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Original Article

Crocin enhances antioxidative and cardioprotective effects of sitagliptin in streptozotocin-induced diabetic rats

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Abstract: Objective: The aim of this study was to explore the efficacy of a combination of sitagliptin (SGL) and crocin for treatment of diabetes and diabetic cardiomyopathy. Methods: A rat diabetes model was established by intraperitoneal injection of streptozotocin (STZ). The effects of co-administration of SGL and crocin on serum glucose levels and oxidative stress markers, including lactate dehydrogenase (LDH), malondialdehyde (MDA), nitric oxide (NO), and superoxide dismutase (SOD) were evaluated. Cardiomyopathy indicators, myocardial enzymes release, and cardioprotective signaling activation were also evaluated. Results: It was demonstrated that co-administration of SGL and crocin can better control blood glucose levels than SGL or crocin alone. SGL and crocin, in combination, remarkably suppressed the release of LDH, MDA, and NO while tremendously elevating release of SOD in diabetic rats. Moreover, myocardial hypertrophy levels between cardiomyocytes was significantly reduced by the combination of SGL and crocin in diabetes-induced heart tissues. Enhanced levels of myocardial enzymes (Mb, CK-MB, and cTnI), induced by diabetes, were markedly inhibited by co-administration of SGL and crocin. Furthermore, decrease in levels of p-PI3K and p-AKT and increase in levels of GSK3 and caspase-9, induced by diabetes, were strongly suppressed by the combination of SGL and crocin. Conclusion: This present research demonstrates the synergistic effects of SGL and crocin in treatment of diabetes and diabetic cardiomyopathy.

Keywords: Crocin, sitagliptin, diabetes, antioxidant, cardioprotective

Introduction

Diabetes mellitus, a serious and complicated metabolic disease with high morbidity (4-5%), is characterized by variable and aberrant glucose metabolism. Moreover, high levels of blood glucose contribute to oxidative stress and diabetic heart disease [1]. Therefore, controlling blood glucose levels is very important. Diabetes can be controlled by multiple therapeutic approaches. People with diabetes are typically instructed how to monitor blood sugar, control diet, get regular exercise, take antidiabetic agents properly, and inject insulin. Unfortunately, the hypoglycemic effects of traditional therapeutic drugs have been unstable, with frequently occurring side effects remaining a big problem. Thus, a novel therapeutic regime is urgently needed for the treatment of diabetes.

The pathological change of diabetes mellitus is complicated. According to one published study, enhanced oxidative stress and damaged antioxidant defense systems were observed in patients with diabetes, seem to result in the initiation and development of various diabetes-induced complications [2]. According to a previous report, oxidative stress can lead to cell damage, mitochondrial dysfunction, and DNA injury, further affecting the energy metabolism in cardiomyocytes and inducing heart injury [3]. Besides, diabetes-induced myocardial injury is the leading cause of morbidity and mortality in the diabetic population [4]. Therefore, prevention of oxidative stress-induced myocardial injuries is extremely important for diabetic patients.

Results obtained from published reports have indicated that phosphatidylinositol 3-kinase

(PI3K)/serine-threonine kinase (Akt) is closely related to regulation of Nrf2, which protects cells from oxidative stress due to hyperglycemia insult in mammals [5, 6]. Additionally, activation of PI3K/Akt can defend myocardium against damage via reducing apoptosis [7]. Therefore, PI3K/Akt pathways may be promising targets for treatment of diabetes and diabetes-induced myocardial injury.

Crocin, a unique water-soluble carotenoid, is a kind of pharmacologically active ingredient in Crocus sativus L. (saffron) [8]. In recent years, accumulated studies have demonstrated crocin to have anti-atherosclerotic, anti-hyperlipidemic, antioxidant, immunity-enhancing, and cardioprotective abilities, playing an important role in the management of diabetes and its complications [9, 10]. Thus, crocin might act as a candidate for treatment of diabetes.

Sitagliptin (SGL), a selective dipeptidylpeptidase-4 inhibitor, has been widely used in patients with diabetes. It exerts hypoglycemic effects via enhancing incretin hormones concentrations, thereby elevating insulin secretion [11]. Anti-inflammatory, antioxidant, and anti-apoptotic properties of SGL have also been documented [12]. Although SGL monotherapy is often effective at the beginning of treatment, its effects on blood sugar control are limited [13]. Thus, a second agent is often required for most patients. This study explored the complementary advantages of crocin and SGL, with an aim of making the combination of these two drugs for treatment of diabetes possible.

This present study investigated whether crocin could enhance the antioxidative and cardioprotective effects of SGL in streptozotocin-induced diabetic rats, while also examining underlying mechanisms.

Material and methods

Animal and ethics

SPF Sprague Dawley (SD) male rats (weighing 200-220 g) were purchased from Laboratory Animal Center of Sichuan University. Operations related to animal studies were approved by the Institutional Animal Care and Use Committee at Sichuan University.

Diabetes model and grouping

The diabetes model was established as follows. Rats were under starvation treatment for 8 hours before intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO, USA) at a dose of 60 mg/kg/d for 4 days. Two weeks later, tail blood samples were detected by a hand-held glucometer (UltraEasy, Johnson, USA). Rats with fasting plasma glucose >13 mM were considered diabetic.

A total of 32 diabetic rats, based on this model, were used and further randomized into 4 groups: diabetes only (STZ group), diabetes treated with crocin (Sigma Aldrich Co., USA, CAS, 60 mg/kg/d in normal saline, intraperitoneally, STZ+ crocin group), diabetes treated with sitagliptin (Januvia, Merck & Co., Inc., NJ, USA, 10 mg/kg/d in normal saline, intraperitoneally, STZ+ SGL group), and diabetes treated with sitagliptin plus crocin (crocin 60 mg/kg/d and SGL 10 mg/kg/d, intraperitoneally, STZ+ SGL+ crocin group). A total of 8 normal rats, without treatment, were used as control group. Treatment continued for 2 weeks. Afterward, blood samples and heart tissues were collected for subsequent experiments.

ELISA assay

Levels of oxidative stress parameters and myocardial injury markers, including lactate dehydrogenase (LDH), malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), myoglobin (Mb), creatine kinase (CK)-MB, and cardiac troponin I (cTnI), were detected using ELISA kits (Thermo Fisher, MA, USA). The experimental protocol was performed in strict accordance with manufacturer instructions.

HE staining

Left ventricles, isolated from the rats, were dehydrated, embedded, and cut into 4 μm sections on a microtome (Leica, Nussloch, Germany). HE staining was performed as described before [1]. Pictures were taken under an optical microscope (Olym-pus, Tokyo, Japan).

Western blotting

Proteins were extracted from murine cardiac tissues. Concentration of protein was detected using BCA protein assay reagent (Solarbio, Beijing, China). Next, all proteins were transferred to PVDF membranes (Millipore, Billerica, MA). After blocking and washing, membranes were incubated with primary antibodies p-PI3K, p-AKT, GSK3, caspase-9, and GAPDH (Abcam, Cambridge, UK) at 4°C overnight, along with secondary antibody for 1 hour at room temper-

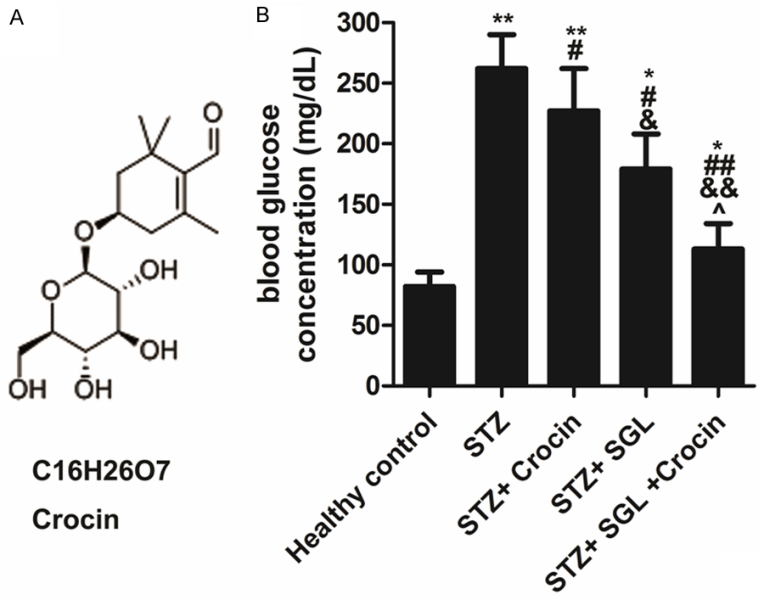

Figure 1. Cronin enhances the hypoglycemic effects of SGL. Rats were randomly divided into 5 groups; Healthy control group: healthy rats; STZ group: diabetic rats without further treatment; STZ+ Crocin group: diabetic rats treated with crocin; STZ+ SGL group: diabetic rats treated with SGL; STZ+ SGL+ Crocin group: diabetic rats treated with crocin and SGL at the same time. A. The structure of crocin. B. Fasting blood-glucose was measured using a hand-held glucometer. Experiments were repeated at least 3 times, and error bars represent ± SD. (*P<0.05, **P<0.01 versus healthy control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 versus STZ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 versus STZ+ crocin group; $^{\wedge}$P<0.05 versus STZ+ SGL group).

ature. Protein bands were analyzed using ImageJ software (NIH, Sacaton, AZ, USA). Blood sugar levels were measured by a hand-held glucometer (Roche, Basel, Switzerland).

Statistical analysis

Data were analyzed using SPSS 17.0 (SPSS, Chicago, IL). One-way ANOVA was used to evaluate statistical differences among 5 groups. Differences between the 2 groups were compared by Bonferroni t-test. Data are represented as mean ± SD. P<0.05 was considered statistically significant.

Results

Crocin enhanced hypoglycemic effects of SGL

To detect the hypoglycemic effects of crocin and SGL, the diabetes model was established by intraperitoneal injection of STZ. As shown in **Figure 1**, blood glucose was remarkably increased in the STZ group compared with control group (P=0.008). Besides, SGL slightly decreased blood sugar compared with the STZ group (P=0.03). Moreover, suppression in the levels of blood sugar induced by SGL was remarkably enhanced through adding crocin (P=0.007). These results demonstrate that the hypoglycemic effects of SGL were remarkably enhanced by crocin treatment.

Crocin enhanced anti-oxidant effects of SGL

To investigate whether SGL exhibits anti-oxidant effects on diabetes-induced myocardial damage, related indicators in the serum were measured using ELISA. As illustrated in **Figure 2**, LDH, MDA, and NO levels were significantly elevated while SOD was decreased in the STZ group, compared with healthy control group (P<0.01). SGL or crocin used alone slightly suppressed this change. Nevertheless, levels of LDH, MDA, and NO were tremendously repressed while SOD remarkably increased in STZ+ SGL+ crocin group compared with STZ group (P<0.01). These results indicate that crocin enhanced the anti-oxidant effects of SGL on diabetes-induced myocardial damage.

Crocin helped SGL alleviate diabetes-induced myocardial damage

To identify whether crocin and SGL play protective roles in diabetes-induced myocardial damage, histologic changes of heart tissue were evaluated by HE staining. Healthy myocardial cells were arranged neatly and tightly, with a clear structure. A small number of fibroblasts were observed in the healthy control group. In the STZ group, the arrangement of cells was irregular and became hypertrophic. Levels of myocardial hypertrophy were slightly ameliorated in STZ+ crocin and STZ+ SGL groups compared with the STZ group. However, myocardial hypertrophy was remarkably alleviated in STZ+

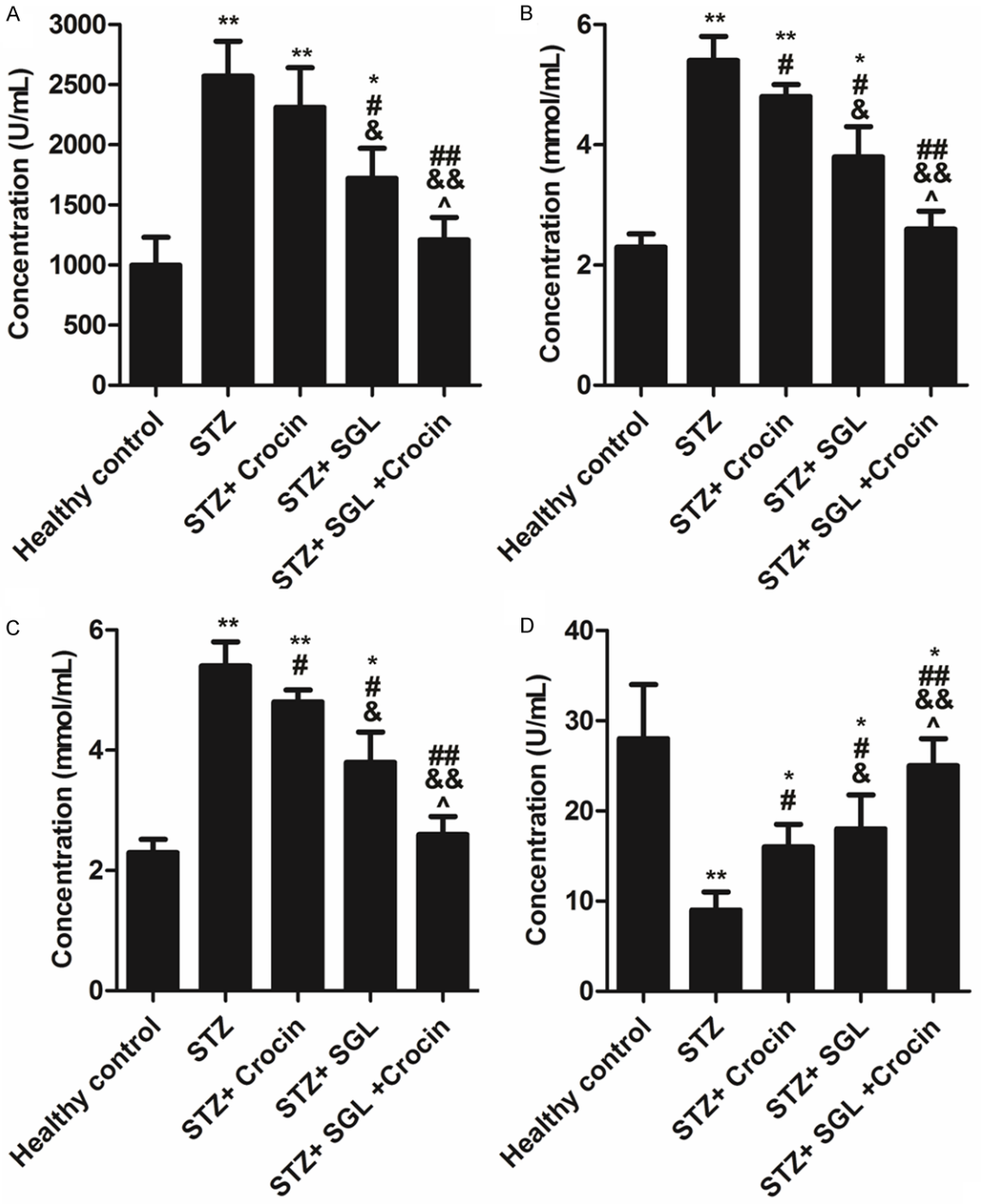

Figure 2. Crocin enhances the anti-oxidant effects of SGL. Rats were randomly divided into 5 groups; Healthy control group: healthy rats; STZ group: diabetic rats without further treatment; STZ+ crocin group: diabetic rats treated with crocin; STZ+ SGL group: diabetic rats treated with SGL; STZ+ SGL+ crocin group: diabetic rats treated with crocin and SGL at the same time. The LDH (A), MDA (B), NO (C), SOD (D) levels in serum were measured using ELISA. Experiments were repeated at least 3 times, and error bars represent ± SD. (*P<0.05, **P<0.01 versus healthy control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 versus STZ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 versus STZ+ crocin group; $^{\wedge}$P<0.05 versus STZ+ SGL group).

SGL+ crocin group compared with STZ group (**Figure 3**). These results indicate that the combination of SGL and crocin produced greater protective effects.

Figure 3. Crocin helps SGL alleviate diabetes-induced myocardial damage. Rats were randomly divided into 5 groups; Healthy control group: healthy rats; STZ group: diabetic rats without further treatment; STZ+ crocin group: diabetic rats treated with crocin; STZ+ SGL group: diabetic rats treated with SGL; STZ+ SGL+ crocin group: diabetic rats treated with crocin and SGL at the same time. HE staining was used to evaluate the injury degree of myocardium. Experiments were repeated at least 3 times (scale bar =100 μm).

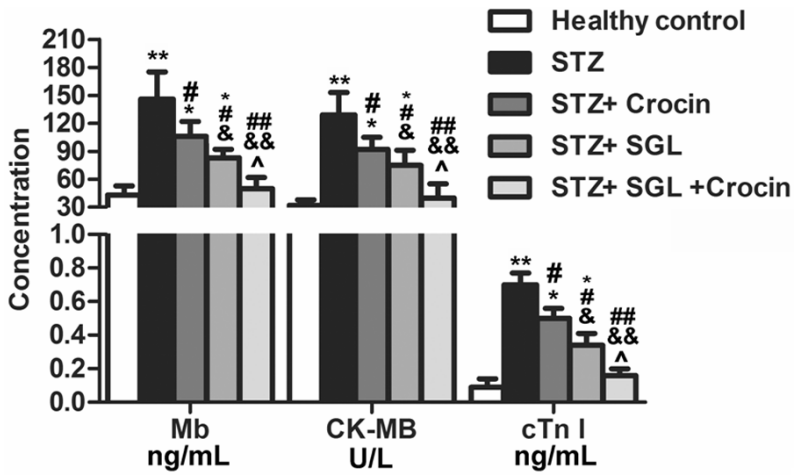

Figure 4. Crocin helps SGL alleviate myocardial injury. Rats were randomly divided into 5 groups; Healthy control group: healthy rats; STZ group: diabetic rats without further treatment; STZ+ crocin group: diabetic rats treated with crocin; STZ+ SGL group: diabetic rats treated with SGL; STZ+ SGL+ crocin group: diabetic rats treated with crocin and SGL at the same time. Expression levels of Mb, CK-MB, and cTnI were detected using Western-blot. Experiments were repeated at least 3 times, and error bars represent ± SD. (*P<0.05, **P<0.01 versus healthy control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 versus STZ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 versus STZ+ crocin group; $^{\wedge}$P<0.05 versus STZ+ SGL group).

Crocin helped SGL alleviate myocardial injury

To explore the protective effects of crocin and SGL on myocardial injury, three myocardium markers (Mb, CK-MB, and cTnI) were measured by ELISA. As illustrated in **Figure 4**, expression of Mb, CK-MB, and cTnI was significantly increased in STZ group compared with the healthy control group (P<0.01). Elevated levels of Mb, CK-MB, and cTnI induced by STZ could be suppressed by crocin or SGL alone (P<0.05). However, combination of the two drugs was more effective (P<0.01).

SGL and crocin activated PI3K/AKT signaling pathways

To determine whether SGL and crocin are related to PI3K/Akt signaling pathways, relative proteins were detected by Western blot. Results indicated that expression of p-PI3K and p-Akt was tremendously reduced, while GSK3 and caspase-9 dramatically increased in the STZ group compared with healthy control group. Crocin or SGL, used alone, slightly enhanced p-PI3K and p-Akt while repressing downstream GSK3 and caspase-9 levels in STZ+ crocin and STZ+ SGL groups, compared with STZ group. However, the combination of SGL and crocin remarkably inhibited reduced p-PI3K and p-Akt while increasing GSK3 and caspase-9 expression, compared with the control group (**Figure 5**). These results demonstrate that PI3K/Akt signaling pathways could be activated by both SGL and crocin. The activation effects were stronger under treatment of SGL together with crocin.

Discussion

Oxidative stress is a common and important factor in the occurrence of diabetes and complications. Emerging evidence has suggested the antioxidant effects of SGL. According to Dalia et al., the activity of antioxidant enzymes was elevated while TNF-α levels were decreased by SGL, protecting kidneys against damage induced by cisplatin in mice [14]. Besides, another study demonstrated that STZ-nicotinamide-induced renal damage was attenuated via treatment with coenzyme Q10 or sita-

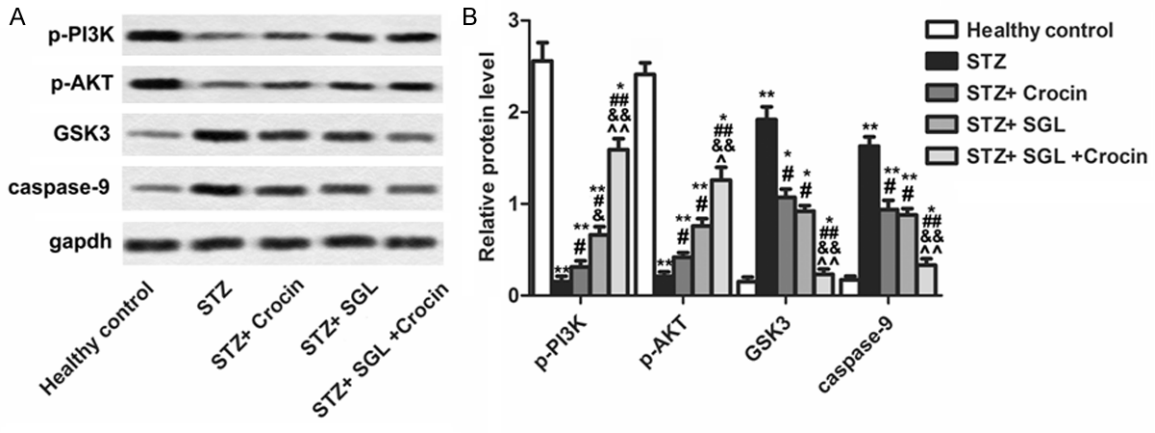

Figure 5. SGL and crocin activate PI3K/AKT signaling pathways. Rats were randomly divided into 5 groups; Healthy control group: healthy rats; STZ group: diabetic rats without further treatment; STZ+ crocin group: diabetic rats treated with crocin; STZ+ SGL group: diabetic rats treated with SGL; STZ+ SGL+ crocin group: diabetic rats treated with crocin and SGL at the same time. A. Expression of p-PI3K, p-AKT, GSK3, and caspase-9 were evaluated using Western-blot. B. The statistical analysis of protein expression. Experiments were repeated at least 3 times, and error bars represent ± SD. (*P<0.05, **P<0.01 versus healthy control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 versus STZ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 versus STZ+ crocin group; $\hat{}$P<0.05, $\hat{}\hat{}$P<0.01 versus STZ+ SGL group).

gliptin or their co-administration in diabetic rats. Evidence included reduced oxidative stress, MPO, TNF-α, TGF-β activity, and nitrite content, along with histopathological changes [15]. In addition to SGL, crocin has also been reported to have antioxidant properties. Yosri and colleagues observed that crocin increased anti-oxidant defenses, suppressed incidence of oxidative stress, and recovered pro-inflammatory cytokines to normal levels in mice with allergic asthma [16]. Oxidative stress was remarkably repressed by crocin administration, reflected by suppressed MDA along with enhanced reductive/antioxidative power in I/R rats [17]. Similarly, in this present research, SGL or crocin or their combination exerted anti-oxidant effects via inhibiting elevated LDH, MDA, and NO while decreasing SOD in diabetic rats. Moreover, simultaneous use of SGL and crocin exhibited greater inhibition effects. Crocin has been widely reported to alleviate oxidative stress and inflammation response via reducing levels of ROS, MDA, and inflammatory factors. Considering the role of SGL in increasing incretin hormones concentrations, thereby elevating insulin secretion, the balanced oxygen levels and immune system treated by crocin allows SGL to exert more anti-oxidant and hypoglycemic effects in diabetic rats.

Diabetes is closely related to heart disease and diabetes-induced cardiovascular complications, including myocardial infarction, cardiomyopathy, and myocardial infarction [18, 19]. Research has indicated that treatment with SGL alleviates diastolic dysfunction, ameliorates hemodynamic indices, and significantly decreases mortality [20]. In addition, heart damage, structural changes in the myocardium, and ventricular function induced by doxorubicin have been tremendously mitigated by crocin treatment [21]. A similar result was drawn in this present research. Structural changes of myocardial tissue along with increase of myocardial enzymes (Mb, CK-MB, and cTnI) were significantly suppressed by SGL, crocin, or their combination. However, combining SGL and crocin produced greater cardioprotective effects.

PI3K/Akt signaling pathways participate in regulating cell growth, proliferation, death, and survival. Su et al. demonstrated that PI3K/Akt pathways play a vital role in modulating cardiomyocyte apoptosis in response to high glucose, *in vitro* and *in vivo* [22]. Additionally, it has been discovered that PI3K/Akt pathways have distinct effects on regulation of antioxidation [23]. Moreover, one study demonstrated that SGL and crocin could regulate PI3K/Akt pathways, respectively. According to a published report, SGL pretreatment could activate PI3K/Akt signaling pathways through GLP-1/GLP-1 receptors to attenuate myocardial damage and improve cardiac function in I/R rats [7]. Additionally, crocin could also activate PI3K/Akt signaling pathways to prevent retinal isch-

emia/reperfusion-induced retinal ganglion cell apoptosis [24]. Similarly, this current research indicated that increased p-PI3K and p-Akt expression, along with decreased GSK3 and caspase-9 expression, were suppressed by SGL, crocin, or their combination in cardiac tissues. Combination of the two produced greater effects.

This study indicates that crocin enhances the protective effects of SGL on diabetic rats through mitigating oxidative stress and PI3K/Akt pathways. Considering the close relationship between oxidative stress and PI3K autophagy pathways, it is worth investigating whether the cardioprotective effects of crocin or SGL in diabetes are involved in autophagy [25].

Taken together, this present study demonstrates that combination of SGL and crocin enhances treatment of diabetes and diabetes-induced myocardial damage. The treatment effects of SGL and crocin on diabetes appear to be related to activation of PI3K/Akt pathways. Results of this present study suggest that combination of SGL and crocin may be a promising therapeutic schedule in the treatment of diabetes and diabetes-induced myocardial damage.

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Disclosure of conflict of interest

None.

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