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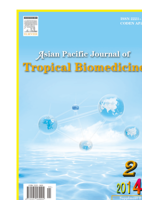

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Lipid peroxidation and oxidative stress in rat erythrocytes induced by aspirin and diazinon: the protective role of selenium

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ABSTRACT

Objective: To investigate the adverse effect of exposure to acetylsalicylic acid (ASA), diazinon (DIA) and their combination on oxidant/antioxidant status in rat erythrocytes and the ameliorating role of selenium (Se).
Methods: Rats were oral administered ASA at the maximum administration dose (1 350 mg/personal/d=2.5 mg/kg body weight/d), DIA at a dose of 20 mg/kg body weight/d and Se at a dose of 200 μg/kg body weight/d and their combinations for 28 consecutive d.
Results: Administration of DIA, ASA and ASA+DIA lead to a significant increment ($P≤0.05$) in lipid peroxidation as evidenced by the increase in erythrocytes MDA levels by 61.8%, 20.79% and 105.62%, respectively. Co-administration of Se to treated rats modulated the augmentation of MDA levels. Administration of DIA, ASA and ASA+DIA lead to significant decreases ($P≤0.05$) in the activities of catalase, superoxide dismutase and glutathione peroxidase enzymes when compared to the control group. The most influence and decreases in the activities of the aforementioned enzymes were observed in the treatments of ASA+DIA by 30.53%, 43.42% and 48.31%, respectively. However, co-administration of Se mitigated the significant decreases of superoxide dismutase, catalase and glutathione peroxidase activities to by 14.47%, 15, 36% and 12.29%.
Conclusions: It can be concluded that DIA and ASA induced oxidative stress and lipid peroxidation in rat erythrocytes. The results reveal the pronounced ameliorating effect of Se in DIA and ASA intoxicated rats. It is supposed that antioxidant supplementation may be beneficial for the people using ASA for longer periods and exposure to pesticides.

1. Introduction

The excessive and unregulated use of pesticides in agriculture and public health has caused severe environmental pollution and severity of risks to human health. There is also a widespread concern about reducing the pesticides residues in food grains, meat, vegetables and milk[1]. Diazinon [DIA: O,O-diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothionate] is one of the organophosphorus insecticides (OPIs) and widely used in agriculture and public health throughout the world[2]. Previous studies showed that DIA-induced tissues injury depends on the increase of oxidative stress and cell death[3,4], which can be directly induced by the parent pesticide or by toxic oxygenated metabolites. DIA induces swelling of mitochondria in hepatocytes[5]. This affects mitochondrial membrane transport in rat liver, and disturbs cytochrome P_{450} system in human liver[6]. It causes toxic effects on blood cells, spleen, thymus and lymph nodes of rats[7] and other organisms[7,8].

Aspirin (acetyl salicylic acid, ASA) represents the prototype of non-steroidal anti-inflammatory drugs (NSAIDs) and has been widely used as analgesic, antipyretic, and anti-inflammatory agent in the world[9]. Previous studies reported that ASA made beneficial effects by making some changes in the antioxidant system and exerted no harmful effects[10-12]. In contrast, NSAIDs may exert the therapeutic effects by chelating various physiologically important metallic cations in the body *e.g.* gastrointestinal irritation, untoward and prolonged bleeding, renal function disturbance, skin eruptions and otic effects[13].

Free radicals have become an attractive means to explain

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the toxicity of numerous xenobiotics and some of these free radicals interact with various tissue components *e.g.* DNA, protein, lipids, resulting in dysfunction[14,15]. Lipid peroxidation (LPO) is one of the molecular mechanisms of pesticides toxicity; it can disturb the biochemical and physiological functions of red blood cell (RBC)[15]. RBCs are highly susceptible to oxidative damage due to the high cell concentration of polyunsaturated fatty acid, hemoglobin and oxygen, which may produce oxidative changes in RBC. To protect itself, RBCs possess effective antioxidative enzyme systems *e.g.* superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which neutralize the reactive oxidants into none or less reactive species[15,16]. They give protection by directly scavenging superoxide radicals and hydrogen peroxide. SOD catalyzes the dismutation of superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and CAT breaks H_2O_2 to water and molecular oxygen. GPx reduces H_2O_2 to water by oxidizing two molecules of glutathione (GSH) into oxidized glutathione (GSSG). It is known that insecticides and drugs may induce metabolic pathways to generate toxic metabolites and drug/insecticide interactions can result in altered response/toxicity[4,17].

In fact, one area of increasing interest is the study of the ability of essential trace mineral to modulate the effects of environmental toxicants. In that respect, several studies have shown that selenium (Se) was of fundamental importance to human health because it is important in many biochemicals and physiological processes[18,19]. As a constituent of selenoenzyme-GSH-Px, Se plays an antioxidant role, it protects cells against damages by free radicals and permits regeneration of a membrane lipid molecule through reacylation[19]. It plays an important role in antioxidant defense systems as well as protects the structure and function of proteins, DNA and chromosomes against oxidation injury[20,21].

Although many studies have evaluated the therapeutic effect of ASA and the toxic effect of DIA, there is paucity of information on the adverse effects of combined exposure to NSAIDs "ASA" and OPI "DIA" in LPO and oxidative damage in rat erythrocytes and the antioxidant role of Se. So, this study amid to investigate the possible effects of ASA and DIA on cellular oxidant/antioxidant system in rat erythrocytes and to assess the adverse effects of combined exposure to ASA and DIA on rat erythrocytes and the antioxidant role of Se.

2. Materials and methods

2.1. Experimental animals

Male Wister rats weighing (97±5) g were obtained from the Animal Breeding House of the National Research Centre, Dokki, Cairo, Egypt, and maintained in clean plastic cages in the laboratory animal room at (23±2) °C. The animals were fed on a standard laboratory pellet diet and water *ad libitum*. Rats were kept at 12 h light 12 h dark cycles at a room temperature of 18-22 °C and acclimatized for 1 week prior to the start of experiments. All animal experiments were approved by the Animal Care & Experimental Committee, National Research Centre, Cairo, Egypt, and international guidelines for care and use of laboratory animals.

2.2. Chemicals

ASA (Aspocid® tablets, The Arab Drug Co., Egypt), each tablet that contains 75 mg ASA, was purchased from local pharmacies. DIA (Nasr-Cidol® 60% EC) was obtained from El-Nasr Mediate Chemical Co., Egypt. Sodium selenite (Na_2SeO_3) was purchased from Mallinckrodt. Inc. (Paris, France). Thiobarbituric acid (2, 6-dihydroxypyrimidine-2-thiol; TBA) was obtained from Merck (Germany). The assay kits used for biochemical measurements of CAT (EC 1.11.1.6), SOD (EC 1.15.1.1) and GPx (EC 1.11.1.9) were purchased from Biodiagnostic Company, 29 Tahrir Str., Dokki, Giza, Egypt. All other chemicals were of reagent grades and were obtained from the local scientific distributors in Egypt.

2.3. Experimental design

2.3.1. Animal treatment schedule

The animals were randomly divided into eight groups and each consists of six rats. DIA, ASA and selenium (sodium selenite, Na_2SeO_3) were prepared in distilled water and given via oral route for 28 consecutive d. Animals in Group 1 were served as control and given only distilled water (0.5 mL/rat). Animals in Group 2 were given Se (Na_2SeO_3) at a dose of 200 μg/kg body weight/d[22]. Animals in Group 3 were given DIA at a dose of 20 mg/kg body weight[23]. Animals in Group 4 were given ASA at a dose of 22.5 mg/kg body weight. The selected dose of ASA was corresponded to the maximum administration dose of 1 350 mg/personal/day based on the manufacture pamphlet. Animals in Group 5 were given simultaneously DIA (20 mg/kg body weight) and ASA (22.5 mg/kg body weight). Animals were co-administered Se with DIA, ASA and ASA+DIA for 5, 6 and 8 Groups, respectively.

2.3.2. Blood collection and erythrocytes preparation

At the end of experimental period, blood samples were withdrawn from the animals under light ether anaesthesia by puncturing the retero-orbital venous plexus of the animals with a fine sterilized glass capillary and collected in EDTA tubes. Within 10 min of blood collection, the erythrocytes were sedimented by centrifugation at 3 500 r/min for 10 min at 4 °C, using Hereaeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany. The erythrocytes were washed for three times (5 mL, each) with cold isotonic saline and the buffy coat was discarded. Then, 0.5 mL of the erythrocytes suspension was destroyed by osmotic pressure, using the same volume of deionized water and centrifugation at 10 000 x *g* for 10 min at 4 °C. The supernatant was then obtained and stored at -20 °C until measurements within one week.

2.4. Oxidative stress biomarkers in rat erythrocytes

The biochemical measurements, except that of LPO, were performed according to the details given in the kit's instructions. The principals below of different methods are given for each concerned biochemical parameter.

2.4.1. Determination of antioxidant enzymes activities

2.4.1.1. SOD (EC 1.15.1.1)

SOD activity in erythrocyte lysate was determined according to the method of Nishikimi *et al*[24]. The method is based on the ability of SOD enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. Briefly, 0.05 mL erythrocyte lysate was mixed with 1.0 mL buffer (pH 8.5), 0.1 mL nitroblue tetrazolium and 0.1 mL NADH. The reaction was initiated by adding 0.01 mL phenazine methosulphate, and then increased in absorbance was read at 560 nm for five minutes. SOD activity was expressed in μmol/mg protein.

2.4.1.2. CAT (EC 1.11.1.6)

CAT activity in erythrocyte lysate was determined according to the method of Abei[25]. The method is based on the decomposition of H_2O_2 by CAT. The sample which contains CAT is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly one minute, the reaction is quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture is then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid in the presence of H_2O_2 and catalyzed by horseradish peroxidase. The resulting quinoneimine dye [N-(4-antipyrl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine] is measured at 510 nm. The CAT activity was expressed in μmol/mg protein.

2.4.1.3. GPx (EC 1.11.1.9)

GPx was assayed by the method of Paglia and Valentine[26]. The activity was measured based on the principle that GSSG produced by GPx is reduced at a constant rate by glutathione (GSH) reductase with NADPH as a cofactor. The NADPH allows the maintenance of predictable levels of reduced GSH. The oxidative rate of NADPH was monitored at 340 nm. The activity of GPx was measured expressed as nmoles of GSH (oxidized/min)/mg protein.

2.4.2. Determination of LPO

Malondialdehyde (MDA), as a marker for LPO was determined in serum by the double heating method of Draper and Hadley with some modifications[27]. The principle of the method is based on spectrophotometric measurement of the color produced during the reaction of TBA with MDA. For this purpose, 2.5 mL of 100 g/L trichloroacetic acid solution was added into 0.5 mL serum in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling under tap water, the mixture was centrifuged at 600 *g* for 10 min, and 2 mL of the supernatant was transferred into a test tube containing 1 mL of 6.7 g/L TBA solution and placed again in a boiling water bath for 15 min. The solution was then cooled under tap water and its absorbance was measured spectrophotometrically at 532 nm. The concentration of MDA was calculated using the follow equation MDA (nmoL/mL)=[(Absorbance of sample/Absorbance of standard)×100].

2.5. Protein concentration

Protein concentrations in the hemolysates were determined spectrophotometrically based on the colorimetric biuret method by standard kits according to Bradford[28].

2.6. Spectrophotometric measurements

The spectrophotometric measurements were performed by using a Shimadzu UV-VIS Recording 2401 PC (Japan).

2.7. Statistical analysis

The results were expressed as means±SE. All data were done with the Statistical Package for Social Sciences (SPSS 17.0 for windows). The results were analyzed using One way analysis of variance (ANOVA) followed by Duncan's test for comparison between different treatment groups. Statistical significance was set at $P \leq 0.05$.

3. Results

Our results revealed that DIA, ASA and DIA+ASA caused a statistically significant decrease ($P \leq 0.05$) in SOD activity in rat erythrocytes (Figure 1). Compared to the control value, the change in SOD activity accounted to −29.39%, −20.61% and −43.42% of DIA, ASA and DIA+ASA-treatment, respectively. Supplementation of Se modulated SOD activity and the change accounted to −11.89 %, −3.95% and −14.47% of DIA, ASA and DIA+ASA-treatment, respectively.

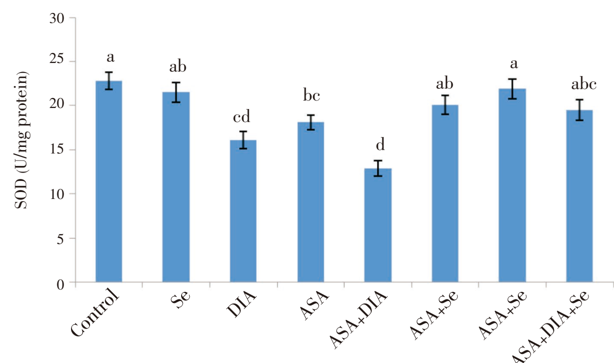

Figure 1. SOD activity in erythrocytes of male rats exposed to DIA, ASA, Se and their combination.

Values are expressed as mean±SE (n=6). Values not sharing the same superscripts letters differ significantly at $P \leq 0.05$.

Administration of DIA, ASA and ASA+DIA led to

significant decreases ($P\leq0.05$) in the activities of CAT enzyme when compared to the control group (Figure 2). The most influence and decreases in the activities of the aforementioned enzyme was observed in the treatments of ASA+DIA by −30.53%. Compared to control values, co-administration of Se mitigated the significant decreases of CAT activity in DIA (−9.04%), ASA (−4.78%) and DIA+ASA (−15.36%), respectively.

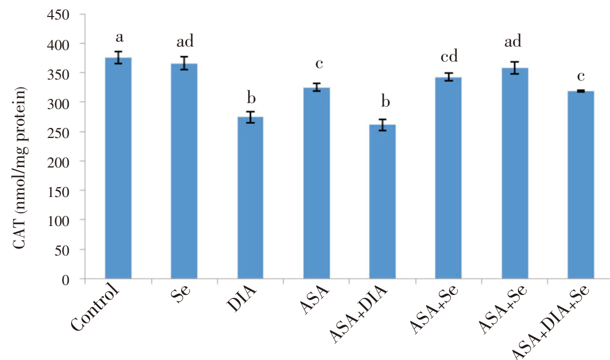

Figure 2. CAT activity in erythrocytes of male rats exposed to DIA, ASA, Se and their combination.

Values are expressed as mean±SE (n=6). Values not sharing the same superscripts letters differ significantly at $P\leq0.05$.

Figure 3 shows that DIA, ASA and DIA+ASA caused significant decrease in GPx activity in erythrocytes of rats and the change accounted to −38.35%, −17.37% and −48.31%, respectively. In contrast, Se with DIA and ASA-treated animals retained the levels of GPx at the normal values and the change accounted to −6.99% and 2.33%, respectively.

MDA level in DIA, ASA and DIA+ASA treatments were significantly ($P\leq0.05$) higher than that in control group and the change accounted to 61.80%, 20.79% and 105.62%, respectively (Figure 4). Co-administration of Se to DIA and ASA-treated rats retained the levels of MDA at the normal values and the change accounted to 4.49% and 3.93%, respectively. Treatment with Se alone did not result in significant alteration in SOD, CAT and GPx activity and MDA level compared to control treatment.

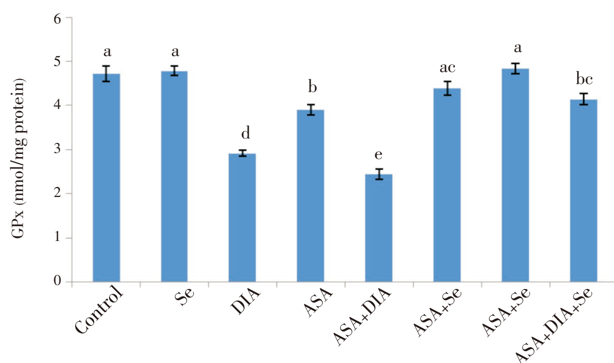

Figure 3. GPx activity in erythrocytes of male rats exposed to DIA, ASA, Se and their combination.

Values are expressed as mean±SE (n=6). Values not sharing the same superscripts letters differ significantly at $P\leq0.05$.

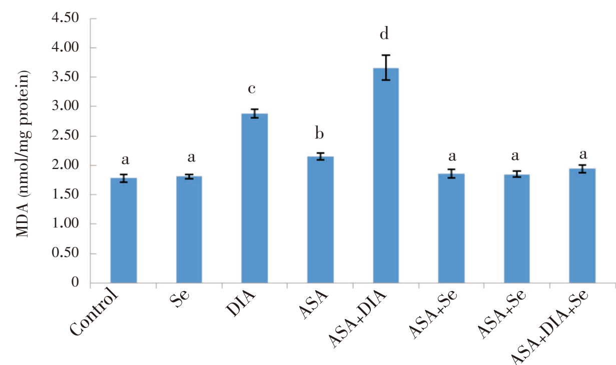

Figure 4. MDA levels in erythrocytes of male rats exposed to DIA, ASA, Se and their combination.

Values are expressed as mean±SE (n=6). Values not sharing the same superscripts letters differ significantly at $P\leq0.05$.

4. Discussion

Free radicals and reactive oxygen species are toxic to biological system, the toxicity is related in particular to hydroxyl radical, which in turn, can react with the molecular components of the cell and generates second radicals that interact with other molecules to continue the radical chain reaction. Free radicals have become an attractive means to explain the toxicity of several xenobiotics *e.g.* pesticides and drugs[4,15,16]. Previous studies reported that free radicals interact with various cells components (*e.g.* DNA, protein, lipids), resulting in modifications and loss of function[4,15,29]. It can disturb the biochemical and physiological functions of RBC[15], cause LPO and result in membrane fluidity[29]. RBCs are highly susceptible to oxidative damage due to the high cell concentration of polyunsaturated fatty acid, hemoglobin and oxygen, which may produce oxidative changes in RBC. To protect itself, RBCs possess effective antioxidative enzyme systems *e.g.* SOD, CAT and GPx, which neutralize the reactive oxidants into none or less reactive species[15,31].

In fact, SOD, CAT and GPx are antioxidant enzymes that function as blockers of free radical process[32]. SOD destroys the free radical superoxide ($O_2^{\cdot-}$) by converting it to hydrogen peroxide (H_2O_2) that can in turn be destroyed by CAT or GPx reactions to water and molecular oxygen. The results of the present work have shown that DIA, ASA and DIA+ASA could decrease SOD activity in rat erythrocytes. The decrease in SOD activity accounted to −29.39%, −20.61% and −43.42% and Se restored SOD activity to −11.89 %, −3.95% and −14.47% of DIA, ASA and DIA+ASA-treatment, respectively. The decrease in SOD activity in treated rats may be due to the use of this enzyme in converting the $O_2^{\cdot-}$ to H_2O. Peixoto *et al.*[33] showed that xenobiotics (*e.g.* pesticide) can induce mitochondria $O_2^{\cdot-}$ production and if additionally SOD was inhibited the amount of $O_2^{\cdot-}$ formed in cell could make hazardous levels.

CAT (hydrogen peroxide oxidoreductase, EC 1.11.1.6) is ubiquitously present in a wide range of aerobic cell types,

with the highest activities in mammals's liver, kidney and RBC[34]. Even though CAT is not essential for some cells type under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells[35]. It is found as a soluble protein in erythrocytes, where it may protect hemoglobin from peroxidation[29]. In the present study, rats treated with DIA, ASA and ASA+DIA showed significant decreases in CAT activity and the most influence and decreases were observed in ASA+DIA-treatments (-30.53%). Compared to the controls, co-administration of Se mitigated the significant decreases of CAT activity in DIA (-9.04%), ASA (-4.78%) and DIA+ASA (-15.36%), respectively. Some studies have indicated that superoxide radicals are potent inhibitors of CAT[36], and the increased H_2O_2 resulting from CAT inhibition could finally inhibit SOD activity. This indicates the rate of free radicals formation[37]. The increase of CAT activity following Se treatment may be due to the decrease of superoxide radicals as the result of increasing the selenoenzyme-GPx.

The selenoenzyme-GSH-Px (*e.g.*, GPx, EC 1.11.1.1.9), catalyze the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thus protecting animals cells against oxidative damage[38]. Although GPx shares the substrate, H_2O_2, with CAT, it can react effectively with lipid and other organic hydroperoxides alone[39]. Results in the present study showed that DIA, ASA and DIA+ASA caused significant decrease in GPx activity in rat erythrocytes and co-administration of Se retained the levels of GPx at the normal values. The decrease of GPx activity induced by DIA and/or ASA may be attributable to a direct inhibitory oxidative effect on the enzyme. The inhibition of GPx by ASA may result in the accumulation of H_2O_2 with subsequent oxidation of the lipids. In fact, the GSH redox cycle is a major source of protection against low levels of oxidant stress, whereas CAT becomes more significant in protecting against severe oxidant stress[39]. In animals cells, especially in human erythrocytes, the principal antioxidant enzyme for the detoxification of H_2O_2 has been considered to be GPx for a long time, as CAT has much lower affinity for H_2O_2 than GPx[40]. However, reduction of antioxidant enzyme activity could be caused by a direct effect on the enzyme by DIA-induced reactive oxygen species generation, depletion of the enzyme substrates and down-regulation of transcription and translation processes[41].

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of LPO[42]. It has been suggested as one of the molecular mechanisms involved in xenobiotics (*e.g.* pesticides) that induced toxicity[15,16]. In fact, normal RBCs function depends on the intactness of the erythrocyte membrane, which is the target for many toxic, including the OPIs[15,43]. Our results revealed that DIA, ASA and DIA+ASA treatments induced significantly in increased MDA level and the change accounted to 61.80%, 20.79% and 105.62%, respectively. Co-administration of Se retained these levels of MDA at the normal values and the change accounted to 4.49% and 3.93%, respectively.

OPIs induce oxidative stress, which play an essential role in its induced toxicity in rats[4,15,16], in mice[41], in human[44] and *in vitro* study[45,46]. Previous studies[3,4,47] reported that DIA-induced tissues injury initially depends on the development of oxidative stress and cell death, which can be directly induced by the parent pesticide or by toxic oxygenated metabolites. It has been reported that DIA induced erythrocyte LPO and changed the activities of antioxidant enzymes *in vitro*[48].

In a previous study, ASA was reported to cause LPO and change antioxidant enzyme activities in erythrocytes and liver samples of rats[49]. Nair *et al*.[50] reported that ASA (40 mg/kg) caused significant decrease in the activity of reduced GSH, SOD, glutathione-s-transferase and CAT activities in intestine and colon of female rats. It impairs the antioxidant system resulting in worsening the clinic symptoms and prognosis of the disease[51,52]. Galunska *et al*.[52] showed that administration of ASA (300 mg/kg) to rats induced gastric mucosal damage, which was accompanied by the development of oxidative stress, evidenced by the accumulation of MDA and concomitanted initial activation of cell antioxidant defenses. GPx and SOD were inhibited in male C57 BL/6 mice feed with diet contain ASA (1%, w/w) for two weeks[51]. It has been reported that normal dose ASA [approximately (10 mg/kg per day for 30 d] may cause peroxidation in the human erythrocytes due to its oxidant potential[53]. ASA at concentration of 5.0 mmol/L inhibited the activity of antioxidant enzymes (*e.g.* SOD and CAT) in rat liver homogenate and erythrocytes *in vitro*[54]. It changes oxygen free-radical metabolism (*e.g.* SOD, CAT, GPx) in liver and kidney of rats[55]. The mechanism of how ASA impairs the antioxidant system is not clear, it may be due to the decreased levels of trace elements (selenium, zinc, *etc*.), which functioning as cofactors of antioxidant enzymes[56,57]. Supporting this hypothesis is the increase in the antioxidant enzyme levels with trace element supplementation during long-term treatment[58]. In addition, co-administration of free radical scavengers, such as the antioxidants, vitamin E[59] and vitamin C[60] with ASA results in markedly less gastric mucosal damage compared with ASA alone[61]. These results indicated that oxygen radicals are generated in the development of ASA-induced damage to the mucous, parietals and endothelial cells.

The mechanisms of protective action of Se against oxidative damage induced by DIA and/or ASA could be explained by stimulating free radical scavenging antioxidant enzymes activities, *e.g.* SOD, CAT and GPx. This might be coupled with the ability of antioxidants such as N-acetyl cysteine and stimulated several antioxidative enzymes against damages from free radicals insult[62,63]. Se increased antioxidant capacity in the cells by the increased activity of GSH reductase which enhances the availability of GSH "one of the most intrinsic antioxidants that prevents cell damage"[63].

It can be concluded that DIA and ASA induced oxidative stress and lipid peroxidation in rat erythrocytes. The results revealed the pronounced ameliorating effect of Se in DIA and ASA intoxicated rats. We supposed that antioxidant supplementation may be beneficial for the people using ASA for longer periods and exposure to pesticides.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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