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The Effect of Trolox on Oxidative Stress Index and Nitric Oxide Levels

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ABSTRACT: Free radicals, which are formed as a consequence of endogenic and exogenic factors in cells, that cause oxidative stress in living organisms can be neutralized through catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), A, E, C vitamins, glutathione, ubiquinone, and flavonoids. The aim of this study is to investigate the effect of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a type of vitamin E, on rabbits regarding the total oxidant and antioxidant capacity (TOC, TAC) levels together with the NO levels. In this study, 0.5 ml physiological saline and 1 µmol kg^{-1} trolox were given respectively to control and experiment rabbits via intraperitoneal (i.p.) route, Plasmas of blood samples, which were obtained in the 1st, 3rd, and 6th hours following injection, were separated and stored at -20 ^{o}C until to be analyzed. Plasma TOC, TAC and NO levels were determined spectrophotometrically. When the TOC, TAC, NO levels and OSI values of rabbits that were given trolox were compared to those of the control group, statistically, it was observed that the NO levels were high ($p< 0,01$) in the 1st, 3rd, and 6th hours; however, there was no alteration in their TAC, TOC levels and OSI values. As a result, it was concluded that trolox given as a single dose to healthy rabbits did not affect TAC TOC levels and OSI value, but the increasing levels of NO might be due to trolox's increasing activity of eNOS.

Keywords: Trolox, antioxidant capacity, oxidant capacity, nitric oxide

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INTRODUCTION

Free radicals which form endogenously as the products of normal metabolism in the cell or those generated exogenously by such factors as foods, ionizing radiation, and exposure to xenobiotics can cause oxidative stress in living organisms (Pham-Huy et al., 2008). Free radicals are inactivated by such enzymes as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and by non-enzymatic antioxidants such as vitamins A, E and C, glutathione, ubiquinone and flavonoids (Urso and Clarkson 2003). Free radicals can cause tissue damage when they form above the antioxidative capacity of the cell. There is a growing assertion that oxidative stress plays a crucial role in tissue damage concerned with assorted diseases such as cancer, rheumatoid arthritis, osteoporosis, polycystic ovary syndrome, Alzheimer's and Parkinson's (Phaniendra et al., 2015; Deveci et al., 2017a; Deveci and Karapehlivan 2018). NO inhibits superoxide ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$), lipoxygenase, copper and macrophage-dependent lipid and lipoprotein oxidation (Rubbo et al, 2000). When overproduced, it plays a role in ischemia-reperfusion injury, chronic inflammatory bone disease and neurodegenerative diseases (Ozcan and Ogun, 2015; Kükürt et al., 2021). As evidence of the link between diseases and oxidants has increased, an attempt has been made to gain insight into diseases by measuring biomarkers of oxidative stress and antioxidant defense in biological samples (plasma, serum, urine, tissue, etc.). It has been reported that antioxidant capacity parameters decline, and oxidative stress parameters raise in many disease cases (Kusano and Ferrari, 2008; Deveci et al., 2017b; Kukurt et al., 2021). Serum (or plasma) concentrations of different oxidant and antioxidant species can be measured in laboratories separately, but the measurements are time-consuming, labor-intensive and costly and require complicated techniques. It has been noted that it is more appropriate to measure the total oxidant and antioxidant capacity rather than measuring the concentration of each oxidant and antioxidant separately in the sample, therefore the TAC and TOC measurement method developed by Erel is an easy, precise, reliable and cheap method (Erel, 2004; Erel, 2005)

The vast majority of the population are affected by free radical-induced oxidative stress, and the role of antioxidants in oxidative stress comes to the fore in maintaining health (Sochor et al., 2010). Water-soluble antioxidants have drawn the attention of researchers particularly due to the reactive oxygen species that form in the aqueous environment. For example, a peroxyl radical which is not scavenged by a water-soluble antioxidant can initiate lipid peroxidation by diffusing into the lipid phase (Sagach et al., 2002). Trolox, which is a water-soluble vitamin E analogue, is a synthetic material. Having the hydrophilic carboxyl group instead of the hydrophobic phytyl group in vitamin E, trolox provides ease of operation in water-based environments as it is capable of penetrating the lipid membrane (Carlotti et al. 2004; Yushkova et al., 2018). *In vivo* and *in vitro* studies show that trolox protects against cardiac ischemia-reperfusion injury (Sagach et al., 2002), reduces oxidative stress (Salgo and Pryor, 1996; Utrera and Estevez, 2013; Bai et al., 2014) and inhibits cancer metastasis (Lee et al., 2014) as well as being used in TEAC-based (trolox equivalent antioxidant capacity) antioxidant measurement methods as standard (Miller et al, 1993) and as a protective agent in cosmetic products (Carlotti et al. 2004). During the literature searches, *in vitro* and *in vivo* studies with trolox revealed antioxidant activity against certain free radical species (Salgo and Pryor, 1996; Sagach et al., 2002; Utrera and Estevez, 2013; Bai et al., 2014), but no data on how a healthy individual made changes in TOC, TAC and NO levels were found. Therefore, this study aimed to investigate the effects of trolox on total oxidant and antioxidant capacity (TOC, TAC and nitric oxide (NO) levels in healthy rabbits.

MATERIALS AND METHODS

The study was confirmed by the Ethics Committee of the Animal Experiments of the Kafkas University (Decision no. KAU-CAE / 2012-86). The study was conducted on 18 New Zealand rabbits (*Oryctolagus cuniculus*) with an average live weight of 3.41± 0.4 kg and 12 to 18 months of age. The rabbits were classified into two groups: Control group (n=9) and experimental group (n=9). The control group was conducted 0.5 ml kg^{-1} physiological saline while the treatment group was conducted 1 µmol kg^{-1} (Utrera and Estevez, 2013) trolox (Sigma-Aldrich, Germany, Product No: 238813-1MG) intraperitoneal (i.p.) Blood samples were taken from the rabbits on hours 1, 3 and 6 following the injection. Their plasma was separated and stored at -20 ^{o}C until analysis. TAC and TOC levels in the samples were determined by Rel Assay Diagnostics Assay (Gaziantep-Turkey, Catalog No. RL0017, RL0024) commercial kits while nitric oxide (NO) levels were measured using a spectrophotometer based on the method described by Miranda et al (2001) Oxidative stress index (OSI) is accepted as the percentage (%) of TAC and TOC values. First, TAC values were turned into µmol/L and then OSI values were calculated concerning the following formula (Aycicek and Erel, 2007).

$$OSI\ (ArbitraryUnit) = TOC\ (\mu molH_2O_2Equiv.\ L^{-1})/TAC\ (\mu molTroloxEquiv.\ L^{-1}) \times 100 \quad (1)$$

Statistical Analysis

Statistical analysis of the data obtained from the study was performed using the SPSS.16 package program (SPSS, SPSS, 2008. SPSS for windows release 16.0.2. SPSS Inc., Chicago). Means between the times were determined by one-way analysis of variance (ANOVA) and differences between the times were determined by the Wilcoxon test. The results were presented as; mean (±) and standard error (x ± Sx). $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

When the TOC, TAC, NO levels and OSI values of rabbits given trolox were compared to those of the control group, it was observed that the NO levels were significantly higher ($p<0.01$), while there was no statistically significant change in TAC, TOC levels and OSI values (Table 1).

Table 1. Plasma TAC, TOC, NO and OSI values of trolox given rabbits

Parameters	Control Group	Experimental Group			P values
		Hour 1	Hour 3	Hour 6	
TAC (mmolTroloxEquiv. L^{-1})	0.59±0.02^{a}	0.48±0.05^{a}	0.56±0.06^{a}	0.48±0.06^{a}	ns
TOC (µmolH_2O_2Equiv.L^{-1})	11.21±1.77^{a}	5.87±1.04^{a}	6.84±0.76^{a}	5.15±0.32^{a}	ns
OSI (ArbitraryUnit)	1.80±0.18^{a}	1.30±0.22^{a}	1.30±0.14^{a}	1.14±0.12^{a}	ns
NO (µmol L^{-1})	16.97±1.73^{a}	27.82±2.54^{b}	31.12±3.41^{b}	33.93±3.69^{b}	*

***:** Differences in the same line are statistically significant ($p<0.01$), ns: Differences in the same line are statistically insignificant.

Vitamin E has the efficacy of recovering the detrimental effects of free radicals particularly on lipids (Azzi, 2007). However, being a fat-soluble vitamin, it cannot pass through the cell membrane easily which makes its efficiency limited (Kaur et al., 2010). Some studies have suggested that trolox may function better as an antioxidant than vitamin E (Huang et al., 1996; Kaur et al., 2010), due to its stoichiometric properties (Barclay and Vinqvist, 1994), such as the ability to reach intracellular hydrophilic compartments better and to retain two molecules of lipid peroxyl radicals per molecule. Sagach et al. (2002) have claimed that trolox provides better protection against myocardial oxidative damage than vitamin E.

Various derivatives of trolox have been synthesized to enhance its antioxidant activity and it has been claimed that the antioxidant activity of these derivatives is higher than that of trolox (Farmanzadeh

and Najafi, 2016) and that they exhibit good activity *in vitro* against reactive oxygen and nitrogen species such as $OH^{\cdot}$, $ONOO^{-}$, $NO^{\cdot}$ and $ROO^{\cdot}$, and against lipid peroxidation (Balogh et al., 2005).

In this study, it was observed that 1 μmol kg^{-1} dose of trolox did not affect TAC, TOC levels and OSI value in healthy rabbits. Due to differences in measurement methods and study design, it is possible to obtain inconsistent results (Niki, 2010). Gallego-Villar et al. (2014) showed that trolox reduces the formation of reactive oxygen species in propionic acidemia patients-derived fibroblasts. Again, Milatovic et al. (2011) reported that trolox protects against manganese-induced oxidative stress in neuronal cell culture. In another *in vivo* study, it was observed that trolox reduced MDA levels and increased SOD and CAT activity in rats with diabetic neuropathy (Sharma and Sayyed, 2006). Contrary to these studies, Diaz et al. (2005) claimed that trolox contributes to the cytotoxic effect of arsenic by increasing oxidative stress. Also, Zheng et al. (2012) showed that trolox increased the cytotoxic effect of curcumin by creating reactive oxygen species.

Also, the TOC and TAC give a summary of the different oxidants and antioxidants. The fact that a living organism is composed of cells with almost completely different redox potential can also lead to different results from a substance whose effectiveness has been demonstrated *in vitro* (both in chemical measurement methods and in cell culture studies) (Collins, 2005).

The studies stated above generally address the effects of trolox on oxidative stress which is usually caused by a certain factor. However, no paper in the literature focuses on the effects of trolox *in vivo* on healthy individuals. This study shows that a 1μmol/kg dose of trolox does not affect TAC, TOC levels and OSI values of healthy rabbits. The results of this study are not compatible with the findings in the literature (Sagach et al., 2002; Balogh et al., 2005; Milatovic et al., 2011; Gallego-Villar et al., 2014). The fact that alterations were not observed in TAC and TOC levels may be due to experimental design.

NO is enzymatically generated via the action of NO synthase isoforms (NOS). The enzymatic activity of NOS depends on some cofactors. This enzyme is regulated by tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), and nicotinamide adenine dinucleotide phosphate (NADPH) (Förstermann and Sessa, 2012). NO is used in favor of the organism for signal transduction, regulation of blood pressure and fighting disease-causing agents at low concentrations whereas it plays a role in neurodegenerative diseases and chronic inflammatory bone disease when produced at high concentrations (Ozcan and Ogun, 2015). Therefore, it is important to keep NO under control. It is reported that NO forms a feedback inhibition mechanism on stimulated NOS activity by various agents (TNF-alpha, IFN-gamma, lipopolysaccharide, etc.) and that this inhibition can be managed by antioxidants, including trolox (Galley et al., 1996).

In vitro and *in vivo* studies have stated that NO formation also depends on BH_4 which is one of the intracellular cofactors, that BH_4 deficiency may be related to vascular oxidative stress and that an increase in the intracellular antioxidant capacity can increase NO formation by protecting BH_4 from oxidation (Heller et al., 2001; d'Uscio et al., 2003). Even though the effect mechanism of trolox on NO formation is not fully clarified, Heller et al. (2004) claimed that trolox increased the amount of NO by protecting BH_4 from oxidation in their *in vitro* cell culture study.

In the current work, the fact that the NO levels of the experimental group (at the 1^{st}, 3^{rd} and 6^{th} hours) were statistically higher than those in the control group is consistent with the studies (Galley et al., 1996, Heller et al., 2004) reporting that effect of trolox on NO levels, and it is thought that trolox probably shows its effect on NO via BH_4.

CONCLUSION

As a result, it was concluded that trolox given as a single dose to healthy rabbits did not affect TAC, TOC levels and OSI values, but the reason for the increase in NO levels may be that trolox increased eNOS activity by protecting BH_4 from oxidation.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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