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Pesticides-induced oxidative damage: Possible in vitro protection by antioxidants

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This study was designed to investigate the effect of selenium (Se) and a combination of vitamin E (VE) vitamin C (VC) on pesticides induced biochemical alterations in rat erythrocytes and hepatocytes in vitro. Vitamin E and C and selenium are potential antioxidants, known to be able to protect cells against oxidative damage. In vitro changes in antioxidant systems and protective role of selenium and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes and hepatocytes induced by atrazine (AT), dimethoate (DM), or endosulfan (ES) at three different levels of 10, 20, and 30 mM for each in rat were investigated. Levels of lipid peroxidation (nmoles MDA/mg protein), glutathione content (µmole GSH/mg protein) and glutathione peroxidase level (µmoles NADPH/min/mg protein) were determined in erythrocytes and hepatocytes following treatment. In comparison with the control, pesticides stimulated thiobarbituric acid reactive substances (TBARS) activity and glutathione peroxidase activity, enhanced the glutathione contents. Treatment with selenium and a combination of vitamin E and/or vitamin C potentially reduced the free radicals in erythrocytes or hepatocytes and ameliorated the oxidative stress induced by such pesticides. The results suggested that pesticides treatment increases in vitro lipid peroxidation, glutathione peroxidase level and glutathione content by increasing oxidative stress in erythrocytes and hepatocytes of rats and selenium and a combination of vitamin E and vitamin C can reduce this lipoperoxidative effect.

Key words: Oxidative stress, red blood cells (RBCs), hepatocytes, atrazine, dimethoate, endosulfan, lipid peroxidation, glutathione, glutathione peroxidase.

INTRODUCTION

Pesticides are used to protect plants and numerous plant products. These compounds are highly toxic to living organisms. Recently, data indicate that the toxic action of pesticides may include the induction of oxidative stress and accumulation of free radicals in the cell. A major form of cellular oxidation damage is lipid peroxidation, which is initiated by hydroxyl free radical through the extraction of hydrogen atom from unsaturated fatty acids of membrane phospholipids (Farber et al., 1990). As a consequence, these compounds can disturb the biochemical and physiological functions of the red blood cells (RBCs) (Akhgari et al., 2003). The susceptibility of RBC to oxidative damage is due to the presence of polyunsaturated fatty acid, heme iron and oxygen, which may produce oxidative changes in RBC (Kale et al., 1999). The increased oxidative stress resulted in an increase in the activity of antioxidant enzymes such as superoxide dismutase and catalase. The enhancement of release of lactate dehydrogenase (LDH) is also indicative of cellular and membrane damage, while inactivation of superoxide dismutase and catalase are expected to enhance the generation of reactive oxygen species and consequently, pose an oxidative stress upon the system (Tabatabaie and Floyed, 1996). The increase in reduced glutathione content in erythrocytes may probably be an initial adaptive response to increase oxidative stress (Krechniak
Chemicals were purchased from Sigma Chemicals (USA). Ad libitum study. Rats were kept to be acclimatized at room temperature.

Analytical grade standards for atrazine, dimethoate and endosulfan pesticides against lipid peroxidation are vitamin E and vitamin C, well-known free radical scavengers (Kumar et al., 2009). Vitamin E is a lipid-soluble, chain-breaking antioxidant playing a major protective role against oxidative stress and prevents the production of lipid peroxides by scavenging free radicals in biological membranes. Some investigators reported that administering Vitamin E may be useful in controlling the toxic effect of insecticides and chemicals (Atessahin et al., 2005). Malathion-induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E was in vitro investigated (Durak, 2009). Vitamin C has an anti-radical activity indicating that it could provide an important dietary source of antioxidants. Vitamin C is also a well-known low molecular weight antioxidant that protects the cellular compartment from water-soluble oxygen nitrogen radicals (Jurczuk et al., 2007). Selenium is an essential element for biological systems, which improves the activity of the seleno-enzyme. It is present in the active center of glutathione peroxidase (GPx), an antioxidant enzyme, which protects lipid membranes and macromolecules from oxidative damage produced by peroxides (Rotruck, 1973; Harman, 1993). Yuan and Tang (1999) and Akhtar et al. (2009) reported that selenium has the ability to counteract free radicals and protect the structure and function of proteins, DNA and chromosomes against oxidation injury. The level of selenium in the body is plays a significant role since it controls the deficiency of vitamin E and facilitates its absorption (Machlin, 1991). The protection effect of antioxidants such as vitamin E, selenium, vitamin C and other natural materials have been investigated by many investigators (Al-Othman et al., 2011; Saafi et al., 2011; Singh et al., 2008; Adesiyan et al., 2011; Singh et al., 2011; Ozdem et al., 2011; Saxena et al., 2011). The aim of the present study was to establish the antioxidant role of selenium and a combination of vitamin E and vitamin C on oxidative stress induced in rat RBCs and hepatocytes by atrazine, dimethoate, or endosulfan pesticides.

**EXPERIMENTAL METHODS**

**Chemicals**

Analytical grade standards for atrazine, dimethoate and endosulfan with high purity were provided by the US (EPA). Kits were purchases from Boehringer Mannheim GmbH, Germany. All other chemicals were purchased from Sigma Chemicals (USA).

**Animals and treatments**

Ten male Wistar albino rats weighing 180±30 g were used in the study. Rats were kept to be acclimatized at room temperature. Animals were fed with standard commercial pelleted feed and water ad libitum. Blood (about 3 ml) was obtained from rat by heart puncture using ethylenediaminetetraacetic acid (EDTA)-sodium salt as the anticoagulant for assays. The blood was centrifuged at 3000 rpm for 5 min at 4°C. RBCs were taken and washed with phosphate buffered saline (PBS), pH 7.2. The final red cell suspension was taken in test tubes for treatment (Saxena et al., 2011). The liver was also dissected out and homogenized in saline solution (1:10 w/v). The homogenates were taken in a test tube for treatment. Pesticides, vitamins and selenium (atrazine, dimethoate, endosulfan, vitamin E, vitamin C, selenium, atrazine + vitamin E, dimethoate + vitamin E, endosulfan + vitamin E, atrazine + vitamin C, dimethoate + vitamin C, endosulfan + vitamin C, atrazine + vitamin E + selenium, dimethoate + vitamin E + selenium, endosulfan + vitamin E + selenium, atrazine + vitamin C + selenium, dimethoate + vitamin C + selenium, endosulfan + vitamin E + selenium) were dissolved in 5% DMSO. Also, 5% dimethyl sulfoxide (DMSO) was dissolved/mixed in the control group. RBCs or liver homogenate were incubated for 3 h at 37°C in a shaking water bath. At the end of incubation, the tubes were removed and were subjected to biochemical analysis.

**Biochemical analysis**

Erythrocytes and their membranes were isolated from the control and experimental groups according to the method of Dodge et al. (1963). Different aliquots of packed cells were thoroughly washed with tris-buffer, pH 7.4. All these were used for the assay of various biochemical parameters. Then, another aliquot of packed cells were subjected to hemolysis by adding hypotonic tris-buffer, pH 7.2. After 4 to 6 h, the erythrocytes ghosts were sedimented by centrifugation at 12000 rpm, for 40 to 45 min at 4°C. The supernatant was used for the analysis of antioxidants. Liver homogenates were taken from the control and experimental groups and were used for the assay of the antioxidants.

**Measurement of lipid peroxidation**

Lipid peroxidation (LPO) was determined according to Ohikawa et al. (1979). The data were expressed as nmole of malondialdehyde formation/mg protein.

**Measurement of reduced glutathione**

Glutathione content (GSH) was estimated by the method of Ellman (1959) and the amount of glutathione is expressed as µmoles/mg protein.

**Determination of glutathione peroxidase activity**

The determination of GSH-Px activity was based on the method of Paglia and Valentine (1967). Glutathione peroxidase levels (GSH-Px) were determined as µmoles nicotinamide adenine dinucleotide phosphate-oxidase (NAPDH)/min/mg protein.

**Determination of protein contents**

The total protein content of liver homogenate was determined by the method of Lowry et al. (1951).

**RESULTS**

**Lipid peroxidation**

Biochemical analysis showed that there was an increase in malondialdehyde (MDA) levels in erythrocytes and...
Table 1. Lipid peroxidation level (nmoles MDA/mg protein), GSH content (µmole/mg protein) and glutathione peroxidase level (µmoles NADPH/min/mg protein) of control or treated erythrocytes and hepatocytes with vitamin E, vitamin C or selenium.

| Treatment   | Lipid peroxidation level (nmoles MDA/mg protein) | GSH content (µmole/mg protein) | Glutathione peroxidase level (µmoles NADPH/min/mg protein) |
|-------------|--------------------------------------------------|-------------------------------|------------------------------------------------------------|
|             | Erythrocytes                                      | Hepatocytes                   | Erythrocytes                                               | Hepatocytes                   |
| 5% DMSO     | 8.96±.0.19                                       | 12.54±.9.6                   | 52.55±6.10                                                | 233.14±0.88                  |
| VE (5 mg)   | 8.55±.11                                         | 11.84±.1.1                  | 60.22±9.10                                                | 244.70±10.15                 |
| VC (5 mg)   | 9.16±.23                                         | 13.22±.77                   | 55.17±8.88                                                | 255.00±10.70                 |
| Se (1 mg)   | 8.92±.44                                         | 13.16±.9.0                  | 60.10±7.56                                                | 247.30±10.20                 |

Table 2. Lipid peroxidation level (nmoles MDA/mg protein) of treated erythrocytes and hepatocytes with atrazine alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment   | Erythrocytes | Hepatocytes |
|-------------|--------------|-------------|
|             | 10 mM        | 20 mM       | 30 mM       | 10 mM        | 20 mM       | 30 mM       |
| AT          | 12.10±.76    | 14.10±.11   | 14.99±.12   | 16.11±.20    | 18.31±.21   | 19.60±.66   |
| AT + VE     | 9.57±.16     | 8.55±.11    | 8.55±.11    | 13.57±.16    | 13.66±.81   | 13.67±.61   |
| AT + VE + Se| 8.58±.99     | 9.50±.90    | 9.99±.16    | 11.20±.46    | 11.58±.22   | 12.44±.44   |
| AT + VC     | 8.99±.02     | 9.38±.12    | 9.58±.22    | 12.52±.22    | 11.77±.02   | 13.54±.29   |
| AT + VC + Se| 7.11±.88     | 8.58±.99    | 8.18±.29    | 11.44±.60    | 11.99±.20   | 13.88±.77   |

Table 3. Lipid peroxidation level (nmoles MDA/mg protein) of treated erythrocytes and hepatocytes with dimethoate alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment   | Erythrocytes | Hepatocytes |
|-------------|--------------|-------------|
|             | 10 mM        | 20 mM       | 30 mM       | 10 mM        | 20 mM       | 30 mM       |
| DM          | 10.20±.10    | 11.20±.11   | 11.33±.20   | 18.66±2.24   | 20.81±1.41  | 23.60±0.68  |
| DM + VE     | 9.10±.40     | 10.91±.21   | 10.60±.88   | 13.44±.70    | 17.31±.81   | 17.10±.77   |
| DM + VE + Se| 8.11±.80     | 10.31±.10   | 10.00±.10   | 10.11±.60    | 14.31±.99   | 17.00±.66   |
| DM + VC     | 8.11±.40     | 8.01±.01    | 10.60±.16   | 10.01±.40    | 11.01±.91   | 16.90±.96   |
| DM + VC + Se| 7.11±.40     | 10.01±1.11  | 9.90±.96    | 9.11±.40     | 10.01±.43   | 15.60±0.16  |

Vitamin E (VE), vitamin C (VC) and sodium selenite (Se).

Table 4. Lipid peroxidation level (nmoles MDA/mg protein) of treated erythrocytes and hepatocytes with endosulfan alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment   | Erythrocytes | Hepatocytes |
|-------------|--------------|-------------|
|             | 10 mM        | 20 mM       | 30 mM       | 10 mM        | 20 mM       | 30 mM       |
| EN          | 11.20±.11    | 11.33±.20   | 18.66±2.24  | 20.81±1.41   | 23.60±0.68  |
| EN + VE     | 10.91±.21    | 10.60±.88   | 13.44±.70   | 17.31±.81    | 17.10±.77   |
| EN + VE + Se| 10.31±.10    | 10.00±.10   | 10.11±.60   | 14.31±.99    | 17.00±.66   |
| EN + VC     | 8.01±.01     | 10.60±.16   | 10.01±.40   | 11.01±.91    | 16.90±.96   |
| EN + VC + Se| 10.01±1.11   | 9.90±.96    | 9.11±.40    | 10.01±.43    | 15.60±0.16  |

Dimethoate (DM), vitamin E (VE), vitamin C (VC) and sodium selenite (Se).

hepatocytes of rats treated with atrazine, dimethoate, or endosulfan at the three different levels of 10, 20 or 30 mM compared to the control group. MDA levels in erythrocytes were increased by 35.04, 13.84 and 57.37% as following treatment with 10 mM of atrazine, dimethoate, or endosulfan, respectively. Also, these values were increased to 57.36, 25.00, and 79.69% following exposure to 20 mM of atrazine, dimethoate, or endosulfan, respectively. At the level of 30 mM, these values rose to 67.30, 26.45, and 97.54%, respectively when compared with the control animals. MDA levels in hepatocytes were also found to be increased following the treatment of pesticides at the three levels for each compound. At the level of 10 mM of atrazine, dimethoate, or endosulfan, the values increased to 46.01, 65.95 and 52.31%. At the level of 30 mM of pesticides they increased to 56.30, 88.20 and 76.24%, respectively. Table 1 presented the levels of MDA of control or treated erythrocytes and hepatocytes with vitamin E, vitamin C or selenium. Tables 1 to 4 showed the antioxidant effect of selenium, vitamin E and vitamin C on erythrocyte and hepatocyte toxicity induced by atrazine, dimethoate and endosulfan.

**Glutathione content**

Biochemical analysis showed that there was an increase in GSH contents in erythrocytes and hepatocytes of rats treated with atrazine, dimethoate, or endosulfan at the three different levels of 10, 20 or 30 mM compared to the control group. MDA levels in erythrocytes were increased by 35.04, 13.84 and 57.37% following treatment with 10 mM of atrazine, dimethoate, or endosulfan, respectively. Also, these values were increased to 57.36, 25.00, and 79.69% following exposure to 20 mM of atrazine, dimethoate, or endosulfan, respectively. At the level of 30 mM, these values rose to 67.30, 26.45, and 97.54%, respectively when compared with the control animals. MDA levels in hepatocytes were also found to be increased following the treatment of pesticides at the three levels for each compound. At the level of 10 mM of atrazine, dimethoate, or endosulfan, the values increased to 46.01, 65.95 and 52.31%. At the level of 30 mM of pesticides they increased to 56.30, 88.20 and 76.24%, respectively. Table 1 presented the levels of MDA of control or treated erythrocytes and hepatocytes with vitamin E, vitamin C or selenium. Tables 1 to 4 showed the antioxidant effect of selenium, vitamin E and vitamin C on erythrocyte and hepatocyte toxicity induced by atrazine, dimethoate and endosulfan.
Table 4. Lipid peroxidation level (nmoles MDA/mg protein) of treated erythrocytes and hepatocytes with endosulfan alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment | Erythrocytes | Hepatocytes |
|-----------|--------------|-------------|
|           | 10 mM | 20 mM | 30 mM | 10 mM | 20 mM | 30 mM |
| ES        | 14.10±0.60 | 16.10±0.76 | 17.70±0.78 | 18.10±0.76 | 19.10±0.76 | 22.10±0.76 |
| ES + VE   | 10.10±0.11 | 14.70±0.16 | 14.90±0.99 | 15.10±0.44 | 17.10±0.33 | 20.10±0.99 |
| ES + VE + Se | 7.10±0.46 | 8.10±0.11 | 13.10±0.60 | 11.10±0.22 | 13.10±0.76 | 15.10±0.88 |
| ES + VC   | 11.10±0.00 | 12.10±0.06 | 12.10±0.70 | 13.10±0.88 | 16.10±0.11 | 17.10±0.06 |
| ES + VC + Se | 9.10±0.10 | 10.10±0.44 | 11.10±0.80 | 11.10±0.16 | 13.10±0.99 | 14.70±0.76 |

Endosulfan (ES), vitamin E (VE), vitamin C (VC) and sodium selenite (Se).

Table 5. GSH content (µmole/mg protein) of treated erythrocytes and hepatocytes with atrazine alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment | Erythrocytes | Hepatocytes |
|-----------|--------------|-------------|
|           | 10 mM | 20 mM | 30 mM | 10 mM | 20 mM | 30 mM |
| AT        | 100.80±12.80 | 122.10±22.45 | 132.40±17.12 | 554.41±15.60 | 612.10±24.11 | 689.80±44.16 |
| AT + VE   | 88.10±5.10 | 100.70±9.90 | 105.00±15.11 | 452.17±55.16 | 489.77±25.51 | 500.69±33.11 |
| AT + VE + Se | 40.18±6.19 | 60.90±7.06 | 61.90±9.66 | 300.80±20.14 | 335.58±22.22 | 435.44±0.44 |
| AT + VC   | 60.19±5.02 | 76.85±6.12 | 88.98±12.20 | 306.92±33.12 | 458.07±24.50 | 446.54±23.19 |
| AT + VC + Se | 33.00±4.68 | 57.58±8.19 | 30.88±11.20 | 167.64±18.90 | 189.95±21.90 | 224.88±9.17 |

Atrazine (AT), vitamin E (VE), vitamin C (VC) and sodium selenite (Se).

Table 6. GSH content (µmole/mg protein) of treated erythrocytes and hepatocytes with dimethoate alone or combined with vitamin E, vitamin C and/or selenium.

| Treatment | Erythrocytes | Hepatocytes |
|-----------|--------------|-------------|
|           | 10 mM | 20 mM | 30 mM | 10 mM | 20 mM | 30 mM |
| DM        | 178.70±21.20 | 212.60±17.12 | 222.73±16.29 | 516.77±18.24 | 588.91±21.91 | 586.90±18.99 |
| DM + VE   | 140.80±12.10 | 171.61±20.61 | 188.60±19.18 | 377.14±16.90 | 407.88±7.91 | 518.10±24.18 |
| DM + VE + Se | 111.99±9.60 | 135.88±21.88 | 144.18±8.15 | 299.10±18.50 | 226.51±19.77 | 400.00±12.12 |
| DM + VC   | 123.91±0.10 | 132.21±9.01 | 133.10±13.12 | 254.01±20.10 | 145.91±13.71 | 256.70±12.16 |
| DM + VC + Se | 55.31±7.19 | 44.81±11.19 | 51.90±0.16 | 77.91±0.10 | 47.91±0.13 | 145.60±0.16 |

Dimethoate (DM), vitamin E (VE), vitamin C (VC) and sodium selenite (Se).

control group. GSH contents in erythrocytes were increased to 91.82, 240.05, and 194.01% as following treatment with 10 mM of atrazine, dimethoate, or endosulfan, respectively. Also, these values were increased to 132.35, 304.57, and 197.05% following exposure to 20 mM of atrazine, dimethoate, or endosulfan, respectively. At the level of 30 mM, these values rose to 151.95, 323.84, and 261.37%, respectively when compared with control animals. GSH contents in hepatocytes were also found to increase following the treatment of pesticides at three levels for each compound. At the level of 10 mM of atrazine, dimethoate, or endosulfan, the values were increased to 137.80, 121.66 and 95.63% as compared to the control group. At the level of 20 mM of pesticides at the same order were 137.80, 121.66 and 95.63% as compared to the control group. At the level of 20 mM of pesticides at the same order were 137.80, 121.66 and 95.63% as compared to the control group. At the level of 30 mM of pesticides, they were 195.87, 151.74 and 156.97%, respectively. Tables 1, 5, 6 and 7 showed the antioxidant effect of selenium, vitamin E and vitamin C on erythrocyte and hepatocyte toxicity induced by atrazine, dimethoate and endosulfan via declining the level of GSH contents.

**Glutathione peroxidase activity**

GSH-Px activity recorded an increase of 160.87, 160.87 and 204.35% in erythrocytes following treatment with 10 mM of atrazine, dimethoate, or endosulfan, respectively. Also, these values were increased to 204.35, 218.84, and 784.06% following exposure to 20 mM of atrazine, dimethoate, or endosulfan, respectively. At the level of 30 mM, these values rose to 333.33, 295.65, and 871.01%, respectively when compared with control animals. GSH-Px activity in hepatocytes was found to increase following...
The treatment of pesticides at the three levels for each compound. At the level of 10 mM of atrazine, dimethoate, or endosulfan, the values were increased to 152.80, 216.36 and 185.05% as compared to the control group. At the level of 20 mM of pesticides, the values increased to 231.78, 316.36 and 231.78%. At the level of 30 mM of pesticides, they increased to 311.21, 409.35 and 465.42%, respectively (Tables 1, 8, 9 and 10).
DISCUSSION

Erythrocytes and hepatocytes are useful model to study the interaction of pesticides with biological membranes. Pesticides are thought to exert damaging effect on biomembranes through free radical generation; therefore, antioxidants can play a crucial role in offering protection against pesticide induced oxidative damage. The in vitro lipoperoxidative effect in erythrocytes and hepatocytes induced by atrazine, dimethoate, or endosulfan in male albino rat was studied. The antioxidant effect of selenium, vitamin E and vitamin C on erythrocyte and hepatocyte toxicity induced by pesticides was also investigated.

Our results concerning the lipid peroxidation levels indicate that the level of MDA increment is pesticide concentration dependent. Evidence from in vitro and in vivo studies with many toxicants including pesticides supports the concept that free radicals e.g. hydroxyl radicals (•OH), H₂O₂ and others, are important mediators of tissue injury and formation of these radicals result in increased LPO (Farber et al., 1990; Akhgar et al., 2003). The results indicate that vitamin E, vitamin C and their combination with selenium play a major protective role against oxidative stress and prevent the production of lipid peroxides by scavenging free radicals in biological membranes. Atessahin et al. (2005) and Durak (2009) reported that vitamin E may be useful in controlling the toxic effect of pesticides. Jurczuk et al. (2007) reported that vitamin C protects the cellular compartment from water-soluble oxygen nitrogen radicals. Rotruck (1973) and Harman (1993) indicated that selenium improves the activity of the seleno-enzyme which protects lipid membranes and macromolecules from oxidative damage produced by peroxides. Yuan and Tang (1999) and Akhtar et al. (2009) also reported that selenium has the ability to counteract free radicals and protect the structure and function of proteins, DNA and chromosomes against oxidation injury.

Our findings in case of GSH contents indicate that the level of GSH enhancement is pesticide concentration dependent. Krechniak and Wrzesniowska (1999) and Kale et al. (1999) mentioned that the increase in reduced glutathione content in erythrocytes may probably be an initial adaptive response to increased oxidative stress. In comparison to the control group, the activity of glutathione S-transferase (GST) was higher in all pesticide treated rat erythrocytes or hepatocytes. It is well known that GSTs are detoxifying enzymes that catalyze the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms (Saxena et al., 2011). The increase of GST activity in the rat erythrocytes or hepatocytes following atrazine, dimethoate, or endosulfan treatment may indicate sufficient detoxification of pesticide while the use of selenium and its combination with vitamin E or vitamin C with such pesticides approaches the control group. The results also indicated that GSH-Px activity trends were found to be similar to lipid peroxidation and glutathione contents.

In conclusion, the results indicated that atrazine, dimethoate or endosulfan treatment increases in vitro lipid peroxidation, glutathione content and GSH-Px activity by increasing oxidative stress in erythrocytes and hepatocytes of rats. Selenium and its combination with vitamin E or vitamin C can reduce this lipoperoxidative effect. Treatment with selenium and a combination of vitamin E and/or vitamin C potentially reduced the free radicals in erythrocytes or hepatocytes and ameliorated the oxidative stress induced by such pesticides.

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