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PAGES: 26-33

ORIGINAL PDF URL: <https://dergipark.org.tr/tr/download/article-file/1921170>

# The ameliorative effect of acetylsalicylic acid plus ascorbic acid against renal injury in corn syrup-fed rats

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## Key Words:

acetylsalicylic acid  
ascorbic acid  
corn syrup  
renal injury

Received : 12.08.2021  
Accepted : 19.10.2021  
Published Online : 29.04.2022  
Article Code : 981913

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This study's histological result was presented as a poster at the 5th International Congress on Cell Membranes and Oxidative Stress: Focus on Calcium Signaling and TRP Channels, 9-12 September 2014, Isparta/ Turkey.

## ABSTRACT

Dietary consumption of commercially prepared (often through processing) corn syrup can activate reactive oxygen species and the inflammatory pathways observed in kidney damage in humans and experimental animals. The study sought to assess the effects of the antioxidant properties of acetylsalicylic (ASA) and ascorbic acid (AA) on corn syrup (CS)-induced kidney damage. The rats (male Sprague-Dawley) were classified into 5 groups as follows: control (C), CS, CS+ASA (ASA-10 mg/kg/day/po), CS+AA (AA-200 mg/kg/day/po) and CS+ASA+AA (combination therapy with ASA and AA doses). Biochemical, histopathological and immunohistochemical analyzes were performed on blood and tissue samples. The levels of malondialdehyde (MDA) were risen in the CS-fed rats compared to control ( $p<0.001$ ) and were reduced in the ASA, AA and ASA+AA treated groups ( $p\leq 0.001$  for all) compared to the CS group. The catalase (CAT) activities were reduced in the CS-fed group ( $p\leq 0.001$ ) compared to the control ( $p<0.001$ ) and significantly risen in ASA+AA-treated group ( $p<0.001$ ) compared to the CS-fed group. Significant histopathological changes including tubular vacuoler degeneration, tubular dilatation, cortical and medullar haemorrhage, mononuclear cell infiltration, and increased inducible nitric oxide synthase (iNOS) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) staining were observed in the CS group. Combination therapy reversed all these changes. The administration of ASA and AA for the treatment of kidney damage due to corn syrup consumption provides an ideal target for improving oxidative stress and potential therapeutic treatments.

## INTRODUCTION

Corn syrups (CS) obtained by hydrolysis of corn starch with  $\alpha$ -amylase and glycoamylase are commercially added to food and widely used in the preparation of packaged foods (1). CS is an additional source of sugar for daily consumption in most western countries that is consumed in ready meals (2). Since CS significantly replaces sucrose in processed foods, the amount of daily fructose consumption by humans has increased significantly (3). Fructose is naturally found in the fruits and vegetables; however, it is added to many convenience foods such as sucrose (from sugar cane and sugar beet) and high fructose corn syrup (HFCS). HFCS is a modified sweetener formed by mixing free fructose and free glucose at varying concentrations. Although fructose and glucose are similar molecules, fructose-derived intermediate metabolites in the liver cause lipogenesis as they cannot be controlled physiologically (4).

Studies have shown that the excess consumption of CS and

fatty foods above daily requirements and low physical activity lead to the development of obesity, metabolic syndrome, non alcoholic fatty liver disease, acute pancreatitis, type 2 diabetes, hypertension, various cancer diseases, and renal disease (5-8). The metabolic syndrome resulted from high fructose consumption varies depending on fructose concentration and feeding time. Metabolic disorders caused by fructose loading cause abnormalities in lipid metabolism (2). Excess lipid accumulation in adipose tissue brings about adipocyte dysfunction, hypoxia, oxidative stress, and chronic inflammation with the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) (9). However, increased reactive oxygen species (ROS) production causes high levels of the lipid peroxide and their end products such as malondialdehyde (MDA) in the organism (10). The potential mechanisms of comprehensive experimental and clinical results are the increasing levels of fructose and uric acid which may induce insulin resistance and metabolic syndrome (11-14). Hemodynamic changes resulting from loss of renal function lead to progressive kidney disease (15). Fructose increases oxidative

stress and inflammation, adversely affecting renal function and morphology (16,17), thereby increasing the progression of chronic renal failure (18).

Antioxidants protect cells against the potential destructive effects of ROS or free radicals by delaying or inhibiting cellular damage. Acetylsalicylic acid (ASA) is one of the most widely used drugs worldwide that inhibits cyclooxygenase enzyme activities, thereby irreversibly blockade the conversion of arachidonic acid to prostanoid and consequently reducing inflammation (19). The antioxidant properties of ASA can be clarified by its ability to decrease the production of free oxygen radicals. The different physiological and pharmacological effects of ASA can be explained by this ability (20). Ascorbic acid (AA) is a potent inhibitor of lipid peroxidation in human plasma and is involved in a variety of important hydroxylation reactions (21). Laboratory animals are used in many experimental studies (22-24). ASA and AA has been studied in several therapeutic research areas and different studies have evaluated the relationship between consumption of ASA-AA and ROS (25, 26).

The present study sought to compare the consequences of the use of ASA and AA against renal damage caused by corn syrup consumption.

## MATERIAL and METHODS

### *Animals and Experimental Design*

The experiments were applied in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Animal Experiments Local Ethics Committee at Suleyman Demirel University (23.02.2012-01).

Fourty male Sprague-Dawley rats, aged 14–16 weeks and weighing between 230–350 g, were provided from Suleyman Demirel University Experimental Animals and Medical Research Application and Research Center. Each of the rats were placed in EU type 2-cages during the 6 weeks of the study under standard light (12 h light/12 h dark cycle) at 25 °C in a well ventilated area. The rats were fed standard commercial redent chow diet and were procured ad libitum (unlimited access) to food and water. CS form (30% of F30) were supplied with drinking water.

### *Chemicals*

F30 form of CS, contained approximately 24% fructose and 28% dextrose in 73% syrup total solids, was purchased from Toposmanoglu (Isparta, Turkey). During the study, F-30 solution (30% fuctose) was added to drinking water for each CS-fed rat.

The commercial form of ASA (Aspirin, Bayer, Turkey), 500 mg ASA tablets and the commercial form of ascorbic acid (Redoxon, Bayer, Turkey), 1000 mg AA tablets, were used for the treatment.

10 mg/kg/d ASA and 200 mg/kg/d AA in an orally single dose were calculated according to the formula (C.f.B.E.a.R. (2002)) for a 60 kg person.

### *Experimental Protocol*

Rats were randomly divided into five different groups with eight rats in each group as follows;

**Control group:** Normal drinking water

**CS group:** F-30 in drinking water (d.w.)

**CS+ASA group:** F-30 in d.w.+ 10 mg/kg/day ASA, orally (p.o.)

**CS+AA group:** F-30 in d.w.+ 200 mg/kg/day AA, (p.o.)

**CS+ASA+AA group:** F-30 in d.w.+ 10 mg/kg/d ASA + 200 mg/kg/d AA, (p.o.)

F30 corn syrup solution (30% fructose) was provided to the rats in d.w. during 6 weeks, except for control group. Oral 0.5 cc normal saline was applied to the controls.

At the end of treatment, 24h after administration of the drugs, all rats were sacrificed by surgical exsanguination under the ketamine HCl (Ketalar; Alfamin) (90 mg/kg) and xylazine (Alfaz's, Alfaz IBV) (10 mg/kg) anesthesia administered intramuscularly in groups. The blood and renal tissue samples were acquired. A portion of the renal tissues were fixed in 10% neutral buffered formalin solution after dividing equally into two longitudinal sections for histochemical and immunohistochemical examinations. Remaining the renal tissues were kept at -80°C frozen until tissues were taken for biochemical analysis. The blood samples were taken to determine the serum levels of blood urea nitrogen (BUN) and creatinin (Cr) parameters.

### *Biochemical Analysis*

The blood samples were collected in serum tubes, all of them were centrifuged at 4000 rpm for 10 min under temperature control and acquired the supernatants of the blood samples. The levels of BUN and Cr were evaluated photometrically method in a biochemistry autoanalyser (Beckman Coulter AU680, California, USA) and presented as mg/dl. The kidney tissues removed from -80°C were thawed and weighed. Then the homogenization process was carried out with a scientific tissue homogenizer (IKA Ultra-Turrax T25 Basic, Germany) and sonicator (UW-2070, Bandelin Electronic, Germany) with pH 7.4 phosphate buffer. Following this, centrifugation was applied at 10,000 rpm for 10 min. at +4 °C. Tissue MDA levels were defined from the supernatant by following the spectrophotometrically method of Draper and Hadley (27). The tissue protein levels in the supernatant were defined according to the method of Bradford assay (28). CAT activity was evaluated using the method of Aebi (29) and described as kilo-units/gram protein.

### *Histochemical Analysis*

The kidney tissues were fixed in 10% neutral buffered formalin solution for at least 24h and washed in running tap water for 24h. Routine tissue follow-up was applied. Tissues were dehydrated through increasing concentrations of alcohol (70%, 80%, 90%, 96% and 100%) and cleared in xylene. The

renal tissue samples were embedded in paraffin and were cut in 4–5  $\mu\text{m}$  sections, after the routine procedure. All tissue sections were stained by Hematoxylin–Eosin (H–E) and analyzed under a light microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The structural changes in the renal tissue sections were examined and assessed according to the scoring method was suggested by Refaiy et al. (30).

#### Immunohistochemical Analysis

Inducible nitric oxide synthase (iNOS) and TNF- $\alpha$  receptor activity were determined using immunohistochemical methods in renal sections. The sections obtained were stained with iNOS primary ab (ab15323, rabbit anti-iNOS antibody, Abcam, Cambridge, USA) and TNF- $\alpha$  primer ab (ab66579, rabbit anti-TNF- $\alpha$  antibody, Abcam, Cambridge, USA). The tissue samples in 3–4  $\mu\text{m}$  section were deparaffinized and dehydrated. Then, the preparations were incubated with respectively 3% hydrogen peroxide (ScyTek Laboratories), primary antibodies (Abcam), Super Block (ScyTek Laboratories), horseradish peroxidase-conjugated streptavidin (ScyTek Laboratories), bio-

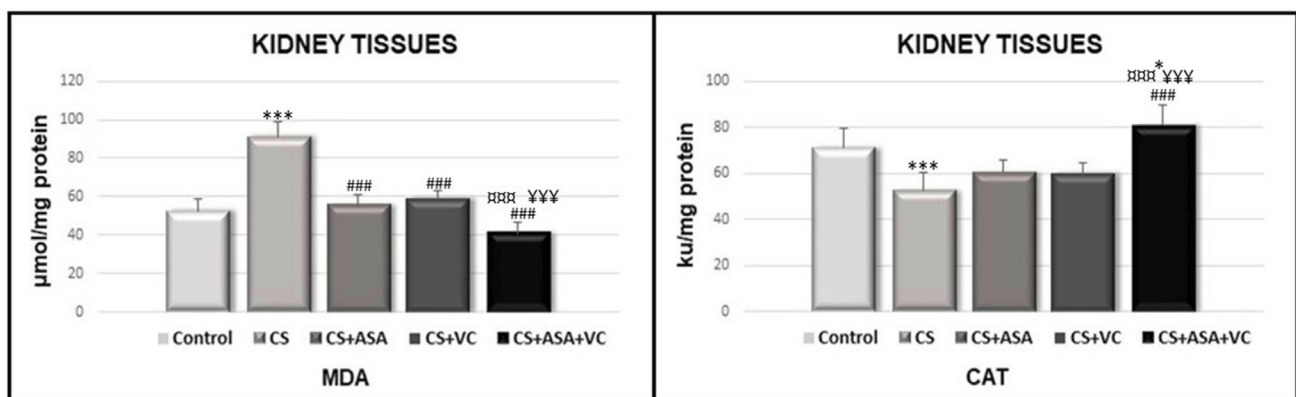
#### Statistical Analysis

The statistical analyzes were performed using IBM SPSS 20.0. Kruskal-Wallis test was used for semi-qualitative assessment in histological analysis. Nonparametric Mann-Whitney U test was used for paired group comparisons. One way ANOVA (post-hoc LSD test) was used for comparison between the groups in biochemical analyzes. Results are given as mean  $\pm$  standard deviation (SD) and p-values below 0.05 were considered significant.

## RESULTS

#### Biochemical Analyses

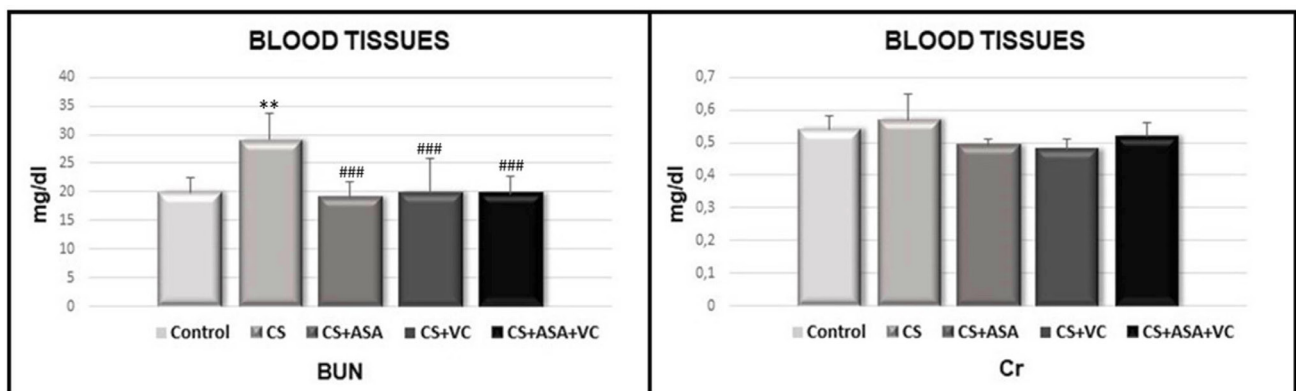
The levels of MDA were risen in the CS-fed group ( $p < 0.001$ ) compared with control and were reduced in the ASA, AA and ASA+AA treated groups ( $p < 0.001$  for all) compared with CS group. The decrement of MDA level in ASA+AA was more pronounced compared to other treatment groups ( $p \leq 0.001$  for all) (Figure 1).



**Figure 1.** Oxidative stress markers of renal tissues; ASA- Aspirin; CS - corn syrup; AA - Ascorbic Acid; CAT - catalase; MDA - malondialdehyde. Results are presented as means  $\pm$  SD. The relationships between groups and results of oxidative stress markers are assessed by one-way ANOVA. \*:  $p < 0.05$ ; \*\*\*:  $p \leq 0.001$  vs control group, ###:  $p \leq 0.001$  vs CS, ###:  $p \leq 0.001$  vs CS+ASA, ###:  $p \leq 0.001$  vs CS+AA

tinylated goat anti-polyvalent (Abcam), 3,3-diaminobenzidine solution (ScyTek Laboratories) and were covered with entellan. Finally, the immunoreactivity of the preparations was estimated with a photomicroscope and the density of receptors were determined using the semiquantitative evaluation method (30).

Decreased CAT activities were in the CS-fed group compared to the control ( $p < 0.001$ ) and increased CAT activities were in the ASA, AA, and ASA+AA treated groups compared to the CS-fed group. But the only statically significant increment was seen in the combination treatment group ( $p < 0.001$ ) (Figure 1). Also in combination group CAT activities were higher



**Figure 2.** Biochemical parameters of blood samples in the serum; ASA - Aspirin; CS - corn syrup; AA - Ascorbic Acid; BUN - Blood Urea Nitrogen; Cr - Creatinine. Results are presented as means  $\pm$  SD. The relationships between groups and results of biochemical markers are assessed by one-way ANOVA. \*\*:  $p \leq 0.01$  vs control group, ###:  $p \leq 0.001$  vs CS

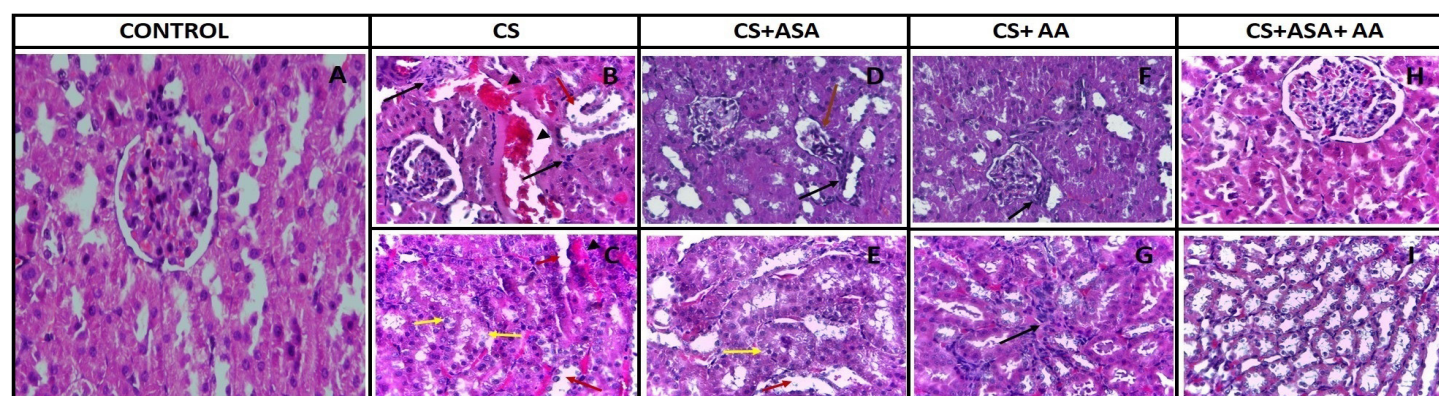


than all other groups ( $p=0.017$  for control group and  $p<0.001$  for others)

Levels of the serum blood urea nitrogen (BUN) were raised in the CS-fed group compared with the control ( $p=0.003$ ) and reduced in all the treatment groups significantly. Increased serum creatinine (sCr) levels were in the CS-fed group compared to control and decreased sCr levels were in all the treatment groups compared to CS, but none of these changes were found statically significant ( $p>0.05$  for all) (Figure 2).

#### Histochemical Analyses

Normal histology was observed in the control rats (Figure 3A). The histopathological changes were detected in the CS-fed rats significantly, including tubular vacuoler degeneration, tubular dilatation, cortical and medullar haemorrhage, and mononuclear cell infiltration (Figure 3 B,C). No significant decrease in these histopathological changes was observed in CS+ASA group (Figure 3D,E) and CS+AA group (Figure 3 F,G). Significant decrease was observed in combined group (Figure 3 H,I).



**Figure 3.** Rat renal tissue section; A) Normal histology of the kidney tissue in control. B, C) tubular vacuoler degeneration (yellow arrow), tubular dilatation (red arrow), cortical and medullar congestion (arrow head), mononuclear cell infiltration (black arrow) in CS group. D, E) Mild histopathological changes in CS+ASA group F, G) Mild histopathological changes in CS+AA group H, I) Significant histopathological changes in CS+ASA+AA group. H&E, x400

#### Immunohistochemical Analyses

All immunohistochemical evaluations were listed in Table 1. While no iNOS and TNF- $\alpha$  staining was detected in control rats (Figure 4A,B-5A,B), intense staining was detected in CS-fed rats (Figure 4C,D-5C,D). While moderate iNOS and TNF- $\alpha$  staining were determined in CS+ASA group (Figure 4E,F-5E,F) and CS+AA group (Figure 4G,H-5G,H) weak staining

was observed in combined group (Figure 4I,J-5I,J).

## DISCUSSION

It has been reported that, over the past few years, excessive consumption of CS has caused metabolic syndrome and several organ damages by hyperlipidemia and insulin resistance (16, 31-36). In numerous experimental studies reported that ASA and AA has beneficial like antioxidant, improving diseases also has a protective effect (37-41).

Oxidative damage is caused by uncontrolled oxidative stress that results in injuries of cells, tissues and organs. It has been long known that free radicals or ROS can directly damage to lipids of cell membrane (42). The increase of MDA, that is produced as the end product of peroxidation of polyunsaturated fatty acids, reflects the presenting of oxidative stress in cells (43). In this study, the increasing levels of MDA in CS group shows that oxidative stress occurs on the basis of damage. The decrease in the level of these markers by ASA and AA, which are used for therapeutic purposes, indicates that tissue damage

does not progress and drugs protect kidney tissue. The most significant improvement in the combined drug group shows that the antioxidant effects of both drugs may result from the cumulative sum (Figure 1). This protective activity needs to be proven by an increase in antioxidant enzyme activity.

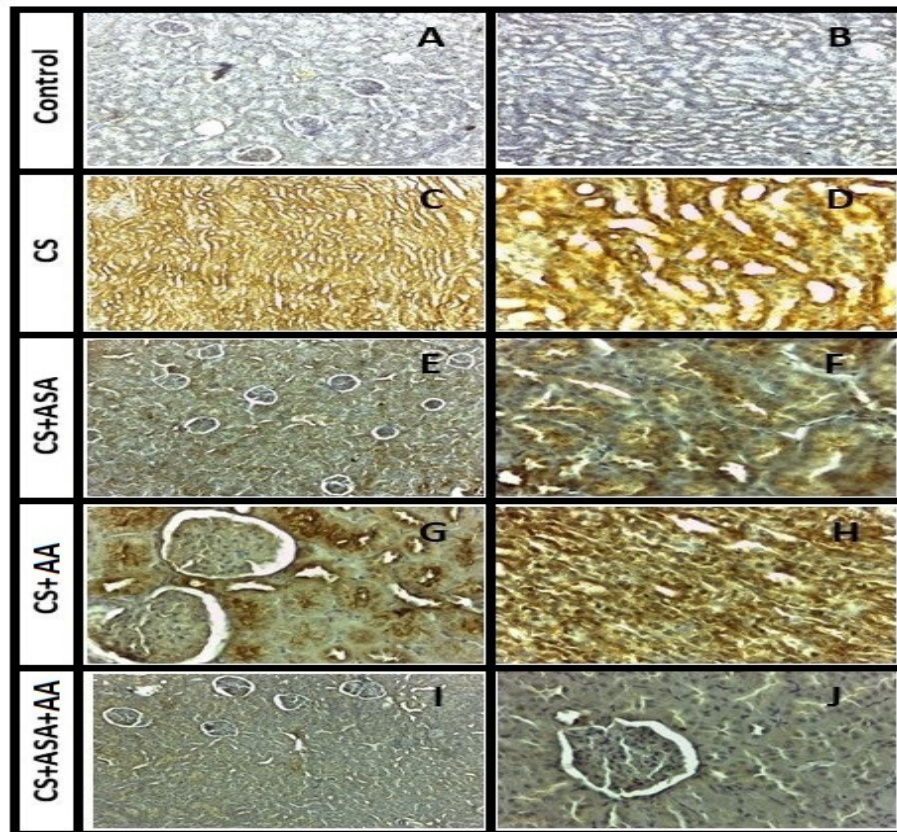
CAT investigated in this study is a potent antioxidant enzyme that is known to catalyze the conversion of hydrogen

**Table 1.** NOS and TNF- $\alpha$  immunoreactivity grades in groups of renal tissue

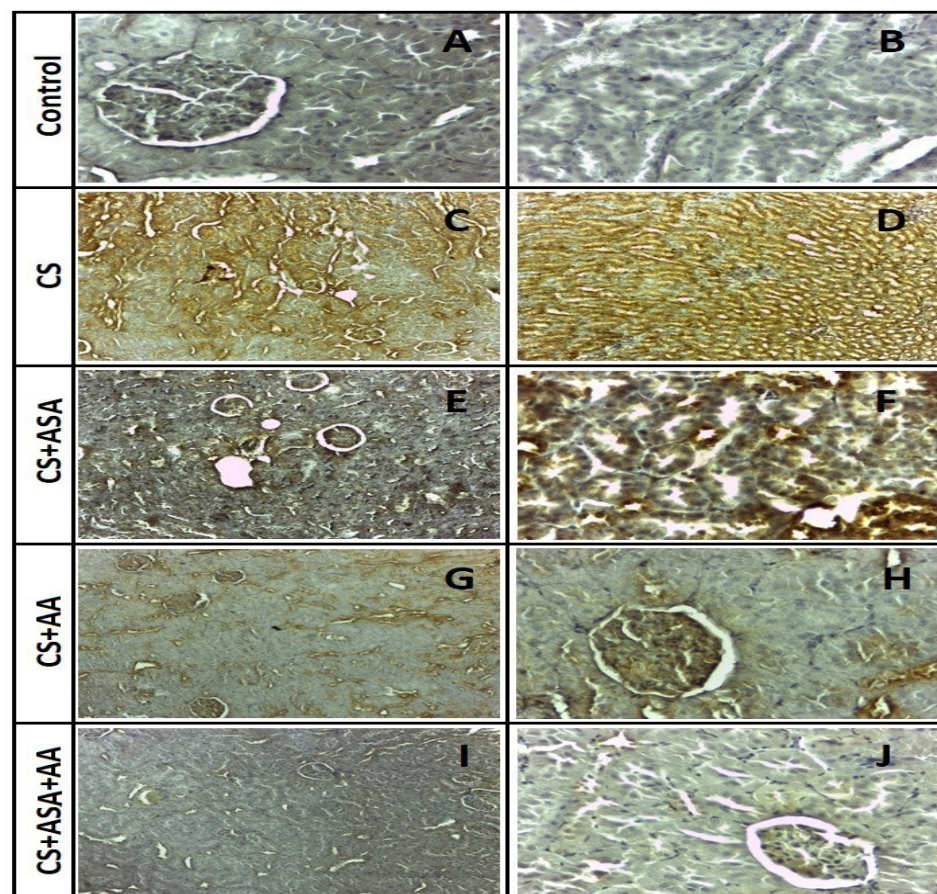
	Control	CS	CS+ASA	CS+AA	CS+ASA+AA
iNOS	(-)	(+++)	(++)	(++)	(+)
TNF- $\alpha$	(-)	(+++)	(++)	(++)	(+)

ASA - Aspirin; CS - corn syrup; AA - Ascorbic Acid; iNOS - Inducible nitric oxide synthase; TNF- $\alpha$  - Tumour Necrosis Factor alpha





**Figure 4.** iNOS immunostaining; A, B) Control group-kidney, No staining C, D) CS group-kidney, Intense staining is observed E, F) ASA group-kidney, Moderate staining is observed. G, H) AA group-kidney, Moderate staining is observed. I, J) CS+ASA+AA group-kidney, Weak staining is observed. H&E, x40, x400



**Figure 5.** TNF- $\alpha$  immunostaining; A, B) Control group-kidney, No staining C, D) CS group-kidney, Intense staining is observed E, F) ASA group-kidney, Moderate staining is observed. G, H) AA group-kidney, Moderate staining is observed. I, J) CS+ASA+AA group-kidney, Weak staining is observed. H&E, x40, x400.



peroxide to water and molecular oxygen. Oxidative compounds like superoxide radicals are detoxified by antioxidant enzymes such as CAT, glutathione peroxidase, and superoxide dismutase (44-46). As seen in CAT activities for this purpose, CAT levels decreased in the CS-fed group and increased in the treatment groups. Again, the highest increase observed in combined drug use supports this cumulative effect (Figure 1). Also these reductions in CAT levels can be caused by excessive use of antioxidant enzymes during oxidative stress. The present study suggested that ASA and AA were influential to ameliorate renal damage induced by CS through suppressing oxidative stress. ASA and AA have protective effects against fructose-induced cardiac damage, renal failure in streptozotocin induced diabetic rats, and methotrexate-induced nephrotoxicity via inhibiting inflammation, oxidative stress, and apoptosis as shown in our previous studies (35, 47, 48).

Fructose overconsumption was resulted in biochemical consequences such as increased urinary fructose levels, higher Cr clearance, and marked proteinuria. Therefore, renal histopathology has changed (17). Kidney-specific markers such as BUN and Cr from blood are used in routine clinical practise to understand the degree of damage in kidney tissue. The BUN and sCr are valuable screening tests of renal function and they essentially reflect glomerular filtration rate (17; 49). In present study, no significant change in serum Cr levels was found in all experimental groups. On the other hand, BUN levels were risen in CS group and reduced in all treatment groups (Figure 2). Although sCr levels did not change significantly in our study, BUN and Cr levels have been found to be increased in previous studies regarding kidney damage due to high fructose consumption (18, 50). In a study by Manitus et al., Cr clearance was found to be 15% higher in fructose-fed rats and microscopic data supported this finding in animals of the same group (51). The results of another study showed no difference in serum cholesterol, uric acid, fasting glucose levels, BUN or sCr (17). Based on especially BUN levels, the combined use of ASA and AA used for therapeutic purposes have preserved kidney tissue.

Basic cell damage mechanisms such as oxidative stress and inflammation have been proven to be effective on the basis of development of metabolic diseases such as hypertension, diabetes mellitus, and chronic kidney failure (52). In addition to oxidative stress, inflammation also plays an important role in nephrotoxicity. Oxidative stress act some internal mechanism that leads to inflammation. In addition to biochemical parameters mentioned above, histopathological and immunohistochemical evaluations can show these damages (53). In addition to H-E staining, by evaluating some parameters as iNOS and TNF- $\alpha$  immunohistochemically can be used for this purpose. In a study evaluating the effects of long-term fructose, sucrose, and glucose consumption on renal function, high Cr clearance and significant proteinuria were observed in the fructose group and supported this in histopathological findings (43).

In this study significant histopathological changes were observed in the CS group, including tubular vacuolar degeneration, tubular dilatation, cortical and medullar haemorrhage, mononuclear cell infiltration (Figure 3). Previous studies have

shown that fructose-induced metabolic syndrome leads to renal hypertrophy with tubular cell proliferation, proteinuria, oxidative stress and renal dysfunction (12, 47, 54).

Nitric oxide (NO) derived from iNOS plays an important role in physiological and pathophysiological conditions (55, 56). TNF- $\alpha$  is a proinflammatory cytokine that becomes amplified in chronic inflammatory states such as hypertension and renal injury. TNF- $\alpha$  increases NO formation in proximal tubular cells due to increased iNOS (57). In a study investigating the possible role of NO in the pathogenesis of glomerular changes induced by high fructose diet, an increase in iNOS expression was observed (Figure 4-5) (58). The reversal of the increase in iNOS and TNF- $\alpha$  levels in the groups by combined treatment shows that the treatment group can prevent corn syrup-induced kidney damage in this study.

## CONCLUSION

Our experimental protocol is specifically designed to identify and characterize the effects of CS on the kidney. In the present work, the adverse effects of inflammation and oxidative stress in rats fed by CS were found, and these effects were restored by ASA and AA combined therapy via reducing the levels of Cr, BUN and ROS. Many studies have reported a reduction in endogenous antioxidants in many disease states. Therefore, the intake of antioxidants in the diet becomes vital to ameliorate the dangerous effect of decreased antioxidants and increased free radicals in pathological conditions. These results suggested that the administration of ASA and AA as an antioxidant agent for the treatment of kidney damage due to CS consumption will provide a protective effect for the prevention of oxidative stress and the promotion of potential therapeutic treatments. In accordance with our findings, the use of antioxidants reduced oxidative damage but pathophysiological mechanisms of corn syrup on the kidney should be investigated by future investigations.

## DECLARATIONS

### Ethics Approval

The study was approved by the Animal Experiments Local Ethics Committee at Suleyman Demirel University and was performed entirely according to ethical rules (23.02.2012-01).

### Conflict of Interest

The authors declare that they have no conflicts of interest.

### Author contribution

Idea, concept and design: SY, EC

Data collection and analysis: SY, MS, MO, IA

Drafting of the manuscript: SY, HA, MS

Critical review: SY, HA, MO, MS, IA, EC

### Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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