Original Article

Effects of Aqueous Saffron Extract on Glucoregulation as Well as Hepatic Agt and TNF-α Gene Expression in Rats Subjected to Sub-Chronic Stress

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Abstract

Background: Stress and saffron seem to affect glucoregulation mechanisms and insulin resistance in different ways. Impacts of the aqueous saffron extract were investigated on serum glucose levels, serum insulin levels, the homeostatic model assessment of β-cell function (HOMA-B), the homeostatic model assessment of insulin resistance (HOMA-IR), adrenal weight, and hepatic gene expression of angiotensinogen (Agt) and tumor necrosis factor-α (TNF-α) in rats under sub-chronic stress.

Materials and Methods: Forty-two male rats were divided into six groups: control, restraint stress (6h/day for seven days), saffron (30 and 60 mg/kg) treatments for seven days, and post-stress saffron (30 and 60 mg/kg) treatments for seven days. The serum glucose and insulin levels, hepatic gene expressions of Agt and TNF-α, HOMA-IR, HOMA-B, and adrenal gland weight were measured.

Results: One-week recovery following sub-chronic stress led to non-significant hyperglycemia, hyperinsulinemia, and insulin resistance. The hepatic Agt and TNF-α mRNA levels increased significantly in this group. Saffron administration led to enhanced hepatic Agt mRNA in the non-stressed subjects. In addition, serum glucose levels, insulin resistance, and hepatic Agt gene expression significantly increased in stress-saffron groups. The hepatic TNF-α gene expression was reduced only in the stress-saffron 60 group.

Conclusion: Saffron treatment after sub-chronic stress not only did not improve glucose tolerance but also enhanced insulin resistance. It indicated the interaction of saffron and sub-chronic stress to promote renin-angiotensin system activity. In addition, the saffron treatment decreased TNF-α gene expression after sub-chronic stress. The synergistic stimulating effect of saffron and sub-chronic stress on gene expression of hepatic Agt led to insulin resistance and hyperglycemia.

Keywords: Angiotensinogen, crocus, hyperglycemia, insulin resistance, psychological stress, tumor necrosis factor-alpha

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Submitted: 14-Feb-2022; **Revised:** 17-Apr-2022; **Accepted:** 18-Apr-2022; **Published:** 15-May-2023

Introduction

Stress has become an inevitable component of modern human life that is growing in quantity to alarming and qualitatively threatening levels.[1] Stress impacts the neuroendocrine system by stimulating the hypothalamus-pituitary-adrenal (HPA) and axis the sympathetic nervous system. Stress increases blood glucocorticoids and catecholamine, as the major predisposing factors of type 2 diabetes and metabolic syndrome.[1,2] Glucocorticoids affect hepatic gluconeogenesis, hepatic glycogenolysis, glucose uptake, and pancreatic function; thereby, they can deteriorate insulin resistance.[2-4] Catecholamines are released due to stress and rapid renin secretion and the renin-angiotensin system (RAS) activation.[5]

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_51_22

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How to cite this article: Yazdani Y, Radahmadi M, Roustazade R. Effects of aqueous saffron extract on glucoregulation as well as hepatic agt and TNF-α gene expression in rats subjected to sub-chronic stress. Adv Biomed Res 2023;12:117.

Systemic RAS plays a significant role in glucose metabolism by increasing hyperglycemia and hyperinsulinemia as well as decreasing insulin sensitivity.[6] Angiotensinogen (Agt), as the precursor of angiotensin II, is mainly synthesized in the liver and contributes to the pathogenesis of metabolic syndrome and diabetes mellitus.[6,7] It has been indicated that the hepatic Agt mRNA levels are significantly increased by glucocorticoids.[7] Stress promotes the pro-inflammatory cytokine productions containing the TNF-α, IL-6, and IFN-γ,[8] which aggravate stress hormone productions and hepatic Agt expression.[9,10] These factors lead to a vicious cycle, exacerbating stress-induced hyperglycemia.[11] It seems that the activation of both the HPA and RAS dysregulates the insulin receptors functions. Therefore, these activations can compromise the function of hepatocytes and aggravate insulin resistance.[5,12]

On the other hand, food elements and supplements are essential in activating protective mechanisms. Saffron (*Crocus sativus* L.) is an indigenous plant in some countries such as Iran, Greece, Spain, and China. It is widely grown for its nutritional value and traditional medicine.[13] Flavonoids, such as crocin, crocetin, picrocrocin, and safranal, comprise its main active constituents of saffron.[14,15] Therefore, there are many proposed pharmaceutical and therapeutic properties for saffron, such as anxiolytic, antioxidant, anti-inflammatory, antidepressants, and neuroprotective effects. Also, saffron is served as a hypoglycemic agent, as saffron can improve insulin sensitivity in muscles, adipose tissue, and liver cells of rats.[16] In addition, the aqueous saffron extract could be readily available as a daily supplement for individuals. It may be beneficial to counteract the harmful effects of chronic exposure to daily stressors due to modern societies. Moreover, despite the numerous studies conducted on the different effects of saffron, the role of two doses of aqueous saffron extract, and recovery period after sub-chronic stress on glycemic control (such as serum glucose levels, serum insulin levels, HOMA-IR, and HOMA-B), adrenal weight, hepatic gene expressions of Agt, and TNF-α in sub-chronic stress condition seems to await further elucidation. Therefore, the current study is implemented to investigate them.

Materials and Methods

Animals

Experiments were done using 42 male Wistar rats (200-250 g, approximate 49-56 days) from Pasteur Institute (Tehran, Iran). All experiments were approved by the Ethics Committee of Animal Use of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1394.1.399). Animals were housed in ventilated cages at 23 ± 2°C and with a humidity of 50 ± 5% under 12h/12h light cycle/dark cycle with lights between 07:00 and 19:00. Water and food were available, except during the restraint stress period. After two weeks of adaptation, the animals were divided into six groups (n = 7), randomly as follows: Control (Co), in which the rats were not exposed to restraint-stress during the first week and received a drug vehicle (saline) for the next seven days; Stress (St) group, in which the animals were subjected to restraint stress 6h/day for seven days before they received saline for the next seven days; Saffron (Saf30 and Saf60) groups, in which there was no stress before administering saffron (30 and 60 mg/kg) for the next seven days; and Stress-Saffron (St-Saf30 and St-Saf60) groups in which rats were subjected to restraint stress 6 h/day for seven days before saffron was administered (30 and 60 mg/kg) for the next seven days [Figure 1].

Experimental procedures

Sub-chronic stress paradigm

The rats in the sub-chronic stress groups were kept in cylindrical Plexiglas restrainers for 6 h/day for seven days (8:00–14:00). Based on stress timing, sub-chronic or mid-stress (seven days of stress) is considered a potent stressor in rats to evoke unavoidable endocrine responses.[17,18] Restraint stress is known as a demanding physical and psychological stressors in rodents.[18] During restraint, animals were not physically compressed and did not experience pain.

Aqueous saffron extract preparation

Saffron red stigmas were purchased from Abbaszadeh Co., Iran. The main active components of its extract have been previously standardized and used in previous studies.[19] About 9.4 g of saffron red stigmas were perfectly ground to a fine powder and macerated in 250 cc_s of distilled water in containers covered with aluminum foil. This mixture was subsequently stirred in a shaker at room temperature in the dark for 72 hours, filtered using a Buckner funnel and a vacuum pump to obtain a transparent filtered solution, and finally poured into Petri dishes and frozen at –22°C for 24–48 hours. Each petri dish contained an adequate amount of the solution to obtain as thin layers of the frozen solution as possible. Then, the frozen mixture was stored in a freeze-dryer for 48 hours. The main active components of saffron (e.g., crocin, crocetin, etc.) are particularly sensitive to heat. Therefore, the aqueous saffron extract was prepared using freeze-drying for the optimal preservation of the flavonoid contents and antioxidant activity of them. Whereas, other extraction methods (e.g., the rotary evaporator) are based on the heating of the solution and cannot produce safely saffron extract.[20] The obtained pure dried red saffron extract was subsequently collected using a razor and stored at –22°C in a dark-tinted glass bottle before use. Finally, saffron (30 and 60 mg/kg/day for seven days) was dissolved in the sterile normal saline for intraperitoneally (i.p.) injection. Both doses of saffron were prevalently used in animal experiments. These doses of saffron had the least toxic effects

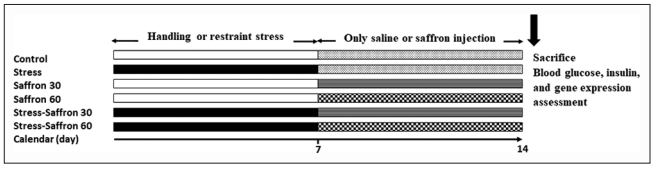

Figure 1: Schematic diagram of the different experimental groups.

in rodents. Some studies demonstrated that saffron and its components (at doses of 15-80 mg/kg; i.p. injection) did not show any biochemical, hematological, and histopathological toxicity in rodents due to chronic administration.[21,22] Also, it was reported that saffron extracts did not have any toxic effect up to 160 mg/kg/day by i.p. injection in rats.[21] Therefore, the doses of saffron were chosen in the present study following previous studies.

Assessment of serum glucose and insulin levels

The animals were decapitated at 8:00–10:00 after 12 hours of fasting. The trunk blood was collected for fasted blood samples. Then, the serum was separated (by centrifugation with 6000 rpm for 20 min) and then stored at −80°C until analysis. Commercial Enzyme-Linked Immuno-Sorbent Assay kit with intra-assay precision CV <10% (ZellBio GmbH Co., Marburg, Germany) measured serum insulin. Also, glucose levels were determined via the glucose oxidase method (Parsazmun Co., Iran).

Insulin resistance and beta-cell function assessments

The homeostatic model assessment (HOMA) indexes are the homeostasis model assessment of insulin resistance and beta-cell function (HOMA-IR and HOMA-B, respectively). In this study, were used the formulas of HOMA-IR = Fasting insulin (μU/ml) × Fasting glucose (mg/dl)/405,[23] and HOMA-B = (360 − Fasting insulin [mU/ml])/(Fasting glucose [mg/dl] −63).[23] Moreover, the relationship between insulin and glucose reflects the equipoise between insulin secretion and hepatic glucose output in the basal condition. This equipoise and/or balance was retained by a feedback loop between the B-cells and liver. It seems that there were a close correlation and positive feedback between pancreatic insulin-sensitive tissues and β-cells. It indicated a higher insulin secretion from β-cells for the insulin-sensitive tissues.[24]

Adrenal gland weight variation

Given the pivotal role played by the adrenal gland in stress-coping mechanisms, the animals adrenal glands were resected at the end of the experiments, weighted (for adrenal gland hypertrophy), and reported as a stress index and/or an indicator of HPA axis activity.[25] It indicated that the different types of chronic stress led to cellular hypertrophy and hyperplasia in the medulla and cortex of the adrenal gland. Therefore, these changes are associated with increased adrenal activation in response to stress.[26]

Assessment of gene expressions

Genes expressions of TNF-α and Agt in the liver were determined based on real-time polymerase chain reaction (PCR). Total RNA was isolated from the liver tissues according to the manufacturer's instructions by using the Hybrid-R™ kit (GeneAll Biotechnology Company, Seoul, Korea). Then, the purity and concentration of messenger RNA (mRNA) were measured using the absorbance of NanoDrop at a ratio of A260/A280 and 260 nm, respectively. Next, 5 ng of total RNA was used for reverse transcription and complementary DNA (cDNA) synthesis with random hexamer primers using the Revert Aid First Standard cDNA Syn. Kit (Fermentas, US) according to the manufacturer's manual. The RealQ Plus 2x Master Mix Green with high ROX™ (Ampliqon) and the primer sequences used in the study are reported in Table 1. Real-time PCR was performed using the StepOnePlus real-time PCR System (Applied Biosystems). Each mRNA input was normalized to the respective Beta-actin (ACTB) RNA as an internal control. Cycle parameters for real-time PCR included 95°C for 15 s and 60°C for 1 min. The fractional cycle number at which the fluorescence passed the fixed threshold was defined as the Ct value. The fold expression changes of experimental groups were calculated using the method of after normalization to ACTB endogenous in their corresponding control group.[27]

Statistical analysis

In this study, data were estimated as means ± SEM and subjected to a one-way analysis of variance (ANOVA) followed by an LSD post-hoc test for different comparisons. The LSD post-hoc test was employed to detect the minimal effect of treatment on experimental groups. *P* values <0.05 were declared statistically significant. Ultimately, the calculations were performed using SPSS 24 (SPSS Inc. Chicago, IL, USA).

Results

Serum glucose and insulin levels

As observed in Figure 2a, neither the St group nor the Saf30 or Saf60 groups exhibited any significant differences in their serum glucose levels relative to the Co group. While, the administration of the saffron extract (30 and 60 mg/kg) during the week after stress led to a significant ($P < 0.001$, in both) enhancements in serum glucose levels relative to that of the Co group.

Serum glucose had significant ($P < 0.05$) enhancement in the St-Saf30 group compared to the St group. In addition, serum glucose levels significantly ($P < 0.001$, in both) increased in St-Saf30 and St-Saf60 groups compared to Saf30 or Saf60 groups, respectively [Figure 2a].

Finally, the comparison of serum glucose levels showed no significant differences between the St-Saf30 and St-Saf60 ones [Figure 2a].

Table 1: Primer sequences employed in real-time PCR

Primer		Sequence (5'-3')
ACTB	Forward	AGGCCCCTCTGAACCCTAAG
	Reverse	CCAGAGGCATACAGGGACAA
Agt	Forward	GGTGTGATGCCTCCTGTGTA
	Reverse	GCCTCTGCCTTGCTGGAA
TNF-α	Forward	ACGTCGTAGCAAACCACCAA
	Reverse	CAAGGGCTCTTGATGGCAGA

PCR, Polymerase chain reaction; ACTB, Beta-actin; TNFα, Tumor necrosis factor α; Agt, angiotensinogen

Figure 2b shows serum insulin levels across all the experimental groups. There were no significant differences in this parameter among all experimental ones. In other words, neither sub-chronic stress nor saffron administration produced significant changes in the insulin levels measured in the non-stressed and stressed groups with this protocol.

HOMA-IR and HOMA-B indices

There were no significant differences in HOMA-IR among the St, Saf30, and Saf60 groups in comparison to that observed for the Co group [Figure 3a], similar to glucose level data in those groups.

As shown in Figure 3a, saffron administration (30 and 60 mg/kg) following sub-chronic stress significantly ($P < 0.05$ and $P < 0.01$, respectively) increased HOMA-IR, in comparison to the Co group. In addition, HOMA-IR significantly ($P < 0.01$) increased in the St-Saf60 group with respect to that in the Saf-60 group.

In addition, the HOMA-B had a significant ($P < 0.05$) decrease in the St-Saf60 group, in comparison to the Saf60 group. Overall, the other experimental groups did not show significant differences in the HOMA-B [Figure 3b].

Adrenal gland weight

As shown in Figure 4, sub-chronic stress had a significant ($P < 0.01$) enhancement in adrenal gland weight/100 gr of body weight compared to the Co group. Also, the normal subjects receiving saffron showed no change in their adrenal gland weights with respect to the Co group. In a comparison of the St-Saf-30 group with the stress ones, there was a significant ($P < 0.05$) decline in adrenal gland hypertrophy as a result of saffron administration (30 mg/kg). Whereas, there was no such effect in the St-Saf 60 group. Moreover, adrenal gland weight showed a significant ($P < 0.01$) enhancement in the St-Saf60 compared to that in the Saf60 group [Figure 4].

Hepatic TNF-α gene expression

In Figure 5a, the TNF-α gene expression exhibited a significant ($P < 0.01$) enhancement in the stress group in comparison with the Co group. Moreover, non-significant reductions were detected in the gene expression of TNF-α in both Saf30 and Saf60 groups due to receiving saffron in normal subjects.

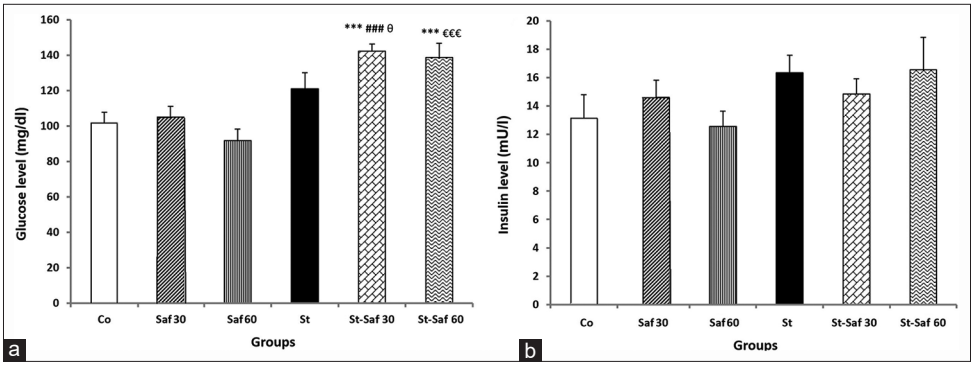

Figure 2: (a) Serum glucose levels and (b) serum insulin levels at the end of the experiment in all experimental groups (n = 7). Results are expressed as means ± SEM (ANOVA test, LSD post-hoc test). ***$P < 0.001$ compared to the control; $^{\theta}P < 0.05$ compared to the stress group; $^{\#\#\#}P < 0.001$ compared to the saffron 30 mg/kg group; and $^{€€€}P < 0.001$ compared to the saffron 60 mg/kg group. No significant differences were observed in insulin levels among the groups. **Co:** Control group, **St:** stress group, **Saf30:** Saffron at dose of 30 mg/kg group, **Saf60**: Saffron at dose of 60 mg/kg group, **St-Saf 30:** Stress-Saffron (30 mg/kg) group, **St-Saf 60:** Stress-Saffron (60 mg/kg) group

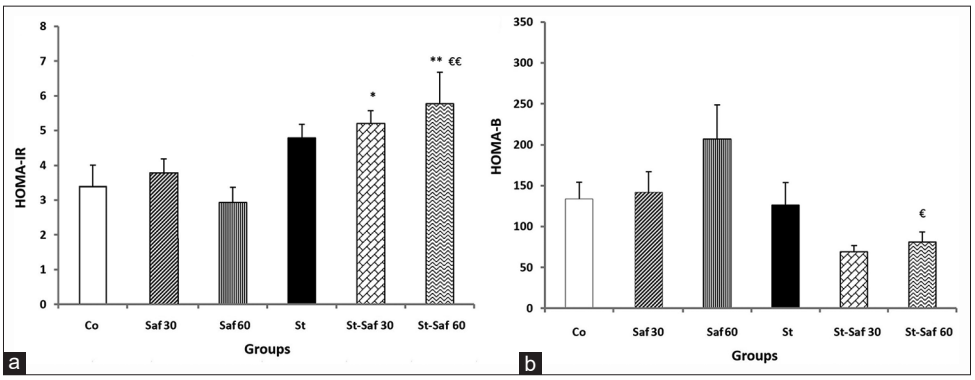

Figure 3: (a) HOMA-IR and (b) HOMA-B values based on day-14 serum glucose and insulin levels in all experimental groups (n = 7). Results are expressed as means ± SEM (ANOVA test, LSD post-hoc test). *P < 0.05 and **P < 0.01 compared to the control; $^{€}$P < 0.05 and $^{€€}$P < 0.01 compared to the saffron 60 mg/kg group. **Co:** Control group, **St:** stress group, **Saf30:** Saffron at dose of 30 mg/kg group, **Saf60**: Saffron at dose of 60 mg/kg group, **St-Saf 30:** Stress-Saffron (30 mg/kg) group, **St-Saf 60:** Stress-Saffron (60 mg/kg) group

As regards the hepatic gene expression of TNF-α, the St-Saf30 and St-Saf60 groups showed significant ($P < 0.01$ and $P < 0.05$, respectively) enhancements in comparison to the corresponding Saf30 and Saf60, respectively. Finally, TNF-α mRNA level significantly ($P < 0.05$) declined in the St-Saf60 when compared to the stress group as a result of a dose of 60 mg/kg of saffron administration [Figure 5a].

Hepatic Agt gene expression

According to Figure 5b, the hepatic Agt gene expression increased significantly in Saf30 Saf60 and stress groups in comparison to the Co group ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively). Also, hepatic Agt gene expression was significantly ($P < 0.001$ in both) higher in the St-Saf30 and St-Saf60 groups than in the Co group.

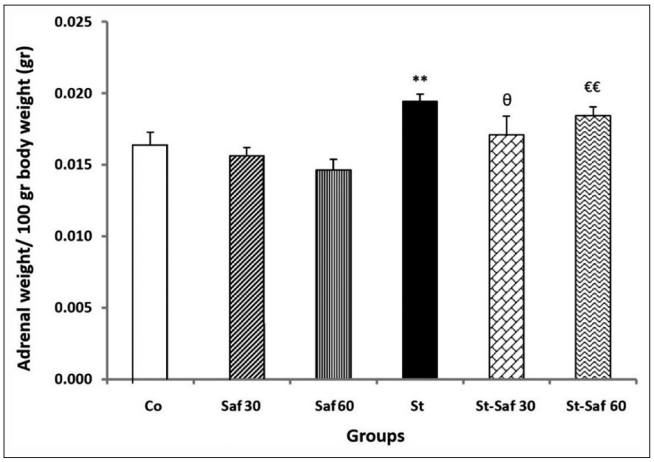

Figure 4: Adrenal weight/100 gr of body weight at the end of the experiment in all experimental groups (n = 7). Results are expressed as means ± SEM (ANOVA test, LSD post-hoc test). $^{**}P < 0.01$ compared to the control; $^{\theta}P < 0.05$ compared to the stress group, $^{\epsilon\epsilon}P < 0.01$ compared to the saffron 60 mg/kg group. **Co:** Control group, **St:** stress group, **Saf30:** Saffron at dose of 30 mg/kg group, **Saf60**: Saffron at dose of 60 mg/kg group, **St-Saf 30:** Stress-Saffron (30 mg/kg) group, **St-Saf 60:** Stress-Saffron (60 mg/kg) group

Likewise, this parameter exhibited significantly ($P < 0.01$ and $P < 0.001$, respectively) higher levels in the St-Saf 30 and St-Saf60 groups than in the corresponding Saf30 and Saf60 ones. Finally, the hepatic Agt gene expression was significantly higher in the St-Saf30 and St-Saf60 groups than in the stress group ($P < 0.001$ and $P < 0.01$, respectively).

Discussion

Effects of two doses of the saffron aqueous extract were investigated on glucoregulation, serum glucose levels, serum insulin levels, HOMA-IR, HOMA-B, adrenal weight, hepatic gene expression of Agt, and TNF-α in rats subjected to sub-chronic stress.

Based on the findings, serum glucose levels, serum insulin levels, HOMA-IR, and HOMA-B recorded no significant changes in the stressed subjects compared to the control group. However, it is reported that stress leads to insulin resistance, and hyperglycemia via complex and interrelated mechanisms, including hormonal pathways (e.g., cortisol, catecholamines, growth hormone, and glucagon), the activation of RAS, and the production of pro-inflammatory cytokines like TNF-α.[11,28] The observed normoglycemia might be due to a one-week recovery period following sub-chronic restraint stress. A previous study reported normalized hyperglycemia, hyperinsulinemia, and insulin resistance in rodents in response to 7- and 14-day recovery periods administered after the noise stress treatment.[29] The post-stress recovery period allows regulatory mechanisms to normalize stress-induced hyperglycemia via different hormonal compensatory mechanisms, attenuated function, and expression of insulin receptors to enhance glucose uptake into body tissues from the blood.[29] In contrast, other studies demonstrated persistent hyperglycemia despite a period of post-stress recovery due to metabolic effects of chronic unpredictable mild stress.[12,30] These paradoxes may be explained by different stress types, recovery durations, and the mechanistic pathways of stress-related disorders in various studies.[12,31]

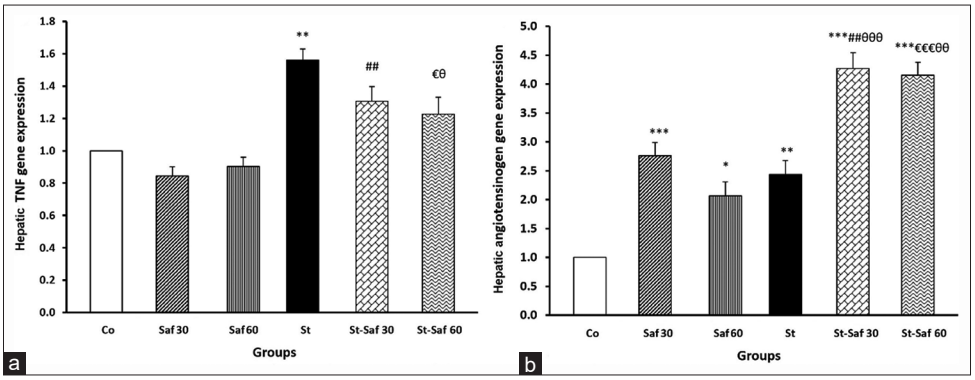

Figure 5: (a) Hepatic TNF-α and (b) angiotensinogen gene expressions measured by RT-PCR. Results are expressed as means ± SEM (ANOVA test, LSD post-hoc test). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control; $^{\theta}P < 0.05$, $^{\theta\theta}P < 0.01$ and $^{\theta\theta\theta}P < 0.001$ compared to the stress group; $^{\#\#}P < 0.01$ compared to the saffron 30 mg/kg group; $^{\epsilon}P < 0.05$, and $^{\epsilon\epsilon\epsilon}P < 0.001$ compared to the saffron 60 mg/kg group. **Co:** Control group, **St:** stress group, **Saf30:** Saffron at dose of 30 mg/kg group, **Saf60**: Saffron at dose of 60 mg/kg group, **St-Saf 30:** Stress-Saffron (30 mg/kg) group, **St-Saf 60:** Stress-Saffron (60 mg/kg) group

It was, however, notable that stress-induced adrenal gland hypertrophy and increased hepatic gene expression of TNF-α and Agt persisted despite the recovery period. In agreement with the present findings, Roy *et al*. (2010) showed that the recovery period following stress failed to restore elevated HPA activity and serum cortisol to normal levels.[32] Moreover, some research studies reported the adaptation of the HPA axis happened after 5–14 days of stress exposure.[27,33,34] Meanwhile, a study reported that one-week recovery period after stress returned serum corticosterone concentration to the baseline levels.[35] Previous studies described that the HPA axis activation and catecholamine release upregulated expression of angiotensinogen and other RAS components (in both mRNA and peptide levels) in response to restraint stress.[5,36]

Based on other data, serum glucose and insulin levels, insulin resistance, and β-cell function were not affected via both doses of saffron in the normal subjects. Consistent with present results, Shirali *et al*. (2013) also reported that neither fasting blood glucose nor insulin levels in healthy subjects did not alter following crocin treatment.[37] In contrast, another study showed that one-week saffron extract administration significantly decreased serum glucose levels; while, it had no such effects on insulin levels.[38] Further exploring the results, adrenal gland weight and hepatic TNF-α gene expression levels remained unchanged in response to both doses of saffron treatment in normal conditions. Consistent with these findings, a recent study reported unaffected serum cortisol levels following aqueous saffron extract treatment in healthy subjects.[27] Akin to our results, Asbaghi *et al*. (2020) found that saffron attenuated the TNF-α levels only in subjects with non-physiologically higher TNF-α levels, but not in those with a regular expression of this inflammatory marker.[36] Some studies have reported attenuated hepatic TNF-α gene expression following saffron treatment in healthy subjects.[39] Collectively, TNF-α gene expression may be linked to saffron dosage, the duration of administration, and baseline levels of TNF-α in subjects.[40] In addition, saffron treatment strongly stimulated hepatic Agt gene expression in healthy animals, with the lower dose having a more powerful effect. Crocin and quercetin are two active constituents of saffron extract, which have been shown to inhibit angiotensin receptors (AT1) by interfering with the calcium signaling pathway.[41] Moreover, it is well established that the AT1 receptor blocking leads to increased plasma levels of angiotensin II,[42] which in turn stimulates angiotensinogen synthesis and secretion.[43]

Saffron administration at both low and high-doses following sub-chronic stress exposure led to marked hyperglycemia and elevated HOMA-IR and hepatic gene expression of Agt compared to the control group. Nonetheless, there were no such effects on serum insulin levels, HOMA-B, adrenal weight, and TNF-α gene expression in the same comparison. Therefore, both doses of saffron extract exert relatively the same effects on the studied parameters in stress-saffron groups compared to normal states. Only low dose of saffron administration after stress exposure resulted in hyperglycemia with unchanged insulin levels, HOMA-IR, and HOMA-B indices compared to the stress-only group. However, both doses of saffron in the present study impaired glucoregulation to some degree in rats exposed to sub-chronic stress. Some previous studies reported that saffron and its active components facilitate insulin signaling, decrease insulin resistance, and high glycemic in rats with diabetes as metabolic stress.[44,45] Furthermore, saffron at both doses triggered the hepatic Agt gene expression in the stressed subjects in comparison to the control and stress-only groups. It has been reported that local RAS activation is involved in physiological and pathophysiological (e.g., inflammation) processes, cellular proliferation, and apoptosis in various tissues. Hence, it played a significant role in hyperglycemia induction.[46] Also, the RAS activation plays a significant role in inducing systemic insulin resistance and glucose intolerance via cross-talking insulin signal transduction, promoting inflammation, oxidative stress, and expression of TNF-α in hepatocytes.[47] Therefore, it is suggested that the remarkable Agt expression and RAS activation might be credible for hyperglycemia and insulin resistance in St-Saf groups.

Based on other results, comparing saffron treatment following stressful and normal conditions demonstrated higher serum glucose levels in the stress-saffron animals. Moreover, saffron treatment at a dose of 60 mg/kg in stressed subjects led to insulin resistance, β-cell dysfunction, and adrenal hypertrophy compared to its non-stressed counterpart (Saf 60 group). Hyperglycemia may be responsible for β-cell dysfunction in the St-Saf 60 group compared to its non-stressed counterpart.[15] Also, both saffron doses upregulated hepatic gene expression of Agt in the sub-chronically stressed subjects compared to their non-stressed counterparts. Thus, it seems that subsequent stimulation of hepatic Agt gene expression could synergistically lead to a decisive enhancement in the Agt gene expression by stress and saffron treatment.

Finally, the dose of 30 mg/kg of saffron alleviated adrenal gland hypertrophy in the sub-chronically stressed subjects compared to the post-stress recovery period group, which indicated the modulating effect of low-dose saffron on adrenal gland activity. Asalgoo *et al*. (2017) confirmed that saffron modulates the HPA axis activity via decreased serum glucocorticoid levels and corticotropin-releasing hormone.[48] Besides, saffron treatment only at a dose of 60 mg/kg recorded attenuated hepatic gene expression of TNF-α in the sub chronic-stressed rats compared to rats subjected to a recovery period following stress. TNF-α interferes with insulin signaling and upholds stress hormone production resulting in worsening of insulin resistance.[14,39] The attenuated TNF-α gene expression due to high dose saffron may be accountable for the non-significant lower gene expression of Agt in the St-Saf60 group in comparison to the St-Saf30 group. Also, decreased TNF-α gene expression in this group might be explained by saffron's radical scavenging properties[15] and/or its attenuating effects of saffron on the upregulation of Agt gene expression. Yang *et al*. (2017) showed that crocin (as the main active component of saffron)

improved insulin signaling pathways, blocked reactive oxygen species generation, and down streamed TNF-α production.[49] Finally, the main limitation of the current study was the lack of prior studies on the effects of saffron on glycemic control under stressful conditions, which warrants further studies to address the interplay between saffron, stress, and their impact on mechanisms involved in glycemic control. Moreover, some possible *in vitro* and *in vivo* mechanisms requires further by using different saffron doses administered with varying durations of stress.

Conclusion

To sum up, one week of the recovery period after stress restored serum glucose levels and insulin sensitivity. It may need to alleviate stress-induced adrenal gland hypertrophy, elevated hepatic TNF-α, and Agt gene expression for a longer time. Saffron administration following sub-chronic stress exposure effectively abolishes adrenal activity with low-dose saffron. It attenuates the production of the pro-inflammatory cytokines like TNF-α with high-dose saffron treatment. The interaction of saffron with sub-chronic stress dominantly promoted RAS activity. Thus, one week of saffron treatment after sub-chronic stress not only did not improve glucose tolerance but also enhanced insulin resistance.

Acknowledgments

The authors would like to thank Dr. Mohammad Kazemi and Mrs. Arezou Dabiri for their valuable assistance. This research study was supported by the Isfahan University of Medical Sciences, Isfahan, Iran.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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