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ORIGINAL ARTICLE

Inflammatory responses bridge comorbid cardiac disorder in experimental model of IBD induced by DSS: protective effect of the trigonelline

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Abstract

Pathogenesis of the inflammatory bowel disease (IBD) involves the combination of immunological and inflammatory factors. IBD is associated with several extra-intestinal manifestations. The exact underlying bridge between the probable cardiac diseases in IBD patients is undetermined. Trigonelline is an alkaloid with several therapeutic potential properties. In this study, we aimed to assess the probable underlying mechanisms of this comorbidity as well as protective effect of trigonelline focusing inflammatory response and oxidative state in mouse model of colitis. Dextran sodium sulfate (DSS) was used for induction of colitis in mice. Trigonelline (10, 50 and 100 mg/kg) was administrated via intraperitoneal rout (i.p.) for 14 continuous days. Heart, intestine and serum samples were taken for assessment of total antioxidant capacity, malondialdehyde (MDA), gene expressions of inflammatory markers including tumor necrosis factor alpha (*Tnf-α*), interleukin 1-beta (*Il/1β*), toll- like receptor 4 (*Tlr4*) as well as for evaluation of histopathological alterations. Results demonstrated that trigonelline effectively attenuated the cellular/molecular and histopathological adverse effects of colitis in the intestine and heart tissues. In this regards, we found that trigonelline decreased the MDA level, attenuated the expression of *Tnf-α*, *Il/1β* and, *Tlr4* as well as modulated the histopathological alterations in the intestine. Furthermore, trigonelline increased the antioxidant capacity in the related experimental groups. We concluded that IBD (colitis) is associated with comorbid cellular/molecular modifications in the heart and for the first time, we found that trigonelline has potential therapeutic effects (at least partially) to attenuate the cardiac manifestations of the colitis.

Keywords Inflammatory bowel disease · Cardiac complications · Comorbidity · Trigonelline · Mice

Introduction

Inflammatory bowel disease (IBD) is categorized into ulcerative colitis (UC) and Crohn's disease (CD), and leads to long-term and sometimes irreversible disturbances of gastrointestinal structure and function (Xavier and Podolsky 2007; Blumberg and Strober 2001). The common hypothesis on the cause of IBD is the combination of immunological and inflammatory factors (Bouma and Strober 2003). In this regards, it has been determined that gastrointestinal microflora acts as initiators for activation of the inflammation in the intestine (Pavli et al. 1996). In addition, ectopic activation of the immune system has a critical role in the IBD pathogenesis such that immunosuppressant such as steroids and azathioprine are effective in treatment of IBD (Danese et al. 2005).

It has been well known that IBD leads to several extra-intestinal complications, including eye, kidney, liver, muscle and pulmonary complications. There is an ample evidence showing that IBD patients are at high risk of developing cardiovascular diseases (Thapa et al. 2015; Schicho et al. 2015). In this concept, inflammatory markers which are increased in patients with IBD have a direct association with cardiovascular diseases. Previous studies have demonstrated that inflammation involves in the pathogenesis of both IBD and cardiovascular disorders (Danese et al. 2005). However, current evidences are insufficient to confirm that IBD patients are at increased risk

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of developing cardiovascular disorders (McKenzie et al. 1996). Thus, further studies warranted to clarify possible pathogenic links between cardiovascular disorders with IBD.

The intestinal mucosa has receptors called toll- like receptor 4 (Tlr4) identifying bacteria-derived LPS. The activation of *Tlr4* activates the *NF-κB* signaling pathway resulting in the production of cytokines and inflammatory markers such as *Tnf-α* and *Il-1β* (Laird et al. 2009). *Tnf-α* and *Il-1β* increase the permeability of vasculature as well as gut mucosa leading to migration of LPS from the intestinal lumen to the mucosa and subsequently to the circulation (Al-Sadi and Ma 2007; Popivanova et al. 2008).

Trigonelline is an alkaloid present in fenugreek and coffee. Various pharmacological effects have been reported for trigonelline including blood glucose lowering, antihyperlipidemic, antihyperglycemic and antiinflammatory effects (Antonisamy et al. 2016; Zhou et al. 2017). It has been determined that trigonelline exerted a potential therapeutic effect on gastrointestinal ulcers via reduction of inflammation as well as potentiation of antioxidant capacity (Antonisamy et al. 2016). Literature suggested that trigonelline potently reduced the levels of *Il-6*, *Il-1β*, and *Tnf-α* (Yoshinari et al. 2013; Zhou et al. 2017).

Considering there are evidences which demonstrated that (1) IBD patients are at high risk for developing cardiac disorders, (2) trigonelline possessed various pharmacological properties in inflammatory states, in the current study, we aimed to examine the possible protective effect of trigonelline against cardiac disorders which are comorbid with the IBD in the mice model of IBD disease induced by DSS.

Materials and methods

Chemicals

Trigonelline, dextran sulfate sodium (DSS) (MW 36,000–50,000) were purchased from the Sigma (Sigma, USA).

Animals

Seventy-two male NMRI mice (20–25 g) (Pasteur Institute of Iran, Tehran, Iran) were used in this study. Animals were kept under standard laboratory conditions. All procedures were carried out in accordance with the National Institutes of Health (NIH) Guideline for the Care and Use of Laboratory Animals (NIH publication #80–23) and institutional guideline for animal care and use (Shahrekord University of Medical Sciences, SKUMS) with ethical code: IR.SKUMS.REC.1395.302.

Induction of experimental IBD

To induce chronic experimental colitis, dextran sodium sulfate (DSS) was dissolved in drinking water (concentration of 5%). Mice were given DSS solution for 7 days, in four cycles. The intervals between cycles are 10 days (Kojouharoff et al. 1997).

Study design

Mice were divided into nine experimental groups and treated continuously for 14 days (Fig. 1). Groups were included as follows: group 1: IBD was induced and received normal saline for 14 days, group 2: control group (without colitis) received saline for 14 days, groups 3–5: intact mice (without colitis) received trigonelline at doses of 10, 50 and 100 mg/kg for 14 days, groups 6–8: experimental colitis mice (IBD was induced) received trigonelline at doses of 10, 50 and 100 mg/kg for 14 days. Treatments were begun at 58th day of the study for 14 constant days. At the end of the study (day of 72), mice were killed under light ether anesthesia, and heart, colon, and blood samples were immediately collected (Liu et al. 2018). We have collected our samples from the distal section of colon (Swidsinski et al. 2005) and midpart of the heart (Gabriels et al. 2012).

Trigonelline was dissolved in the saline and injected via the intraperitoneal (ip) route. Dose and time of drug administration were chosen according to our pilot study as well as previous studies (Antonisamy et al. 2016, Mirzaie et al.

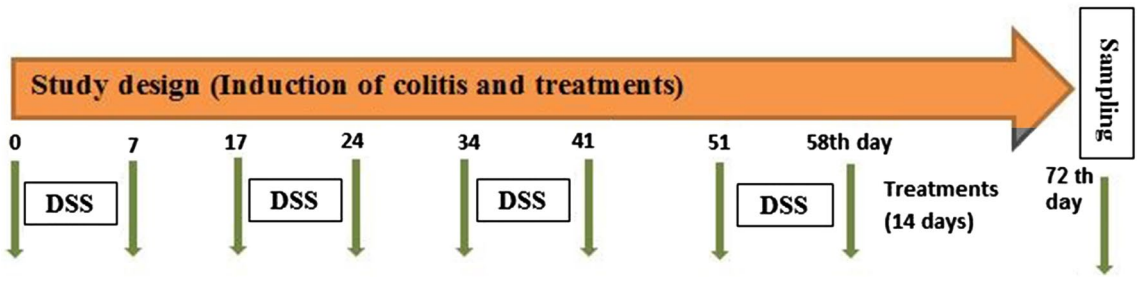

Fig. 1 Schematic of the study design

2016). Mice were divided into nine groups ($n=8$). All trial groups involved eight mice and we tried to minimize the use of animals and to improve their well-being.

Histopathological examination

Colon and heart samples were dissected out and then fixed in 10% formaldehyde and embedded in paraffin. For each sample, 5-µm sections from the paraffin blocks were obtained and processed for routine hematoxylin–eosin (H&E) staining. Of each experimental group, eight samples ($n=8$) were prepared for histopathological assessment. Five sections from each sample were examined for grading.

The microscopic scoring for colon tissue was performed as follows: Epithelium (E): 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the L. submucosa. The total histological score represents the sum of the epithelium and infiltration scores (total score = E + I) (Haj-Mirzaian et al. 2017; Amini-Khoei et al. 2019).

Microscopic grading for heart samples was performed according to the method described previously. The extent of cardiac inflammation was scored as follows: minimal (grade 1) changes involved 1–10% of the section; mild (grade 2) involved 11–40%; moderate (grade 3) involved 41–80%; and severe (grade 4) involved 81–100% (Zhang et al. 2007; Nyska et al. 2004).

Determination of gene expression

RNA extraction was carried out using RNX-plus isolation reagent according to the manufacturer's instructions. RNA was quantified using Nanodrop technologies. Alterations in the mRNA expression of genes were also performed by Real-time polymerase chain reaction (PCR). After reverse transcription of mRNA with PrimeScript RT reagent kit (Takara) according to the manufacturer's instruction, the qRT-PCR experiment was done on a light cycler apparatus (Rotor gene Diagnostics) using the SYBR Premix Ex Taq technology (Takara). The thermal cycling program profile was 95 °C for 30 s and followed by 45 cycles of denaturation for 5 s at 95 °C, and annealing step for 15 s at 60 °C and extension for 15 s at 72 °C. Melting curve analysis was applied to confirm whether all primers yield a single PCR product. Histone H2A variant, *H2afz*, was amplified as a normalizer and fold changes in expression of each target mRNA relative to H2afz were calculated based on $2^{-\Delta\Delta Ct}$ relative expression formula, as described earlier. The primer sequences are demonstrated in Table 1.

Determination of total antioxidant capacity

Ferric reducing/antioxidant power (FRAP) assay was performed to measure total antioxidant capacity in the serum, colon and heart samples according to the previous studies. In brief, the antioxidant power of samples was determined by measuring its ability to reduce Fe3+ to Fe2+ with FRAP (ferric reducing antioxidant power) test. FeSO4 (100–1000 µM concentration range) was used as a standard in FRAP assay (Rahnama et al. 2015; Luque-Sierra et al. 2018).

Measurement of the malondialdehyde (MDA)

The malondialdehyde (MDA) concentration in the serum, colon and heart samples was measured spectrophotometrically as described previously (Zou et al. 2016; Wong et al. 1987). Briefly, trichloroacetic acid and a TBARS reagent were added to the supernatant, then mixed and incubated in boiling water for 90 min. After cooling on ice, the samples were centrifuged at 1000*g* for 10 min, and the absorbance of the supernatant was read at 532 nm. The TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Statistical analysis

Comparison between the groups was analyzed using one-way ANOVA followed by Tukey's post hoc test in the GraphPad Prism software (version 7). $p < 0.05$ was considered as statistically significant.

Table 1 Primer sequences

Primer	Forward sequence	Reverse sequence
H2afz	TCATCGACACCTGAAATCTAGGA	AGGGGTGATACGCTTTACCTTTA
TNF-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
Il-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA

Results

Trigonelline increased the antioxidant capacity in the intestine, heart and serum samples

Findings (Fig. 2) showed that experimental colitis significantly decreased the antioxidant capacity in the intestine ($p < 0.01$) and heart ($p < 0.001$) tissues as well as serum samples ($p < 0.001$) when compared with the control group. In addition, we found that trigonelline at doses of 50 and 100 mg/kg significantly increased the antioxidant capacity in comparison with the saline treated colitis group in cardiac, intestinal tissues and serum samples significantly increased the antioxidant capacity in comparison with the saline-received colitis counterpart. Furthermore, trigonelline at the dose of 10 mg/kg significantly increased the antioxidant capacity of the serum sample compared with the saline-received colitis counterpart ($p < 0.05$). In aspect of the control counterparts, our results showed that trigonelline at the dose of 100 mg/kg increased the antioxidant capacity in the heart tissue when compared with the saline-receive control counterpart ($p < 0.01$). Trigonelline at doses of 10 mg/kg ($p < 0.05$), 50 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.001$) significantly increased the antioxidant capacity of the intestine tissue in comparison with the saline-received counterpart. Moreover, trigonelline at doses of 10 mg/kg ($p < 0.01$), 50 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.001$) significantly increased the antioxidant capacity of the serum samples in comparison with the saline-received counterpart.

Trigonelline decreased the levels of MDA in heart, intestine and serum samples

The MDA level of experimental colitis (the intestine ($p < 0.01$), heart ($p < 0.001$) tissues and serum samples ($p < 0.001$)) was significantly increased when compared with the control group (Fig. 3). In addition, we found that trigonelline at dose of 50 mg/kg [for heart ($p < 0.01$) and intestine tissue ($p < 0.001$) and at dose of 100 mg/kg (for heart ($p < 0.001$), intestine tissue ($p < 0.001$) and serum samples ($p < 0.01$)] significantly decreases the MDA level in comparison with the saline-received colitis counterpart. Furthermore, trigonelline at the dose of 10 mg/kg significantly decreased the MDA level of the intestine in compared with the saline-received colitis counterpart ($p < 0.05$). Considering the control counterparts our results showed that trigonelline at the dose of 100 mg/kg decreased the MDA level in the

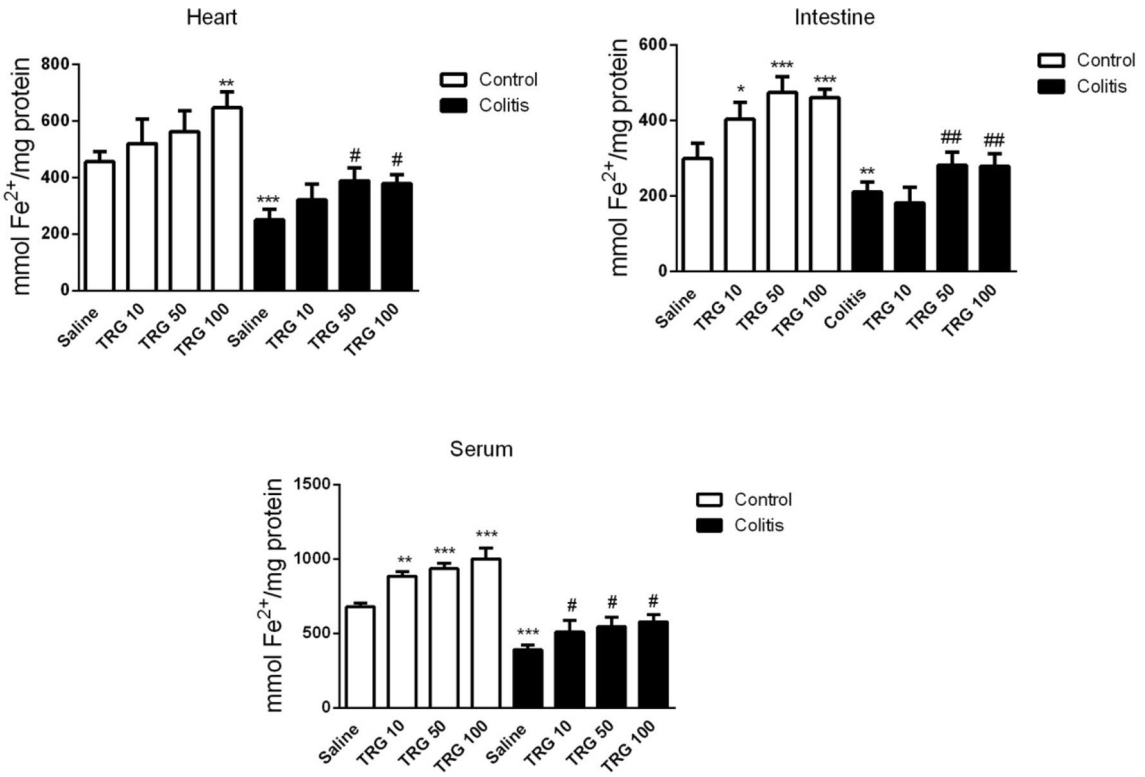

Fig. 2 Antioxidant capacity was evaluated in the colon, heart and serum samples using FRAP method. Data are expressed as mean ± SEM and analyzed with one way ANOVA followed by tukey's post- test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the saline-received control group, #$p < 0.05$ and ##$p < 0.01$ compared with saline-received colitis group. *TRG* trigonelline

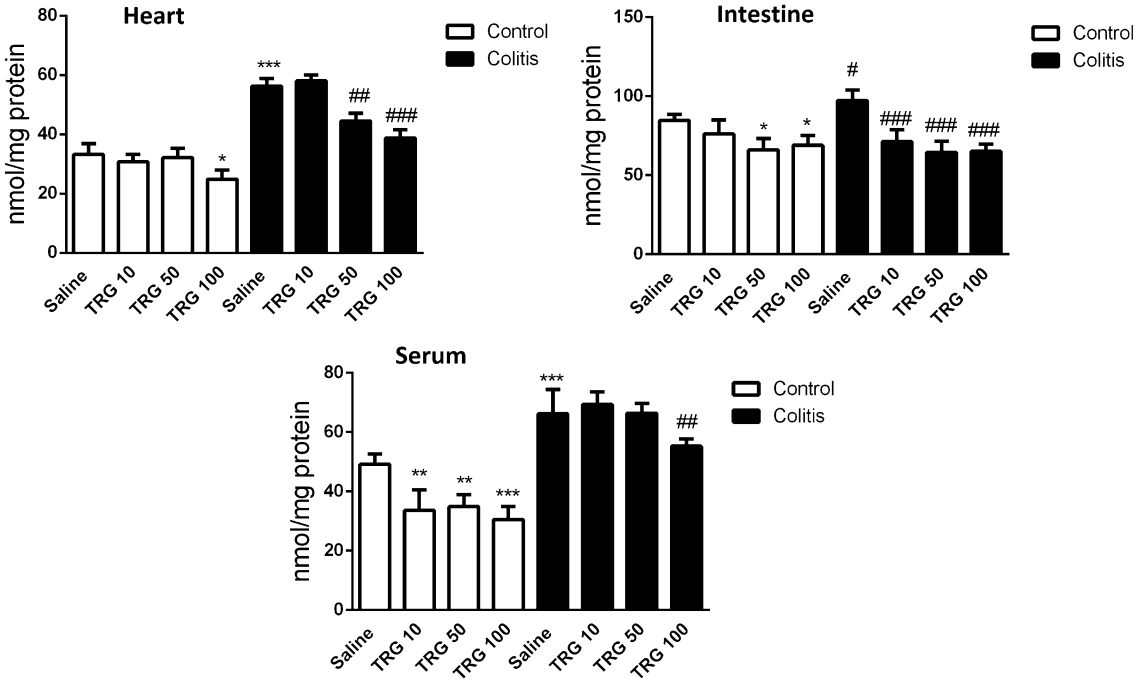

Fig. 3 The level of MDA was evaluated in the colon, heart and serum samples. Data are expressed as mean ± SEM and analyzed with one way ANOVA followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the saline-received control group, #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with saline-received colitis group. *TRG* trigonelline

heart tissue when compared with the saline-receive control counterpart ($p < 0.01$). Trigonelline at doses of 50 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.05$) significantly decreased the MDA level of the intestine tissue in comparison with the saline-received counterpart. Moreover, trigonelline at doses of 10 mg/kg ($p < 0.01$), 50 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.001$) significantly decreases the MDA level of the serum samples in comparison with the saline-received counterpart.

Trigonelline decreased the expression of inflammatory genes of *Tnf-α, Il/1β*, and *Tlr4* in the intestine and heart tissues

Experimental colitis led to a significant increase in the gene expression of *Tnf-α* ($p < 0.001$), *Il/1β* ($p < 0.001$) *and Tlr4* ($p < 0.001$) in the intestine and heart samples in comparison to their control groups (Fig. 4). In addition, a significant reduction at doses of 10 mg/kg, 50 mg/kg and 100 mg/kg of trigonelline for *Tnf-α*, *Il/1β and Tlr4* in heart and in the intestine samples was seen when compared with the saline-received colitis group.

Histopathological findings

We observed no significant differences among experimental groups in case of the histopathological scores for the extent of cardiac inflammation. As shown in Fig. 5, epithelial damage and inflammatory cell infiltration were detected in the colitis group. Neutrophilic permeation to the mucosa, goblet cell, and crypt loss was clear, indicating colonic damage. The histopathological scores were significantly greater in the colitis group in compared to the control group ($p < 0.01$, Table 2). Moreover, trigonelline at doses of 50 ($p < 0.05$) and 100 ($p < 0.01$) mg/kg significantly decreased histopathological scores in comparison with the saline-received colitis group. However, treatment with trigonelline did not make significant differences in control groups when compared with the saline-received control counterpart.

Discussion

Findings of the present study demonstrated that experimental colitis significantly increased the lipid peroxidation (MDA) and expression of inflammatory genes as well as decreased the antioxidant capacity in the serum, intestine and heart samples. We determined that treatment with trigonelline significantly increased the antioxidant capacity, restores the MDA levels and decreased the expression of inflammatory genes in the intestine and heart tissues.

The interaction between the immune system with intestine plays a major role in the pathophysiology of colitis (IBD) (Bouma and Strober 2003). Infiltration of immune

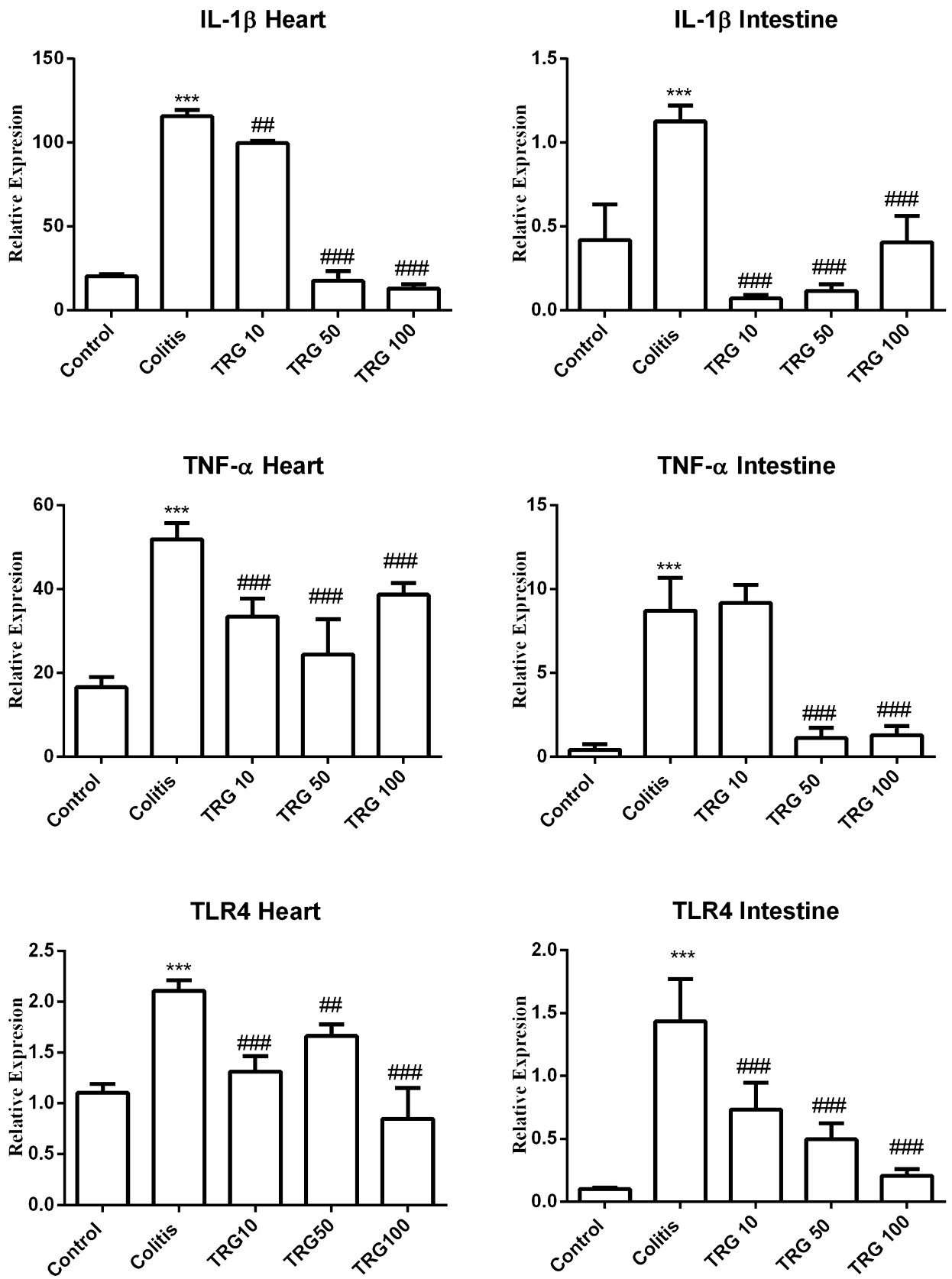

Fig. 4 The expressions of inflammatory genes of *Tnf-α, Il/1β* and *Tlr4* in the intestine and heart tissues were measured using RT-PCR. Data are expressed as relatively and analyzed with one way ANOVA followed by tukey's post- test. ***$p < 0.001$ compared with the saline-received control group, ##$p < 0.01$ and ###$p < 0.001$ compared with saline-received colitis group. *TRG* trigonelline

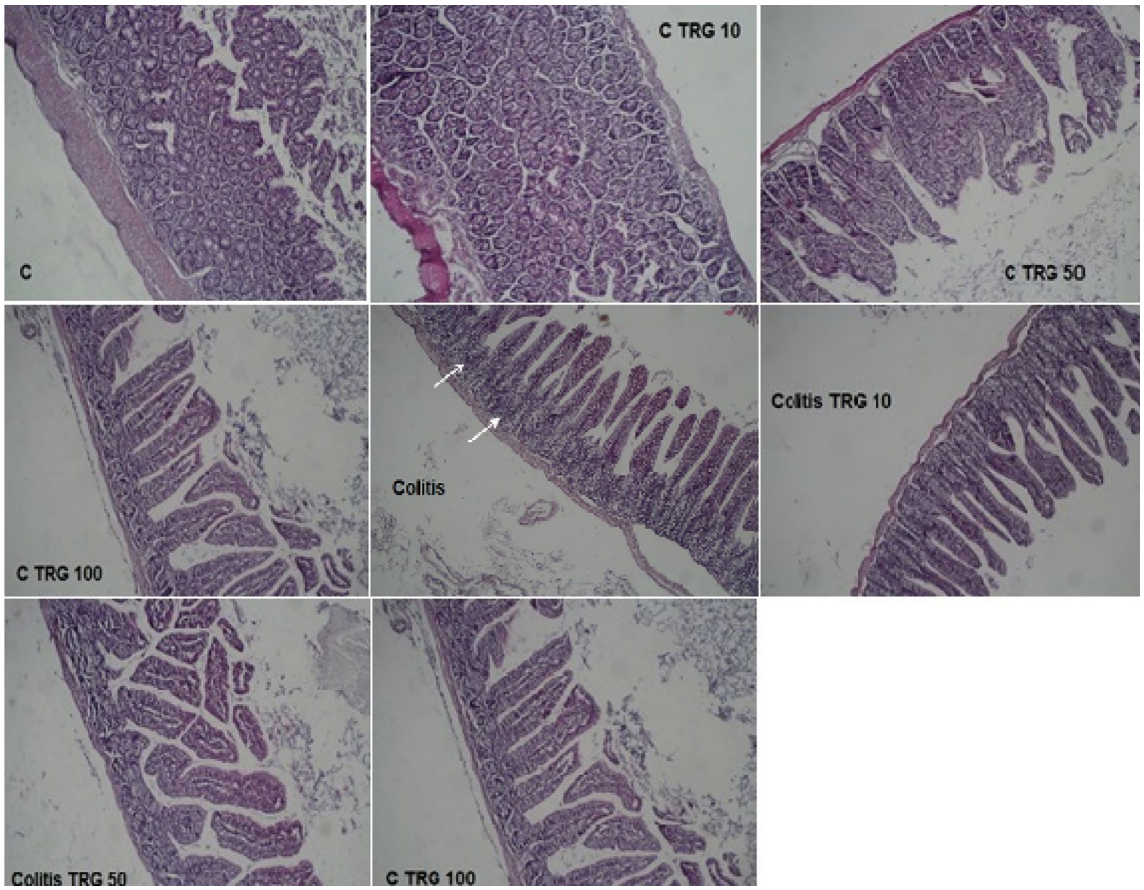

Fig. 5 Representative features of histopathologic evaluations provided from H&E-stained colon sections (×100). The normal mucus layer and crypts without leucocyte infiltration observed in the control group, while the mucosal layer with leucocyte infiltration observed in the colitis group (white arrow). *C* control group

Table 2 Histopathologic scores of the colon samples

Group	Pathologic score Median (min–max)
Control	1 (0–2)
Control + TRG 10 mg/kg	1 (0–1)
Control + TRG 50 mg/kg	1 (0–2)
Control + TRG 100 mg/kg	1 (1–1)
Colitis	3 (3–4)**
Colitis + TRG 10 mg/kg	3 (3–3)
Colitis + TRG 50 mg/kg	2 (2–2)*
Colitis + TRG 100 mg/kg	1 (1–2)***

Histopathologic changes were scored semi-quantitatively. Values are expressed as median and min–max ($n=8$) and were analyzed using one-way ANOVA

**$p<0.01$ compared to the control group, *$p<0.01$ and ***$p<0.01$ compared to the colitis group

cells such as neutrophils and macrophages to the intestine tissue provokes an oxidative damage state which is combined with the initiation of an inflammatory response in the intestine (Pavli et al. 1996). Activation of the Toll-like receptors (*Tlr4*) in the intestine results in the production of pro-inflammatory cytokines such as *Tnf-α* and *Il/1β*, (Laird et al. 2009). In this regards, it has been determined that *Il/1β* overexpression can cause disturbance of the intestinal function (Al-Sadi and Ma 2007). In addition, overexpression of *Tnf-α* increases vascular permeability and exacerbates inflammation. In this context, previous studies have shown that suppression of *Tnf-α* by its receptor antagonists potently reduced the symptoms of the IBD (Popivanova et al. 2008). Clinical investigations declared that immune suppressants such as steroids and azathioprine are effective in treatment of the IBD. Clinical and preclinical studies have demonstrated that the expression of inflammatory cytokines significantly increased in the bowel tissue of the IBD patients (Danese et al. 2005). In line with the above-mentioned studies, we found that the expression of *Tnf-α*, *Il/1β* and, *Tlr4* significantly increased in intestine tissue of the colitis group when compared with the control group.

Ample evidences showed that inflammatory bowel disease is associated with several extra-intestinal complications

including heart, eye, kidney, liver, muscle and pulmonary complications (Thapa et al. 2015). Growing evidences demonstrated that IBD patients have a high risk of developing cardiovascular complications, although the exact mechanisms that link cardiovascular disorders with IBD remain poorly understood which warranted further studies to determine the exact underlying mechanism of this comorbidity (McKenzie et al. 1996). Several inflammatory markers which are involved in the pathophysiology of cardiovascular diseases, such as *Tnf-α*, *IL-6*, *IL-18*, homocysteine and C-reactive protein, have been identified in patients with IBD (Danese et al. 2005; Schicho et al. 2015). Vascular alterations and coronary artery disease are common manifestations in IBD patients. Arterial wall alterations and endothelial dysfunction have been observed in IBD patients, which increase the risk of development of atherosclerosis (Roifman et al. 2011; Schicho et al. 2015). It has been demonstrated that IBD was associated with an increased risk of hospitalization for heart failure (Kristensen et al. 2014).

In this study, the lipid peroxidation level (MDA) in colon and heart tissues, as well as serum sample, was significantly higher than the control group. In addition, we found that the antioxidant capacity of the heart and colon tissues, as well as serum sample, was significantly lower than the control group. In case of inflammatory response, our findings showed that the expression of inflammatory cytokines including *Tnf-α*, *Il/1β* and *Tlr4* was significantly increased in the heart and colon tissues of the colitis group in comparison with the control group. Considering the aforementioned findings, the comorbidity between IBD and cardiovascular disorder might have represented a clue for subsequent IBD complications. In agreement with the previous studies (Amini-Khoei et al. 2016; Antoni et al. 2014; Menconi et al. 2015; Yazbeck et al. 2011), our histopathological evaluations showed that there are epithelial damage, neutrophilic permeation to the mucosa as well as goblet cell and crypt loss in the colitis group.

Several studies have reported that trigonelline exhibited antioxidant, anti-free radical, neuroprotective, and anti-apoptotic effects (Yoshinari et al. 2013; Dutta et al. 2014). It has been shown that trigonelline has potential therapeutic effects in diabetic neuropathy and reduced the levels of inflammatory cytokines (Zhou et al. 2012; Tohda et al. 2005; Gaur et al. 2013). It has also suggested that trigonelline via inhibition of Nrf2 transcription factor in the pancreatic cancer cells increases the apoptosis (Arlt et al. 2013). In addition, previous studies have demonstrated that modulation of the Nrf2 transcription factor exerted antioxidant properties (Vicente et al. 2013; Cardozo et al. 2013). Results of the present study showed that trigonelline effectively reduced the cellular/molecular and histopathological adverse effects of colitis in the intestine tissue. Interestingly, we showed that trigonelline significantly mitigated the negative effects of colitis on the heart tissue in which following treatment with trigonelline levels of the MDA and expression of inflammatory genes decreased in compared with the saline-received colitis group. Furthermore, we found that trigonelline increased the antioxidant capacity of the heart tissue. In addition, we found that trigonelline significantly reversed the adverse effect of the colitis in serum samples.

Conclusion

Overall, findings of the present study demonstrated that, in part at least, trigonelline via attenuation of oxidative stress (decrease in the MDA level and increase in the antioxidant capacity), as well as mitigation of inflammatory response, decreased the histopathological and cellular adversative effects of experimental colitis in the colon and heart tissues. Our results provide indicators that IBD (colitis) is associated with cellular/molecular alterations in the heart and for the first time, we found that trigonelline has potential therapeutic effects to attenuate the colitis signs as well as probable cardiac complications.

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