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Effects of Sodium Benzoate and Citric Acid on Serum, Liver and Kidney Tissue Total Sialic Acid Levels: An Ultrastructural Study

Elvan BAKAR[1*] Tülin AKTAÇ[2]
[1] Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Edirne Turkey
[2] Trakya University, Faculty of Science, Department of Molecular Biology, Edirne, Turkey

*Corresponding Author:
E-mail: elvanbakar@hotmail.com

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Abstract

In this study, it was aimed to investigate the morphologic effects of sodium benzoate (SB) and citric acid (CA) , additives used in food, on rat liver and liver tissues and changes in total sialic acid (TSA) levels. A total of 30 Wistar albino rats weighing 200-250 gr were divided to 3 groups with 10 in each. Rats in control group, SB group and CA group were administered on by gavage tap water, Na-benzoate (2442 mg/kg body weight) and citric acid (576 mg/kg body weight) once daily respectively for 10 days. At the end of the experiment, animals in control and experiment groups were opened and heart blood, hepatic and renal tissues were obtained. Removed tissues were examined under light and electron microscopy following routine follow up procedures. TSA amounts in serum and tissues were measured with spectrophotometric method. In SB and CA groups, disruption was observed in hepatic tissue engineering, vacuolization was observed in hepatocytes and also pyknotic nuclei, nucleus losses and hypertrophy were observed. In SB and CA groups, pyknotic nuclei were observed in renal tubular cells, membrane injury was observed in apical and basal surfaces of tubular cells, degeneration was observed in glomerular structures and irregularity was seen in tubular structures. Plasmarhexis, losses in mitochondrial crista, degeneration in outer membrane and nuclear membrane of mitochondria, vacuolization in hepatocytes and myelin figures were observed electromicroscopically due to SB and CA. In renal tissues, electronmicroscopic degenerative changes included electron-dense granules, crista losses in mitochondria, irregular microvilli, vacuolization and plasmarhexis in cytoplasma of tubular cells, irregularity in chromatin material, disruption in basal membrane, enlargement in ER sacs. In SB groups, serum TSA amount was seen to decrease significantly ($p<0.05$) and a significant difference was not seen in CA group. It was seen that sialic acid amounts significantly decreased both in liver and kidney tissues in SB group ($p<0.0001$); in CA group, while a significant difference was not seen in hepatic tissues, renal TSA level was detected to increase significantly ($p<0.0001$). When effects of SB and CA on TSA amount and morphologic degenerations in liver and renal kidney were evaluated together, we consider that more detailed studies are required about the use of these food preservatives.

Key words: sialic acid, citric acid, sodium benzoate, liver, kidney

INTRODUCTION

Sialic acids are a monosaccaride family which are located as bound to N-terminal of glycoproteins and glycolipids [1] and [2] which has negative electric load, derivated from noraminic acid and has about 50 members [2], [3]and [4]. External positions of sialic acids, their negative electric load and proper localization on outer surface of the cell membrane are important for cell biology. Sialic acids which consist the main compound of protein and lipids in cell membrane contribute to complex carbohydrate formation by adding much structural difference [5] and [6].

These acidic monosaccarides may easily interact with other cell surface compunds, extracellular substances and effector molecules. Sialic acids play an important role in intercellular communication and cell adhesion [7], [8], [9] and [10].

Increase or decrease of sialic acid amount on cell surface or change in molecular properties cause different effects in cells and tissues [7] and [8]. Increased glycolization in cell structure is important in various pathologic processes [11].

Increased expression of sialic acid in cells results in increased cell surface sialization. This condition is important for defining these cells as cancer cell [12].

In addition, serum sialic acid levels are seen as a risk factor in cardiovascular diseases [13], [14] and [15] and accepted as a potential tumor marker [16]. An increase in total sialic acid (TSA) level leads to emergence of some cancer types including colon, prostate, ovarian, breast, lung cancer and lymphoma [16], [17], [18], [19] and [20]. That some chemical substances known to have toxic and carcinogenic effects were reported to increase serum sialic acid levels in previous studies [21] and [22].

Increase, decrease of sialic acid amounts in cell surface or change in properties of sialic acid reveal the change in cells or tissues. Sialic acids are seen to have both pulling effect in their binding to positive loaded molecules and transport and pushing effect both for cells and molecules [9].

Cell surface polysaccaride chains show structural differences related with metabolic changes occuring in the cell. Most of these changes occur as the result of changes in enzymatic reactions in the cell [11]. A genetic defect in glycolization is responsible for various systemic diseases.

These molecular enzymatic defects may significantly affect the lives of the living [23] and [24]. Cell surface sialization has gradually gained importance for prevention of increase in tumor cells, metastasis development, protecting cells from apoptosis and for resistance to therapy. For this purpose, sialic acids are accepted as potential therapeutic targets [12]. In addition, proper glycolization of cell surface is of great importance for therapeutic glycoproteins [25].

Sialic acid is also suggested to be functional as an antioxidant. H_2O_2 is widely generated in phagocytic immunity where they are used against microoorganisms and as the result of aerobic metabolism. However H_2O_2 is quite toxic and leads to cellular aging and disesaes which accelerate aging. Therefore it should be eliminated when unnecessary. H_2O_2 is known to be eliminated by enzymes like catalase, glutathion, peroxidase. In the reaction, H_2O_2 and sialic acid are converted to H_2O and non-toxic carboxyllic acid. Therefore sialic acid is considered to be a reactive oxygen scavenger. Sialic acid is proposed to protect the cell through covering cell surface against the injury caused by oxygen radicals and being in mucus [26] and [27].

Preservatives which are added in food, drugs and other pharmaceutical products for prolonging their shelf life and preventing microbial contamination are not included in the main product, they are added in the course of production [28], [29] and [30].

Preservatives are added into the products for preventing, delaying the losses arising from microbiologic, enzymatic or chemical changes and for prolonging shelf life [31].

Preservatives which have a quite large field of use are the leading chemicals which individuals expose in daily life. Therefore studies investigating the potential toxic effects of preservatives gain great importance today. Some food preservatives were previously reported to be able to be toxic on the living [32], [33], [34], [35], [36] and [37]. Although preservatives play an important role in food safety, many studies have revealed that they have genotoxic and mutagenic effects when used for a long time [19], [38], [39] and [40]. In addition, food additives were stated to lead to eczema, urticaria, diarrhea, migraine [41]. In recent years, studies are available investigating the effects of preservative susbtances on biofilm formation. However, in these studies, SB was seen not to prevent biofilm formation in the studied species. Therefore dose increase was stated to be unnecessary with regard to toxic effects [42] and [43].

SB (E211) which is in preservative class enables to prolong shelf life of the food through preventing contamination [44]. SB which is the sodium salt of benzoic acid is an antimicrobial substance which is used in prepared foods. Today, it is widely used in many prepared foods including margarine, non-carbonated beverages, fruit juices, souces, cacao products, biscuits, wafer, cake and creams [21] and [39].

CA (E 330) which is used for preventing antimicrobial growing in processed foods [36] and [40]. CA which also has an antioxidant effect is widely used in many prepared foods including biscuits, cakes, soupmixes, baby foods, margarines, food and fish products [29] and [40]. Citric acid which is used as acid regulator in conservated vegetables, dairies, particularly in cheese is known to have a flavor enhancer effect. It is widely used in many medications in drug sector and in shampoo production in cleaning sector.

Antioxidants are known to prevent tumor formation and protect tissues from the effects of free radicals when used in low doses [22]. However they were reported to have carcinogenetic effects when used in high doses [40].

Free radicals are formed as the result of oxidation/reduction reactions occuring during biotransformation of many endogenous and exogenous substances in the liver and these radicals lead to various injuries including membrane damages, DNA damages, enzyme inactivations when exceed the amount which antioxidiant defence system can eliminate [45].

Researches about the effects of food preservatives on serum and tissue TSA amounts could not be encountered in literature. However given the importance of sialic acids for cellular metabolism, it is clear that exposure of these molecules to the effects of food preservatives would affect cellular metabolism and organ functions.

MATERIALS AND METHODS

Animals and Experimental Procedure

Approval for our study was obtained from Trakya University's Animal Experiments Local Ethics Committee.

Wistar albino male rats with a weight of 200-250 g and with an age of 10-12 weeks were supplied to be used in experiments from Trakya University's Test Animals Unit. Our all test subjects were fed, in addition to daily potable water, with pellet feedstuff (Purina) containing 21% raw protein under suitable laboratorial conditions (at 22±1 0C with a cycle of 12 hrs light/dark) in the test period. Rats were divided into 3 groups through the method of random sampling so that each contains 10 animals. The following procedures were performed on the animals in the experimental groups considering the doses mentioned in the literature.

The control group (n:10) had been feed on by gavage once a day for 10 days with tap water.

The NB group (n:10) had been feed on by gavage once a day for 10 days with Na-benzoate (2442 mg/kg body weight).

The CA group (n:10) had been feed on by gavage once a day for 10 days with citric acid (576 mg/kg body weight).

Tissues were taken from animals with anesthesia (10mg/kg Xylazin and 50 mg/kg Ketamine, intramuscular) at the end of the test period. The tissues were stored at -80 ^{O}C until the biochemical analysis process.

Serum and Tissue TSA Measurement

Blood samples obtained from the animals were centrifuged at 2500 rpm for 10 min and sera were separated and stored at -80 C until the time of study [13].

TSA amounts in tissue homogenates were measured at 525 nm in spectrophotometer. According to this method, tissue homogenate were incubated in perchloric acid solution (0.2 ml sample + 1.5 ml 5% perchloric acid) at 100 ^{O}C for 5 minutes and centrifuged at 2500X g for 4 minutes. Then, 0.2 ml of Ehrlich's reactive was added to the produced supernatant and incubated at 100 ^{O}C for 15 minutes before the measurements were conducted at 525 nm in a spectrophotometer. Tissue and serum TSA levels were calculated in mg/ml by using the prepared standard graphic [6]. The results had been evaluated with the biostatistical methods.

Morphologic Analysis of Liver and Kidney Tissues

Tissues, which had been fixed for 24 h in Bouin solution, were embedded into paraffin after dehydration and transparency processes and sections with a thickness of

4-5 µm, were stained with hematoxylin-eosin to be investigated under light microscope (Nikon E-100).

For investigating the dissected tissues under electron microscope, they were fixed with 4% glutaraldehyde for 2 h and washed with buffer. Then, they were made exposed to post-fixation with 1% osmic acid. The tissues were passed through acetone and propylene oxide series and embedded into Epon 812 [3], [32] and [33]. Thin sections taken by Leica EM UC 6 make ultramicrotome (serial no: 522637) were investigated under FEI Company-TecnaiTM G2 Spirit/Biotwin (serial no: 12TN47B/1043) make Transmission Electron Microscope at ultrastructural level.

Statistical Analysis

Statistical analysis was done using STSTIASTICA AXA Program with serial number of SN: AXA 507C775506: FAN 3. Descriptive measurements of variables were given as X ± SD. Single sample Kalmagorov Smirnov test was applied to normality distribution, one way variance analysis was applied for inter-group comparisons as they were normally distributed, for paired comparisons, Bonferroni t test was applied for the ones whose variances were homogenous and Dunnet T3 test was applied to the ones whose variances were not. A p level of <0.05 and <0.0001 were accepted as statistically significant.

RESULTS

Light Microscopic Findings

Degenerative changes were detected in overall histologic structures of tissues when subjects were compared with control group.

When compared to control group tissue (Fig. 1A), vacuolization and nucleus losses, pyknotic nuclei, hypertrophic hepatocyte, irregular hepatocyte cell columns, degeneration and disorganisation of hepatocytes, were seen in liver tissue (Fig. 1B).

In CA group, vacuolization was observed in hepatocytes, pyknotic nuclei, nucleus losses, hypertrophy, irregular hepatocyte cell columns were observed (Fig. 1C).

In SB group, the control group showed normal appearance (Fig. 2D) membrane injury in apical surfaces of tubular cells. Tubular cells which lost the nuclei, basal membrane injury, degeneration in glomerular structure and visseral epithelial injury were detected in renal tissues when compared to control group (Fig. 2E).

In CA group, pyknotic nuclei, membrane injury in apical surfaces of tubular cells, tubular irregularity, tubular cells which lost nuclei, degeneration in basal membrane were detected (Fig. 2F).

Electron Microscopic Findings

In liver tissue, control group was showed normal appearance (Fig. 3A). In SB group, membrane injury was observed in hepatocytes, and also crista loss in mitochondria, fusing in outer membranes of mitochondriai vacuolization in cytoplasm (plasmarhexis) and degradation in nuclear membrane were observed (Fig. 3B).

In CA group, mitochondrial crista loss in hepatocytes, injury in outer membrane of mitochondria and vacuolization in hepatocytes, myelin figures were observed. In addition, invagination in nuclear membrane (Fig.3C).

In kidney tissue, control group was showed normal appearance. In SB group, crista loss in mitochondria, fusing in outer membranes, plasma rhexis and vacuolization, irregular chromatine material, (Fig. 4E).

In CA group, crista losses in mitochondria, vacuolization in cytoplasms of tubular cells, plasma rhexis and disordering of basament membrane were seen in renal tissues (Fig. 4F).

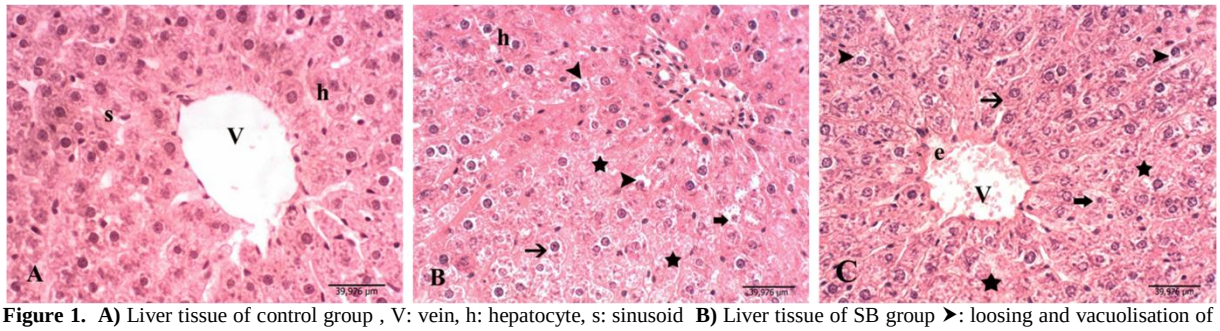

Figure 1. A) Liver tissue of control group , V: vein, h: hepatocyte, s: sinusoid **B)** Liver tissue of SB group ➤: loosing and vacuolisation of cytoplazm, →: hypertrophic hepatocyte, ★: degeneration and disorganisation of hepatocytes, ➡: picnotic nukleus , **C)** Liver tissue of CA group, V: vein, →: hypertrophic hepatocyte, ★: degeneration and disorganisation of hepatocytes, ➤: loosing and vacuolisation of cytoplazm, e: damage in endotel, ➡: picnotic nukleus. (H&E, bar represent 40 µm)

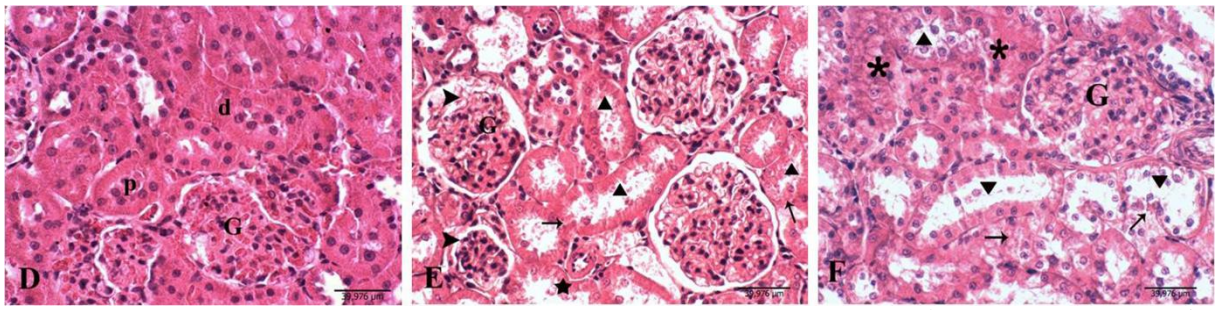

Figure 2. D) Control kidney, G: Glomerulus, p: proximal tubule, d: distal tubule, **E)** Kidney tissue of SB group, G: Glomerulus, ➤: degeneration in glomerulus and visseral epithelial sheet, *: loss of integrity tubules →: damage in basement membrane, ▲ :degeneration of membrane, **F)** Kidney tissue of CA group, G: Glomerulus, ▲ :degeneration of membrane, *: loss of integrity tubules, →: damage in basement membrane. (H&E, bar represent 40 µm)

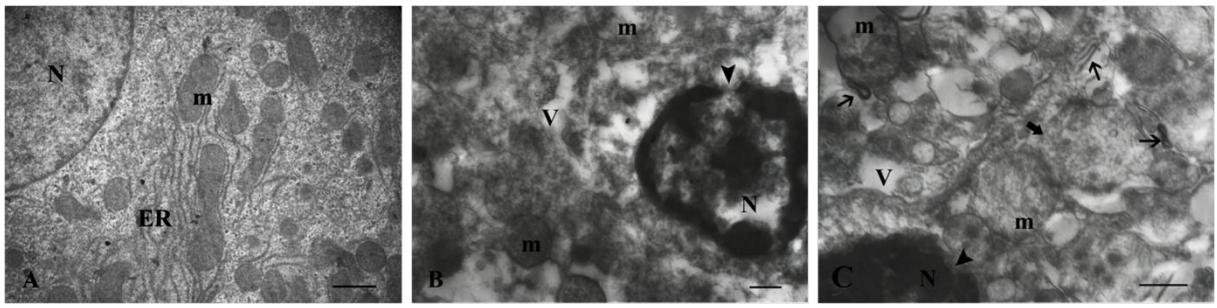

Figure 3. A) Control group, N: nucleus, m: mitochondria, ER: Endoplasmic Reticulum, **B)** NB Group, N: disorganization of chromatin in nucleus, m: loosing of cristae in mitochondria, v: vacuolization ➤: degradation in nuclear membrane **C)** CA group, N: disorganization of chromatin in nucleus, m: loosing of cristae in mitochondria, v: loosing of cytoplasm, ➤: invagination in nuclear membrane, ➡: injury in outer membrane of mitochondria, →: myelin figures, (bars represent 1µm 500nm and 500nm respectively).

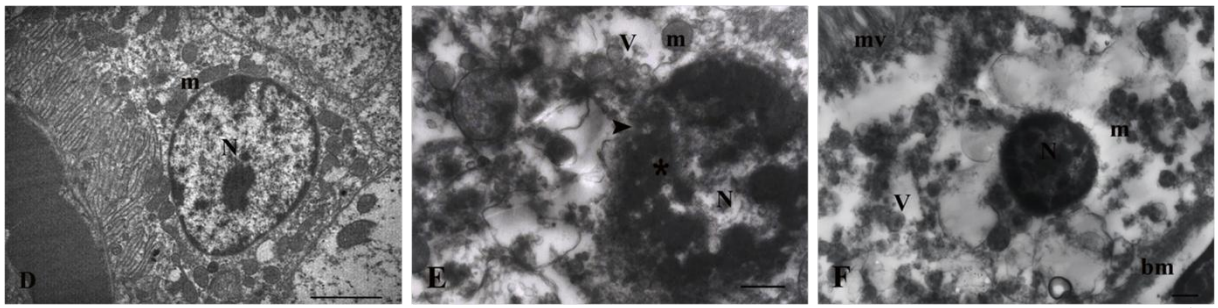

Figure 4. D) Control group, N: nucleus, m: mitochondria, **E)** NB group, N: nucleus, m: mitochondria, v: vacuolization in cytoplasma ➤: invagination of nucleus membrane, *: disorganisation of chromatine in nucleus **F)** CA group, N: nucleus, m: disordering in mitochondria v: vacuolisation in cytoplasma, bm: disordering of basament membrane, (bars represent 2µm 500nm and 1µm respectively).

Biochemical Findings

According to spectrophotometric measurements, serum TSA amounts of the animals in SB group were seen to significantly decrease compared to control group ($p<0.05$) (Table 1). A significant difference was not observed in citric acid group. TSA amounts were seen to significantly decrease both in hepatic and renal tissues of animals in SB group (Table 1) ($p<0.0001$). In CA group, while a significant change was not seen in liver, TSA level was seen to increase significantly in renal tissue ($p<0.0001$).

DISCUSSION

Food preservatives used for prolonging shelf life of prepared foods is the leading chemical which is exposed by humans every day. That some food preservatives could be toxic on the living was reported in previous studies [3], 32], [33], [42], [46] and [47].

In our study, both BS and CA were seen to cause degenerative changes in tissues. In the studies in rats, CA administered via peroral route [29] and [32] and intraperitoneally [32, 33] were seen to cause necrotic changes in liver and kidney. SB administered via peroral route was reported to cause dose dependent hepatic degeneration in rats [3] and [42]. These results are similar to those of previous studies.

In our study, both preservtaives were detected to lead to degenerative changes both in hepatic and renal tissues as the result of light micrsocopic and electron micrsocopic examinations.

On electron microscopic examinations, degeneration and necrotic changes were observed in hepatic and renal tissues both in SB and CA group. Degenerative changes in hepatic tissues included injury in hepatocyte membrane, crista loss in mitochondria, degradation in nuclear membrane; membrane degeneration, basal membrane injury and degenerative changes in renal tissue included crista loss in mitochondria, membrane degeneration, injury in basal membrane and nuclei. These results are similar to those of previous studies conducted with SB [3] and CA [32] and [33].

In the study, serum, hepatic and renal tissue TSA levels were analysed together with degenerative changes resulted from SB and CA administered through gavage.

Table 1. Serum and tissues TSA levels.

Groups	Serum TSA (mg/ml)	Liver TSA (µg/ml)	Kidney TSA (µg/ml)
Control	14.11 ± 3.66	2.36 ± 0.51	2.94 ± 0.47
SB	9.57 ± 1.43[a]	0.87 ± 0.67[b]	1.17 ± 0.32[b]
CA	14.29± 1.46	2.57 ± 0.81	3.99 ± 0.56[b]

Values are presented as the mean ± SD. and n:10 for all groups
SB: Sodium Benzoate, CA: Citric Acide
Compare with the control group, [a] ($p<0.05$), [b]($p<0.0001$)

Beside degenerative changes observed in the cells, serum and tissue TSA amounts were observed to decrease significantly as the result of short term SB administration (Table 1). This reduction was suggested to be related with disorders in sialic acid biosynthesis developing as the result of SB-related tissue injury. Cell surface sialic acid levels are known to play an important role in cell adhesion. It is considered that the reduction in tissue TSA levels could alter cell surface sialization and thereby affect adhesion. Sialic acids which are bounded to complex carbohydrates in cell membrane play important roles in many cellular functions due to this location. They protect particularly cells and macromolecules from enzymatic and immunologic attack [9]. SB-related degenerative changes observed in membranes are of great importance with this aspect.

Sialic acids are accepted as an important marker for diagnosis of many diseases today[2], [16], [37] and [43]. Elevations in tissue and serum sialic levels are associated with various cancer types [2], [27], [48] and [49]. Association between cancer and sialic acid gradually gains importance.

In our study, TSA level was observed to increase in kidney tissue due to CA administration (Table 1). This elevation in TSA levels was considered to arise from lipid peroxidation and cell membrane degradation. TSA elevation in tissues was reported to be related with lipid peroxidation and could alter the structural integrity of glycoproteins in membranes [15] and [50]. It is suggested that glycosaminoglycans (GAG) consisting extracellular matrix significantly increased physiologic levels of plasma GAG as the result of tissue injury [51] and this increase was originated from the injury in hepatic mesenchymal tissue [41].

In our study, increased sialization in kidney tissue caused by CA is important given the relationship between sialic acid levels and carcinogenesis. In addition, serum TSA concentrations were reported to increase in some liver diseases [37].

SB and CA were determined to cause degeneration in organels in hepatic and renal tissues and cellular metabolism could be impaired as the result of these degenerations. Integrity of cytoplasm is quite important for maintenance of cellular vital functions. Integrity of cytoplasm is important also for regular functioning of intracellular transport mechanisms. These transport mechanisms are considered to be impaired due to cytoplasmic vacuolization. Structural changes observed in nucleus and ER which play an important role in biosynthesis of glucoconjugates could probably lead to impairment in glycolization mechanisms. Potential injuries are likely to affect cell surface sialization as it arises both in transport mechanisms and glycolization mechanisms.

Integrity of mitochondrial membrane is important for maintanence of vital functions and determination of apoptotic process. So these degenerations in mitochondrial membranes and crista structure could have negatively affect oxidative metabolism and vital balance limits of the cell.

Food preservatives used for prolonging shelf life of prepared foods is the leading chemical exposed by the humans in daily life. Previous studies have revealed that some food preservatives could be toxic for the living [3], [32], [33], [42], [46] and [47]. these substances had also genotoxic and mutagenic effects [23], [35] and [39]. However studies investigating the effect of food preservatives on serum and tissue TSA levels were not encountered. Therefore potential effects of SB and CA, the widely used food preservatives in many fields, on TSA were investigated. The elevation in TSA level in renal tissue is important particularly due to CA administration.

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