Prooxidative effects of aspartame on antioxidant defense status in erythrocytes of rats

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Since aspartame (L-aspartyl-L-phenylalanine methyl ester, ASP) is one of the most widely used artificial sweeteners, the aim of the present study was to investigate its effects on serum glucose and lipid levels as well as its effects on oxidative/antioxidative status in erythrocytes of rats. The experiment included two groups of animals: the control group was administered with water only, while the experimental group was orally administered with ASP (40 mg/kg b.w.) daily, for a period of six weeks. When compared with the control group, the group administrated with ASP indicated higher values of serum glucose, cholesterol and triglycerides. Significantly increased concentrations of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and lipid peroxides (LPO) were recorded in the erythrocytes of ASP treated group in comparison to the control group. In the course of chronic ASP administration, the following was observed: the concentration of reduced glutathione (GSH) and the activity of catalase (CAT) increased. Thus, these findings suggest that long-term consumption of ASP leads to hyperglycemia and hyperlipidemia, as well as to oxidative stress in erythrocytes.

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1. Introduction

Sweeteners are alternate substances for sugar, used as sucrose substitute (Magnuson *et al*. 2007). Aspartame (L-aspartyl-L-phenylalanine methyl ester, ASP) is a methyl ester of dipeptide used as a synthetic non-nutritive sweetener in over 90 countries worldwide and in over 6000 products (Butchko *et al*. 2002; Magnuson *et al*. 2007). It is 200 times sweeter than sucrose.

After oral administration, ASP is completely decomposed into three components: two amino acids (aspartic acid and phenylalanine) and methanol. These constituents are metabolized using the same pathways, as they are derived from food, such as meat, milk, fruits and vegetables (Butchko *et al*. 2002). Aspartame may be hydrolysed into its components in the gastrointestinal lumen, to be later absorbed into the circulation. At times, methanol is hydrolysed in the intestinal lumen with transportation of the aspartylphenylalanine dipeptide into mucosal cells, where it is metabolized to aspartate and phenylalanine and then absorbed into circulation. Aspartame may also be absorbed by intestinal mucosal cells, where it is hydrolysed to its component and then absorbed into the circulation (Stegink 1987). Approximately 50% of the aspartame molecule is phenylalanine (Phe), 40% is aspartic acid (aspartate, asp) and 10% is methanol (MeOH). After consumption of ASP,

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it is found that their concentrations are increased in the bloodstream. Of all ASP metabolites, methanol is the most toxic and causes systematic toxicity (Humphries *et al.* 2008).

Phenylalanine is an essential amino acid necessary for normal growth and development (Butchko *et al.* 2002). It is also a precursor in the synthesis of tyrosine (Hawkins *et al.* 1988), DOPA, dopamine, norepinephrine, epinephrine and phenylethylamine (Magnuson *et al.* 2007; Humphries *et al.* 2008). After the intake of aspartame, the concentration of phenylalanine in blood increases (Stegink 1987). An increased phenylalanine level may affect brain levels of dopamine and norepinephrine, thus modifying brain functions (Fernstrom *et al.* 1983).

Aspartate, a metabolite of aspartame is an excitatory neurotransmitter, normally found in high levels in the brain. Brain levels of aspartate are controlled by the blood–brain barrier, which protects neural tissue from large fluctuations of aspartate in plasma (Maher and Wurtman 1987).

On the other hand, a relatively small amount of aspartame can significantly increase methanol levels (Davoli *et al.* 1986). Methanol has low toxicity, but its metabolites are very toxic. In the liver, methanol is oxidized to formaldehyde, then to formic acid and finally to water and CO_2 (McMartin *et al.* 1980; Ells *et al.* 2000). During methanol metabolism, NADH level is elevated, which leads to the formation of superoxide anion radical ($O_2^{\bullet-}$) (Castro *et al.* 2002). Methanol metabolites react with cytochrome c oxidase in mitochondria, cause damage and increase microsomal proliferation, resulting in increased production of oxygen radicals (Parthasarathy *et al.* 2006; Humphries *et al.* 2008).

Clinical studies have shown that administration of ASP is responsible for neurological and behavioural disturbances in sensitive individuals (Walton 1988; Camfield *et al.* 1992; Moser 1994; Van den Eeden *et al.* 1994; Goerss *et al.* 2000). Chronic use of ASP might contribute to hypersensitivity reactions and atherosclerosis (Walton 1988; Van den Eeden *et al.* 1994; Jang *et al.* 2011). Many concerns have been raised about the side effects of ASP consumption and its safety, since different studies that investigated the effects of ASP revealed controversial results (Stegink 1987; Magnuson *et al.* 2007). A number of studies indicates that a mechanism of ASP toxicity in different tissues is based on the induction of oxidative stress (Abhilash *et al.* 2011; Mourad and Noor 2011; Iyyaswamy and Rathinasamy 2012; Abhilash *et al.* 2013).

Oxidative stress is characterized by an increased production of prooxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS include $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). Superoxide anion radical can react with nitric oxide (NO) to form high toxicity peroxynitrite ($ONOO^-$), which belongs to the RNS. Overproduction of ROS and RNS is prevented in physiological conditions by the components of antioxidative defense system (AOS). AOS include non-enzymatic and enzymatic components (Matés 2000; Halliwell and Gutteridge 2007). The increased levels of ROS can be caused by their increased production and/or decreased elimination by nonenzymic and enzymic antioxidants (Halliwell and Gutteridge 2007). Elevated concentrations of prooxidants could lead to cell dysfunction and degradation (Matés 2000; Halliwell and Gutteridge 2007).

The aim of this study was to investigate chronic effects of ASP administration on serum glucose, cholesterol and triglycerides levels, as well as its effects on oxidative/antioxidative status in erythrocytes.

2. Material and methods

2.1 *Chemicals*

For this study, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany). All reagents and chemicals were of analytical grade or higher purity.

2.2 *Animals and treatment*

Male *Wistar albino* rats (about 2 months old, weighing 210–220 g) were used. The animals were kept under standard laboratory conditions (12 h light, 12 h dark and 21±2°C). All rats were housed in individual cages and given standard diet. The University Committee of the Ethics of Animal Experimentation approved all animal experiments. The animals were divided into two groups (n =5 per group) and treated as follows: the animals in control group were orally administered with distilled water daily throughout the experimental protocol while the animals in treated group were orally administered with ASP (40 mg/kg) dissolved in distilled water daily for 6 weeks. The European Food Safety Authority (EFSA) established 40 mg/kg as an Acceptable Daily Intake (ADI) for aspartame. The animals were anesthetized with ether and sacrificed after the treatment.

2.3 *Analytical procedures*

The blood samples were collected in tubes using K-EDTA as anticoagulant and in tubes without anticoagulants. Biochemical parameters were measured on the day of sacrifice. Plasma and serum were removed by centrifugation for 10 min at 4000 rpm. The erythrocytes were washed three times with an equal volume of cold saline (0.9%, v/v). One milliliter of washed-out erythrocytes was lysed with 3 ml dH_2O (1:3, v/v) at 0°C for 30 min. All samples were extracted from lysates. Analyses of reactive oxygen species and reactive nitrogen species were completed within 1–2 h after

extraction. Furthermore, lipid peroxides (LPO) and reduced glutathione (GSH) were analysed within 6 h after extraction. The antioxidant enzymes activity assays were performed in erythrocyte lysate according to McCord and Fridovich method (1969). The samples were collected and stored at −20°C (not longer than 7 days).

2.3.1 *Biochemical assays of serum:* Serum concentrations of glucose, total cholesterol and triglycerides were measured on autoanalyser (C 8000 Architect, Germany) using diagnostic kits (Abbott laboratories). Concentrations of all biochemical parameters were expressed as mmol/L.

2.3.2 *Oxidative stress parameters:* The concentration of ROS and RNS in erythrocytes was determined after extraction using the following protocol: ½ vol 3M perchloric acid and 2 vol 20 mM EDTA were added to 1 vol of lysate. After extraction on ice and centrifugation for 10 min/4000 rpm, the extracts were neutralized using 2M K_2CO_3. The spectrophotometric determination of superoxide anion was based on the reduction of nitroblue tetrazolium (NBT) in the presence of $O_2^{\bullet-}$ (Auclair and Voisin 1985). The determination of the hydrogen peroxide concentration was based on oxidation of phenol red (PR) in the presence of Horseradish peroxidase (HRPO) as catalyst (Pick and Keisari 1980). The spectrophotometric determinations of nitrites (NO_2^-, as an indicator of NO concentration) were performed using the Griess method (Green *et al.* 1982). The concentration of 3-nitrotyrosine (3-NT) as an indicator of the peroxynitrite ion (Herce-Pagliai *et al.* 1998) was determined using Riordan and Valle's method (1972). The concentrations in all reactive species were expressed in nmol/ml erythrocytes.

The determination of lipid peroxide level was based on the reaction between products of lipid peroxidation (malondialdehydes) and TBA (thiobarbituric acid reactive substances – TBARS analysis) using the method of Ohkawa *et al.* (1979). These results were expressed in nmol MDA/ml erythrocytes using a molar extinction coefficient for MDA of 1.56×10^5 $M^{-1}cm^{-1}$.

2.3.3 *Determination of reduced glutathione:* The level of reduced glutathione was based on GSH oxidation with 5.5′-dithiobis-2-nitrobenzoic acid (DTNB) using Beutler method (1975), and concentrations were expressed in nmol/ml erythrocytes.

2.3.4 *Antioxidative enzymes activity assays:* Superoxide dismutase (SOD) activity was determined using pyrogallol as a substrate by the method of Marklund and Marklund (1974). This method is based on pyrogallol oxidation by the $O_2^{\bullet-}$ and its dismutation by SOD. The enzyme activity was expressed as units/g Hb.

Catalase (CAT) activity was measured by the method of Beutler (1982). The method is based on the rate of H_2O_2 degradation by the action of CAT contained in the examined samples and followed spectrophotometrically at 230 nm in 5 mM EDTA, 1 M Tris-HCl solution, pH 8.0. The enzyme activity was expressed in µmol H_2O_2 /min/g Hb.

2.4 *Statistical analysis*

All analyses were done using windows based SPSS statistical package (SPSS/13 software). The results were expressed as mean±S.E.M. and were evaluated by using one-way ANOVA (analysis of variance) test with $p<0.05$ as the criterion for significance.

3. Results

3.1 *Biochemical results*

The results of biochemical analyses are given in table 1. The concentrations of glucose, cholesterol and triglycerides in serum show significant increase ($p<0.05$) in the group treated with ASP in comparison to the control group.

3.2 *Oxidative stress parameters*

Figures 1–3 present the effects of ASP on oxidative stress parameters in rats. The results show that treatment with ASP caused a significant increase ($p<0.05$) in the concentrations of $O_2^{\bullet-}$ and H_2O_2 in the erythrocytes (figure 1).

$ONOO^-$ concentration in erythrocytes showed a significant increase ($p<0.05$) in the ASP treated group in comparison to the control group (figure 2). However, no significant change in nitrite concentration was observed in the experimental group when compared to the control group (figure 2).

The level of lipid peroxide in erythrocytes significantly increased ($p<0.05$) in the ASP treated rats in comparison to the control rats (figure 3).

Table 1. Biochemical parameters in serum of control and experimental groups

Parameters	Experimental groups	
	Control	ASP
Glucose (mmol/L)	5.84 ±0.31	7.76 ±0.40*
Cholesterol (mmol/L)	1.58 ±0.04	1.92 ±0.06*
Triglycerides (mmol/L)	0.56 ±0.02	0.88 ±0.03*

Each value represents mean ± S.E.M. (n =5 animals).
*Statistically significant when compared to control group ($p<0.05$).

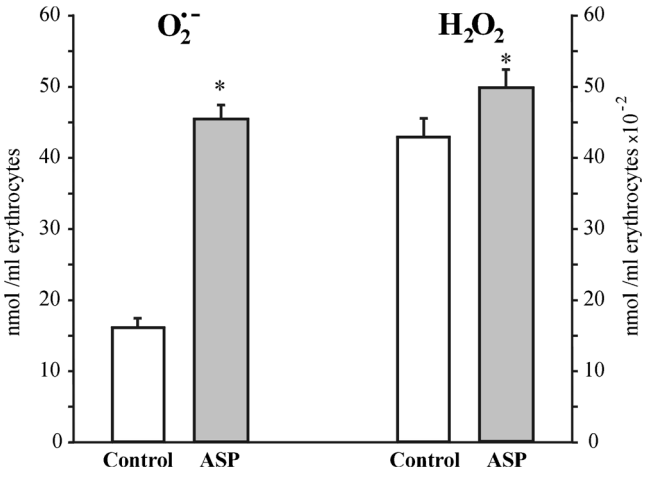

Figure 1. Effects of aspartame (ASP) treatment on superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) concentrations in erythrocytes. Data are expressed as mean ± S.E.M. (n =5 animals). * p<0.05 statistically significant compared to control group.

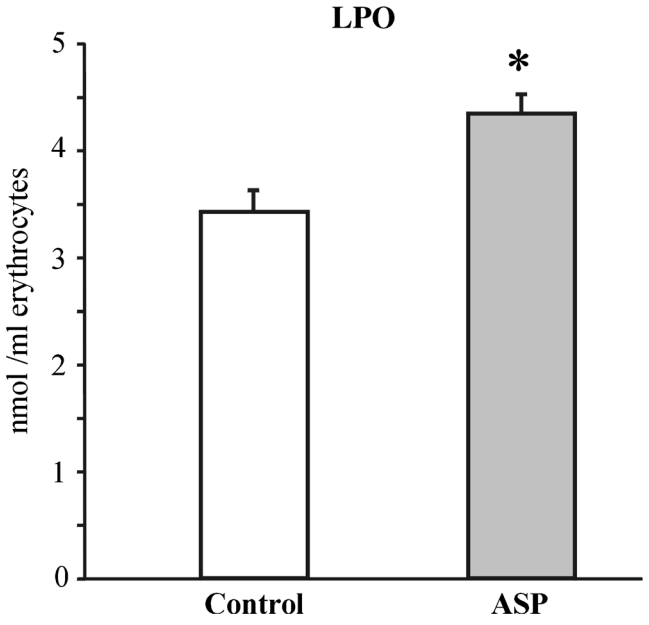

Figure 3. Effects of aspartame (ASP) treatment on lipid peroxides (LPO) concentration in erythrocytes. Data are expressed as mean ± S.E.M. (n =5 animals). * p<0.05 statistically significant compared to control group.

3.3 *Components of antioxidative defense system*

The changes in the levels of GSH and the activity of antioxidative defense enzymes (CuZn SOD and CAT) in the erythrocytes of control and experimental rats are shown in figures 4 and 5. The data in figure 4 shows significantly increased (p<0.05) GSH concentration in ASP treated group. Also, ASP administration significantly increased (p<0.05) CAT activity in erythrocytes (figure 5). However, no significant change in the CuZn SOD activity was detected in the ASP treated group when compared to the control group (figure 5).

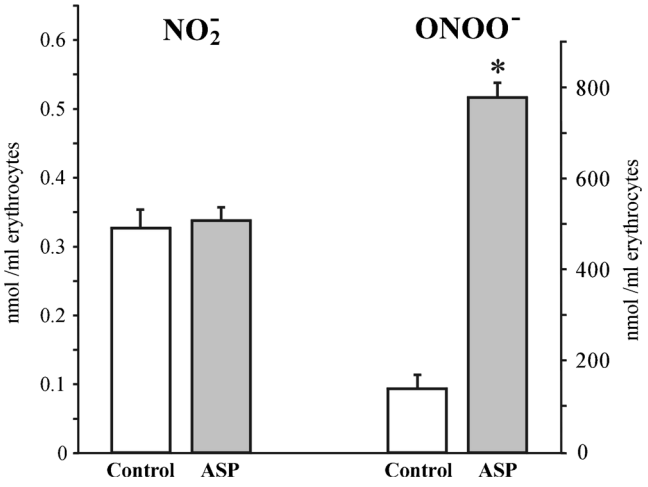

Figure 2. Effects of aspartame (ASP) treatment on nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) concentrations in erythrocytes. Data are expressed as mean ± S.E.M. (n =5 animals). * p<0.05 statistically significant compared to control group.

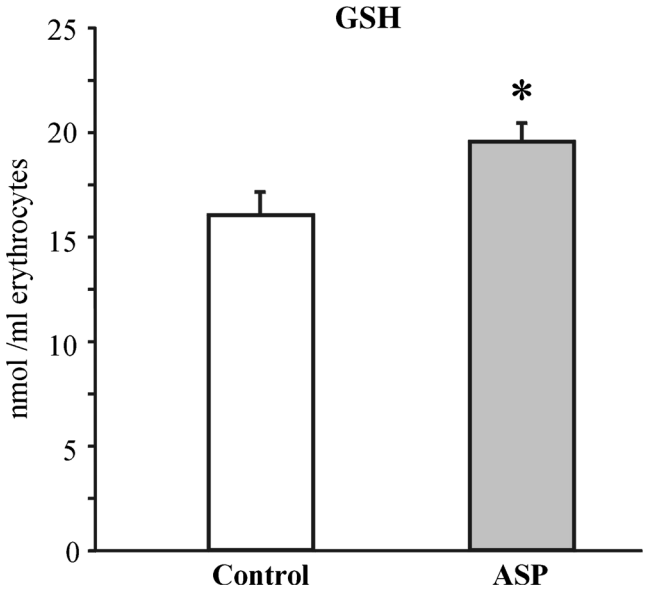

Figure 4. Effects of aspartame (ASP) treatment on reduced glutathione (GSH) concentration in erythrocytes. Data are expressed as mean ± S.E.M. (n =5 animals). * p<0.05 statistically significant compared to control group.

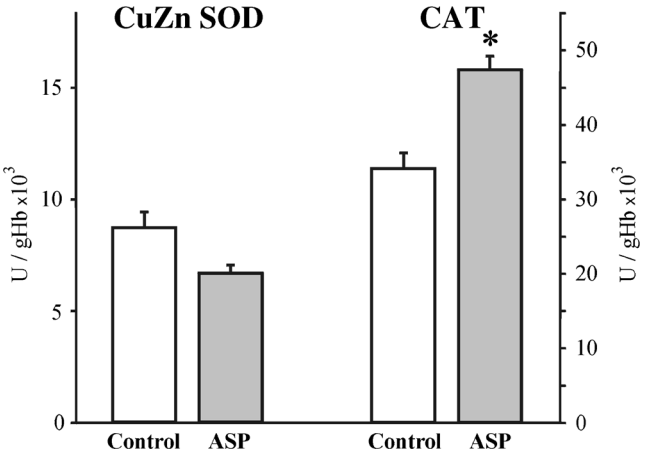

Figure 5. Effects of aspartame (ASP) treatment on CuZn superoxide dismutase (CuZn SOD) and catalase (CAT) activities in erythrocytes. Data are expressed as mean ± S.E.M. (n =5 animals). * p<0.05 statistically significant compared to control group.

4. Discussion

Aspartame is used in over 6000 packaged consumer goods and in nearly 500 pharmaceutical products, including children's medicines. The acceptable daily intake levels of aspartame established by US Food and Drug Administration and European Food Safety Authority are 50 and 40 mg/kg/day, respectively (Magnuson *et al*. 2007).

It is known that the chronic use of ASP causes various harmful effects on human health, such as different neurological and behavioural disturbances (Walton 1988; Camfield *et al*. 1992; Moser 1994; Van den Eeden *et al.* 1994; Goerss *et al.* 2000). Furthermore, long-term consumption of artificial sweeteners, such as ASP, might contribute to hypersensitivity reactions (Walton 1988; Van den Eeden *et al.* 1994) and atherosclerosis development (Jang *et al*. 2011). In this study, we have examined the effects of chronic treatment with ASP on glucose, cholesterol and triglycerides levels in serum. We also examined the effects of ASP on erythrocytes oxidative/antioxidative status as markers of endothelial cells dysfunction (Djordjević *et al*. 2008).

The results of the present study indicate that serum concentrations of glucose, cholesterol and triglycerides increased during ASP treatment. According to the literature data, hyperglycemia, hypercholesterolemia and hypertriglyceridemia activated neutrophils, which increased adhesion to endothelium and promoted oxidative burst (Petnehazy *et al*. 2006; Kummer *et al*. 2007; Alipour *et al*. 2008). Large amount of ROS produced from activated neutrophils can damage the endothelium (Sugano *et al*. 2005) and can also pass through the erythrocytes membrane and induce oxidative stress in these cells (Lynch and Fridovich 1978; Winterbourn and Stern 1987).

Oxidative stress and oxidative damage of the tissue could lead to development of some chronic diseases, such as diabetes. During diabetes, persistent hyperglycemia causes increased production of free radicals due to glucose autooxidation and protein glycosylation (Chaitanya *et al*. 2010).

The results of this work show that during the ASP treatment the concentrations of $O_2^{\bullet-}$ and H_2O_2 in erythrocytes significantly increased. The activation of neutrophils, which released high ROS concentrations caused by hyperlipidemia and hyperglycemia during ASP treatment, resulted in influx of $O_2^{\bullet-}$ and H_2O_2 in erythrocytes. The accumulation of these ROS induced oxidative stress in erythrocytes. Superoxide radical was converted to a far less reactive product H_2O_2 by SOD activity (Matés 2000). However, in conditions of oxidative stress NO has to compete with the SOD for $O_2^{\bullet-}$ to form $ONOO^-$ (Wink and Mitchell 1998). Our results indicate that the concentration of the $ONOO^-$ increased while the activity of SOD showed no significant change in erythrocytes during the ASP treatment.

Aspartame may act on the *N*-Methyl-D-aspartate (NMDA) receptors (Humphries *et al*. 2008). These receptors are present in the erythrocytes and their activation causes NO production through nitric oxide synthase (NOS) activation (Makhro *et al*. 2010). NO reacts with oxyhemoglobin to form nitrate, while a part oxidizes to NO_2^- (Wang *et al*. 2004; Dejam *et al*. 2005). According to our results, ASP treatment did not result in the change of the concentration of NO_2^- in erythrocytes. Considering that NO is scavenger of $O_2^{\bullet-}$, the increased NO production induced by ASP treatment via NMDA receptors (Humphries *et al*. 2008) led to formation of $ONOO^-$.

In the presence of iron, $O_2^{\bullet-}$ and H_2O_2 can react (Haber-Weiss and Fenton reactions) to form highly reactive $OH^{\bullet}$, which is also produced from $ONOO^-$. Hydroxyl radical oxidizes polyunsaturated fatty acids in biological membranes inducing formation of lipid peroxides (Halliwell and Gutteridge 2007). Our results indicate that ASP treatment increased concentration of LPO in erythrocytes. Other authors have also shown that after chronic ASP treatment, the concentrations of LPO increase in the brain (Mourad and Noor 2011; Iyyaswamy and Rathinasamy 2012), liver and renal tissue (Mourad 2011). The formation of LPO leads to cellular membrane damage which results in the reduction of membrane fluidity, essential for normal functioning and homeostasis of erythrocytes (Dargel 1991).

Oxidative stress has been implicated in several diseases including cancer, neurodegenerative diseases, rheumatoid arthritis, atherosclerosis, etc. The increased ROS levels in human heart are associated with aortic valve stenosis (Chaitanya *et al*. 2010). It was also observed that hyperglycemia triggered the generation of ROS in both mesangial and tubular cells of human kidney and induced structural and

functional changes in glomeruli, therefore causing diabetic nephropathy (Pacher *et al*. 2007; Chaitanya *et al*. 2010).

Reduced glutathione is a primary defense against oxidative stress. Antioxidative role of GSH is based on its ability to scavenge free radicals, to reduce peroxides and to participate as a co-substrate in the activity of GSH-dependent enzymes of AOS (Hayes and McLellan 1999). In this study, GSH concentration in erythrocytes increased during ASP treatment in order to protect the cells from oxidative damage.

The obtained results show that, during the ASP treatment, the activity of CAT increased, while the activity of CuZn SOD decreased, but that decrease was not statistically significant. In physiological conditions, SOD converts $O_2^{\bullet-}$ to H_2O_2 and protects cells from superoxide-induced damage (Matés 2000). The reaction between NO and $O_2^{\bullet-}$ which results in $ONOO^-$ production is three times faster than the reaction in which SOD catalyses the dismutation of $O_2^{\bullet-}$ to H_2O_2. Peroxynitrite can react with CuZn SOD, which leads to formation of nitrogen dioxide and copper-bound hydroxyl radical species that react with histidine residues, thus forming histidinyl radical and inactivating CuZn SOD (Alvarez *et al*. 2004). The literature data show that long-term administration of ASP decreases SOD activity in liver and renal tissue (Mourad 2011), spleen, thymus, lymph nodes and bone marrow of rats (Choudhary and Devi 2014).

CAT is an enzyme that catalyses the conversion of H_2O_2 to H_2O and molecular oxygen (Matés 2000). Chronic administration of ASP increases CAT activity in rat cerebral cortex and brain tissue (Mourad and Noor 2011; Iyyaswamy and Rathinasamy 2012). According to Matés (2000), free radicals may induce the expression of antioxidative enzymes, thereby enhance the resistance to oxidative challenges. Tephly (1991) also proved that a catalase system was one of the major pathways of methanol oxidation in rat hepatocytes. In this study, the increased production of ROS induced increased CAT activity.

5. Conclusions

The obtained results indicate the toxic nature of aspartame when it is administered orally to rats for 6 weeks. The presented study reveals that chronic aspartame administration indicates increased concentration of serum glucose, cholesterol, triglyceride and markers of oxidative stress in erythrocytes, such as $O_2^{\bullet-}$, H_2O_2, $ONOO^-$, GSH and CAT. The increased concentration of LPO results in oxidative injury of erythrocytes during chronic ASP administration. Further studies are required to evaluate other effects of aspartame and to elevate the awareness regarding the usage of this artificial sweetener, since it is consumed widely.

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