THE EFFECT OF VITAMIN C AND VITAMIN E ON DNA DAMAGE, OXIDATIVE STATUS, AND SOME BIOCHEMICAL PARAMETERS IN RATS WITH EXPERIMENTAL FLUOROSIS

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ABSTRACT: Excess fluoride intake may lead to metabolic, functional, and structural damage in soft tissue and organs as well as in teeth and bones. The aim of the present study was to investigate the protective and therapeutic properties of vitamins C (vit C) and E (vit E) on the oxidative status and balance, DNA damage, and some biochemical parameters in rats with experimentally-induced chronic fluorosis. Seventy-two Wistar-Albino rats were divided into 9 groups, each containing 8 rats. Control groups were designed as real control and vehicle control groups. Vit C (100 mg/kg), vit E (300 mg/kg) and vit C + vit E (100 mg/kg + 300 mg/kg, respectively) in water that contained 150 ppm NaF were administered daily to three protection groups over 16 weeks. Three treatment groups received *ad libitum* water with 150 ppm NaF for 16 weeks, followed by the administration of normal drinking water plus vit C (100 mg/kg), vit E (300 mg/kg), and vit C + vit E (100 mg/kg + 300 mg/kg, respectively) on alternate days for 4 weeks. Serum biochemistry, total antioxidant status (TAS), total oxidant status (TOS), and analyses were conducted on sera obtained at the end of the study. To determine DNA damage, the comet assay and 8-hydroxy-2'-deoxyguanine (8-OHdG) analysis were used. Although the therapeutic and protective vitamin administration of vitamins C and E to the rats with experimentally-induced chronic fluorosis had only a limited effect on the oxidative status, the biochemical results for renal and hepatic function showed that the vitamins had positive effects and the comet assay and 8-OHdG analysis showed that DNA damage was reduced. In conclusion, significant positive results were obtained with protective and therapeutic treatment with vitamins E and C in rats with experimentally-induced chronic fluorosis. It is suggested that further studies are conducted to clarify the molecular mechanisms involved in producing these positive outcomes.

Keywords: DNA damage; Fluorosis; Serum biochemistry; Total antioxidant status (TAS); Total oxidant status (TOS); Vitamin C; Vitamin E.

INTRODUCTION

The first study on fluorosis in Turkey was conducted by Prof. Dr. Pertev Ata in Isparta in 1955 and reported above normal fluorine levels (4.03 ppm) in drinking water. In later studies, endemic fluorosis was reported in people and animals in Eskişehir-Kızılcaören, Doğubeyazıt district and villages in Ağrı province, and Muradiye and Çaldıran districts and villages in Van province due to high fluorine concentrations in natural drinking water sources.[1,2]

There are issues that need to be clarified in the fluorosis induction mechanism in excessive fluorine consumption and in the pathogenesis of the functional and structural changes observed in tissues and organs. Furthermore, recent studies demonstrated that lipid peroxidation damage may have a role in these variations, and increases in the MDA (TBARS) level, which is an indicator of lipid peroxidation damage, have been demonstrated in various tissues.[3-7] Consistent with these

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findings, significant changes have been reported in the antioxidant system, although the enzymatic and non-enzymatic parameters varied based on the tissue and organs involved. Antioxidant vitamins are the leading substances that prevent cellular damage caused by oxidative damage.[5,8] In fluoride-induced toxicity, it has been claimed that active oxygen and free radicals lead to DNA damage and adverse effects on protein synthesis [7,9,10].

DNA is a susceptible molecule and DNA damage could occur due to various reasons. In cases where the damage is high, or the repair systems are inadequate, DNA damage leads to necrosis or cellular mutation, and plays an important role in mutagenesis, carcinogenesis, and aging.[9,11,12] Free radicals can attack any type of macro molecule including DNA.[13] These radicals can affect lipid, protein, and DNA molecules, leading to lipid peroxidation, protein oxidation, and DNA damage.[7,14] DNA damage is characterized by structural damage such as degradation of the chromatin structure, oxidation of DNA bases, mismatch and suppression of tubulin polymerization, chemical modification of the bases, chromatin anomalies, breakage of the chain, DNA-DNA and DNA-protein crosses, and mutation.[15,16]

The present study aimed to analyse the beneficial effects of vitamin E and vitamin C administration on the protection and treatment of experimentally-induced chronic fluorosis in rats, especially with regard to the effects on DNA damage.

MATERIALS AND METHODS

Animal material: The study material involved 1-month-old Wistar-Albino rats. The animals were kept in an environment with 20–24ºC temperature and 55–60% humidity. All groups were fed *ad libitum* with standard rodent feed. The study was conducted with 9 groups of 8 animals. All study phases were conducted in accordance with the relevant rules and regulations, under the supervision of Van Yuzuncu Yil University Animal Ethics Committee.

Experimental design: NaF-drinking water solutions (150 mg/kg) were administered *ad libitum* during the study. Since corn oil was used to administer vitamin E, a further corn oil group was also designed, and 0.2 mL corn oil was administered orally every other day to this group (Table 1).

For the three protection groups, for 16 weeks, water containing 150 ppm NaF was administered *ad libitum* and vitamin C (100 mg/kg), vitamin E (300 mg/kg), and vitamin C + vitamin E (100 mg/kg + 300 mg/kg) were administered to the respective groups every other day. For the three treatment groups, for 16 weeks, water containing 150 ppm NaF was administered *ad libitum* and then, for 4 weeks, normal drinking water was administered *ad libitum* and vitamin C (100 mg/kg), vitamin E (300 mg/kg), and vitamin C + vitamin E (100 mg/kg + 300 mg/kg) were administered to the respective groups every other day.

Fluoride analysis: Urine samples were collected as explained below to determine the presence of fluorosis. Fluoride levels in collected samples were measured with a fluoride electrode (Mettler Toledo, USA).

Blood sample collection: The control, NaF, and protection group rats were sacrificed at the end of the 16th week and the treatment group rats were sacrificed at the end of the 20th week under ketamine HCL + xylazine anesthesia after the blood

samples were obtained. The blood samples were centrifuged at 3,000 rpm for 10 min and the serum samples were separated and utilized in biochemical tests and analyses of the total antioxidant status (TAS) and total antioxidant status (TOS). For the comet analysis, 2 mL of whole blood was obtained.

Table 1. Experimental groups

Experimental group	Group design
Control group1	Normal drinking water for 16 weeks
Control group 2	Normal drinking water + corn oil* for 16 weeks
NaF group	150 ppm NaF in drinking water for 16 weeks
Protection group vit C	150 ppm NaF in drinking water + vitamin C† for 16 weeks
Protection group vit E	150 ppm NaF in drinking water + vitamin E‡ for 16 weeks
Protection group vit C+E	150 ppm NaF in drinking water + vitamin C† + vitamin E‡ for 16 weeks
Treatment group vit C	150 ppm NaF in drinking water for 16 weeks and then normal drinking water + vitamin C† for 4 weeks
Treatment group vit E	150 ppm NaF in drinking water for 16 weeks and then normal drinking water + vitamin E‡ for 4 weeks
Treatment group vit C+E	150 ppm NaF in drinking water for 16 weeks and then normal drinking water + vitamin C† + vitamin E‡ for 4 weeks

*Corn oil was administered orally in a dose of 0.2 mL every other day.
†Vitamin C was administered orally in a dose of 100 mg/kg bw every other day.
‡Vitamin E was administered orally in corn oil in a dose of 300 mg/kg bw every other day.

Biochemical analyses: Routine biochemical analyses were conducted on serum samples using an autoanalyzer device (BM / HITACHI-911, Germany). Furthermore, TAS and TOS were determined with a commercial kit (RelAssay: AT15053A-JY15060O, Turkey) and the OSI value was calculated.

DNA DAMAGE TESTS

(i) Determination of the oxidative DNA damage: Oxidative DNA damage in the collected blood samples was analysed with an adequate procedure using a commercial assay kit. The DNA damage detected included 8-hydroxy-2'-deoxyguanine (8-OHdG, a product of oxidative DNA damage by hydroxyl radicals) (ADI-EKS-350 - Enzo Life Sciences).

(ii) Comet assay:[17-20] The comet assay technique was used to determine the DNA damage with single cell gel electrophoresis. The DNA damage score was determined using a fluorescence microscope (Oxion Microscope for Fluorescence, Netherlands).

Statistical analyses: The data obtained at the end of the study were analysed with the analysis of variance. The Duncan test was applied for multiple comparisons. The

differences were considered as statistically significant at $p<0.05$ (SPSS 22.0, IBM Corporation, USA).

RESULTS

The results obtained for the serum biochemical parameters for renal and hepatic function are summarized in Table 2.

Table 2. Statistical findings based on the biochemical analyses (renal function: creatinine and urea; liver function: alanine aminotransferase [ALT], aspartate aminotransferase [AST], and LDH [lactate dehydrogenase], NaF=sodium fluoride, vit C=vitamin C, vit E=vitamin E, control 1=normal drinking water, control 2=normal drinking water+corn oil)

Group	Creatinine	Urea	ALT	AST	LDH
Control 1	0.59±0.012 a	58.00±1.476 b, c	47.00±7,510 a, b	150.80±16.521 b, c, d	1512.80±114.540 c
Control 2	0.59±0.027 a	58.88±2.199 c	30.29±1.614 a	106.88±5.104 a, b	862.14±89.243 a, b
NaF	0.605±0,009 a	48.88±1.025 a	50.00±6.459 b	175.00±17.205 d	1676.14±87.392 c
Protection vit C	0.60±0.020 a	53.50±2.952 a, b, c	40.80±2.672 a, b	120.80±3.308 a, b, c	995.83±64.154 b
Protection vit E	0.60±0.31 a	55.00±2.280 a, b, c	52.25±11.564 b	168.00±25.043 c, d	677.75±36.926 a
Protection vit C+E	0.58±0.036 a	49.00±1.852 a	53.00±4.074 b	188.40±31.621 d	609.33±79.577 a
Treatment vit C	0.62±0.016 a	52.33±2.108 a, b	37.17±2.104 a, b	117.83±6.215 a, b, c	1496.25±116.731 c
Treatment vit E	0.60±0.021 a	54.29±1.924 a, b, c	47.17±6.488 a, b	99.80±11.910 a	870.75±97.560 a, b
Treatment vit C+E	0.56±0.01 a	58.00±1.528 b, c	53.67±6.677 b	158.80±17.072 c, d	600.20±57.080 a

Different letters in the same column indicate a statistically significant difference ($p<0.05$).

It was determined that for the liver enzymes LDH and AST the highest levels were present in the NaF group. Compared to the NaF group, the LDH levels were significantly decreased ($p<0.05$) in the Protection+vitamin C, Protection+vitamin E, and Protection+vitamin C+vitamin E groups and in the Treatment+vitamin E and Treatment+vitamin C+vitamin E groups. Compared to the NaF group, the AST activity was significantly decreased ($p<0.05$) in the treatment groups with the single administration of vitamins E and C. There were no significant differences in the ALT levels in the NaF, protection, and treatment groups (Table 2).

Compared to the Control 1 and Control 2 groups, the urea was significantly decreased ($p<0.05$) in the NaF and Protection+vitamin C+vitamin E groups. There were no significant differences in the creatinine levels between the groups.

The oxidative status index for the groups was calculated from the TAS and TOS levels and is shown in Table 3.

Table 3. Statistical findings based on oxidative damage indicators (TOS=total oxidant status, TAS=total antioxidant status, OSI=oxidative status index, NaF=sodium fluoride, vit C=vitamin C, vit E=vitamin E, control 1=normal drinking water, control 2=normal drinking water+corn oil)

Group	TOS	TAS	OSI
Control 1	3.82±0.24 b, c, d	0.21±0.03 a, b	5.3425±0.42136
Control 2	3.94±0.33 c, d	0.235±0.016 b	5.5700±0.57279
NaF	4.09±0.26 c, d	0.202±0.05 a	7.3044±1.10889
Protection vit C	3.72±0.19 a, b, c, d	0.221±0.003 a, b	6.3183±0.42231
Protection vit E	3.29±0.47 a, b, c	0.209±0.006 a, b	6.1817±0.92526
Protection vit C+E	4.50±0,54 d	0,191±0,011 a	5,1957±,78882
Treatment vit C	3,27±0.22 a, b, c	0.208±0.005 a, b	5.9086±0.63286
Treatment vit E	2.79±0.10 a	0.201±0.009 a	7.2943±0.52885
Treatment vit C+E	2.88±0.18 a, b	0.205±0.013 a, b	7.2683±0.66441

Different letters in the same column indicate a statistically significant difference ($p<0.05$).

There was no significant difference between the NaF and control groups based on the TOS. However, the TOS was significantly higher ($p<0.05$) in the NaF group compared to the Treatment+vitamin E and Treatment+vitamin C+vitamin E groups. There was no difference in the TAS between the NaF group and the protection and treatment groups. Although the oxidative stress index was the highest in the NaF group, there was no statistically significant difference between the groups.

The oxidative DNA damage results determined from the comet assay and the 8-OHdG levels are given in Table 4. Based on the comet findings, the DNA damage was the highest in the NaF group. Vitamin C and vitamin E, both individually and in combination, reduced the DNA damage parameters in both the protection and treatment groups. The level of 8-OHdG, an oxidative DNA damage parameter, was significantly decreased in the vitamin treatment groups.

Table 4. Statistical findings based on DNA damage indicators (DCP=damaged cells percentage, GDI=genetic damage index, 8-OHdG=8-hydroxy-2'-deoxyguanine, NaF=sodium fluoride, vit C=vitamin C, vit E=vitamin E, control 1=normal drinking water, control 2=normal drinking water+corn oil)

Group	Comet assay		8-OHdG
	DCP	GDI	
Control 1	157 a	0.421891 a	4.41±0.49 b
Control 2	247 c	745.026975 c	6.76±0.62 c
NaF	629 b	1683.064516 b	5.80±0.31 c
Protection vit C	450 d	1205.035573 d	2.21±0.27 a
Protection vit E	578 e	1433.021622 e	3.38±0.57 a, b
Protection vit C+E	360 f	996.0147059 f	3.00±0.56 a, b
Treatment vit C	597 b, e	1447.026341 e	2.81±0.20 a
Treatment vit E	483 d	1253.017637 d	2.66±0.24 a
Treatment vit C+E	270 c	796.0075472 g	2.62±0.038 a

Different letters in the same column indicate a statistically significant difference ($p<0.05$).

DISCUSSION

It has been demonstrated in several recent experimental and epidemiological studies that an excess fluoride intake may lead to metabolic, functional, and structural damage in kidney, thyroid, lung, liver, endocrine gland (pituitary gland, thyroid, and parathyroid), muscle, testes, and soft tissue and organs that contain nervous tissue in addition to damage in the teeth and bones.[8,18-24]

The emergence of 8-OHdG as a measure of DNA oxidation has led to a new method to monitor oxidative DNA damage, often observed in diabetes, cardiovascular diseases, and cancer.[24] Nuclear and mitochondrial DNA is a common target for oxidative damage. Guanine is the nucleic acid most prone to oxidation by the addition of a hydroxyl group to the 8th position of the molecule.[22]

Comet assay (single-cell gel electrophoresis, SCGE), one of the methods used to identify DNA damage, is a fast, simple, sensitive, and widely used technique. The comet assay method is based on the differences in the migration of DNA molecules,

with different molecular weights and different electrical charges, in an electrical field. While healthy DNA does not form a comet during transport, damaged DNAs move at different speeds in the electrical field forming a tail.[25] DNA degradation, antioxidant status, and DNA repair levels in lymphocytes can also be measured with a comet assay.[11]

Several studies have reported DNA damage in various tissues of individuals with experimentally-induced fluorosis. High doses of fluoride break the double or single DNA strands in the cells, leading to damage.[8,17,21] NaF, a well-known protein inhibitor, is frequently used to induce experimental fluorosis.[18] DNA damage has been demonstrated with the comet assay technique in experimental studies where NaF was administered in various doses and methods, in various organs and tissues, such as mucosa and peripheral lymphocytes,[21] brain,[8] oral mucosa,[20] liver,[26] embryonic hepatocytes,[17] ovaries,[27] and osteoblasts.[28]

Although it was reported in a previous study[29] that there was no association between DNA damage and lipid peroxidation, several researchers have demonstrated that oxidative stress leads to DNA damage in experimental diabetes and in *in vitro* studies.[30,31] In the present study, it was determined that DNA damage was significantly higher in the NaF group based on 8-OHdG when compared to the control group ($p<0.05$). It was also determined that therapeutic and protective vitamin administration reduced the DNA damage ($p<0.05$).

The comet assay has been widely used in recent studies to investigate the role of vitamin E in combating the adverse effects of genotoxic substances on DNA.[8,32] In the present study, there were significant differences between the control, NaF, and other groups based on the comet analysis findings. Our results showed that the therapeutic administration of vitamin C and vitamin E was effective in the treatment of fluorosis. Furthermore, it was determined that the vitamins administered for protection could be effective when administered with 150 mg/kg NaF. We consider that future studies implementing longer protection periods may reveal even more positive results.

Various studies have reported that vitamin E has protective effects in arsenic and fluorine toxicity due to its antioxidant properties.[33] Vitamin C and E have protective effects in the prevention of fluoride-induced apoptosis[34,35]and therapeutic effects on the oxidative damage to gametogenesis and steroidogenesis in fluorosis-induced testicular tissue. However, the administration of vitamin E alone led to a partial but effective recovery.[35-37]

It has been reported that, following the administration of fluoride at a toxic dose in a cell culture, the administration of vitamin D, E, and C administration was effective in preventing oxidative DNA damage.[38] However, vitamin A was ineffective.[38] It was found that the administration of vitamin C and E in combination in fluorosis-induced endometrial apoptosis did not lead to a full recovery.[39] However, it contributed to a histopathological improvement.[39] Although it has been reported that vitamin C and E administration partially reduced organophosphorus pesticide- and fluorosis-induced oxidative DNA damage[40] and vitamin E and C administration is beneficial for human lymphocytes despite H_2O_2-induced oxidative DNA damage, another study reported that vitamin E was not effective for DNA damage.[41] In other

studies, protective effects by antioxidant vitamins against DNA damage have been reported for vitamin C[42] and vitamin E.[43,44] Using the comet assay, Duthie et al.[45] reported that daily vitamin C (100 mg/day), vitamin E (280 mg/day), and β-carotene (25 mg/day) supplements significantly reduced lymphocyte DNA base collapse.

CONCLUSIONS

In conclusion, it was determined that, for the dose of NaF used in the present study (150 ppm NaF in drinking water for 16 weeks), and using for assessment the oxidative state determined by the TOS, TAS and OSI values, vitamin C and vitamin E had only a limited beneficial impact. However, by using for assessment the biochemical analysis of kidney and liver function, positive effects of vitamin C and E administration were demonstrated. The present study demonstrates that in experimentally-induced chronic fluorosis in rats, fluoride-induced DNA and oxidative DNA damage can be both prevented and treated to a significant extent by treatment with vitamins C and E, both individually and in combination. Further studies are required to elucidate the molecular mechanisms involved in producing the positive results obtained with the vitamin C and E treatment.

ACKNOWLEDGEMENTS

The present research was sponsored by the Van Yuzuncu Yil University Research Projects Directorate (Project no: 2015-VF-B096).

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