HACETTEPE JOURNAL OF BIOLOGY AND CHEMISTRY
Volume 35, Issue 2, June 2007
Research Article
Hacettepe J. Biol. & Chem., 2007, 35 (2), 111-116

Biochemical Effects of Pesticide Contaminated Drinking Water on Lipid Peroxidation and Free-Radical Scavenger

Hüseyin Tunçmen[1], M. Nalan Tüzmen[2*]
[1]Department of Chemistry, Süleyman Demirel University, Arts and Science Faculty, Isparta, Turkey
[2]Deparment of Chemistry, Dokuz Eylül University, Arts and Science Faculty, İzmir, Turkey

Abstract

In agriculture, pesticides are commonly used for achieving better quality prouducts, increased production rate and controling pest population. However, they also lead to generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide(O_2^{-}•), and hydroxyl (•OH) radicals, which have harmful effect on human health. Each year about 15000 tons of fertilizer and 500 tons of pesticides contaminate the groundwater of Büyükkabaca where agriculture and fruit growing are commonly widespread. The drinking water of Büyükkabaca is obtained from 2 aquifers which are 150 m below surface of this plain. Thus, the aim of this study was to investigate the biochemical effects of Büyükkabaca's drinking water on oxidant/antioxidant system in the blood, brain, liver and kidney of the rats. The level of thiobarbituric acid reacting substances and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were increased and GSH level and catalase (CAT) activity (only in blood and brain) were decreased in the experimental group of rats. The present results suggest that free radicals like superoxide and peroxyl radicals formed as a result of excessive ethion level determined in Büyükkabaca's drinking water can be responsible for the incresase in LPO and alteration in antioxidative defence mechanisms. Therefore, further studies are needed to investigate the effect of the increased lipid peroxidation on human health in this region.

Key Words: Free radical, antioxidant enzyme, lipid peoxidation, pesticide.

Introduction

Agricultural and industrial chemicals, pollutants and toxicants are now widely dispersed in the environment. Among them, pesticides such as organophosphorus and organochlorine are used extensively in agriculture to enhance food production by eradicating unwanted insects and controlling diease vectors (1,2). Atmospheric emissions of pesticides result from volatilization of the chemical during manufacturing and formulation, from soil and vegetation resulting from their use as an insecticide and acaricide etc., or from drift during pesticide application or careless mixing and cleanup procedures during or after application. Pesticides like ethion can be released to surface waters directly by point source dischargers, from drift during pesticide applications, and by nonpoint source runoff from agricultural and urban areas (3). Over the last decades, concern about the contamination of water sources has been raised due to increasing pesticides concentrations. Uncontrolled use of agricultural chemicals especially in intensive agricultural areas of Turkey causes serious soil, surface and groundwater pollution. Thus, regulations for drinking water are required in order to limit human risks and environmental pollution (4). Because these chemicals are known to have a cumulative hazardous effect on humans and animals.

Most pesticides do not show any immediate adverse effect (at least at concentrations normally used) but may pose a significant long term hazard to man. Pesticide poisoning is an important cause of morbidity and mortalitiy in developing countries although only one-fourth of the total world consumption of pesticides has been reported from these region. Nevertheless, every year there are 3 million cases of severe poisoning and 220.000 deaths; the majority of these poisoning and 99 % of the resulting deaths occur in the third world (5).

Undesirable side-effects that result from the indiscriminate use of organochlorine, organophosphate, and carbamate pesticides are widespread. These pesticides are known to disturb the biochemical and physiological function of erythrocytes and lymphocytes (5). It has been previously suggested that several pesticides also exert their biological effects mainly through electrophilic attack of cellular constituents with simultaneous generation of reactive oxygen species (ROS). ROS may, therefore, be involved in the toxicity of various pesticides (6). Pesticide chemicals may induce oxidative stress leading

* Correspondence to: Dr. M. Nalan Tüzmen,

Department of Chemistry, Dokuz Eylul University, Faculty of Art and Science, 35160 Buca-İzmir, Türkiye

Tel: + 90 232 453 50 73 (ext: 2261). Fax: +90 232 453 41 88
E-mail: nalan.tuzmen@deu.edu.tr

to generation of free radicals and alteration in antioxidants or oxygen free radical (OFR) scavenging enzyme systems (5). It is important to note that many environmental contaminants, such as organochlorine pesticides, accumulate in fatty tissues (7). Tissue degeneration is a free-radical-medited process that involves lipid peroxides and lipid peroxidation of polyunsaturated fatty acids (PUFA) of the mammalian tissue (1). Therefore, lipid peroxidation has been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity (5).

In the region of Büyükkabaca-Isparta, Turkey, agricultural production and fruit growing are widespread and each year about 1500 tons of fertilizers and 500 tons of pesticides contaminate the groundwater. The drinking water of Büyükkabaca is obtained from 2 aquifers which are 150 m beneath the Uluborlu-Senirkent-Büyükkabaca plain. Thus, in the current study, we aimed to determine the effects of Büyükkabaca's drinking water on LPO and the antioxidant enzyme activities such as SOD, GSH-Px and CAT in erythrocytes and different tissues of rats.

Materials and Methods

Chemicals

The following chemicals were used: Dithionitrobenzoic acid (DTNB), β-nikotinamid adenin dinucleotide phosphate (β-NADPH), reduced glutathione, 2-thiobarbituric acid (TBA), xanthine, xanthine oxidase (XOD), glutathione reductase (GR), cumene hydoperoxide and bovine serum albumin (Fraction V). They were all obtained from Sigma, St.Louis, MO, USA. Pesticide analytical standards (Methamidophos, Diazinon, Parathion Methyl, Cyprodinil, Captan, Methidathion, Kresoxim Methyl, Ethion, Fenazaquin, Cypermethrin, Deltamethrin) were supplied by RDH. All other reagents used were of analytical grade.

Animals and Treatment

Sixteen, male Wistar albino rats (3 month-old; weghing 180 ± 30 g) were used in the present experiment. The animals were kept in a room in which the humidity and temperature were environmentally controlled. The rats were randomly divided into two groups each comprising eight animals and treated for 16 weeks.

Group I (control group): Rats were fed with standard commercial pelleted feed and water ad libitum.

Group II (experimental group): Rats were fed ad libitum with a standard commercial pellet diet and drinking water of Büyükkabaca.

The animals were sacrificed under ether anaesthesia after 16 weeks treatment. Blood samples were collected by cardiac puncture into heparinized vials. Plasma was separated by centrifugation and stored at -20°C. Erythrocyte pellets were prepared by washing erythrocytes three times with cold isotonic saline. Washed erythrocytes were diluted twice with isotonic saline, and the hemoglobin concentration was determined using a hemocounter (Coulter STKS, USA). The erythrocyte MDA and GSH levels, and SOD, GSH-Px, and CAT activities were determined. Liver, kidney and brain tissues were minced separetly and homogenized with 50 mM phosphate buffer (pH 7.4) under ice-cold condition. The homogenates (10%) were centrifuged for 10 min at 8000 rpm and the supernatants were stored at 4°C for determining different biochemical parameters.

Pesticide Residue Analysis of Water

50 ml of water sample was extracted in 10 ml of dichlormethane for three times and extracts combined. Then the solvent was evaporated until dryness. Then residue dissolved in 500 µl of toluene. Individual stock standard solutions were prepared by exact weighting of high-purity substances (Methamidophos, Diazinon, Parathion Methyl, Cyprodinil, Captan, Methidathion, Kresoxim Methyl, Ethion, Fenazaquin, Cypermethrin, Deltamethrin) and dissolving them in toluen. Stock standard solutions were stored in a freezer whereas working standard solutions were prepared daily by appropriate dilution in ultrapure Milli-Q water (Millipore, Molsheim, France). Chromatographic analyses were carried out in a Perkin Elmer model Auto System XL gas chromatograph equipped with nitrogen–phosphorus detection (NPD) and a 30 m x 0,32 mm i.d., 0.25-µm thickness, TRB5 capillary column. Flow rate of helium used as carrier gas was 6 mL/min. The chromatographic temperature conditions was as follows: 80°C for 1 min, increased at 20°C / min to 270°C, then 4°C/min to 295°C final temperature, held for 10 min. The injector temperature was maintained at 295°C. The detector temperature was 295°C. The injection volume was 1 µl.

Biochemical Studies

Estimation of lipid peroxidation (assay of MDA value). MDA, as a marker for LPO, was determined by the double heating method of Draper and Hadley (8). The principle of the method was spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g/l triochloroacetic

acid (TCA) solution was added to 0.5 ml erythrocytes in a centrifuge tube and placed in a boiling water-bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000g for 10 min, and 2 ml of the supernatant was added to 1ml 0.67% TBA (w/v) solution in a test-tube and placed in a boiling water-bath for 15 min. The solution was then cooled and the absorbance was measured using a Shimadzu UV-1601 spectrophotometer (Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56x105 $cm^{-1}M^{-1}$ and expressed in nmol/g Hb.

Assay of antioxidant and antiperoxidant enzymatic activities.

SOD activity: The measurement of SOD was based on the principle in which xanthine reacts with xanthine oxidase to generate superoxide radicals which react with 2-(4-iodo-phenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride (INT) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction (9).

CAT activity: CAT activity was measured according to the method of Aebi (10). The principle of the assay is based on the determination of the rate constant k (s^{-1}) of hydrogen peroxide decomposition by catalase enzyme.

GSH-Px activity: The determination of GSH-Px activity was based on the method of Paglia and Valentine (11). GSH-Px catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance of NADPH was measured at 340 nm.

Quantification of reduced glutathione. GSH content was assayed using Ellman's reagent (DTNB) by the method of Tietze (12).

Statistics

Data are given as mean ± standard deviation. Statistical analysis of data was performed on a computer by using SPSS Version 11.0. One-way ANOVA was firstly done to confirm normal distribution and variances between experiment and control groups. Then an unpaired t-test was done to determine the significance of differences observed between study groups.

Results

None of the rats treated with Büyükkabaca's drinking water showed signs of morbidity or mortality during the studies. In comparison to the initial body weight, no significant changes were noticed in the experimental group compared to the control group.

Level of pesticide residues in drinking water. There was no any pesticide residues in drinking water of Isparta. The only observed pesticide residue in Büyükkabaca's drinking water was ethion (18 ± 0.12 ppb). The average fortified recoveries of ethion at 0.5 mg l^{-1} ($mgkg^{-1}$) were 99% in water. The detection limit of ethion was 0.005 mg l^{-1} in solution.

Lipid peroxidation. Biochemical analysis showed that there was a significant increase ($p<0.001$) in MDA levels in the erythrocytes of rats treated with Büyükkabaca's drinking water as compared to the control group (Table 1). LPO in brain, liver and kidney of rats was significantly increased by 26.18% ($p<0.01$), 73.45% ($p<0.001$) and 53.94% ($p<0.01$), as a result to exposure of Büyükkabaca's drinking water, respectively, when compared with control animals.

Table 1. Effect of ethion contaminated drinking water on lipid peroxidation (nmol MDA/g Hb or g protein) and glutathione level (mg GSH per dl blood or μg GSH per g tissue) in rats treated with Büyükkabaca's drinking water.

	Blood	**Brain**	**Liver**	**Kidney**
Lipid peroxidation				
Control	175.8±4.8	16.3 ± 1.3	11.4 ± 2.2	14.3 ± 2.0
Experiment	232.2± 23.4[a]	20.6 ± 2.7[b]	19.8 ± 1.8[a]	22.1 ± 2.9[b]
Glutathione level				
Control	3.7 ± 0.3	26.6 ± 4.0	4.7 ± 0.5	15.5 ± 3.8
Experiment	4.7 ± 0.6	12.4 ± 3.2[a]	2.0 ± 0.4[a]	11.4 ± 1.6[c]
[a]Significantly different from control ($p<0.001$)				
[b]Significantly different from control ($p<0.01$)				
[c]Significantly different from control ($p<0.05$)				

The antioxidative and antiperoxidative enzyme systems. The observed activities of antioxidative and antiperoxidative enzymes as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) are shown in Table 2-4.

SOD activity.

SOD activities showed similar trend in erythrocytes and all the defined tissues of rats treated with

Büyükkabaca's drinking water. SOD activity in blood was significantly increased by 39.13% ($p<0.001$) relative to the control animals. SOD activity in brain, liver and kidney tissues also increased significantly by 28.12% ($p<0.001$), 96.48% ($p<0.001$) and 65.50% ($p<0.01$) as a result to exposure of Büyükkabaca's drinking water, respectively, when compared with control animals (Table 2).

Table 2. Effect of ethion contaminated drinking water on SOD activity (U/gram hemoglobin or g protein) in rats treated with Büyükkabaca's drinking water.

	Blood	Brain	Liver	Kidney
Control	2621.9 ± 296.2	0.6 ± 0.03	27.6 ± 5.4	1.2 ± 2.8
Experiment	3647.9 ± 342.4[a]	0.8 ± 0.07[a]	54.1 ± 3.4[a]	18.5 ± 2.7[b]
[a]Significantly different from control ($p<0.001$)				
[b]Significantly different from control ($p<0.01$)				

GSH-Px activity

GSH-Px activity ternds were similar to those of SOD activity variations. GSH-Px activity recorded an increase of 113.87% ($p<0.001$), 34.91% ($p<0.001$), 68.26% ($p<0.001$) and 17.19% ($p<0.01$) in blood, brain, liver and kidney of animals treated with Büyükkabaca's drinking water, respectively (Table 3).

Table 3. Effect of ethion contaminated drinking water on GSH-Px activity (U/gram hemoglobin or g protein) in rats treated with Büyükkabaca's drinking water.

	Blood	Brain	Liver	Kidney
Control	1.4 ± 0.2	3.2 ± 0.3	26.1 ± 4.7	67.5 ± 4.4
Experiment	2.9 ± 0.5[a]	4.3 ± 0.3[a]	43.9 ± 4.2[a]	79.2 ± 3.9[b]
[a]Significantly different from control ($p<0.001$)				
[b]Significantly different from control ($p<0.01$)				

CAT activity

Trends of CAT activity were opposite to SOD and GSH-Px activities. The activity of CAT was significantly decreased in blood and liver of the experiment group of rats by 33.70% ($p<0.01$) and 31.03% ($p<0.01$), respectively, relative to the control animals. CAT activity didn't show any change in brain and kidney of Büyükkabaca's drinking water treated rats when compared with control animals (Table 4).

Table 4. Effect of ethion contaminated drinking water on CAT activity (U/gram hemoglobin or g protein) in rats treated with Büyükkabaca's drinking water GSH-Px.

	Blood	Brain	Liver	Kidney
Control	135.1 ± 23.5	0.01 ± 0.008	0.3 ± 0.04	0.1 ± 0.02
Experiment	89.6 ± 15.5[b]	0.01 ± 0.007	0.2 ± 0.04[a]	0.1 ± 0.02
[a]Significantly different from control ($p<0.01$)				

Glutathione content in erythrocytes

GSH content in erythrocytes of the rats in the experimental group didn't change remarkably. However GSH content in brain, liver and kidney of rats treated with Büyükkabaca's drinking water decreased significantly by 53.33% ($p<0.001$), 56.72% ($p<0.001$) and 26.32% ($p< 0.05$), respectively, when compared to the control animals (Table 1).

Discussion

Water pollution by pesticides from routine agricultural practices is a common and growing problem in the major agricultural areas of the world. The pesticide usage is not equally distributed in all the agricultural areas in Turkey. Uncontrolled use of agricultural chemicals in intensive agricultural areas of Turkey causes serious soil, surface and groundwater pollution (4). Thus, we investigated the effects of drinking water of Büyükkabaca where each year about 1500 tons of fertilizers and 500 tons of pesticides contaminate groundwater on lipid peroxidation, GSH content and antioxidant enzymes such as SOD, GSH-Px and CAT in rat blood, brain, liver and kidney tissues following 16 weeks exposure.

The present observation indicates that treatment with Büyükkabaca's drinking water causes oxidative stress. Oxidative damage caused by this water is initiated by the formation of free radicals, as is evident from the generation of lipid peroxides in the blood and all the defined tissues of rats in the experiment group. Evidence from in vitro and in vivo studies with many toxicants including pesticides supports the concept that free radicals e.g. hydroxyl radicals (•OH), H_2O_2 and others, are important mediators of tissue injuryand formation of these radicals result in increased LPO (13,14). In the present study, we found that treatment with Büyükkabaca's drinking water increases the production of lipid peroxides (Table 1). The observed increases in MDA may be

due to the increase in ROS formed by the interaction of hemoglobin and tissues studied with ethion determined in Büyükkabaca's drinking water. If released to water, ethion has the potential to bio-accumulate, assuming that rapid degradation of the compound by hydrolysis does not ocur (3). Pesticides in drinking water are limited to 0.1 ppb for a single pesitice and 0.5 ppb for the sum of all pesticides, repectively by Turkish Drinking Water Standards and the World Health Organization (15,16). Ethion has been detected only rarely in both surface water and groundwater monitoring studies typically at concentrations below 0.5 ppb (3). The Environmental Protection Agency has concluded that the water concentration of ethion to be used for estimating chronic exposure is 1.0 ppb (17). As can be clearly seen from our results, ethion in Büyükkabaca's drinking water exceeded the level of concern for chronic drinking water exposure. Thus, we may suggest that free radicals like superoxide and peroxyl radicals formed as a result of excessive level of ethion in Büyükkabaca's drinking water can be responsible for the incresase in LPO and alteration in antioxidative defence mechanisms. Because once such free radicals are formed, the cells start some physiological defence mechanisms to prevent the damage (1).

Free radical scavengers such as SOD, CAT and GSH and metabolism regulatory enzymes such as GSH-Px, GR and GSH-S-transferase can protect the cellular system from deleterious effect of free radicals (5,18,19). Obsevations in this study show that in blood, brain, liver and kidney of the rats SOD has increased, CAT has decreased in blood and liver and GSH-Px again has increased with Büyükkabaca's drinking water treatment. This increase in the activity of SOD indicates an increase in $O_2^{\cdot-}$ production (20). The decline in CAT activity could be due to the excess production of $O_2^{\cdot-}$ (21). As indicated in our study, reduction in the CAT activity in blood and liver tissues can be attributed to record high SOD activity due to ethion induced $O_2^{\cdot-}$ production. As it is known, the first barricade for oxygen-free radicals is provided by SOD, but the end product of SOD reaction is H_2O_2, which is again a toxic material for the cell and has to be removed by antioxidant enzymes. This is accomplished by CAT and also by GSH-Px. The second barricade is provided by GSH-Px because of its lower Km for H_2O_2 and the third by CAT (22). In other words, after toxicant exposure, the system first raises its GSH-Px level. The increase in GSH-Px activity observed in this study may be due to enhanced production of H_2O_2 derived from $O_2^{\cdot-}$. After drinking water of Büyükkabaca to rats of rats, SOD and GSH-Px activities in blood and all studied tissues shows that both enzymes were related to the maintanence of lipoperoxide concentration.

There was a significant decrease in the level of GSH in blood and in all the studied tissues compared to control subjects. The decreased GSH level accompanied by increse in SOD and GSH-Px enzyme activities probably indicates an adaptive measure to tackle any insecticide accumulation. GSH together with GSH-Px, SOD and CAT efficiently scavenge toxic free radicals and are partly responsible for protection against lipid peroxidation due to acute/chronic pesticide exposure (23,24). The reduction of GSH observed in blood and all the studied tissues in this study may have resulted from the activity of GSH-Px in reducing lipid hydroperoxides to stable non-radical lipid alcohols, utilizing GSH as the source of reducing equivalents.

In conlusion with this study, we have demonstrated through this work that Büyükkabaca's drinking water causes oxidative stress via increasing lipid peroxidation, SOD and GSH-Px activities and decreasing CAT activity and GSH level. Further studies are needed to determine how these changes affect the people living in Büyükkabaca in terms of the effect of the increased lipid peroxidation on human health. This would help in the development of necessary safety measures and in the design of interventional strategies for warding off toxic hazards to man and other animals.

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