered with Opsite-film (Opsite Flexifix[®]; Smith & Nephew Medical
146 Ltd., Hull, UK) dressings. The dressings were replaced every 3 days.

147

148 *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

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

177

178 *Expression of VEGF and TGF- β 1 in diabetic wound tissue by immunohistochemistry*

179 The paraffin-embedded wound tissue blocks were sectioned, placed on slides, and incubated at
180 60–70°C for 30 min. The sections were deparaffinized in a graded series of xylene and ethanol
181 (100%, 95%, 80%, and 70%) and treated with an endogenous peroxidase blocking agent, which
182 then was diluted with methanol (99%) and a 1.4% hydrogen peroxide solution (FW = 34.01).
183 After 30 min, the slides were washed with 1× Tris-buffered saline (TBS) and sections were
184 blocked with 1.5% normal serum (PK-6102, horse serum, mouse IgG or PK-6101, goat serum
185 rabbit IgG; Vector Laboratories, Burlingame, CA, USA) for 1 h followed by incubation
186 overnight at 4°C with anti-VEGF monoclonal antibody (SC-7269, 1:500 dilution; Santa Cruz
187 Biotechnology) and anti-TGF- β 1 polyclonal antibody (ab92486, 1:1000 dilution; Abcam) in
188 blocking buffer. Then the samples were washed with 1× TBS followed by incubation for 1 h
189 at room temperature with a biotinylated secondary antibody (PK-6102, horse serum, mouse
190 IgG or PK-6101, goat serum rabbit IgG; 1:3000 dilution; Vector Laboratories) in blocking
191 buffer, and washed again with 1× TBS. Incubation with the VECTASTAIN Elite avidin-biotin
192 complex (PK-6102 or PK-6101; Vectastain®; Vector Laboratories) was used to increase the
193 target antigen-antibody reaction during a 30 min incubation. Then the sections were stained
194 with the chromogen diaminobenzidine (C02-100; Liquid DAB + Chromogen Kit; Golden

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

198

199 *Reverse transcription–polymerase chain reaction (RT-PCR)*

200 Total RNA was isolated from wounded skin tissue samples using TRIzol Reagent® (TR118;
201 Molecular Research Center, Cincinnati, OH, USA). mRNA was isolated using chloroform and
202 isopropanol (C2432, I9516; Sigma-Aldrich). After precipitation with isopropanol, the RNA
203 pellet was dissolved in distilled water treated with diethyl pyrocarbonate (DEPC; WR2004;
204 Biosesang, Sungnam, Korea). Total RNA was quantified to 3 µg to react 1 µg/µL random
205 hexamer (C1181; Promega, Madison, WI, USA) at 70°C for 5 min, then incubated at 4°C for
206 5 min. Each reaction contained 1 µL of 10 mM dNTPs, 1 µmol/L MgCl₂, 1 µg/µL RNaseOUT,
207 1 µg/µL 5×reaction buffer, 1 µL GoScript reverse transcriptase (A5003; Promega), and 7 µL
208 RNase-free double-distilled H₂O (N2511; Promega) and was incubated at 25°C for 5 min,
209 followed by incubation at 42°C for 1 h and 70°C for 15 min. The VEGF, TGF-β1, and
210 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer gene sequences were as follows:
211 VEGF forward primer, 5'- CAG GCT GCT GTA ACG ATG AA -3'; VEGF reverse primer,
212 5'- AAT GCT TTC TCC GCT CTG A -3'; TGF-β1 forward primer, 5'- ATT CAG CGC TCA
213 CTG CTC TT-3'; TGF-β1 reverse primer, 5'- TTC TCT GTG GAG CTG AAG CA-3'; and
214 GAPDH forward primer, 5'- CCT TAA ACA GGC CCA CTT GA-3'; GAPDH reverse primer,
215 5'- CCT TCC ACA ATG CCA AAG TT-3' (Table 1). All primers were ordered from

216 Macrogen Oligo (Seoul, Korea). PCR was performed using 1 μ L cDNA in a mixture with 2 \times
217 PCR premix (LGT-1212; Lugen Science Co., Seoul, Korea). The conditions for VEGF
218 included initial denaturation at 95°C for 5 min, melting at 95°C for 30 s, annealing at 60°C for
219 1 min, and elongation at 72°C for 30 s. For TGF- β 1 and GAPDH, annealing was performed at
220 58°C for 30 s. PCR products were visualized using a bioimaging system (C280; Azure
221 Biosystems, Dublin, CA, USA) after electrophoresis on a 1% agarose gel containing Safe Shine
222 Green DNA Staining Solution (GC6051; Biosesang). GAPDH was selected for normalization
223 of specific primer target bands, and density measurements of the target bands were analyzed
224 with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

225

226 *Western blot analysis*

227 The skin tissue was homogenized using complete EDTA free protease inhibitor cocktail
228 (11836170001; Roche Diagnostics GmbH), phosSTOP phosphatase inhibitor (30498800;
229 Roche Diagnostics GmbH), and RIPA buffer (R4200; GenDEPOT, Barker, TX, USA).
230 Dissolved protein was analyzed for protein concentration by micro-scale BCA assay (Micro
231 BCA Protein Assay Kit #23225; Thermo Fisher Scientific Inc., Waltham, MA, USA). Lysates
232 measured at 50 μ g were separated using 10% sodium dodecyl sulfate-polyacrylamide
233 electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane
234 (10600023; GE Healthcare Life Sciences, Chalfont, UK). After blocking with 5% skim milk
235 (232100; Becton Dickinson, Franklin Lakes, NJ, USA) at 4°C for 24 h, the membranes were
236 incubated with anti-VEGF monoclonal antibody (SC-7269; 1:500 dilution; Santa Cruz
237 Biotechnology), anti-TGF- β 1 polyclonal antibody (ab92486; 1:1000 dilution; Abcam), and β -
238 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

261 effects of the red ginseng extract and Rg1 groups were evaluated histologically. Samples of all
262 groups exhibited histological patterns of dermal tissue (consisting of three layers of epidermis,
263 dermis, and subcutaneous tissue). H&E and immunohistochemistry (IHC) staining were
264 performed (Figure 3A–C). The degree of granulation and inflammatory cell infiltration was not
265 different in the three groups at day 4. However, at day 10, granulation tissue was formed in the
266 Rg1 group whereas the control group remained unchanged ($p < 0.05$). On the other hand,
267 inflammatory cell infiltration in the Rg1 group was significantly lower at day 10 compared to
268 the other two groups ($p < 0.05$) (Figure 3D, E). Expression of VEGF and TGF- β 1 was
269 confirmed by IHC staining; VEGF was mainly present in granulation tissues and TGF- β 1 was
270 observed on the wound surface.

271

272 *Expression of VEGF and TGF- β 1 by RT-PCR*

273 We investigated the effects of wound healing on mRNA expression of VEGF and TGF- β 1 in
274 a diabetic wound model. GAPDH was used to normalize the expression level of specific target
275 bands (Figure 4A). At day 7, mRNA expression of VEGF and TGF- β 1 was significantly
276 increased in the red ginseng extract group ($p < 0.05$) and decreased at day 10. The expression
277 of VEGF in the Rg1 group significantly increased on days 4–10 ($p < 0.05$). Expression of TGF-
278 β 1 was observed from day 4 in the red ginseng extract group but gradually increased on days
279 7–10 in the Rg1 group. In the control group, the expression of VEGF and TGF- β 1 was delayed
280 (Figure 4B–C).

281

282 *Expression of VEGF and TGF- β 1 by western blotting*

283 Protein levels of VEGF and TGF- β 1 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- β 1 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- β 1 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- β 1, 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, wound 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

307 in mice [27, 28]. Kim *et al.* [29]. reported that RG promotes angiogenesis by stimulating VEGF
308 and improves the proliferation of epidermal cells by upregulating cytokine expression in
309 keratinocytes. These results suggest that RG accelerates the wound-healing process by
310 increasing TGF- β and VEGF expression in the early stages of wound healing [30]. Diabetes is
311 characterized by the overexpression of factors such as nuclear factor- κ B as well as pro-
312 inflammatory mediators, cytokines, and nitric oxide (NO), which increase intracellular
313 oxidative stress due to insulin resistance and hyperglycemia, resulting in abnormal cells and
314 inhibition of angiogenesis [31]. In addition, high glucose-derived reactive oxygen species
315 affect the onset of diabetic complications [32]. Recent studies have shown that growth-factor
316 regulatory defects in wounds in both diabetic models and diabetic patients exacerbate wound-
317 healing disorders [33, 34]. Although prophylactic treatment with red ginseng contributes to
318 wound healing by controlling the expression of VEGF and TGF- β 1, excessive expression can
319 lead to chronic inflammation and negatively affect wound healing [35, 36, 37]. Therefore, it is
320 important to evaluate the inflammatory response in diabetic wounds and the underlying
321 molecular mechanisms of growth factors. In the present study, we examined whether the
322 administration of red ginseng extract and Rg1 affects expression of VEGF and TGF- β 1 in a
323 diabetic wound model. Our results provide evidence of the wound healing effects of red
324 ginseng extract and Rg1 on the diabetic wound model. To determine whether red ginseng
325 extract and Rg1 are effective for wound healing at mRNA and protein levels, we confirmed the
326 expression of VEGF and TGF- β 1 by RT-PCR and western blotting. Treatment with Rg1 in a
327 diabetic wound model with delayed wound healing resulted in upregulated mRNA expression
328 level of VEGF and TGF- β 1 from the wound-healing inflammation phase to the proliferation
329 phase (4–10 days after the injury) (Figure 4). Treatment with Rg1 also induced protein
330 expression of VEGF and TGF- β 1 during the wound-healing inflammation phase (4–7 days

331 after the injury), resulting in downregulation of expression at day 10 after the wound was fully
332 healed (Figure 5). Other studies have shown that treatment with Rg1 increases angiogenesis
333 and insulin secretion by increasing VEGF expression in the proliferative phase and
334 progressively decreasing VEGF expression over time [38, 39]. In particular, Rg1 treatment
335 reduced wound size compared to the control group (red ginseng extract, Rg1, and control mice
336 were all diabetic). Factors such as TGF- β , PDGF, and interleukin (IL)-1 produced by T cells
337 and macrophages stimulate a variety of growth factors; the expression of VEGF during the
338 inflammatory stage also plays an important role in wound healing [40, 41, 42]. VEGF is an
339 angiogenic cytokine that contributes to the proliferation phase of wound healing by promoting
340 angiogenesis and the formation of fibrous cells, collagen, and granulation tissue around the
341 wound [43, 44]. Many studies have focused on VEGF as a factor promoting angiogenesis and
342 its role in various diseases [45, 46]. In the remodeling phase, VEGF induces cell degranulation,
343 fibroblast proliferation, and migration of existing blood vessels by proteases; fibroblasts are
344 transformed into α -smooth muscle actin bundles similar to smooth muscle cells, resulting in
345 contraction of the wound [47, 48]. Nogami *et al.* [49] reported that VEGF mRNA levels were
346 reduced significantly in diabetic animal models compared to normal models; thus, regulatory
347 defects in VEGF delayed wound healing. Our results also showed that both red ginseng extract
348 and Rg1 were affected by a delayed wound healing due to a lack of VEGF and TGF- β 1
349 expression in untreated diabetic wounds (i.e., in the control group). In our histological analyses,
350 both red ginseng and Rg1 treatment promoted formation of granulation tissue at days 7–10
351 after injury; wound contraction, reduction of inflammatory cells, and promotion of growth
352 factors were observed during reconstruction at day 10. Treatment of tissues with Rg1
353 contributes to wound healing by promoting collagen production and VEGF expression [50,
354 51]. Red ginseng positively affects wound healing by modulating VEGF and TGF- β 1

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- β 1; 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- β 1 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- β 1, 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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378

379 **Availability of data and materials**

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

382

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

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555 **Figure 1.** Protocol for wound induction.

556 STZ-induced diabetic mice were orally administered red ginseng extract (10 mg/kg) and Rg1
557 (10 mg/kg). Wounds were made on day 0 and measured on days 1, 4, and 7. Mice were
558 sacrificed on days 4, 7, and 10. Mice were randomly divided into three groups according to
559 oral-administration treatment. G1: red ginseng extract treatment group (10 mg/kg/day, $n = 27$);
560 G2: Rg1 treatment group (10 mg/kg/day, $n = 27$); G3: control (PBS) group (0.3 mL/kg/day, n
561 = 27). STZ: streptozotocin, Rg1: ginsenoside Rg1.

562

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

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

569

570 **Figure 3.** Histology and IHC analysis.

571 For histological analysis, the slides corresponding to days 4, 7, and 10 for each group were
572 stained with hematoxylin and eosin (H&E). IHC staining of VEGF and TGF- β 1 (important
573 factors for wound healing) was observed. (A) Tissues from mice of each group sacrificed 4
574 days after injury (H&E stain in the left panels, VEGF antibody IHC stain in the middle panels,
575 and TGF- β 1 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

599 **Figure 5.** Effects of red ginseng extract and Rg1 on the expression of VEGF and TGF- β 1 in
600 diabetic wounds indicated by western blot analysis. (A) Immunoblotting was performed for
601 diabetic mouse tissue using VEGF and TGF- β 1 antibodies. β -actin was used to normalize the
602 intensity of the analyzed bands. (B–C) Protein expression of VEGF and TGF- β 1 increased
603 significantly in the red ginseng extract and Rg1 groups during the wound healing period (4–10
604 days after injury) and then decreased with time. Significance was determined using the Mann–
605 Whitney U test. Mice were divided into three groups (red ginseng extract group, Rg1 group,
606 PBS-treated group as a control group). * $p < 0.05$ compared to the control group and # $p < 0.05$
607 compared to the treatment group. TGF: transforming growth factor, VEGF: vascular
608 endothelial growth factor, β -actin: beta-actin.

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626 **Tables**

627

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

629 VEGF, vascular endothelial growth factor; TGF- β 1, transforming growth factor- β 1; GAPDH,

630 glyceraldehyde-3-phosphate dehydrogenase. ¹⁾All primers were designed directly and

631 purchased from Macrogen Corporation (Seoul, Republic of Korea). The primers for VEGF,

632 TGF- β 1, and GAPDH used in this experiment are shown in the table and reverse transcription-

633 polymerase chain reaction was performed using each primer.

634

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- β 1	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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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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663 **Table 3.** Degree of inflammatory cells infiltration score.

664 The degree of infiltration of inflammatory cell was scored in mouse skin tissue. Hematoxylin

665 and eosin staining was performed histologically to evaluate the degree of infiltration of

666 inflammatory cell.

667

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

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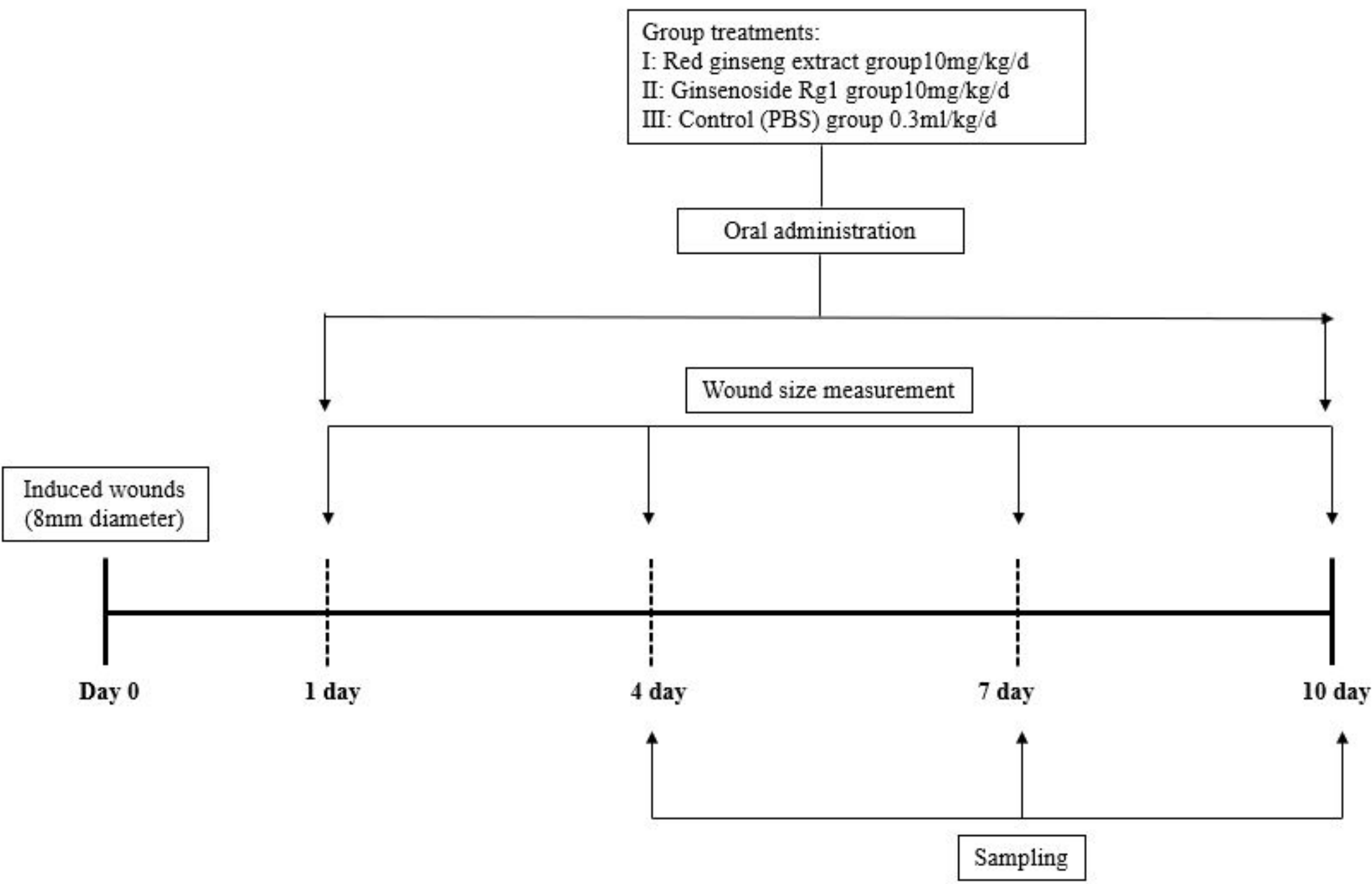


Figure 1

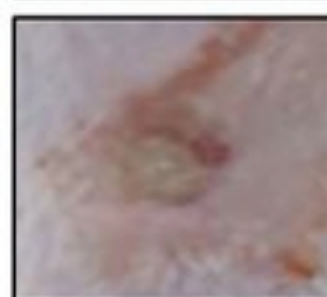
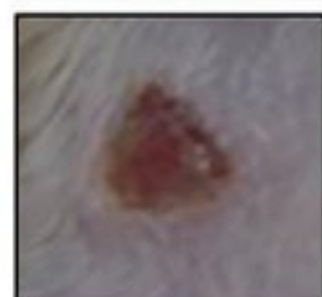
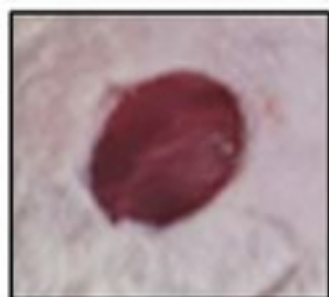
A

Diabetic wounds

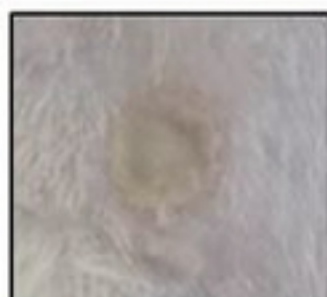
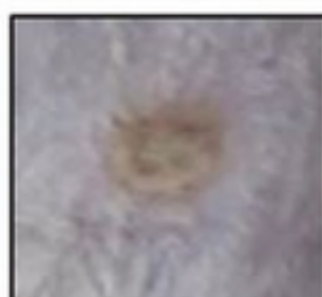
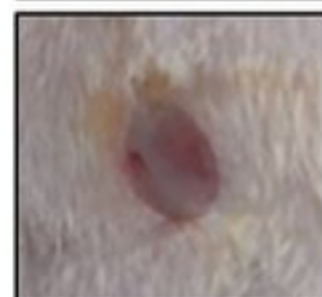
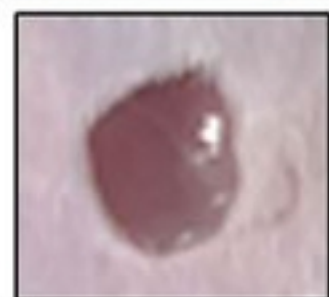
**Control (PBS)
group**



**Red ginseng extract
group**



**Ginsenoside Rg1
group**



0 day

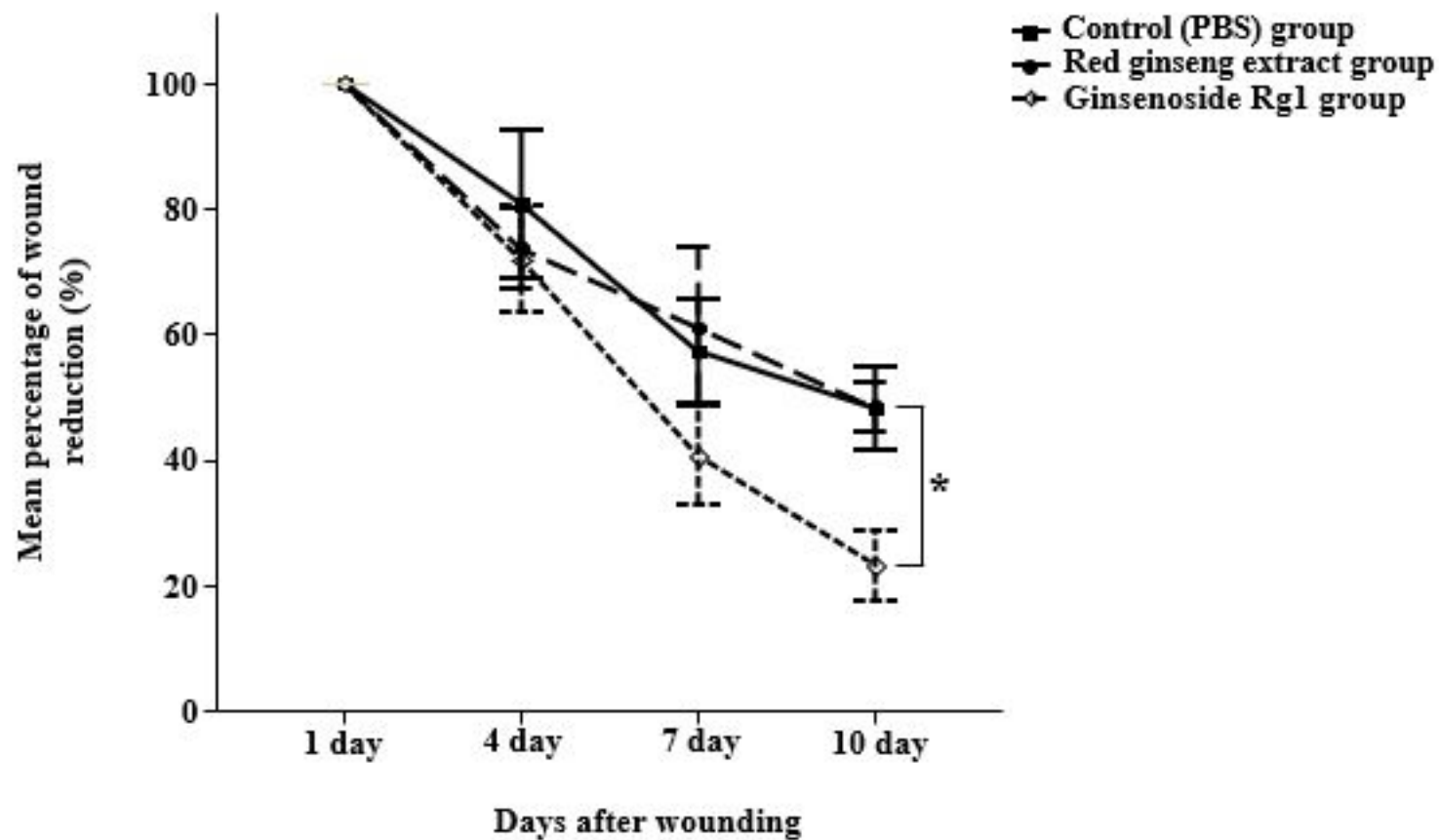
1 day

4 day

7 day

10 day

Figure 2a

B**Figure 2b**

A

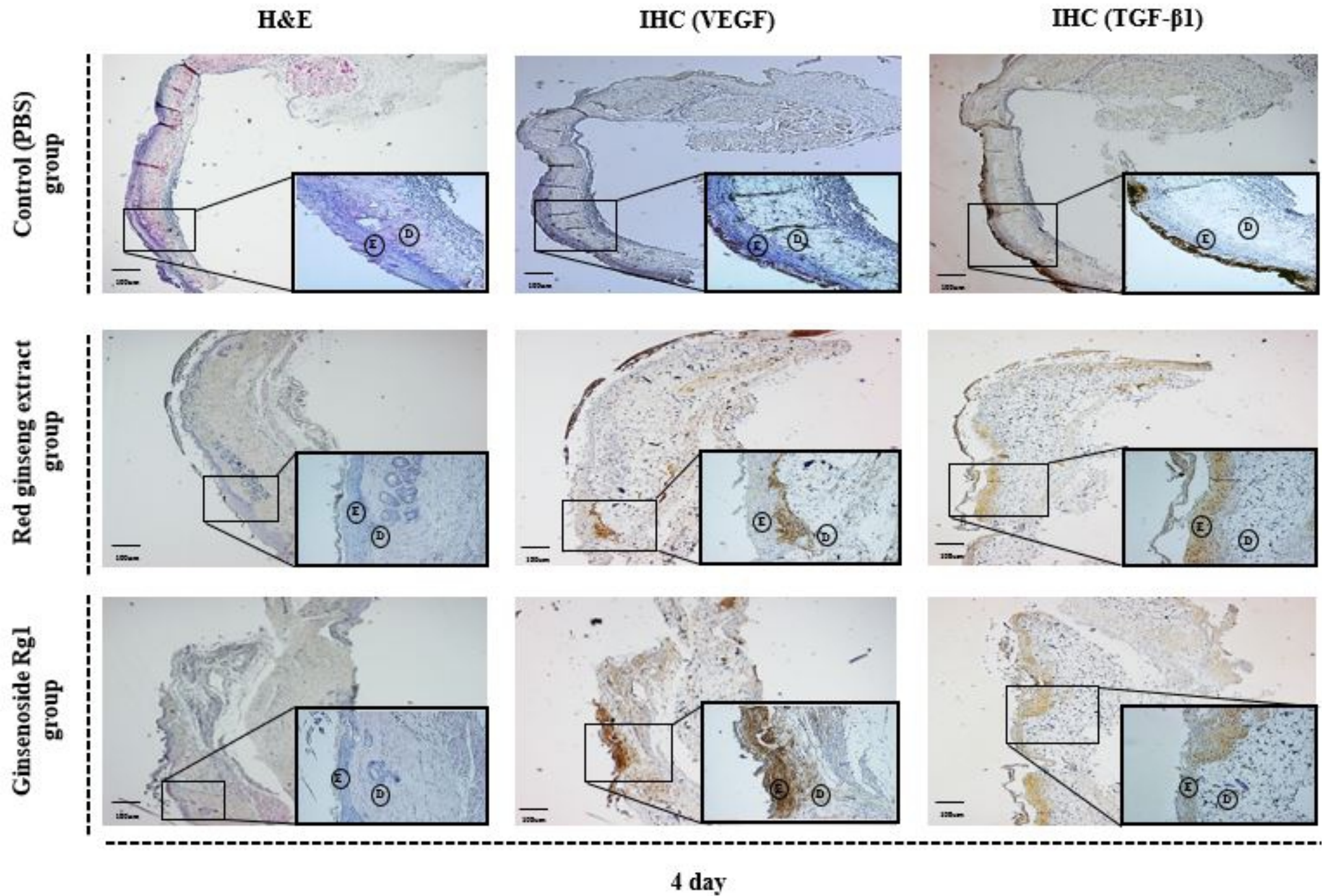
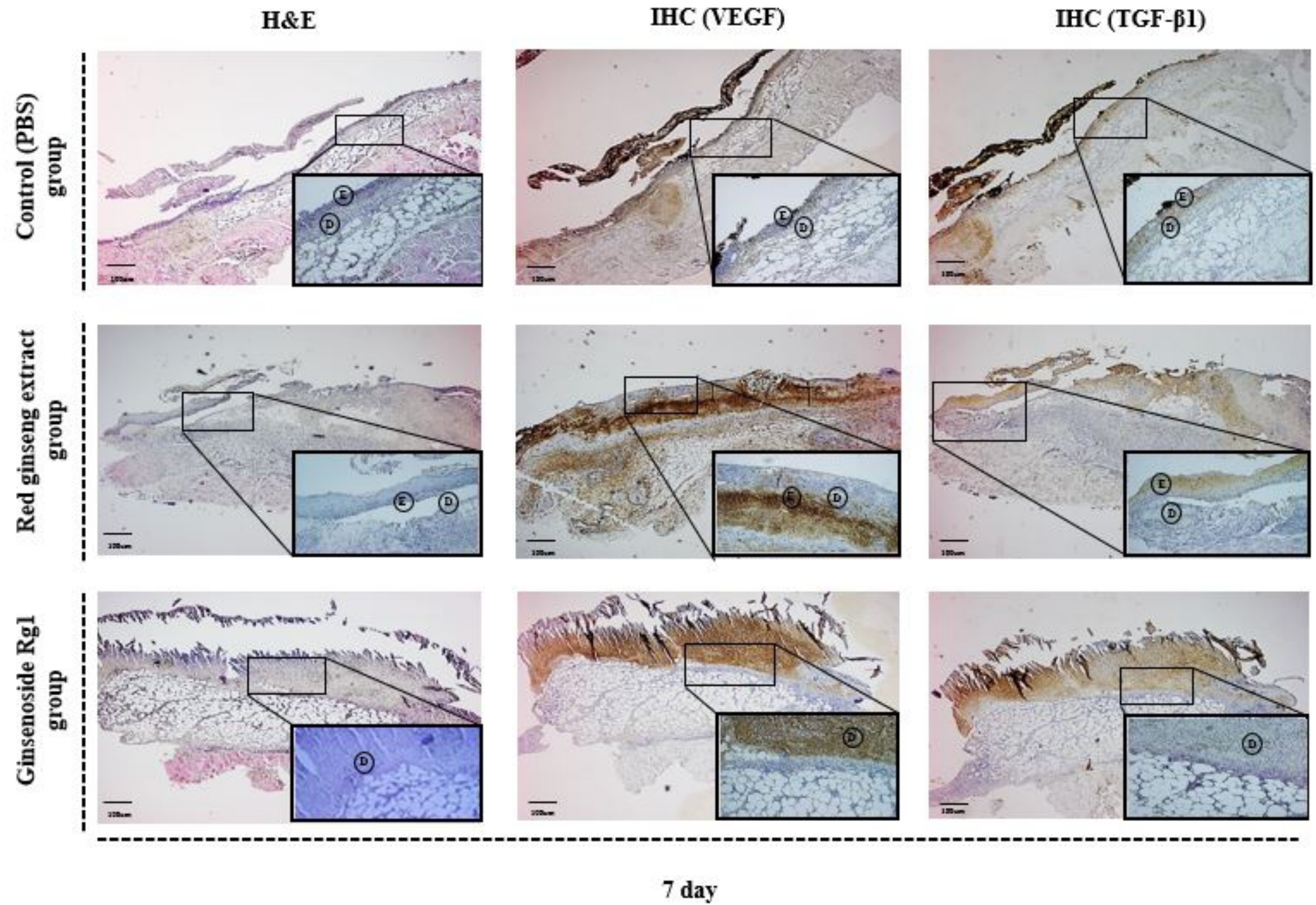


Figure 3a

B**Figure 3b**

C

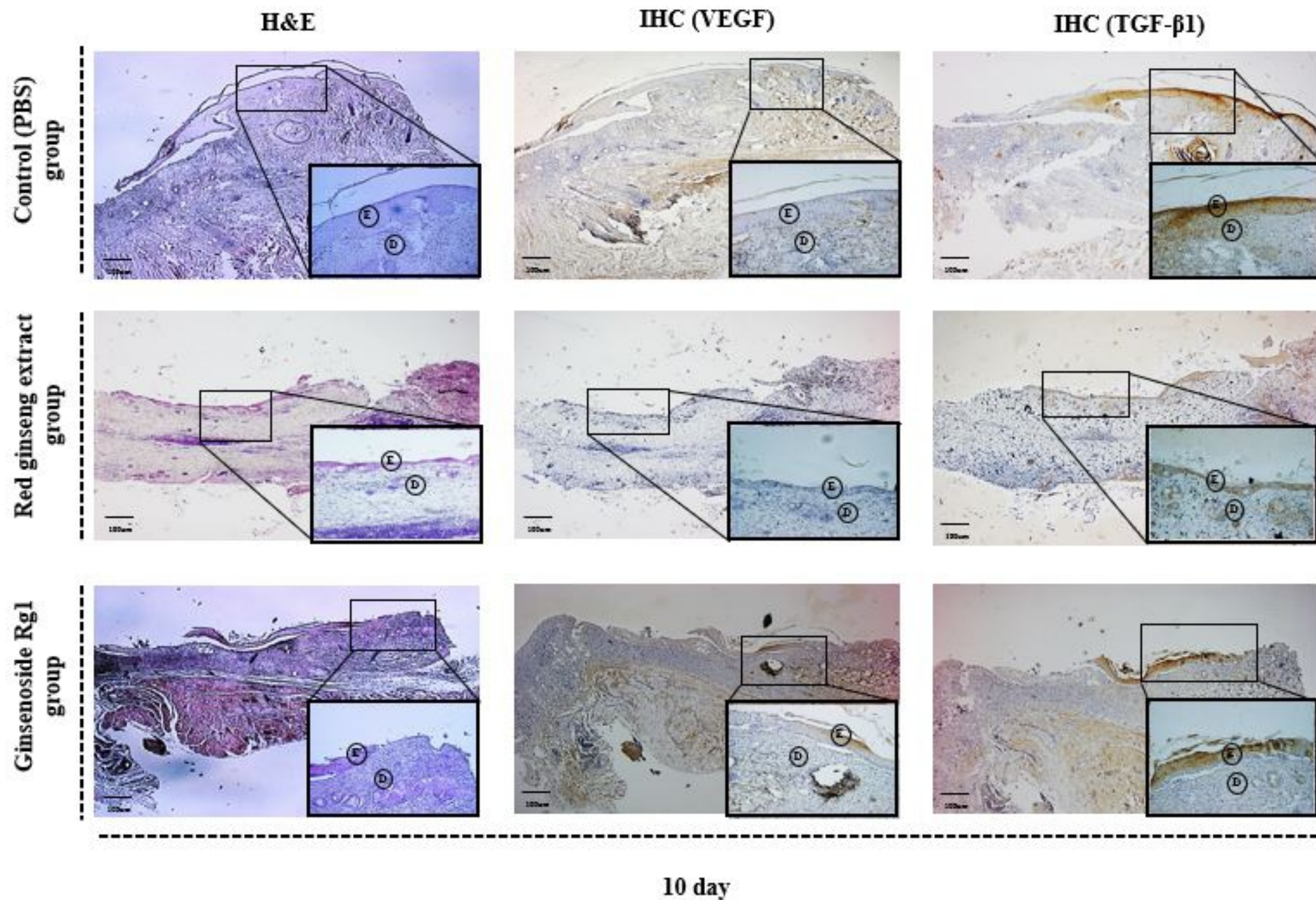
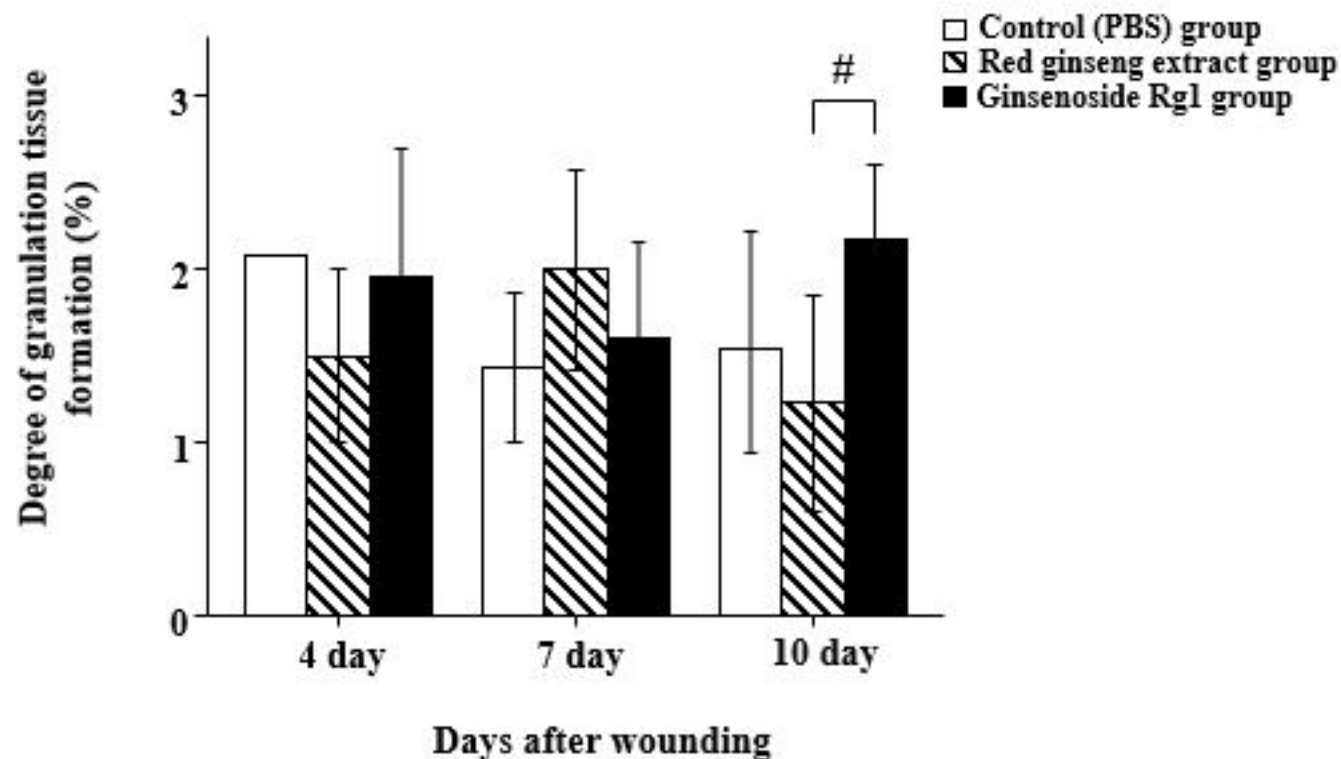
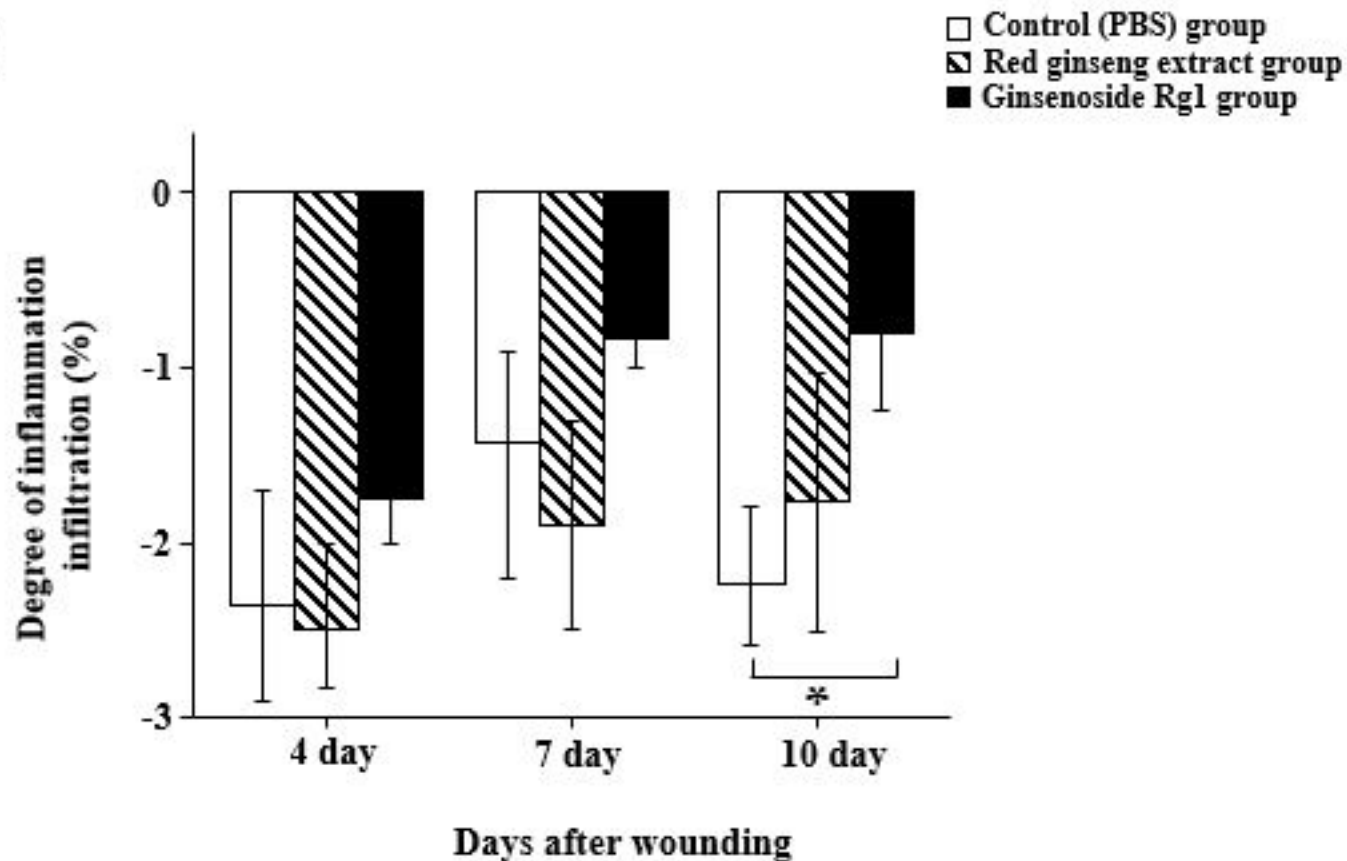
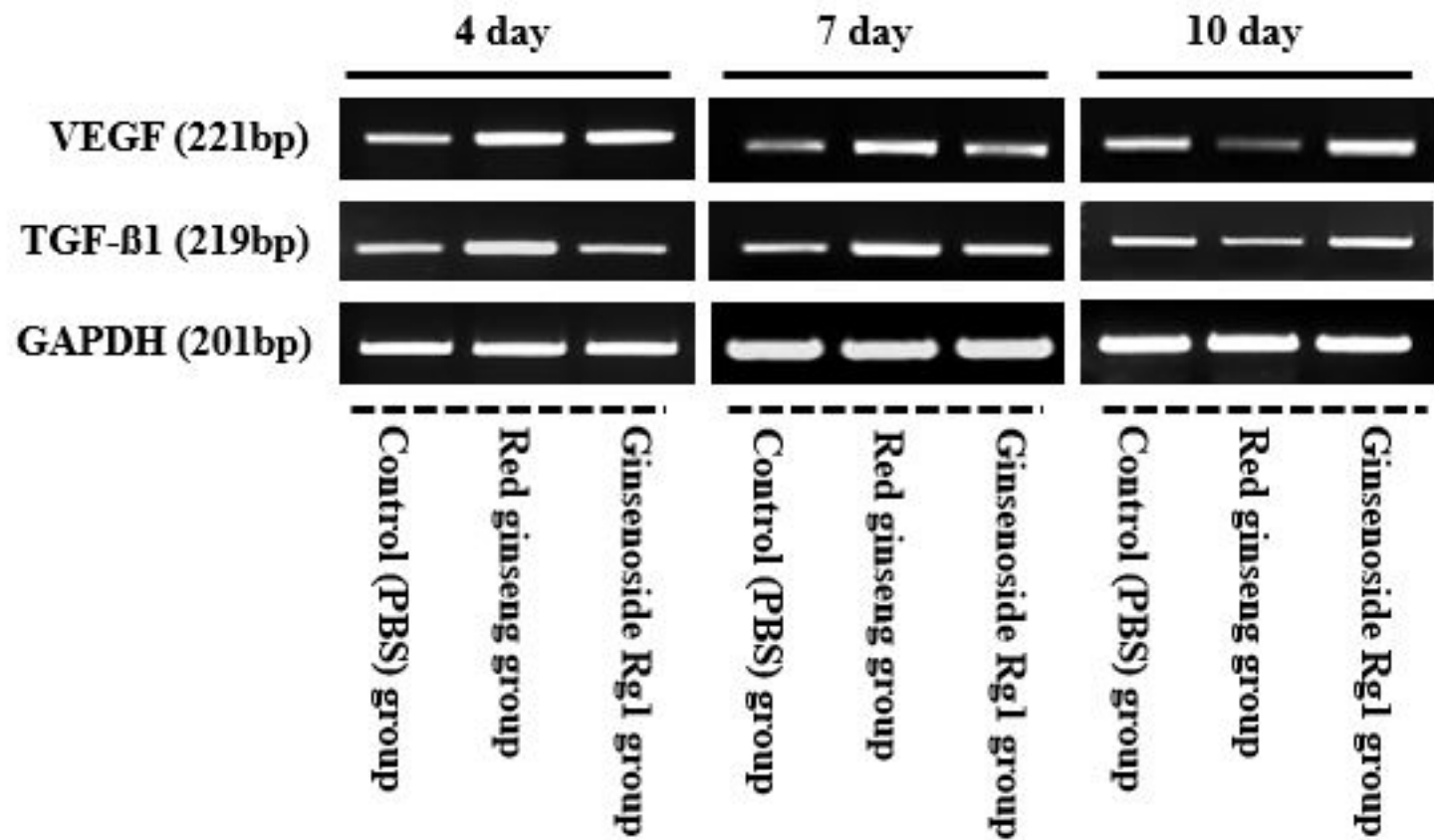


Figure 3c

D**Figure 3d**

E**Figure 3e**

A**Figure 4a**

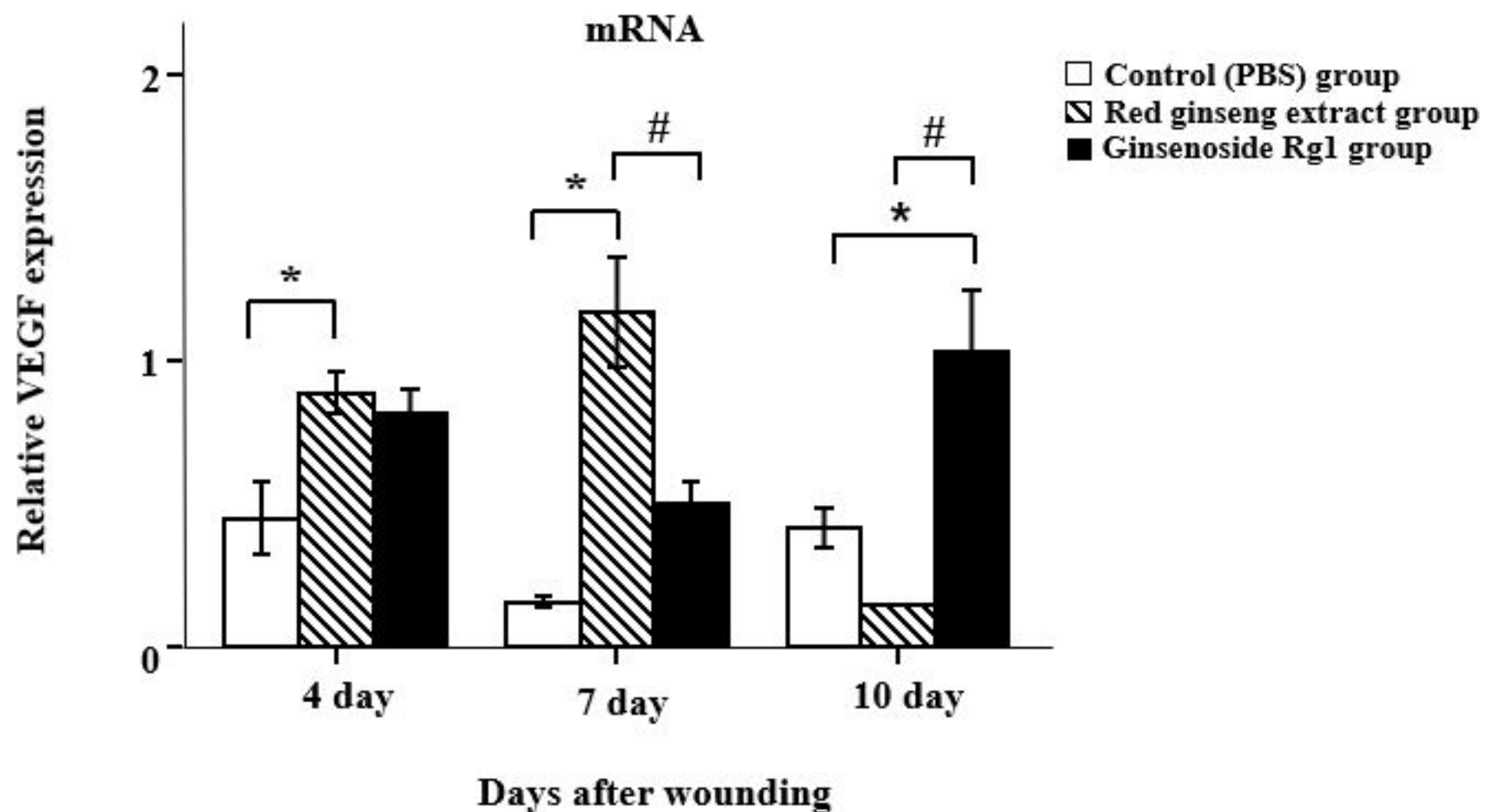
B

Figure 4b

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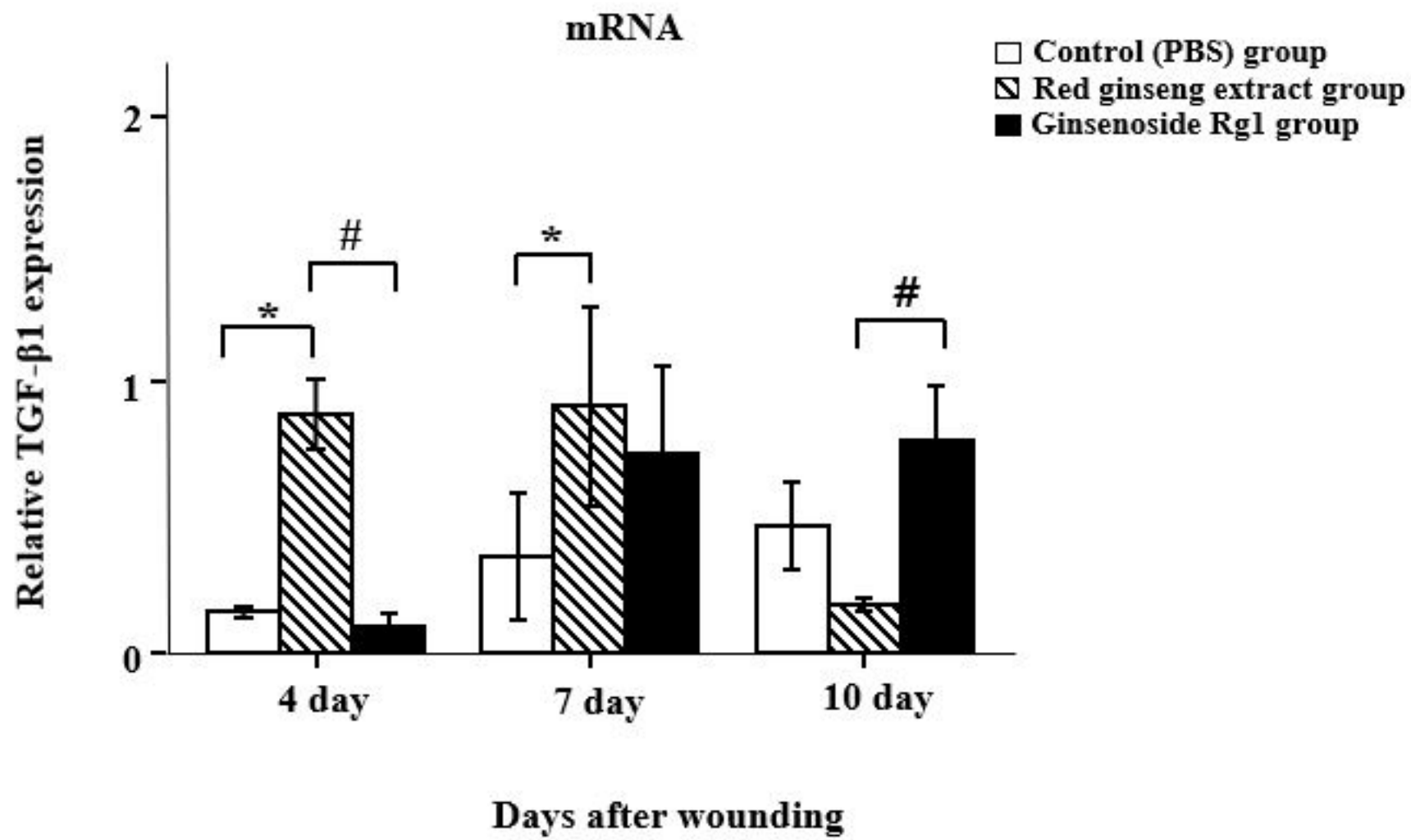


Figure 4c

A

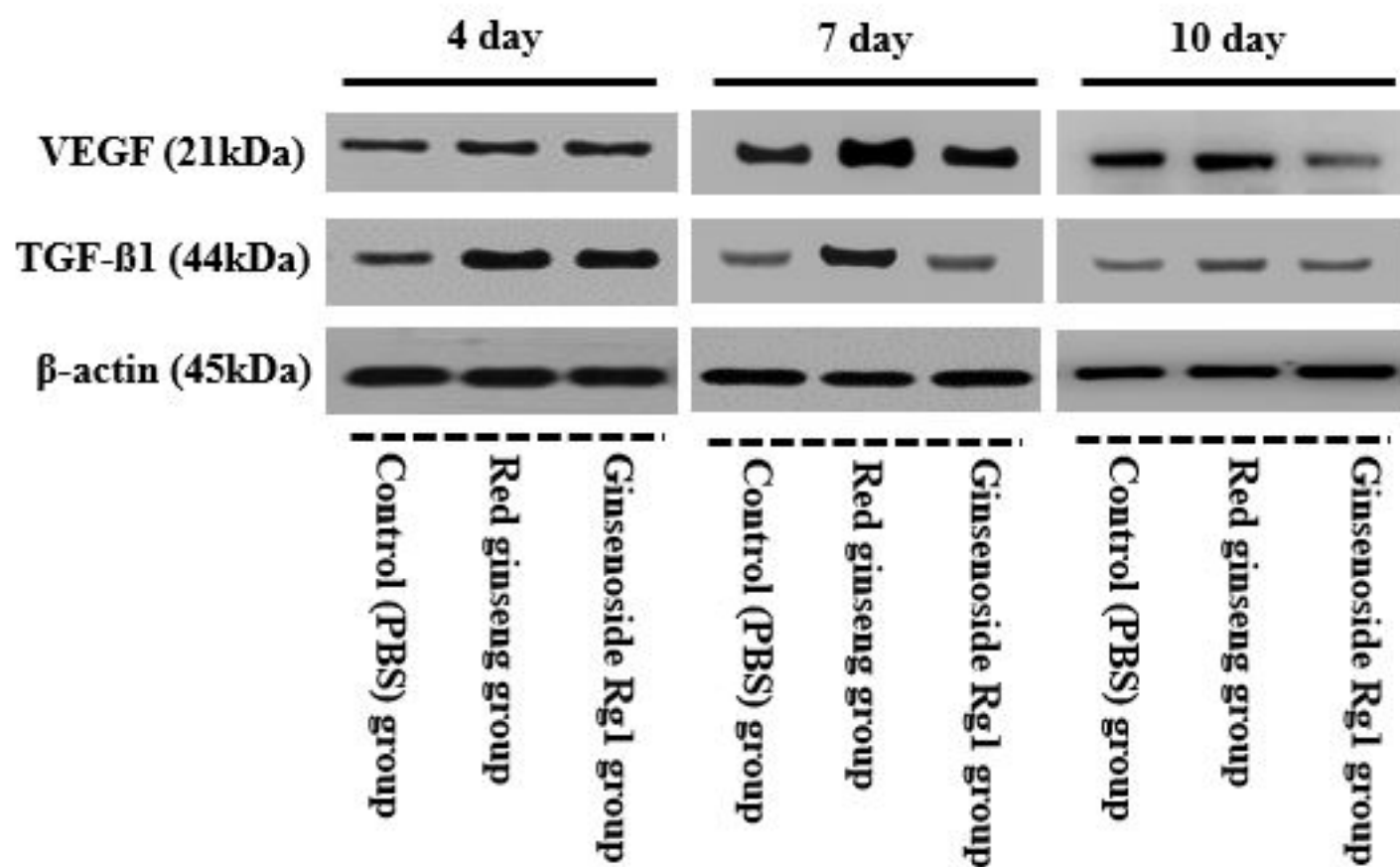


Figure 5a

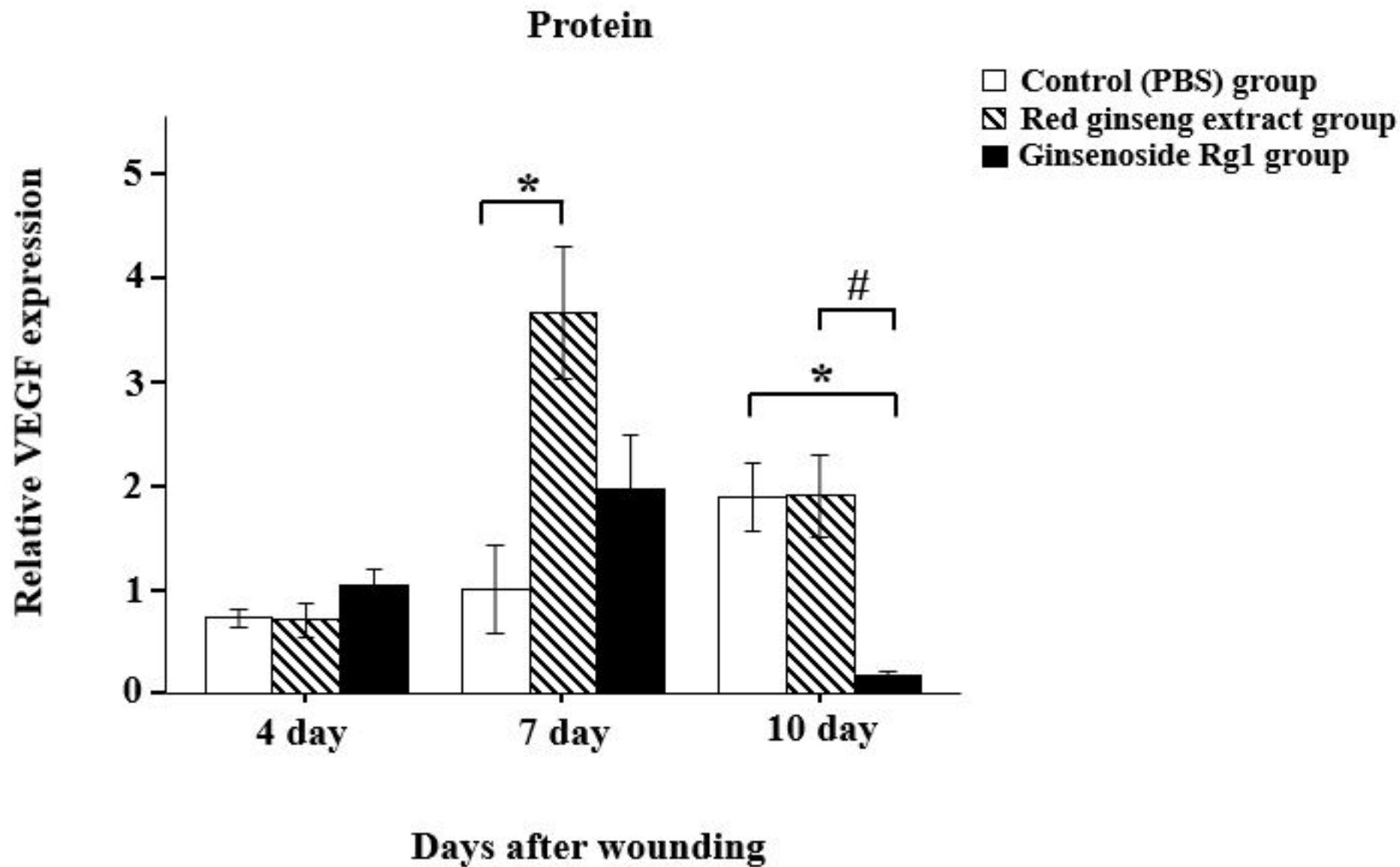
B

Figure 5b

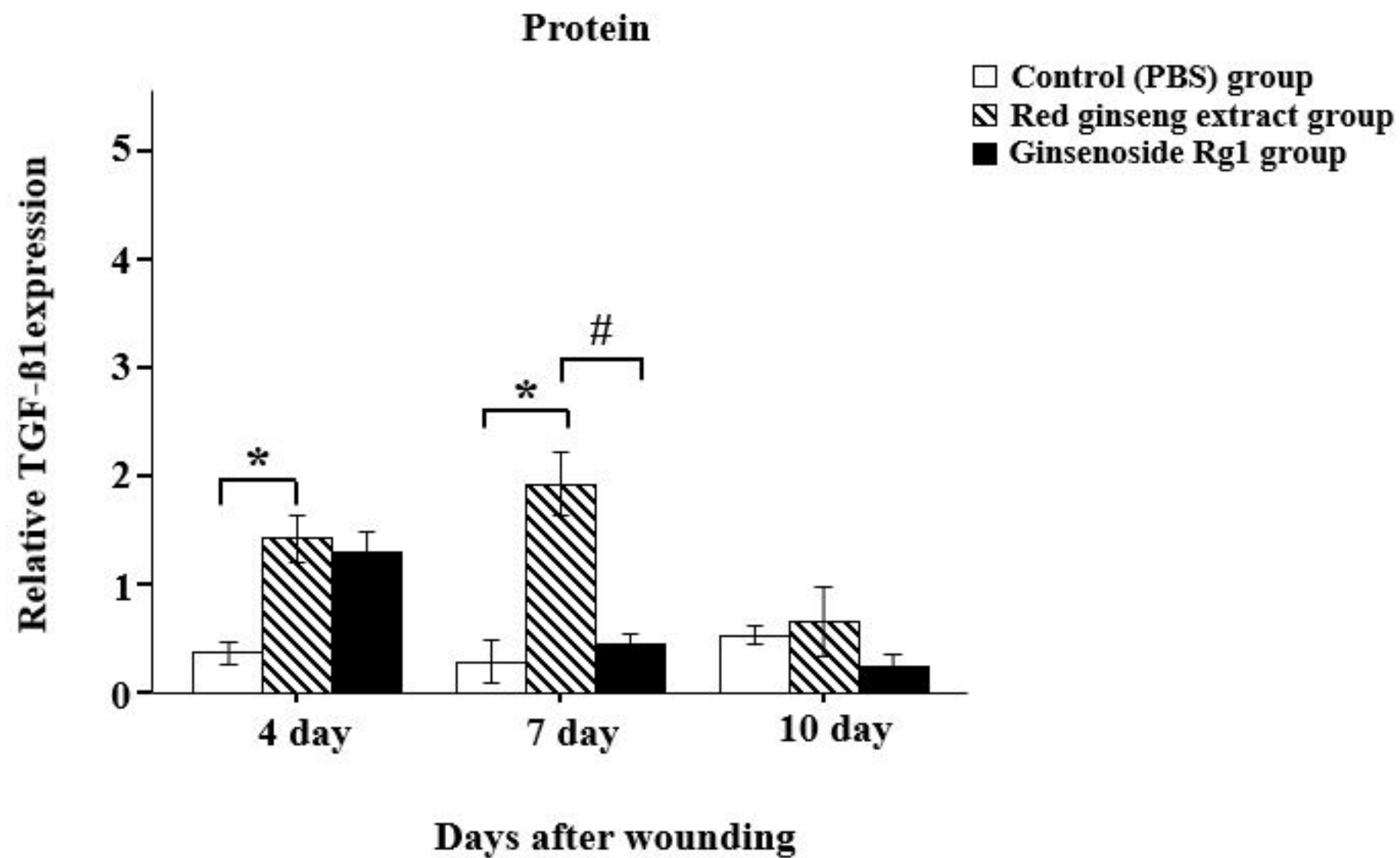
C

Figure 5c