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Original Article

Antioxidant effects of astaxanthin and metformin combined therapy in type 2 diabetes mellitus patients: a randomized double-blind controlled clinical trial

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Abstract

Background and purpose: Since the critical role of oxidative stress in the pathogenesis and complications of type 2 diabetes mellitus (T2DM) has been proven, antioxidant therapy is considered an applicable strategy to control T2DM development. This study aimed at evaluating the effect of astaxanthin (AST) supplementation combined with metformin on oxidative indices and antioxidant defenses in T2DM patients.
Experimental approach: In this randomized, double-blind placebo-controlled trial, 50 T2DM subjects receiving metformin were supplemented with 10 mg/day AST or placebo for 12 weeks. Malondialdehyde concentration and serum total antioxidant capacity (TAC) were assessed as oxidative indices. We also evaluated NF-E2-related factor2 (Nrf2) as the most critical transcription factor of antioxidant defense. Moreover, the activity of antioxidant enzymes, superoxide dismutase (SOD), and catalase were calculated.
Findings/Results: AST supplementation-metformin combination caused a significant increase in SOD and catalase activities, as well as inducing Nrf2 protein expression compared to the placebo group.
Significant changes in serum malondialdehyde and TAC between the AST group and placebo group after supplementation were not observed, although a significant increase was observed in TAC within the AST group after supplementation (32.67 ± 6.73) to before (25.86 ± 5.98). These results remained without change after adjustment for potential confounders.
Conclusion and implications: Our study demonstrated that AST supplementation controlled oxidative stress through a synergistic effect with metformin and ameliorated overall antioxidant capacity by inducing Nrf2 transcription factor and activating SOD and catalase in T2DM patients. As a result, AST and metformin combination therapy can be considered beneficial in modifying oxidative stress and preventing T2DM complications.

Keywords: Astaxanthin; Malondialdehyde; Nrf2; Oxidative stress; Superoxide dismutase; T2DM.

INTRODUCTION

Diabetes mellitus (DM) is introduced as one of the major non-contagious diseases by the World Health Organization (WHO) and its prevalence is increasing worldwide. It is estimated that by 2040, about 642 million people will suffer from type 2 diabetes mellitus (T2DM) as the main type of diabetes (1). Despite various complications such as nephropathy, neuropathy, and retinopathy, T2DM is a potential coronary heart disease risk factor. About 50% of individuals with longtime DM usually develop renal damage and progressive impairment in renal function correlated with a higher risk of cardiovascular events and hospitalizations. Considerably, 50-70% of all DM patients die due to cardiovascular disease (2).

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Experimental studies have highlighted a direct relationship between oxidative stress and diabetes, which is recognized by measuring oxidative stress biomarkers in types 1 and 2 diabetic patients. Oxidative stress has a crucial role in T2DM development and the pathways involved in its pathogenesis (1,3). It has been shown that the production of reactive oxidative species (ROS) is increased and antioxidant capacity is decreased in T2DM patients. It is usually led by some induced hyperglycemic mechanisms, including alteration of eicosanoid metabolism, activation of protein kinase C, the elevation of the polyol pathway flux, and induction of glucose autoxidation. ROS has serious adverse effects on inducing and intensifying diabetes, such as reducing glucose transport channels and insulin secretion, fragmentation and oxidation of protein, DNA damage, free fatty acid generation, and increased vascular permeability. Moreover, oxidative stress induces the formation of advanced glycation end products which develop vascular complications of T2DM (4).

Based on experimental and clinical studies, the disturbance of the natural antioxidant defense system might contribute to oxidative stress. The antioxidant defense system consists of several low molecular weight antioxidants, some metal-binding proteins, and some specific enzymes including superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase (5). It is regulated by many transcription factors. Nuclear factor erythroid 2-related factor 2 (Nrf2) is recognized as one of the most important transcription factors and the master regulator of antioxidant damage and endogenous antioxidant (6). In an increased ROS production situation, Nrf2 is released from the cytoplasm, followed by nuclear translocation and located on the promoter regions of genes that encode defense proteins/enzymes (7). Nrf2 regulates the transcription of enzymatic antioxidant defense proteins including heme oxygenase1, SOD, NAD(P)H dehydrogenase quinone 1, catalase, and others. In this regard, the assessment of the circulatory levels of Nrf2 and measurement of total antioxidant capacity (TAC), malondialdehyde (MDA), and ROS levels are suggested as the important biomarkers for oxidative stress (6,7).

Concerning the role of oxidative stress on T2DM, the use of antioxidants provides an attractive therapeutic strategy to prevent diabetic complications. Antioxidant therapy has been considered as a potential approach to control and reduce T2DM pathogenesis (8). Some natural products have been confirmed as antioxidant interventions to decline oxidative stress through various mechanisms such as enhancing biological enzymatic antioxidant activity and scavenging free radicals in diabetic subjects (9,10).

Astaxanthin (AST), a natural xanthophyll carotenoid pigment, plentifully exists in yeasts, microalgae, trout, salmon, krill, and other seafood with some health benefits. AST has some advantages to diabetic patients, including the repression of oxidative stress and the control of some diabetic complications (11,12). Molecular targets of AST including transcription factors, cytokines, signaling proteins, enzymes, hormones, and receptors are diverse (13). The wide spectrum of biological targets confers several pharmacological activities to AST such as positive effects on lipid and cholesterol metabolism and potential anti-hyperglycemic effects. For example, AST improves glucose metabolism, enhances insulin signaling, and prevents steatohepatitis in insulin-resistant mice (14).

Furthermore, AST is best known for its antioxidative activity. Because of its unique structure, it is able to incorporate into the cellular membranes without destroying them. Interestingly, AST is one of the rare antioxidants that can make a move all over the body and protect all of the body cells and prevent lipid-based oxidation (13,15). Moreover, its bioactivity and ability to scavenge ROS in humans are much more than other carotenoids like α and β-carotene and α-tocopherol. It has been observed that AST improves the activity of enzymatic antioxidants, such as SOD and catalase, and reduces serum MDA content in animal models (13).

Considerably, some reports claim that oxidative stress biomarkers decline in metformin-treated patients (7,16). Metformin co-treatment was found to reduce ROS production rate and maintain redox homeostasis inside the cells by upregulating the antioxidant

proteins and improving the function of antioxidant machinery (17). Co-treatment and intensifying the antioxidative effect of metformin by natural antioxidants can be helpful to prevent and block oxidative stress and its consequences in T2DM. Given these points, it seems reasonable to assume that AST can augment the efficacy of metformin in reducing ROS formation. Up to now, the antioxidant effects of metformin and AST combination on T2DM have not been investigated (18). Therefore, this study aimed to evaluate the antioxidant effects and ROS scavenger mechanisms of AST in T2DM patients receiving only metformin as antidiabetic medication as well as the Nrf2 protein expression along with its downstream targets, antioxidant enzymes SOD and catalase, as a randomized, double-blind placebo-controlled trial.

MATERIALS AND METHODS

Subjects

Adult subjects aged 20-60 were recruited from the Isfahan specialized clinic No1 (Shahid Asghar Shabani Clinic). The inclusion criteria, included in the study, were diagnosis of T2DM based on American Diabetes Association guidelines (FBS > 126 mg/dL or 2-hour post-load plasma glucose > 200 mg/dL or hemoglobin A1C (HbA1C) > 6.5%) (19) and taking only metformin as standard antidiabetic treatment. Exclusion criteria were considered participation in a concomitant trial, pregnancy or breastfeeding, endocrine diseases other than T2DM (*e.g.* hypothyroidism or hyperthyroidism, hypertension, and so on), impossibility to give informed consent, presence of malignancies, chronic liver disease renal failure (being on dialysis or serum creatinine: 2.0 mg/dL), obsessive-compulsive disorder, chronic inflammatory diseases such as rheumatoid arthritis and acute infections, hyperglycemia due to secondary causes, hypersensitivity to the study medication, receiving hormone therapy or other herbal medicines, and lack of compliance with the study medication.

Study design

A total of 200 patients were assessed for eligibility in the trial. Sixty out of 200 subjects met the inclusion criteria (consort diagram, Fig. 1). Sample size in the current study was determined according to the following formula for comparing means between two independent groups. Considering type one error rate of 5%, statistical power 80% for detecting standardized effect size ($\Delta = 0.8$) (20) for study main outcomes and an equal number of patients in each group ($\varphi = 1$) lead to 50 samples. We considered a 20% additional sample for compensating possible attrition. Patients were randomly allocated by using permuted block randomization of block size 4 (1:1) from generated random digits by using SPSS software based on the uniform distribution (0-1) to receive either the AST supplement (n = 30) or the placebo supplement (n = 30) for 12 weeks.

$$m = \left(\frac{1+\varphi}{\varphi}\right)\frac{(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2} + \frac{z^2_{1-\alpha/2}}{2(1+\varphi)}$$

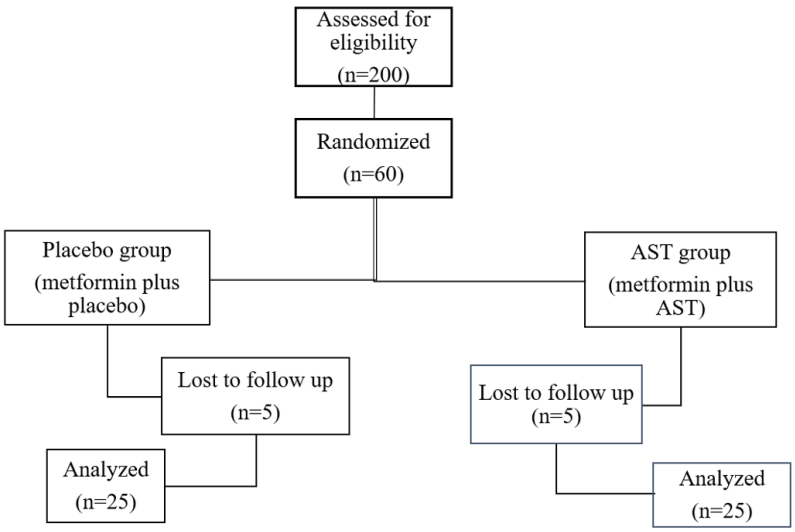

Fig. 1. Flow diagram of patients' recruitment.

This study was planned as a randomized, double-blind (both patients and investigator were blinded about the used treatments by providing the AST supplement and placebo in hard gelatin capsules matched in shape, size, and color.) placebo-controlled trial conducted on 50 T2DM patients taking metformin (at a dose of 1000-2000 mg/day). Based on inclusion criteria, subjects were divided into two groups, including 25 patients each group that randomly were allocated to AST supplement (Waka Tani Health Nutrition Company, USA) or placebo (Barij Essence Pharmaceutical Company, Iran) for 12 weeks (Fig. 1).

The study protocol was approved by the Ethics Committee at the Isfahan University of Medical Science (Ethics ID. IR.MUI.RESEARCH.REC.1398.103), and it was registered in the Iranian Registry of Clinical Trials Center with the number IRCTID: IRCT20190305042939N1 and written informed consent were received from all individuals. The study was carried out between March and May 2020.

Blood sampling and biochemical measurements

Overnight fasting blood samples were collected at the onset and the end of the study. Collected blood samples (4 mL) were centrifuged at 750 *g* for 10 min in vacuum tubes without EDTA to separate serum for biochemical analysis and 6 mL in tubes with EDTA to HbA1C assay and separate peripheral blood mononuclear cells (PBMCs) for western blot analysis. Sera were aliquoted and kept at -70 °C until assessments.

Serum activity of SOD was evaluated by Biorexfars (Iran) SOD kit (No. BXC0531) by WST-1 method. Catalase activity was assessed based on the reaction of undecomposed hydrogen peroxide with ammonium molybdate (21). Plasma MDA level was measured by the thiobarbituric acid reactive substance through 1,1,3,3-tetra methoxy propane as a standard (22). Serum TAC was calculated *via* a previously described colorimetric procedure (23).

Anthropometric and body composition were measured according to standard procedures including weight and body mass index (BMI). BMI was derived from dividing weight (Kg) by square of the body height (m).

Isolation of PBMC and western blot analysis

PBMCs isolation was done by Ficoll-Hypaque density gradient centrifugation of whole blood and they were used instantly after collection (8).

Western blot analyses were performed as previously described with some modifications (24). For western blotting, PBMCs were lysed with RIPA buffer, and the lysates were removed by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentration was determined by the BCA (bicinchoninic acid) protein quantification kit (Cat No: DB9684, DNAbiotech) according to the manufacturer's instructions. The cell lysates were mixed with an equal volume of 2X Laemmli sample buffer. Lysates (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after a 5 min boiling and then transferred to a 0.2 μm immune-blot™ polyvinylidene difluoride (PVDF) membrane (Cat No: 162-017777; Bio-Rad Laboratories, CA, USA). The membranes were blocked with 5% BSA (bovine serum albumin) (Cat No: A-7888; Sigma Aldrich, MO, USA) in 0.1% Tween® 20 for 1 h. After that, the membranes were incubated with anti-Nrf2 (Cat No: ab62352, Abcam) and anti-beta actin-loading control antibodies (Cat No: ab8227; Abcam) for 1 h at room temperature. Then, membranes were incubated with goat anti-rabbit IgG H&L (HRP) secondary antibody (Cat No: ab6721; Abcam); however, they were washed triple with tris-buffered saline Tween® 20 (TBST) before incubation. The membranes were incubated with enhanced chemiluminescence (ECL) for 1-2 min. β-actin was used for protein expression normalization. The gel analyzer version 2010a software (NIH, USA) was performed to densitometry of protein bands, such that the percentage area under the curve of each band was divided by the percentage area under the curve of its corresponding actin band, and then calculated values were compared between groups.

Statistical analysis

The statistical analyses were conducted by SPSS software (version 16; SPSS Inc., Chicago, IL, USA). Quantitative and qualitative variables were presented as mean ± standard deviation and frequency, respectively.

Independent sample t-test and chi-squared test were used to assess the differences between the two groups regarding basic quantitative and qualitative variables, respectively. The normality of continuous variables was assessed by using Kolmogorov-Smirnov and Q-Q plots. Non-normality positively skewed data (lipid indices) were subjected to logarithmic transformation. Statistical analyses for main study variables were conducted in both per protocol and intention to treatment approaches. Changes in variables within groups were evaluated *via* paired student's t-test. Analysis of covariance (ANCOVA) was used for comparing study outcomes at the end of intervention between groups while adjustment was done for baseline value and some unbalanced nutrient intake. The linear mixed effects method was used for conducting intention to treat analysis. A statistically significant level was defined as $P < 0.05$.

RESULTS

Fifty subjects finalized the trial, including 25 in each group. Several anthropometric features of the study groups at baseline are listed in Table 1. Comparison of parameters before *vs.* after intervention did not show significant reductions in weight and BMI in the AST group.

The groups were compared in serum levels of FBS and HbA1c as well as oxidative indices, including MDA and TAC at the beginning of the study (Table 2).

Table 1. The basic characteristics of placebo and astaxanthin groups. Values are presented as mean ± SD.

Variables	Placebo	Astaxanthin	*P*-value
Age	50.48 ± 7.35	52.88 ± 7.23	0.266
Body mass index (baseline)	27.70 ± 3.65	29.64 ± 4.28	0.103
Body mass index (after 12 weeks)	27.73 ± 3.55	29.54 ± 4.30	0.124
Weight (baseline)	79.13 ± 10.53	82.00 ± 16.66	0.486
Weight (after 12 weeks)	79.74 ± 10.97	81.75 ± 17.06	0.635
Hip (baseline)	106.52 ± 5.80	109.68 ± 9.58	0.180
Hip (after 12 weeks)	106.70 ± 6.45	109.25 ± 10.26	0.315
Duration (year)	5.91 ± 2.25	5.97 ± 2.45	0.924
Sex: Male Female	13 (56.5%) 10 (43.5%)	9 (37.5%) 15 (62.5%)	0.191

Table 2. The effects of astaxanthin supplementation on the glycemic indices, lipid profiles, oxidative stress parameters, and blood pressure in type 2 diabetic patients. Values are presented as mean ± SD.

Variables	Placebo group			AST group			
	Baseline	After 12 weeks	*P*-value*	Baseline	After 12 weeks	*P*-value*	*P*-value**
Fasting blood sugar(mg/dL)	121.65 ± 24.38	119.22 ± 28.96	0.49	126.96 ± 26.98	108.96 ± 19.30	< 0.001	< 0.001
Hemoglobin A1C (%)	5.80 ± 0.65	5.68 ± 0.89	0.47	5.82 ± 0.73	5.59 ± 0.60	0.11	0.61
Cholesterol (mg/dL)	138.13 ± 27.24	136.04± 20.27	0.62	152.08 ± 27.13	142.33 ± 25.74	0.01	0.60
Triglyceride (mg/dL)	151.39 ± 47.77	156.87 ± 71.79	0.60	126.54 ± 44.77	122.00 ± 38.01	0.07	0.31
High-density lipoprotein (mg/dL)	41.65 ± 11.13	40.87 ± 10.27	0.65	43.00 ± 9.05	44.42 ± 11.56	0.49	0.30
Low-density lipoprotein (mg/dL)	111.30 ± 17.78	112.22 ± 21.03	0.83	113.42 ± 20.48	111.83 ± 19.71	0.64	0.74
Diastolic blood pressure (mmHg)	8.04 ± 0.86	8.12 ± 0.70	0.70	8.09 ± 0.84	7.58 ± 0.70	0.003	0.56
Systolic blood pressure (mmHg)	12.02 ± 1.36	11.60 ± 0.92	0.09	11.79 ± 1.20	11.58 ± 0.80	0.37	0.78

Although there were no significant differences between the two groups before AST supplementation, FBS significantly reduced in the AST group rather than the placebo group after supplementation. Also, a significant reduction of FBS within the AST group after *vs.* before supplementation was observed (Table 2). HbA1C, high- and low-density lipoprotein (HDL and LDL) were not significantly different between the study groups at baseline and after supplementation (Table 2).

Significant changes in serum cholesterol, triglyceride, MDA (Fig. 2A), and TAC (Fig. 2B) between the AST group and placebo group after supplementation were not observed, although a significant increase was observed in cholesterol, triglyceride, and TAC within the AST group after supplementation in comparison with (32.67 ± 6.73) to before supplementation (25.86 ± 5.98) (Fig. 2B).

Significant elevation in serum SOD was observed in the AST group *vs.* placebo group (34.49 ± 4.20 and 29.37 ± 5.58, respectively). Also, there was a significant difference in SOD within the AST group after supplementation (34.49 ± 4.20) compared to before it (29.98 ± 3.80) (Fig. 3A).

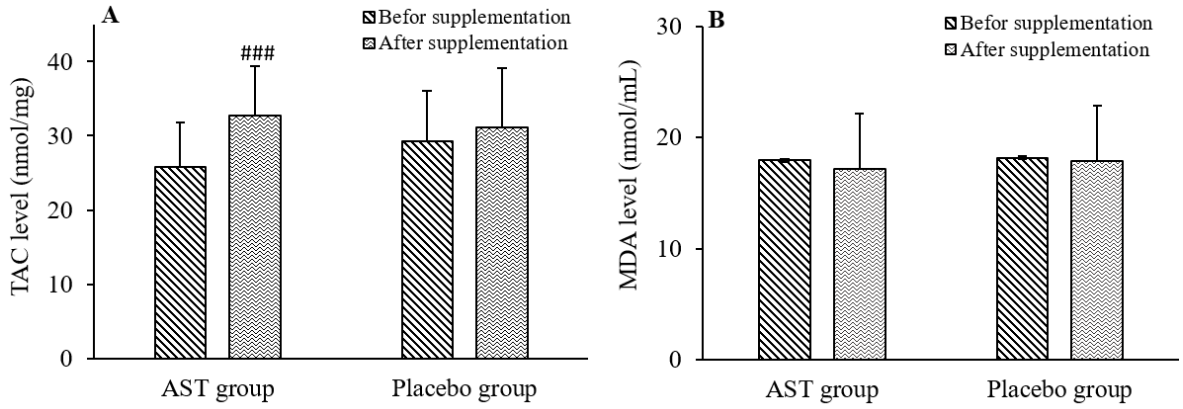

Fig. 2. Evaluation of (A) TAC and (B) MDA levels in AST *vs* placebo groups before and after supplementation. Data are presented as mean ± SD. $^{###}P < 0.001$ Indicates a significant difference within the AST group after supplementation versus before supplementation. AST, Astaxanthin; MDA, malondialdehyde; TAC, total antioxidant capacity.

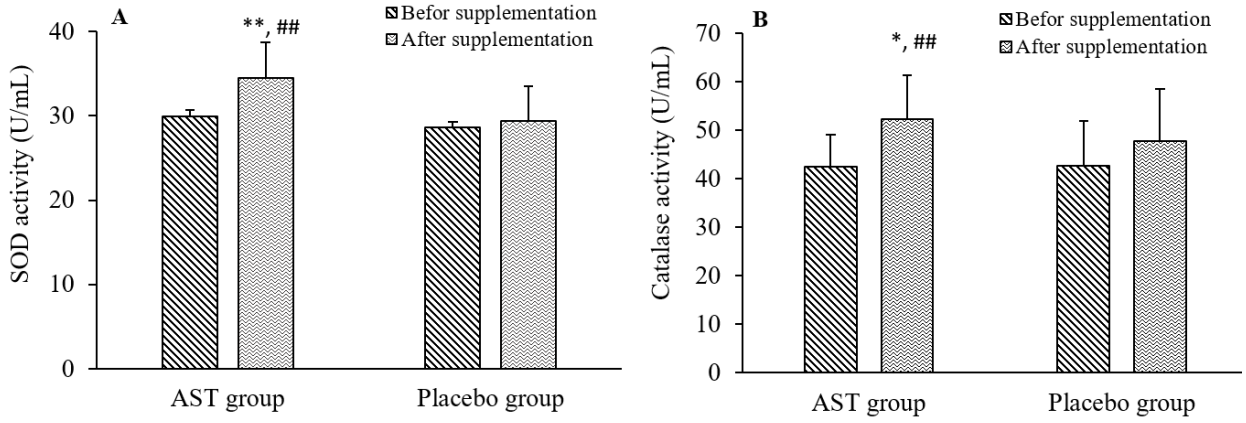

Fig. 3. Evaluation of (A) SOD and (B) catalase activities in AST and placebo groups before and after supplementation. Data are presented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant difference between AST and placebo groups after supplementation; $^{##}P < 0.01$ indicates significant differences within AST group after and before supplementation. AST, Astaxanthin; SOD, superoxide dismutase.

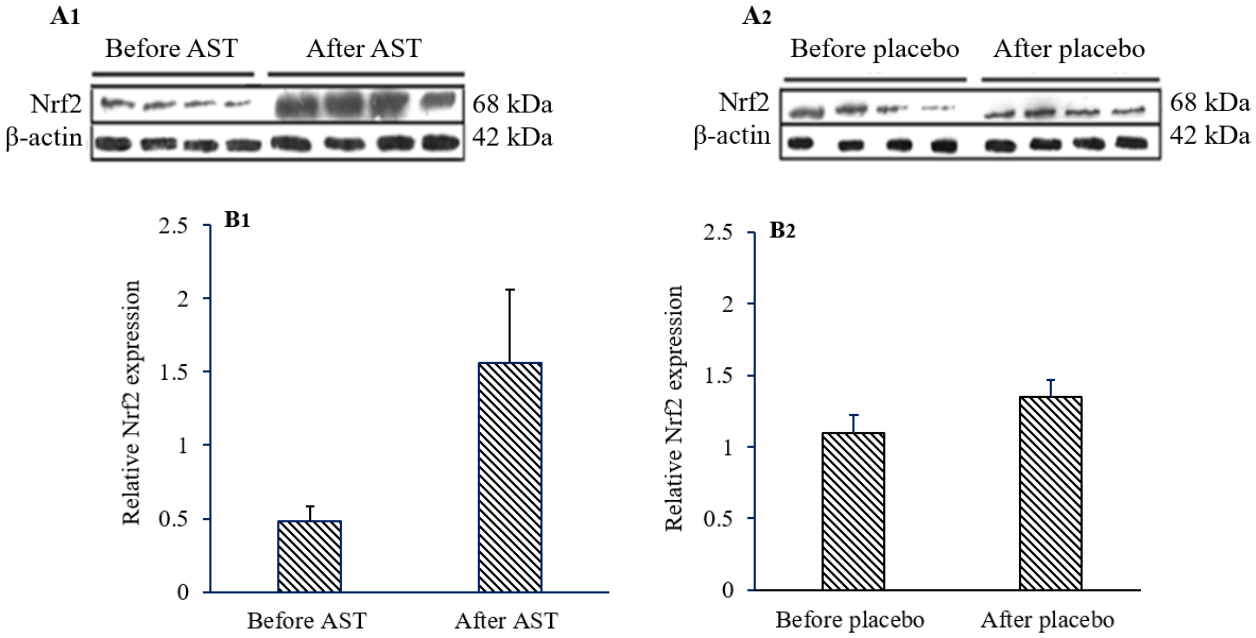

Fig. 4. Expression of Nrf2 was increased in the AST group *vs* the placebo group. (A1 and A2) The expression of Nrf2 protein by western blotting; (B1 and B2) the quantitation of Nrf2 expression. Data are expressed as mean ± SD ****P* < 0.001 Indicates a significant difference. Nrf2, Nuclear factor erythroid 2-related factor 2; AST, astaxanthin.

Equally important, catalase significantly increased in the AST group (52.33 ± 9.02) to the placebo group (47.82 ± 10.75), as well as within the AST group after supplementation (52.33 ± 9.02) relative to before supplementation (42.41 ± 6.73) (Fig. 3B).

Furthermore, the expressions of the Nrf2 transcription factor significantly increased in the AST group (1.55 ± 0.11) compared to the placebo group (0.78 ± 0.13) after supplementation (Fig. 4).

All statistical analyses in the framework of intention to treat for study outcomes were conducted by the linear mixed-effects method. The results did not differ from those obtained in the per-protocol approach.

Compliance, tolerability, and safety

Compliance assessment was based on pill counts. A compliance audit of returned soft-gel capsules indicated 96% compliance in the AST group and 95% in the placebo group. During the treatment period, AST was well tolerated and no patient reported any significant adverse effect.

DISCUSSION

The present study was conducted as a clinical trial to investigate the impact of AST supplementation at a dose of 10 mg/day for 12 weeks on oxidative indices of subjects with T2DM who received only metformin as antidiabetic medication. Most notably, to our knowledge, this was the first study that investigated the synergistic effect of AST supplementation with metformin on oxidative stress in people with T2DM. The results demonstrated that AST with metformin synergistically increased the activity of SOD and catalase by inducing the expression of Nrf2 protein. However, it did not have a significant effect on MDA and TAC levels. These findings demonstrated the ameliorative effect of metformin-AST combination against hyperglycemia-induced oxidative stress.

Basically, disrupted metabolism of carbohydrates and lipids in T2DM contributes to oxidative stress leading to the vascular complications of T2DM. Hyperglycemia enhances ROS production and induces DNA single-strand breaks. Oxidative stress can degenerate pancreatic B-cell function and promote glucotoxicity, lipotoxicity, and the pathogenesis of T2DM. It has been shown that if antioxidant enzymes expression reduces in pancreatic B-cells, the cells are damaged by ROS (9). Higher intracellular glucose levels increase glucose oxidation and NADH and FADH2 entry to the mitochondrial electron

transport chain. Subsequently, electron accumulation in coenzyme Q causes the generation of superoxide radicals. In addition to increased ROS formation, biological antioxidant defenses are depleted in diabetic subjects (9,25). Antioxidant pharmacotherapy is proposed as a useful approach to prevent comorbidities and manifestations of diabetes. In this context, it has been shown that the intake of antioxidants can decrease oxidative damage in diabetic individuals. For example, N-acetylcysteine, ascorbic acid, and α-lipoic acid have been confirmed to be effective in suppressing diabetic complications (25).

Some studies have also emphasized that metformin, the oldest and most commonly used blood sugar reducing drug and the first-line treatment in diabetic patients, has an antioxidant impact in T2DM. Dehkordi *et al*. showed that metformin is efficient in lowering the level of oxidative stress factors by regulating the antioxidant system of the cell (26). Wu *et al*. showed that combination therapy with metformin and salvianolic acid A played more numerous protective roles against vascular and kidney damage in diabetic patients compared with salvianolic acid A or metformin alone (7). Abdelsamia *et al*. found that co-administration of curcumin with metformin has a more significant protective role against cardiomyopathy than metformin alone. Suppression of the JAK/STAT pathway and stimulation of the Nrf2/HO-1 pathway appeared to be one of the mechanisms mediating the effects of curcumin and metformin (16). Esteghamati *et al.* in a randomized controlled trial reported that the use of metformin reduced and regulated oxidative stress indices (27). Therefore, inducing the antioxidant effect of metformin with natural antioxidants has been regarded as a new and considerable approach in managing and controlling oxidative stress in T2DM patients to prevent diabetic complications (28).

AST is a potent natural antioxidant that exerts protective effects such as free radical scavenging *via* the enhancement of cellular antioxidant defense systems. The antioxidant properties of AST are derived from oxygenated groups that exist in ionone rings of its structure (29).

Previous studies have shown that AST can regulate the Nrf2-antioxidant response element pathway and expression of phase II antioxidant enzymes (HO-1 and NQO-1). The Nrf2 antioxidant response element pathway is applied as a remarkable mechanism to inhibit oxidative stress by the cells (29,30). These studies have also presented that treating Nrf2 modulators with different mechanism-based drugs can be a better strategy to prevent the complications of diabetes (7,16,31). As a result, the use of metformin along with AST through the Nrf2 activation mechanism may increase the effectiveness of treatment.

Despite the absence of data from clinical trials, the antioxidant effects of AST have been reported in other conditions closely linked to T2DM. Some *in vitro* and *in vivo* studies have demonstrated that AST prevents cell or tissue damages induced by hyperglycemia and diabetes by reducing oxidative stress (32,33). Ranga Rao *et al.* reported that feeding rats with hematococcal biomass, a rich source of AST, increased thiobarbituric acid reactive substance and the antioxidant enzymes peroxidase, catalase, and SOD in plasma and liver (34). The results of another study indicated that AST from *Haematococcus pluvialis* showed the best antioxidant properties and free radical scavenging in rats compared to β-carotene and lutein (35). In db/db mice, AST not only attenuated the levels of oxidative stress markers including superoxide anion and MDA but also increased SOD in the retinal tissue. Accordingly, it improved oscillatory potentials (36). In the research carried out by Manabe *et al.* AST increased the activity of SOD and catalase and reduced the generated MDA in the serum. it also accumulated in the mitochondria of human mesangial cells and reduced ROS-induced glycemic production (33).

In this research, AST also reduced total blood cholesterol concentrations in the participants, although a significant difference in other lipid profiles such HDL, LDL, VLDL, and triglyceride was not observed. However, in this study, we did not compare the statin used by patients in the two groups. This data extended the results that have been reported for cases with mild hyperglycemia (37). They reported that the administration of 12 mg of

AST significantly increased the HDL cholesterol concentration in non-obese individuals and argued that changes in the adiponectin concentration were positively correlated with changes in the HDL cholesterol concentration (37). Probably, AST dosage and the period of intervention can be considered as a reason for different results. The data of a double-blind, placebo-controlled clinical trial conducted in T2DM patients that randomly received 8 mg/dL of oral AST or placebo for 8 weeks demonstrated that AST supplementation diminished plasma level of MDA and some inflammation factors (20). Consistent with our results, Augusti *et al*. also explained that AST increased SOD activity in hypercholesterolemic rabbits; however, it could not significantly change the level of MDA and other oxidative markers (38). Most of the studies were criticized for being short term, small sample size, and lack of adjustments of proper variables in the statistical analyses. Probably, the differences in results are derived from these factors.

The oral/parenterally administration of AST to T2DM animal models has been shown to activate antioxidant enzymes and reduce oxidative stress, along with an anti-inflammatory effect. Therefore, it may have a notable preventive and protective impact on the beginning and progression of DM and in the development of diabetic complications. However, it should be considered that the factors like the heterogeneity of DM models, dosage, AST source, and outcomes studied and the mode of administration could cause different results (13).

The current study also declared that AST supplementation significantly decreased FBS levels in T2DM patients. This data is in line with the results of Shokri-Mashhadi *et al*. that showed AST marginally decreased FBS concentration in T2DM subjects receiving metformin (20). Along with our study, a meta-analysis article also suggested that AST diminished blood glucose levels in humans (2). However, BMI and weight did not tend to decrease during AST treatment. These findings are in agreement with the study conducted by Yoshida *et al*. that reported AST administration at different doses (0, 6, 12, 18 mg/day) for 12 weeks in subjects with mild hyperlipidemia did not affect BMI and body weight at all doses before and after supplementation (37).

Finally, AST showed cellular protection against oxidative stress by activating antioxidant enzymes such as SOD and catalase through the induction of Nrf2 protein expression (Fig. 5). Differences in results of related studies are because of their specific follow-up time, various sample sizes, and lack of adjustments of proper variables in the statistical analyses. Therefore, more extended and longer-duration trials and testing different doses of AST in combination with various antidiabetic drugs in T2DM are proposed.

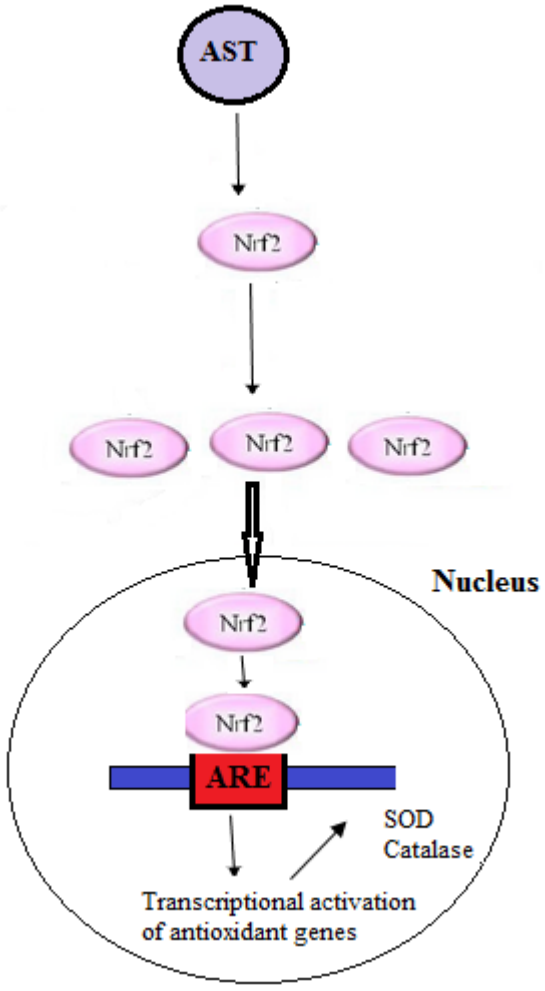

Fig. 5. Schematic representation of AST mechanism in reducing stress oxidative in type 2 diabetes mellitus patients. AST induces the expression of transcription factor Nrf2. Nrf2 translocates into the nucleus and binds to ARE on the gene and increases antioxidant enzymes SOD and catalase. AST, Astaxanthin; Nrf2, nuclear factor erythroid 2-related factor 2; SOD, superoxide dismutase; ARE, antioxidant response element.

CONCLUSION

In this study, we have represented the antioxidant effect of AST and its synergistic effects with metformin on oxidative stress with an associated increase in Nrf2 protein expression along with its downstream antioxidant enzymes, SOD and catalase, in T2DM. As a result, this co-treatment may be a valuable approach to intensify the therapeutic and antioxidant role of metformin and beneficial for managing T2DM. Further studies in the future are recommended with a larger sample size and longer intervention duration and follow-up.

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Conflict of interest statements

The authors declared no conflicts of interest in this study.

Authors' contribution

MH. Aarabi participated in hypothesis generation, study design, data interpretation, and manuscript development (draft, revision, and final editing). A. Movahedian and A. Aminorroaya contributed to the study design and data interpretation. N. Roustaei Rad conducted the experiments, collected the data, and contributed to manuscript development (draft revision, and final editing). A. Feizi performed the statistical analysis, data interpretation, and manuscript development (draft revision, and final editing). All the authors studied and approved the final version of the manuscript.

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