A Combination of Herbal Compound (SPTC) Along with Exercise or Metformin more Efficiently Alleviated Diabetic Complications Through Down-Regulation of Stress Oxidative Pathway upon Activating Nrf2-Keap1 axis in AGEs Rich Diet-Induced type 2 Diabetic Mice

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Research

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Abstract

**Background:** SPTC is a mix of four herbal components (*Salvia officinalis, Panax ginseng, Trigonella foenum-graecum*, and *Cinnamomum zeylanicum*) which may prevent the development of AGEs rich diet-induced diabetic complication and liver injury via activating the nuclear factor erythroid-2-related-factor-2 (Nrf2) pathway. Nrf2, as a master regulator of antioxidant response elements by activating cytoprotective genes expression, decreases oxidative stress associated with hyperglycemia and increases insulin sensitivity. We hypothesized that the combined effect of SPTC along with exercise could efficiently moderate oxidative stress with more favorable effects in the treatment of diabetes.

**Methods:** We induced diabetes in C57BL/6 mice by AGEs using a diet supplementation and limitation of physical activity. After 16 weeks of intervention, AGEs fed mice were compared to control mice. Diabetic mice were assigned into seven experimental groups (n=5): diabetic mice, diabetic mice treated with SPTC (130 mg/kg), diabetic mice treated with *Salvia Officinalis* (60 mg/kg), diabetic mice treated with metformin (300 mg/kg), diabetic mice with endurance exercise training, diabetic mice treated with SPTC+metformin (the same as mentioned), diabetic mice treated with SPTC+exercise training

**Results:** SPTC+exercise and SPTC+metformin reduced diabetic complications like gain weight, water, and calorie intake, blood glucose, insulin, and GLUT4 content more efficiently than each treatmen. These combinations improved oxidative stress hemostasis by activating the Nrf2 signaling pathway and attenuating keap1 protein more significantly.

**Conclusions:** Eventually, combined treatment of SPTC with exercise and metformin as a novel approach had more beneficial effects to prevent the development of diabetes and oxidative stress associated with hyperglycemia.

Introduction

One of the most complex prevalent disorders all over the world is Type 2 diabetes mellitus (T2DM) that is rapidly growing globally. T2DM has been connected with unhealthy dietary habits, including high carbohydrate and advanced glycation end products (AGEs) rich meals and sedentary life-style, and obesity in recent years [1–3]. Heterogeneous pathomolecular cause of diabetes is associated with modulation in several signaling pathways, in which most important of them is stress oxidative. In general, T2DM is characterized by hyperglycemia and hyperinsulinemia that leads to pro-stress oxidative situations. Subsequently, amplified levels of reactive oxygen species (ROS) and inflammatory factors and reduction of the antioxidant defenses cause tissue injury and long term complications of T2DM [4].

The body is equipped with several defense mechanisms against oxidative stress and returning to normal conditions. Nrf2-Keap1 signaling pathway is one of the well-known of these molecular mechanisms. Nrf2 has a cytoprotective role in response to oxidative stress [5, 6] as well as in insulin sensitivity, mitochondrial bioenergetics, glucose and lipid metabolism, drug metabolism [7], and inflammation alleviation. Nrf2 signaling is triggered by detachment from Keap1 (a protein-rich in cysteine and the best-
studied Nrf2 inhibitor), and translocation of Nrf2 to the nucleus. Subsequently, Nrf2 targets the expression of downstream genes with antioxidant response elements (ARE) in their regulatory regions Such as Gpx1, HO1, Nqo1, and Txn [8, 9]. Hierarchy to this pathway, compounds inducers of ARE related genes expression could independently alter Nrf2-Keap1 structure such as reactive cysteine (Cys) residues in Keap1.

Nowadays, herbal medicine and physical activity are used as a natural approach to increase antioxidant and anti-inflammatory abilities to combat the micro- and macrovascular complications of diabetes [1]. In traditional oriental medicine, herbal extracts have been extensively used as effective medication against T2DM for a long time [10]. The herbal mixture of SPTC that we used in the present study consists of leaves and seeds aqueous extract of four plants include Salvia officinalis, Panax ginseng, Trigonella foenum-graeceum, and Cinnamomum zeylanicum with already identified antioxidant potential. Favorable antioxidant impact of SPTC is due to its flavonoids and polyphenolic components such as ginsenoside, salvianolic acid A, carnosic acid. Therefore, SPTC might be accounted for a reduction of T2DM complications through blocking of stress oxidative pathways.

An alternative factor for improving cell metabolic responses and insulin sensitivity is a regular exercise that is prescribed for relieving T2DM. Physical activity is a well-evidenced feasible approach for maintaining metabolic homeostasis and attenuate complications of diabetes [11]. A variety of studies have supported the beneficial effects of exercise, which is mediated through activation of the Nrf2 pathway and increasing endogenous antioxidant defense [12]. Exercise affects through Nrf2 signaling with different mechanisms including increasing ROS lead to oxidation and modification of keap1 cysteine residues [13]. However, moderate exercise-induced ROS could suppress the oxidative damage by increasing adaptive responses of the body and improving antioxidant capacity in tissues. [14]. This adaptive activation have more protective effects when accompanied by antioxidants treatment [15]. Generally, exercise in combination with antioxidants or Nrf2 activator supplementation has more significant effects on oxidative stress situation in the liver [13]. Here, we hypothesized that utilizing the variance capacity of Nrf2-keap1 complex through exercise and medication with SPTC, might be more efficient to stimulate Nrf2 detachment of keap1 and regulate diabetic related oxidative stress.

As both diet type and calorie intake are prominent environmental factors in the pathogenesis and development of T2DM, in the present study we used AGEs rich diet to induce diabetic mice. Since AGEs have an important role in liver fibrosis development [16] and liver dysfunction is one of the principal complication of T2DM [17], we aimed to assess to examine aforementioned pathways in the liver tissue. In this study, we have utilized high-fat advanced glycation end products (AGEs) rich diet to induce T2DM and stress oxidative in a period of 4 months.

**Materials And Methods**

**Experimental animal and treatments**
Four-week-old male C57BL/6 mice were procured from Royan Institute for Biotechnology (Isfahan, Iran) and were housed in a temperature-controlled facility (24 ± 3 °C and humidity of 65% ±5), under a 12 h light-dark cycle. All experiments were conducted following the guidelines of the committee of the Royan Institute (IR.ACECR.ROYAN.REC1398.44). Mice were acclimatized to the Royan animal housing condition for two weeks. They were held ad libitum to access water and foods throughout the study. After adaptation, mice with an approximate weight of 14 ± 2 g, were randomly divided into two groups of control (Ctrl) and diabetic mice (DM) that respectively fed with normal diet and AGEs-reach diet (Abedpour et al., Unpublished data) which were obtained from Royan Institute for Biotechnology, for 16 weeks. After ensuring the emergence of DM, the diabetic group was divided into seven groups randomly (n = 5), as follows: 1. Diabetic mice (DM group), 2. Diabetic mice treated with SPTC (130 mg/kg, DM/SPTC group), 3. Diabetic mice treated with Salvia Officinalis (60 mg/kg, DM/Sal), 4. Diabetic mice treated with metformin (300 mg/kg, DM/Met), 5. Diabetic mice with endurance exercise training (DM/EX), 6. Diabetic mice treated with SPTC + metformin (as the same dose which mentioned, DM/SPTC + Met) 7. Diabetic mice treated with SPTC + exercise training (DM/SPTC + EX).

SPTC (Salvia officinalis 145 mg, Panax ginseng 145 mg, Trigonella foenum-graeceum 60 mg, and 25 mg Cinnamomum zeylanicum) and Salvia were provided as a dried powder by the Goldaru pharmaceutical company (Isfahan, Iran). All chemical and herbal drugs were administered as a gavage supplement, five times per week (day 1 up to 5 /each week) for 8 weeks.

Once a week during the experiment period, body weights, and 24 h, Calorie, and water intake were measured. At the end of experiments, mice were sacrificed under xylazine and ketamine anesthesia, and blood and tissue samples were separated for subsequent tests.

**Biochemical Analyses Of Serum Glucose And Plasma Insulin Levels**

The serum level of insulin was determined by Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, US) according to the protocol. Glucose tolerance test (GTT) and fasting blood sugar (FBS) were measured using an Alpha TRAK glucometer and its standard strips (Zoetis, US) as described elsewhere.

**Exercise Training Protocol**

Endurance exercise was applied as a type of moderate-high intensity exercise on a motorized treadmill with a 0 ° incline during 8 weeks (5 days/week). At first, mice were acclimated to treadmill exercise for two weeks, and after that speed and duration of treadmill training were gradually increased at a rate of 3 m/min from 10 to 25 m/min in the final session for 45 min (~70% VO₂ max).

**Quantitative Real-time Pcr (qrt-pcr)**
Total RNA was isolated from the liver using TRIzol reagent (Thermo Scientific, USA). To remove contaminating genomic DNA, samples were treated with DNaseI (TaKaRa, Japan). mRNA was reverse transcribed with 1 µg of total RNA using the cDNA synthesis kit according to the manufacturer’s instruction (TaKaRa). qRT-PCR was performed with CYBR green (TaKaRa, Japan) on an Applied Biosystems real-time PCR thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). Evaluation of gene expression was carried out according to the $2^{-\Delta\Delta ct}$ method. Accordingly, relative quantification was calculated according to 18 s rRNA as an internal control. Primers were ordered from micro-gene (Korea), and their sequences are shown in Table 1.

### Table 1
Primer list

| Gene | Forward primer (5’-3’) | Reverse primer (5’-3’) | Annealing temperature (°C) |
|------|------------------------|------------------------|---------------------------|
| Gpx1 | TGAGAAGTGCGAAGTGGAATG | TCTCAAAAGTTCCAGGCAATG | 62                        |
| Tnx  | CTCCCCGCAACAGCCAAA    | GCAGTCATCCACATCCA     | 62                        |
| Nqo1 | GCCAATCAGCGTTCGGTA    | AGTTACATGCATAGAGGTC   | 62                        |
| HO1  | GGCTGTGAACTCTGCTC    | ATACCCACCACACACCTG    | 56                        |
| 18 s rRNA | CGGACACGGACAGGATTG | TCGCTCCACCAACTAA | 59                        |

### Western Blot Analysis

Tissue proteins were extracted using TRI reagent, according to the manufacturer protocol. Proteins were resolved by SDS-PAGE (10%) and transferred to PVDF membranes (Bio-Rad, USA). Membranes were blocked with different blocking buffer containing 10% skim milk (Millipore, USA), and 5% TBST. Then, they were respectively incubated with primary antibodies for 1.5 h (Anti-Nrf2 antibody [1:1000, EP1808Y, Abcam, UK], anti-β actin antibody [1:500, Sigma, USA], anti-Keap1 antibody [1:1000, ab119403, Abcam, UK] and secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP) [1:20000, Santa Cruz SC-2301, HRP-conjugated goat antimouse IgG [1:5000, Dako, Japan P0447]), for 1 h at room temperature. Blots were detected by an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, USA). Image J software (National Institutes of Health, Bethesda, MD, USA) was utilized for quantification of the intensity band.

### Histological Studies
Immediately after mice sacrificing, tissues were fixed in 10% buffered formalin and embedded in paraffin. Accordingly, fixed tissues were then cut into slices with 5 µm thickness. After deparaffinization and hydration, tissue sections were stained with hematoxylin and eosin (H&E) and finally were observed under light microscopy.

**Ros Detection**

ROS production in mice liver was measured using 2',7'-Dichlorofluorescin Diacetate (DCFDA) fluorescence method as previously described. Briefly, liver samples (100 mg) were homogenized in 1 mL ice-cooled (4 °C) 40 mM Tris–HCl buffer (pH:7.4). After diluting to 0.25% with the same buffer, the total homogenate of each sample was divided into two equal portions of 2 mL. Approximately 40 µL of 1.25 mM DCFDA was added to one portion, and the same volume of methanol was added to the other portion (Control). After 20 min incubation of all samples in 37 °C, the conversion of DCFH to the fluorescent product DCF was determined at 488 nm excitation and 525 nm emission using BD FACSCalibur Flow Cytometer (Becton Dickinson, USA). The results were evaluated according to DCF fluorescence intensity.

**Total Antioxidant Capacity**

Total antioxidant capacity of tissue and drug samples were determined using Ferric Reducing Antioxidant Power (FRAP) Assay Kit (Naxifer™- TAC Capacity Assay Kit, NS-15012, Iran) as described in manufacturer protocol.

**Statistical analysis**

The statistical analyses were carried out using GraphPad Prism 8.5 software (GraphPad Software, San Diego, CA, USA). The paired samples t-test was performed to evaluate the diabetic group compared to the control group. Also, One-way analysis of variance (ANOVA) was used to make comparisons between all treatment groups. All experimental results are presented as mean ± SD. p-value < 0.05 represents significant difference between the samples.

**Results**

**AGEs diet contributed to the development of diabetic complications**

Our data clearly showed AGEs rich diet contributes to increased calorie intake and water drinking of model mice (Table 2). The weight gain percentile of AGEs diet group was more than the control group (Fig. 1a). Moreover, the measurement of liver/body weight ratio was shown that AGEs diet causes increasing liver/body weight in DM model mice (Table 2).
### Table 2
Liver/body weight ratio, Calorie intake, and water drinking amount

| Groups                  | Relative Liver Weight (g liver/g body weight %) | Water consumption (mL/day/mouse) | Calories intake (Kcal/day /mouse) |
|-------------------------|-----------------------------------------------|---------------------------------|----------------------------------|
| Control                 | 4.8 ± 0.06                                    | 3.65 ± 0.1                      | 3.71 ± 1.45                     |
| DM                      | 6 ± 0.22<sup>a</sup>                          | 5.89 ± 0.29<sup>a</sup>        | 23.89 ± 1.46<sup>a</sup>        |
| DM/Met                  | 4.1 ± 0.12<sup>b</sup>                        | 4.2 ± 0.3<sup>b</sup>          | 5.38 ± 1.33<sup>b</sup>         |
| DM/SPTC                 | 3.8 ± 0.19<sup>b</sup>                        | 3.85 ± 0.26<sup>b</sup>        | 6.22 ± 1.2<sup>b</sup>          |
| DM/ SPTC + Met          | 3 ± 0.3<sup>b</sup>                           | 4.5 ± 0.13<sup>b</sup>         | 5.23 ± 1.39<sup>b</sup>         |
| DM/Sal                  | 3.5 ± 0.09<sup>b</sup>                        | 4.32 ± 0.15<sup>b</sup>        | 4.3 ± 1.26<sup>b</sup>          |
| DM/EX                   | 3.9 ± 0.1<sup>b</sup>                         | 4.85 ± 0.6<sup>b</sup>         | 4.99 ± 1.65<sup>b</sup>         |
| DM/ SPTC + EX           | 3.1 ± 0.16<sup>b</sup>                        | 3.98 ± 0.16<sup>b</sup>        | 4.21 ± 1.38<sup>b</sup>         |

Values are expressed as mean ± SD. (a) Indicates statistically significant difference with control group at *p* < 0.05, (b) indicates statistically significant difference with DM at *p* < 0.05

After 16 weeks of AGES, rich diet consumption, morphological characteristics of liver tissue are significantly changed (Fig. 2). As a result, this certain diet played an essential role in the histopathological characteristics of the liver. HE staining data from diabetic mice demonstrated the development of steatosis, lymphatic infiltration, and vacoulation of the liver (Fig. 2). AGES diet mice also had a larger liver size compare to the normal diet mice (Fig. 2b). Despite such a clear difference in liver characteristics, HE staining of pancreatic and muscle tissue exhibited no vital difference compared with the control group (Data not shown)

## Molecular And Biochemical Assays

Glucose intolerance and hyperinsulinemia are related onsets for diabetes. Our data indicated that plasma insulin and glucose levels were elevated in mice after 16 weeks by AGES diet (Fig. 1b). Plasma insulin and glucose tolerance tests (AUC insulin and AUC glucose) were higher in mice with AGES diet compared to mice with a normal diet (Fig. 1c). Subsequently, we calculated insulin resistance (HOMA-IR) (Fig. 1b). As depicted, the HOMA-IR index for AGES rich diet diet mice was significantly amplified. Moreover, Mice on AGES rich diet displayed significantly higher insulin resistance than mice on normal diet (Fig. 1b).

Insulin resistance triggers Glut4 pathway impairment in skeletal muscle and, finally, glucose uptake deficiency. Determination of Glut4 expression revealed that this protein level was reduced in mice on AGES reach diet (Fig. 1d).
Ages Rich Diet Increased Oxidative Stress In Liver

To investigate whether AGEs rich diet contributes to making oxidative stress, we analyzed some parameters for oxidative stress in diabetic mice. The data showed that AGEs diet raised the amount of ROS in liver compared to control mice. Gated cells with DCF (a marker of ROS) increased from 25.03% in the control group to 63.65% in the AGEs diet group (Fig. 3a).

Consistently, the total antioxidant capacity of liver declined in the AGEs diet group compared to the control (Fig. 3b). Consequently, we also determined keap1 and Nrf2 protein levels and related antioxidant genes expression as one of the main signaling pathways involved in oxidative stress regulation. AGEs diet resulted in enhancement of the keap1 protein levels whereas, it reduced Nrf2 protein levels and downstream antioxidant genes Gpx1, Ngo1, HO1, and Txn at the level of transcription in (Fig. 3c and d). AGEs rich diet emerged to disturb the balance between ROS generation and antioxidant defense system.

Sptc, As Well As Exercise, Diminished Diabetes Complications

After inducing diabetes via AGEs rich diet, mice were treated with either SPTC or exercise and a compound type of both interventions. The main body portion of SPTC was Salvia extract. Therefore, one group of mice was treated with Salvia extract (DM/Salvia). As positive controls, metformin-treated group, and metformin combination with SPTC were also implemented to assess whether SPTC could able to alleviate the diabetes symptoms or not. Despite the reduction of diabetes complications in all groups relative to the control (no received SPTC, metformin or exercise, and Salvia, Table 2). Besides, liver/body weight ratio in DM mice reduced partly after 8 weeks of treatment with SPTC, metformin, Salvia, and endurance exercise compared to the diabetic group (Table 2). Furthermore, the simultaneous treatment of SPTC plus exercise and SPTC plus metformin caused a decrease in the ratio of liver/body weight compared to the diabetic group.

The liver histological findings demonstrated morphological characteristics were changed, mostly by simultaneous treatment. SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) groups attenuated pathological changes induced in diabetic mice significantly. Also, SPTC, endurance exercise (Ex), and metformin (Met) groups showed lower blood glucose levels and glucose areas under the curve than the diabetic group (DM). Nevertheless, this increase was more in combination groups of SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) groups compared to sole treated groups (Fig. 4b).

Moreover, plasma insulin and FBS were significantly decreased after applying all treatment. However, plasma insulin and FBS in SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC-Met) groups declined more compared to the sole treated group. We also calculated HOMA-IR in all groups. It was lower in all treatment groups, and then this reduction was more in combination groups of SPTC plus exercise.
(SPTC + EX) and SPTC plus metformin (SPTC + Met) than groups of SPTC, metformin (Met), *Salvia* (Sal), and endurance exercise (EX).

The protein expression levels of Glut4 were measured in skeletal muscle of all treatment groups. The level of Glut4 was enhanced in all treatment groups compared to the diabetic group (Fig. 4c). Additionally in SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) were significantly increased than SPTC, metformin (Met), *Salvia* (Sal), and endurance exercise (EX).

**Attenuation of oxidative stress was significant in SPTC plus exercise (SPTC + EX), and SPTC plus metformin (SPTC + Met)-treated groups.**

SPTC, endurance exercise, and metformin treatment amplified the total antioxidant capacity of the liver. Of important antioxidant capacity of SPTC was more than metformin (Met) and it was supposed to be mainly due to the constituents of *Salvia* extract (supplementary figure). Nevertheless, SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) restored total antioxidant capacity more than those treatments alone. We observed that combinatory treatments increased the abundance of Nrf2 and decreased Keap1 proteins. Consistently, SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) groups indicated a more increased in Nrf2 levels and antioxidant gene expression compared to each treatment alone. Moreover, transcript levels of liver antioxidant genes, including *Gpx1*, *Nqo1*, *HO1*, and *Txn*, were significantly increased in groups of SPTC, exercise (EX), metformin (Met), and *Salvia* (Sal), whereas they were higher in SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met).

**Discussion**

The present study indicated more pronounced effects of the combination of SPTC with exercise or metformin on diabetic complications and oxidative stress in AGEs-rich diet-induced diabetic mice compared to each treatment alone. In this study, high oxidative stress markers were observed after AGEs rich diet supplementation in C57BL/6 mice liver. These results were in good agreement with the previous studies that exhibited the role of AGEs and RAGE in the progression of metabolic syndrome like DM by activation of oxidative stress and inflammatory pathways [18, 19]. AGEs could be led to ROS production by interacting with its ligand-receptor RAGE and leading to NADPH oxidase activation [20]. It has been explored that in type 2 diabetes, AGE-RAGE signaling inhibited SIRT1 expression and subsequently downregulation of Nrf2 and downstream antioxidant genes [21]. Dysfunction in Nrf2-keap1 signaling as an essential defense system against oxidative stress was indicated in the growing evidence which results in an imbalance between antioxidant and ROS production, and subsequently leads to high blood glucose associated with DM [22].

In the liver, there is a relevant pathway of Nrf2 restricted gluconeogenesis-related gene expression which plays a role in insulin sensitivity, maintenance of normal level of blood glucose and obesity prevention [23]. In the present manuscript we have tried to figure out an interconnection with activation of Nrf2-
keap1 signaling pathway as a multifactorial process and modulation of oxidative stress associates with diabetes.

Additionally, we investigated significant depletion of blood glucose and insulin levels, insulin resistance, and GLUT4 protein level after treatment with SPTC, endurance exercise training, metformin, and combination treatment of SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met). In general diabetic complications decreased in combination treatment of SPTC plus exercise (SPTC + EX) and SPTC plus metformin (SPTC + Met) compared to each treatment of SPTC, exercise (EX), *Salvia* (Sal), and metformin (Met). Moreover, we found that SPTC and exercise (EX) had strong antioxidant effects on AGEs-rich diet-induced diabetic mice as observed by elevation of Nrf2 protein level and *HO1, Gpx1, Txn*, and *Nqo1* genes expressions and depletion of Keap1 protein level in liver. Furthermore, combination treatment had the highest additive effect in the induction of antioxidant capacity of the liver rather than each treatment alone.

Antioxidant therapy as new clinical trials is promisingly used for medicinal purposes and deal with many diseases [24]. Currently, phenolic compounds like flavonoids and polyphenols which have natural origins and lesser side effects compared to synthetic drugs are widely suggested to treat diabetic damage [25]. Given that mixed therapy of antioxidant herb with other antidiabetic applications had more combination effects, we hypothesized that Nrf2 signaling antioxidant activator might be affected with exercise and metformin than each treatment alone. Our results showed that combination treatment of SPTC plus exercise and SPTC plus metformin exerts more capable effects in diabetic injury and modulation of oxidative stress. Also, our findings showed that these combined interventions in this study strongly caused more control oxidative stress by activation of the Nrf2-keap1 signaling pathway. This is possibly mediated by various complementary mechanisms that lead to Nrf2 activation and thereby induction of related downstream cytoprotective gene expression.

*Salvia officinalis* with significant bioactive composition have been extensively used for the treatment of type 2 diabetes in herbal medicine [26]. Carnosic acid is one of the main polyphenolic diterpenes of *Salvia officinalis* and was found as an activator for Nrf2-keap1 transcriptional pathway. Takumi and colleagues observed that Carnosic acid leads to phase 2 enzymes induction by binding to keap1 cysteines and NRF2 nuclear translocation [27]. Salvianolic acid A is another robust antioxidant compounds found in herb *Salvia officinalis* and was reported to activate Akt/mTORC1 signaling which results in Nrf2 phosphorylation [28, 29]. Ginseng, as a traditional anti-diabetic herb was indicated to inhibit pro-inflammatory factors and promotes the Nrf2/keap1 pathway. Ginsenoside Rg1, the main active ingredient of Ginseng, by decreasing NLRP3 leads to down-regulation of ILb, which further restricted NF-κB activation [30]. NF-κB unidirectionally acts as an antagonizing factor against the Nrf2 through depletion of MAF protein, which interacts with Nrf2 [31].

As we reported, exercise training and metformin treatment diminished diabetic complications and induced Nrf2-keap1 signaling pathway. Nonetheless, as mentioned, their effects were more pronounced in combination with SPTC. It is presumable that different mechanisms of each treatment had possibly
additive effects to improve DM and reduced oxidative stress injury. Metformin, as a first-line antidiabetic medicine, was reported to induce Nrf2 gene expression along with BACH1 protein reduction [32]. BACH1 protein was recognized as an Nrf2 repressor by binding to Nrf2 sites and causes inhibition of antioxidant response element genes expression [33].

Exercise with increasing ROS moderately and oxidation of cysteines residues of keap1, leads to the formation of disulfide bridges, alteration of the Nrf2/Keap1/Cul3 Complex structure and Nrf2 detachment from keap1 [12, 13]. In response to inducing ROS formation during physical training, Nrf2 is activated through oxidation of the cysteine residues in Keap1 and subsequently Nrf2 dissociation [34]. Moreover, it has been reported that PGC-1α modulates the systems to tolerate induced ROS production in exercise. Exercise could be induced by PGC-1α protein expression and activation. After that PGC-1α might interact with Nrf2 and causes antioxidant gene expression. This interaction and induction of Nrf2 related pathways are involved in different adaptations to long term training [35].

Besides, our finding showed that the antidiabetic approach combining the protective effects of SPTC in association with exercise training and metformin is more effective in modulating oxidative stress associated with type 2 diabetes. This study generally reported that SPTC plus exercise and SPTC plus metformin dramatically reversed AGEs induced diabetic changes and decreased oxidative stress injury more than each treatment alone. Moreover, all treatments of SPTC, exercise, metformin, Salvia and their combinations reduced blood glucose and insulin levels, insulin resistance and GLUT4 protein level. Also, all treatment amplified Nrf2 protein levels and its antioxidant gene expression of HO1, NQO1, GPX, and Txn and declined KEAP1 protein level in diabetic mice liver. These favorable effects were more remarkable in combination treatment. Thus, we suggested that combination therapy of SPTC plus exercise (SPTC + EX) or metformin (SPTC + Met) could serve as a potential intervention approach through activating the Nrf2-keap1 pathway and alleviating oxidative stress associated with diabetes mellitus. We hope that future studies will obtain further experimental data to support our results.

**Conclusion**

Our data demonstrated more beneficial effects of SPTC supplement in combination with exercise (SPTC + EX) and metformin (SPTC + Met) in AGEs rich diet-induced diabetic mice than each treatment alone, suggesting that SPTC + EX and SPTC + Met could be an accessible and therapeutic approach related to lifestyle in diabetic individuals.

**Abbreviations**

AGEs, advanced glycation end products; ANOVA, One-way analysis of variance; ARE, antioxidant response elements; Cys, Cysteine; DCFDA, 2',7'-Dichlorofluorescin Diacetate; T2DM, type 2 diabetes mellitus; FBS, fasting blood sugar; FRAP, ferric reducing antioxidant power; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GTT, glucose tolerance test; H&E, hematoxylin and eosin; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.
Declarations

- Ethics approval for animal usage

Approval of mouse usage in this study was obtained by the Ethics committee of Royan Institute.

- Conflict of interest

None of the authors has any conflicts of interest to disclose.

- Consent to publish

All authors support submission to this journal.

- Availability of data and materials

All of the raw data and the rest of the materials are remained in Royan Institute for Biotechnology and are available upon request.

- Competing interests

There is no competing of interest to disclose.

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- Authors’ Contributions

The design of study was done by G. R., S.H., K. G., and M.H. N.E. and experiments were performed by G. R., S.H., Analyses and data mining were performed by G. R., S.H., B.R., N. A., I. N., M. P., Z. D., and F.S.F. technical assistance were performed by Z.S, N. A. Interpretation of the obtained information was done by G. R., S.H, K. G., and M.H. N.E. The manuscript was written by G. R., S.H and was approved by K.G., M.H. N.E.

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Figures
Figure 1

Improvement of oxidative stress condition of AGEs rich diet-induced diabetic mice with different treatment groups. Total antioxidant capacity of liver (a). Gpx1, HO1, Nqo1, and Txn mRNA expression were measured using qPCR and normalized to 18s rRNA (b). Nrf2 and Keap1 proteins expression in liver (c). Values were expressed as mean ± SD (n= 5 per group). Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc test.
Figure 2

Improvement of diabetic complications of AGEs rich diet-induced diabetic mice with different treatment groups. Measurement of plasma insulin concentration, HOMA-IR and fasting blood sugar (a). Glucose concentration and area under the curve glucose (b). GLUT4 protein expression in skeletal muscle (c). All values were expressed as mean ± SD (n= 5 per group). Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc test.
Figure 3

AGEs rich diet was impaired by the endogenous redox system in liver. Liver ROS production was measured using DCF fluorescence method (a). Total antioxidant capacity of liver (b). Gpx1, HO1, Nqo1, and Txn expression were measured using qPCR and normalized to 18s rRNA (c). Nrf2 and Keap1 proteins expression in liver (d). Values were expressed as mean ± SD (n= 5 per group). #p<0.05 indicates a
statistically significant difference in diabetic mice in comparison to the control mice. Data were analyzed by t-tests.
Figure 4

AGEs of rich diet induces liver damage in diabetic mice rather than control mice. Liver morphology of control and diabetic mice (a). H & E staining of liver tissue (b). Semi-quantitative evaluation of steatosis, lymphatic infiltration, and vacoulation in liver tissues (c). Values were expressed as mean ± SD (n= 5 per group). ##p < 0.01 indicates a statistically significant difference in diabetic mice in comparison to the control mice. Data were analyzed by t-tests. Liver damages were scored by a pathologist after observing the samples of H & E staining.
Figure 5
AGEs of rich diet exacerbates diabetic symptoms in mice rather than control mice. Control mice (Ctrl) and AGEs rich diet-induced diabetic mice, body weight ratios and weight gain percentile (a). Measurement of plasma insulin concentration, HOMA-IR and fasting blood sugar (b). Glucose concentration and area under the curve glucose (c). GLUT4 protein expression in skeletal muscle (d). All values were expressed as mean ± SD (n= 5 per group). # p < 0.05, ## p < 0.01 indicate statistically significant difference in the diabetic mice in comparison to the control mice. Data were analyzed by t-tests.

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