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Oxidative Damage in Rats Receiving Ethanol and Supplemented with Vitamin E

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Abstract: The metabolism of ethanol is directly related to oxidative stress, and its ingestion leads to the formation of reactive oxygen species (ROS) such as hydroxyl radicals, superoxide and hydrogen peroxide. Vitamin E has been widely used as an antioxidant; when administered in large doses it is deposited in the liver and then excreted in the bile, urine and feces. The objective of the present study was to evaluate the rate of excretion of fecal vitamin E in relation to its concentrations in serum and liver, and its role as a protective antioxidant against DNA damage induced by acute ethanol consumption. Wistar rats were divided into four groups receiving food and water *ad libitum* for 4 days plus the following treatments: Control (CG, n = 10) no treatment; Ethanol (ET, n = 10), receiving an acute ethanol dose intraperitoneally in the amount of 5 g/kg; vitamin E (VE, n = 10) receiving a high oral dose of vitamin E within the first three days in the amount of 100 tocopherol mg/kg body weight; ethanol plus vitamin E (VE + ET, n = 10) receiving both the ethanol and vitamin E doses. Higher concentrations of vitamin E were observed in the blood and liver of the animals in the groups that received vitamin E supplementation, independent of the presence or absence of ethanol. Concomitantly, these groups were also those with the highest concentration of the vitamin in the stool. The rate of DNA damage was higher in the groups that received ethanol with or without supplemental vitamin E. However, the rate of damage was lower in the group that received vitamin E supplementation than in the group that did not. The present results show that vitamin E has a protective effect against DNA damage induced by ethanol by reducing the extent of DNA damage.

Keywords: Vitamin E, Ethanol, Antioxidants, DNA Damage, Supplementation

1. Introduction

Ethanol metabolism is directly related to oxidative stress, In the organism, about 2 to 10% of the absorbed ethanol is eliminated through the kidneys and lungs and the remainder is practically metabolized in hepatic tissue [1, 2], since it cannot be stored, in a process involving two phases. In the first, ethanol is oxidized to acetaldehyde through three possible pathways, i.e., alcohol dehydrogenase (ADH) in the cytosol, microsomal ethanol oxidizing system (MEOS) in the endoplasmic reticulum, or catalase in the peroxysomes. In the second phase, acetyl-CoA and acetate are formed from

acetaldehyde oxidation by aldehyde dehydrogenase, this representing an irreversible transformation [3]. This oxidative pathway of ethanol metabolism favors a high formation of reactive oxygen species (ROS) such as hydroxyl radicals, superoxide and hydrogen peroxide. These, by being highly unstable, may initiate tissue damage by reacting with other molecules such as lipids, favoring increased lipid peroxidation and generating toxic final products such as malondialdehyde and by reacting with cell proteins, forming complexes that act as autoantigens triggering the immune response [4] and inducing DNA damage [5]. Ethanol has no genotoxic effect per se, but its oxidation to acetaldehyde is

related to a single break, i.e., a break in one of the two DNA chains [6]. Acetaldehyde has a genotoxic effect through various mechanisms such as ROS generation [7] cross-linking of proteins to DNA [8] and the formation of DNA and nucleoside adducts [9] The organism has antioxidant defense mechanisms that protect cell structures against the damage provoked by ROS, which may be endogenous like thiols such as glutathione and the enzymes glutathione peroxidase, catalase and superoxide dismutase, or exogenous, such as tocopherol, carotenoids, ascorbic acid and flavonoids from the diet [10].

Vitamin E is present in large amounts in lipids, playing a strong protective antioxidant role against lipid peroxidation of cell membrane polyunsaturated fatty acids. Vitamin E belongs to a group of eight naturally occurring homologous compounds, the tocopherols (TOH) and the tocotrienols, both in the α-, β-, γ- and δ- forms. Alpha-Tocopherol (α-TOH) is the most bioactive in the organism and is considered to be the most potent antioxidant of the liposoluble type [12].

In a study by our group [13] vitamin E supplementation increased the serum and hepatic concentrations of the vitamin in rats, but the hepatic store of the vitamin was reduced 2 hours after acute ethanol administration in the group receiving a vitamin E-deficient diet, after 8 hours in the group receiving a normal diet, and after 24 hours in the group receiving a diet supplemented with vitamin E. In particular, recovery of vitamin E stores occurred during the subsequent period in the animals receiving a normal diet.

Vitamin E is excreted through the bile, urine and feces [14], although vitamin E is liposoluble, Jordão Jr et al [15] detected urinary vitamin E excretion in healthy individuals and in subjects with HIV infection and AIDS, with the last group showing the highest vitamin concentration in urine. In rats, the plasma concentration of α-TOH ranges from 7.2 to 13.0 μmol/L, and is higher than the concentration of γ-TOH, which ranges from 1.3 to 1.7 μmol/L. The hepatic store is also greater for the α isoform compared to the γ form, with respective concentrations ranging from 30.0 to 33.4 nmol/g and from 4.5 a 5.3 nmol/g [16].

The objective of the present study was to determine the rate of vitamin E excretion through the feces in relation to its serum and hepatic concentrations, and the protective antioxidant role of the vitamin against the cytogenotoxicity induced by acute ethanol consumption.

2. Methodology

Male Wistar rats weighing between 150 - 180g were obtained from the Central Animal Facilities of the Faculty of Medicine of Ribeirão Preto – USP. The animals were alocated into four groups receiving commercial diet for rodents (Nuvilab CR-1, Nuvital Nutrientes S/A, Brazil) and water *ad libitum* for 4 days, plus the following treatments: Control (C) (n = 10), received saline solution by gavages for 3 days; Ethanol (ET) (n = 10) received saline solution for 2 days and on the 3rd day received an acute dose of ethanol per gavage in the amount of 5 g / kg body weight; Vitamin E

(VE) (n = 10), received tocopheryl acetate by gavage at the dose of 100 mg / kg of weight for 3 days; Ethanol + Vitamin E (ET + VE) (n = 10) received tocopherol acetate by gavage at a dose of 100 mg/kg of weight for 3 days and ethanol (dose of 5 /kg) on the 3rd day 30 minutes after gavage of vitamin E. The animals were housed in individual cages on a 12 hour light-dark cycle at a mean room temperature of 22ºC. Food intake was controlled by weighing the feeders and the animals were weighed at the end of the experiment, without differences between the groups. The feces were collected from all groups in the last day of experiment and samples contaminated with hair or ration were discarded. All animals were euthanized by decapitation 6 hours after the last gavage, blood was collected for the determination of plasma vitamin E, and the liver was removed, weighed and immediately placed in liquid nitrogen (-196ºC) for later determination of vitamin E. The comet assay was carried out using fresh samples of the right lobe of the liver.

For the determination of vitamin E, a 1 mL serum aliquot and a 200 mg liver mass and stool mass were obtained and homogenized in 2 mL ethanol. Next, the mixture was added to 2 mL n-hexane, shaken for 2 minutes and centrifuged at 1,000 rpm for 10 minutes. The supernatant, 1 mL n-hexane, was extracted and carefully dried under gaseous nitrogen flux The sample obtained was suspended again in 0.5 mL of the mobile phase consisting of acetonitrile, dichloromethanol and methanol (70:20:10, v. v. v.), and 100 μL of the sample were used for analysis. The concentration of α-TOH was estimated by HPLC using a Shimadzu apparatus model LC-10 AT, a 4.6 mm by 25 x cm C18 column, a flow of 1.5 mL/min, UV/Vis of 292 nm, a particle diameter of 5.0 μm, and an injection volume of 20 μL. The apparatus was calibrated with standard solutions of α-tocopherol at concentrations of 10, 20 and 200 μmol/L. Data were obtained by comparison and are reported as μmol/L or μmol/g.

3. Comet Assay

An aliquot of 300 μL was taken from each cell sample after the procedure of mincing liver tissue in Hanks solution (1% DMSO) to test for cell viability by trypan blue exclusion and for the alkaline version of the Comet assay as described (6). Briefly, 300 μL of the cell suspension was centrifuged for 5 minutes (500rpm) in a refrigerated microcentrifuge (Eppendorff). The resulting pellet was homogenized with 80 μL of a low melting point agarose (0.5%), spread onto microscope slides pre-coated with a normal melting point agarose (1.5%), and covered with a coverslip. After 5 min at 4ºC, the coverslip was removed and the slides were immersed in cold lysis solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH 10) for 24 hours. After lysis, the slides were placed in an electrophoresis chamber and covered with electrophoresis buffer (300 mM NaOH per 1 mM EDTA, pH > 13), for an additional 20 min to permit DNA unwinding. The electrophoresis proceeded for 20 min (25V and 300 mA) and the slides were then submerged for 15 min in neutralization

buffer (0.4 M Tris-HCl, pH 7.5), dried at room temperature and fixed in 100% ethanol for 5 min. Slide staining was performed immediately before analysis using ethidium bromide (20 μg/mL). Slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each slide) under a fluorescent microscope (ZEISS, Germany) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm using a 40X objective. The nucleus was classified visually according to the migration of the fragments as: class 0 (no damage); class 1 (little damage with a short tail length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length one or two times the diameter of the nucleus); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus); class 4 (significant damage with a long tail of damage greater than three times the diameter of the nucleus).

The data of the comet assay were compared by analysis of variance (ANOVA) with the level of significance set at $\alpha = 0.05$.

4. Results

Vitamin E concentrations in serum, liver and feces of all groups are showed in Table 1.

Table 1. *Vitamin E concentration in the serum, liver and feces of the different groups.*

Group	Serum (μmol/L)	Liver (μmol/g)	Feces (μmol/g)
Control (C)	7.32 ± 1.56	14.63 ± 2.90	70.63 ± 19.58
Ethanol (ET)	4.91 ± 2.05	19.48 ± 4.68	86.57 ± 15.89
Vit E (VE)	24.31 ± 4.10[a, b]	94.18 ± 32.01[a, b]	309.70 ± 232.88[a, b]
ET + VE	19.22 ± 7.11[a, b]	82.03 ± 34.87[a, b]	246.86 ± 68.14[a]

[a]: $p < 0.05$ compared to C; [b]: $p < 0.05$ compared to ET. C: control; ET: ethanol; VE: vitamin E; VE + ET: vitamin E plus ethanol.

In serum, higher vitamin E concentrations were observed in the VE group, being approximately 3.3 times higher than in the C group and 4.9 times higher than in the ET group, and being 2.6 times higher in the ET + VE group than in the C group and 3.9 times higher than in the ET group. A similar behavior was observed in the liver. The concentrations in the VE group were 6.4 times higher than in the C group and 4.8 times higher than in the ET group, and in the ET + VE group they were 5.6 times higher than in the C group and 4.2 times higher than in the ET group.

A similar behavior was observed in the feces. The highest concentrations were detected in the VE group, being 4.4 times higher than in the C group and 3.6 times higher than in the ET group, and being 3.5 times higher in the ET + VE group than in the C group.

DNA damage was observed with the aid of the comet assay and the results are listed in Table 2.

It can be seen that both the comet frequency and the rate of DNA damage were significantly higher in the ET and EE groups compared to control ($p < 0.01$). However, it can be seen that the EE group showed a significantly lower rate of

DNA damage than the ET group ET ($p < 0.01$)

Table 2. *Comet cells (CC) and DNA damage index (DI) obtained in liver samples from Wistar rats submitted to different treatments with VE and/or ethanol.*

Group	Comet Length					CC Mean ± SD	DI Mean ± SD
	0	1	2	3	4		
C	834	105	38	14	9	16.6 ± 2.4	25.9 ± 5.7
ET	634	141	102	92	31	36.6 ± 4.2[a]	74.5 ± 9.6[a]
VE	836	76	55	20	13	16.3 ± 4.2	29.8 ± 7.1
ET+VE	676	129	134	39	22	32.4 ± 3.1[a]	60.2 ± 5.6[a, b, c]

[a]: $p < 0.05$ compared to C; [b]: $p < 0.05$ compared to ET; [c]: $p < 0.05$ compared to VE. C: control; ET: ethanol; VE: vitamin E; VE + ET: vitamin E plus ethanol.

5. Discussion

In the present study, higher vitamin E concentrations were observed in the blood and liver of the animals receiving vitamin E supplementation both in the presence and absence of ethanol. Concomitantly, these groups also showed the highest concentration of the vitamin in the feces. Thus, we may infer that greater vitamin intake can increase the hepatic store and plasma concentration to a maximum threshold, beyond which the vitamin cannot be utilized and the excess is excreted.

The values of vitamin E in blood observed in the present study confirms the fact that vitamin E supplementation is unable to increase the plasma concentration of the vitamin by more than 2 to 4 times the normal concentration because, by being a fat-soluble vitamin, it requires a specific protein for transport, the α-TOH transporter protein (α-TTP), a fact that limits the amount of incorporation of the vitamin into VLDL lipoproteins for transport in the blood circulation [17].

The rate of DNA damage was higher in the two groups receiving ethanol, whether or not accompanied by vitamin E supplementation. However, the supplemented group showed a lower rate of damage than the group that was not supplemented. Thus, the reduction of the DNA damage mediated by vitamin E supplementation is minimal since this group still showed significant DNA damage compared to the negative control. Other studies also demonstrated that antioxidant agents can prevent or even reverse induced DNA damage [18]. It was demonstrated that derivatives of selenium, an element with an extensively studied antioxidant action, were able to prevent the oxidative damage induced by the chemotherapeutic drug doxorubicin [18]. Moreover, in a model of chronic alcoholism, the level of DNA damage detected as chromosomal aberrations is increased in the group of animals that received ethanol [19].

Vitamin E is a potent antioxidant and has been demonstrated to be able to prevent or even reverse the oxidative damage induced by drugs. Experimental treatment with ethanol diet induces oxidative stress and consequent liver toxicity, independent of the levels of fat in diet, and that tocopherol supplementation has a protective effect against oxidative stress and liver toxicity in rats [20]. Shirpoor et al.

[21] demonstrated that treatment with vitamin E induces a significant amelioration of brain atrophy and DNA damage in the offspring of female Wistar rats that received ethanol during gestation and seven days of lactation. A recent meta-analysis showed that vitamin E significantly improved liver function and histologic changes in patients with hepatic steatosis [22]. Ozkol et all showed relative protection against acute EtOH toxicity, in both single and combined pretreatments of Selenium, N-Acetylcysteine, and Vit E supplementation were seen in brain, liver, and kidney tissues, probably by antioxidant mechanism [23].

Interestingly, in the present study vitamin E was excreted through the feces in both the VE and the EE groups, thus indicating that the maintenance of plasma and hepatic levels were not sufficient to reduce the DNA damage to levels comparable to those of the control group.

6. Conclusion

We may also conclude that vitamin E had a chemopreventive role regarding the DNA damage induced by ethanol, although this effect was not antigenotoxic. The results show that vitamin E had a protective effect against the DNA damage induced by ethanol, reducing the extent of DNA damage. More studies with different doses and duration should be performed to elucidate the metabolism of vitamin E and ethanol when given together, and the effects on DNA damage.

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