Chlorpyrifos Induced Cardiotoxicity in Rats and the Protective Role of Quercetin and Catechin

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ABSTRACT

In this study, chlorpyrifos, catechin, quercetin and catechin- or quercetin+chlorpyrifos were given to Wistar rats for 4 weeks. Chlorpyrifos increased the levels of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT); decreased glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities in heart compared to the control. In catechin- and quercetin+chlorpyrifos groups, we observed the protective effects of flavonoids on examining parameters. Light microscopic investigations revealed that chlorpyrifos induced histopathological alterations in heart. Milder histopathological changes were observed co-treated with catechin- or quercetin+chlorpyrifos. As a result, catechin and quercetin significantly reduce chlorpyrifos induced cardiotoxicity in rats, but they aren’t protect completely.

Key Words: Chlorpyrifos, Flavonoids, Antioxidant Enzymes, Histopathology, Heart

1. INTRODUCTION

Organophosphorus insecticides constitute a large family of pesticides and they are used widely for controlling pests in the household, agricultural and urban environment [1]. Chlorpyrifos is a broad spectrum organophosphate insecticide utilized extensively in agriculture and for residential pest control throughout the world under different registered trademarks [2]. Chlorpyrifos, like other organophosphates, exerts its toxic effects mainly by inhibiting the action of enzyme acetylcholinesterase [3,4]. Xenobiotics, including pesticides, are known to enhance the production of reactive oxygen species (ROS), which in turn generate oxidative stress in different tissues [5]. Chlorpyrifos has multiple effects on cells including generation of reactive oxygen species and induction of intracellular oxidative stress thereby disrupting normal cellular development and differentiation [3,4].

Oxidative stress, the imbalance between free radical production and antioxidant defenses, is associated with many cardiovascular diseases including atherosclerosis, hypertension, heart failure, stroke and diabetes [6]. Reactive free radicals can cause oxidative modification of low-density lipoprotein (LDL) [7]. Cells have several ways to alleviate the effects of oxidative stress. They can either repair the damage or directly reduce the pro-oxidative state via enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), etc.) and nonenzymatic (vitamin E and C, urate, melatonin, flavonoids, etc.) antioxidants [8]. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) in tissues may neutralize the oxidative stress [9]. Many insecticides bind extensively to biological membranes, especially phospholipid bilayers [10,11,12] and they may damage membranes by inducing lipid peroxidation [13,14]. Malondialdehyde (MDA) is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation [9].

Flavonoids are polyphenolic compounds ubiquitously included in natural plants and are used currently for various kinds of foods and beverages as antioxidant additives [7]. Their beneficial effects against degenerative process linked to ageing or oxidative stress, such as cancers or cardiovascular diseases are now well documented [15]. They may scavenge ROS, chelate metal ions, act as chain-breaking antioxidants by scavenging lipid peroxil radicals, or integrate into
the lipid bilayer to prevent lipid damage [16]. Catechins are widely distributed flavonoids, present in various fruits, beverages (green tea, fruit juice), and in chocolate [17]. Catechin is a member of the flavonoids family, which have a variety of pharmacological effects such as cardioprotective, diuretic and hypotensive actions [18]. Catechins have beneficial effects on human health, serving to protect against congestive heart failure and cancer due to their antioxidative activity [16]. Quercetin belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources [19] and it is a powerful bioactive constituent of the human diet as a free radical scavenging agent [7]. Quercetin has many beneficial effects such as cardiovascular protection, anticancer activity, antiallergic activity, antiviral activity and anti-inflammatory effects [20].

The aim of this study was to determine the effect of subacute chlorpyrifos exposure on the heart of male rats and to assess whether these effects can be ameliorated by co-treatment with catechin and quercetin. To achieve this aim, rats were given chlorpyrifos and/or catechin and quercetin for 4 weeks, then their heart tissues were examined for antioxidant activity and pathological changes.

2. MATERIALS AND METHODS

2.1. Animals

Sexually mature male Wistar rats (300–320 g) were obtained from the Lemali Industry and Trade Co. Ltd. Animals were housed in plastic cages, fed ad libitum with a standard laboratory diet and water. Rats exposed to a 12 h light/dark cycle, and maintained at 20 ± 2°C. They were quarantined for 10 days before beginning the experiments. All rats were handled in accordance with the standard guide for the care and use of laboratory animals.

2.2. Chemicals

Chlorpyrifos (%99 purity) was obtained from the National Measurement Institute (Australia). Quercetin and Catechin were supplied by Sigma–Aldrich (Germany), and dimethyl sulfoxide (DMSO) was supplied by Merck (Germany). All other chemicals used were analytical grade and were obtained from Sigma–Aldrich (Germany).

2.3. Animal Treatment Schedule

The rats were divided into two groups, control (n=6) and experimental group (n=30). Experimental group were further divided into five treatment groups: catechin (n=6), quercetin (n=6), chlorpyrifos (n=6), catechin+chlorpyrifos (n=6) and quercetin+chlorpyrifos (n=6) groups. The substances were administered in the morning (between 09:00 and 10:00 a.m) to non-fasted rats. At the end of the 4th week, rats were sacrificed and dissected, and heart tissue samples were taken for histopathological examination via light microscope and assess changes on antioxidative defence constituents and malondialdehyde contents.

2.3.1. Control treatment

For the experimental control group, %0.5 DMSO at a dose of 1 ml/kg bw per animal was administered via gavage once a day.

2.3.2. Catechin-treatment

Catechin was dissolved in %0.5 DMSO and rats were treated once a day via gavage (20 mg/kg bw).

2.3.3. Quercetin-treatment

Quercetin was dissolved in %0.5 DMSO. Rats were treated (20 mg/kg bw) via gavage once a day.

2.3.4. Catechin plus chlorpyrifos treatment

Rats were given catechin dissolved in %0.5 DMSO at a dose of 5.4 mg/kg bw (1/25 of the oral LD50) via gavage.

2.3.5. Chlorpyrifos treatment

Once a day, the rats were given chlorpyrifos dissolved in %0.5 DMSO at 20 mg/kg bw per day. Thirty minute later, chlorpyrifos dissolved in %0.5 DMSO (5.4 mg/kg bw per day) was administered via gavage once a day.

2.3.6. Quercetin plus chlorpyrifos treatment

Once a day, rats were given quercetin dissolved in %0.5 DMSO (20 mg/kg bw per day). Thirty minute later, chlorpyrifos dissolved in %0.5 DMSO (5.4 mg/kg bw per day) was administered via gavage.

2.4. Biochemical Evaluation

The heart tissues were dissected and washed in sodium phosphate buffer (pH 7.2), then samples were taken and stored at -80°C until the analysis. The tissues were homogenized for 3 min in homogenization buffer (pH 7.4) using a teflon homogenizer (Heidolph Silent Crusher M), and then the homogenates were centrifuged at 10000 g for 15 min at 4°C.

2.4.1. Measurement of malondialdehyde (MDA)

MDA is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems. MDA content was assayed using the thiobarbituric acid (TBA) test as described by Ohkawa et al., [21]. MDA reacts with TBA to form a colored complex. Absorbance was measured at 532 nm to determine the MDA content. The specific activity is defined as nanomoles/milligram of protein.

2.4.2. Measurement of superoxide dismutase (SOD)

Total SOD activity was determined according to the method described by Marklund and Marklund [22] by assaying the autoxidation and illumination of pyrogallol at 440 nm for 3 min. One unit of total SOD activity was calculated as the amount of protein that caused %50 pyrogallol autoxidation inhibition. The total SOD activity is expressed as units per milligram of protein (U mg⁻¹). A blank without homogenate was used as a control for nonenzymatic oxidation of pyrogallol in Tris-EDTA buffer (pH 8.2).

2.4.3. Measurement of catalase (CAT)

Before determination of the CAT activity, samples were diluted 1:9 with %1 v/v Triton X 100. Enzyme activity was measured according to the method described by
Aebi [23] by assaying the hydrolysis of H2O2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25°C. CAT activity is expressed as millimoles of H2O2 reduced per minute per milligram of protein using an extinction coefficient of 0.0394 M⁻¹ cm⁻¹. A blank without homogenate was used as a control for enzymatic hydrolysis of peroxide in 50 Mm phosphate buffer, pH 7.0.

2.4.4. Measurement of glutathione-S-transferase (GST)
GST activity was assayed by measuring the formation of GSH (Glutathione) and the 1-chloro 2,4-dinitrobenzene (CDNB) conjugate [24]. Increases in absorbance were recorded at 340 nm for 3 min. The specific activity of GST is expressed as micromols of GSH CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 M⁻¹ cm⁻¹. All assays were corrected for non-enzymatic conjugation using 25 mM CDNB and 20 mM GSH in 50 mM phosphate buffer, pH 7.0.

2.4.5. Measurement of glutathione peroxidase (GPx)
GPx activity was measured using H2O2 as substrate according to the method described by Paglia and Valentine [25]. The reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min. Enzyme activity was expressed as nmol of NADPH consumed per minute per milligram of protein using an extinction coefficient of 6.220 M⁻¹ cm⁻¹. A blank without homogenate was used as a control for non-enzymatic oxidation of NADPH upon addition of hydrogen peroxide in 0.1 M Tris buffer, pH 8.0.

2.5. Histopathology
For histopathological examination, heart tissues were dissected and tissue samples were fixed in %10 neutral formalin for 24 h. Tissue samples were then processed using a graded ethanol series and embedded in paraffin. Paraffin sections were cut into 6 µm-thick slices and stained with hematoxylin and eosin for light microscopic examination. The sections were viewed and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Japan).

2.6. Statistical Analysis
The data were analyzed using SPSS 11.0 for Windows. The significance of differences was calculated using one-way analysis of variance (ANOVA) followed by Tukey’s procedure for multiple comparisons. \( P<0.05 \) was considered statistically significant.

2. RESULTS
At the end of the 4th week, there were no statistically significant changes in MDA, SOD, CAT, GST and GPx activities between the catechin-treated and quercetin-treated groups compared to the control group.

3.1. Malondialdehyde (MDA) Levels
Compared to the control group, there was a statistically significant increased in MDA activity in the chlorpyrifos-, catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated groups at the end of the 4th week of treatment. However, relative to the chlorpyrifos-treated rats, MDA activity was significantly decreased in the catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated rats (\( P<0.05 \), Fig. 1).

3.2. Superoxide Dismutase (SOD) Activity
SOD activity was significantly increased in the chlorpyrifos-treated group compared to the control, while no statistically significant changes were observed in the catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treatment groups. However, SOD activity was significantly decreased in heart tissues at the end of the 4th week in the catechin plus chlorpyrifos-treated group and quercetin plus chlorpyrifos-treated groups compared to the chlorpyrifos-treated group (\( P<0.05 \), Fig. 2).

![Fig. 1. Effects of subacute treatment of chlorpyrifos on MDA (nmol/mg protein) content in the heart tissues of rats.](image-url)
Fig. 2. Effects of subacute treatment of Chlorpyrifos on SOD levels (U/mg protein) in the heart tissues of rats. 
*Comparison of control and other groups. **Comparison of catechin group and other groups. ***Comparison of quercetin group and other groups. ****Comparison of chlorpyrifos group and other groups. Data represents the means ± SD of six samples. Values are means ± SD of six rats in each group. Significance at P < 0.05.

3.3. Catalase (CAT) Activity
A significant increase was observed in CAT activity at the end of the 4th week in the chlorpyrifos, catechin plus chlorpyrifos, and quercetin plus chlorpyrifos-treatment groups compared to the control. CAT activity was decreased significantly in the catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated groups compared to the chlorpyrifos-treatment group at the end of the 4th week (P < 0.05, Fig. 3).

Fig. 3. Effects of subacute treatment of Chlorpyrifos on CAT levels (mmol/mg protein) in the heart tissues of rats. 
*Comparison of control and other groups. **Comparison of catechin group and other groups. ***Comparison of quercetin group and other groups. ****Comparison of chlorpyrifos group and other groups. Data represents the means ± SD of six samples. Values are means ± SD of six rats in each group. Significance at P < 0.05.

3.4. Glutathione-S-transferase (GST) Activity
The chlorpyrifos-treated group showed a significant decrease in GST activity compared to the control group. No statistically significant changes were observed in catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated groups compared to the control. Yet, the catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated rats showed statistically increased levels of GST activity compared to chlorpyrifos-treated rats (P < 0.05, Fig. 4).
3.5. Glutathione Peroxidase (GPx) Activity
GPx activity was significantly decreased in the chlorpyrifos-treated group relative to the control, while no statistically significant changes were observed in the catechin plus chlorpyrifos and quercetin plus chlorpyrifos-treated groups. Compared to the chlorpyrifos treated rats, however, GPx activity increased significantly in the catechin plus chlorpyrifos- and quercetin plus chlorpyrifos-treated rats (P < 0.05, Fig. 5).

3.6. Histopathological Examination of Heart
Histopathological changes of heart tissues were investigated using light microscope. Heart tissues of control group rats were observed in normal structure (Fig.6A). No significant differences in cardiac muscle cell preparations were observed between catechin-treated, quercetin-treated and control groups. Chlorpyrifos caused disorganization and degeneration in myocardial fibers, cytoplasmic vacuolization in cardiac muscle cells, edema in connective tissue and degenerative changes in cardiac muscle cells (Fig. 6B, 6C). Less histopathological changes were observed in catechin plus chlorpyrifos treated (Fig. 6D) and quercetin plus chlorpyrifos treated (Fig. 6E) groups. In catechin plus chlorpyrifos and quercetin plus chlorpyrifos treated groups we observed mild disorganization of myocardial fibers. It was found that the significant protective effects of catechin and quercetin against chlorpyrifos induced cardiotoxicity in rats.
Fig. 6. Heart section of control rats were observed in normal structure x400 (A). Heart sections of chlorpyrifos treated rats showing disorganization (➔) and degeneration (▲) in myocardial fibers, cytoplasmic vacuolization in cardiac muscle cells (◆), edema in connective tissue (●), degenerative changes in cardiac muscle cells (▲) x400 (B,C). Heart sections of catechin plus chlorpyrifos (D) and quercetin plus chlorpyrifos (E) treated rats showing mild disorganization in myocardial fibers (➔) x400.

3. DISCUSSION

Chlorpyrifos is one of the most often used organophosphorus pesticides, which needs metabolic activation to become a potent phosphorylating agent, namely chlorpyrifos oxon [26]. Most of the pesticides interact with living organisms through the lipid rich biomembranes because of being lipophilic in nature [27]. Formation of oxygen free radicals can be a major factor in the toxicity of pesticides [28]. Chlorpyrifos is also known to produce oxidative stress resulting in the accumulation of lipid peroxidation products in different organs of rats [2] and induce histopathologic changes in different tissues [29]. The oral LD50 of chlorpyrifos for male rats is 135 mg/kg body weight [30]. In the present study, even though chlorpyrifos was given at 1/25 of the oral LD50 and none of the rats died during the experimental period, pathological changes were observed in rat heart tissues.

Pesticides act as pro-oxidants and elicit effects in multiple organs [31]. Oxidative damage primarily occurs through production of reactive oxygen species and can damage lipids, proteins, and DNA. Therefore oxidative damage may contribute to loss of enzymatic activity and structural integrity of enzymes and activate inflammatory processes [32].

Organophosphate insecticides cause increase of lipid peroxidation [10]. MDA is a major oxidation product of
peroxidized polyunsaturated fatty acids, and increased MDA content is an important indicator of lipid peroxidation [33]. MDA is a marker of membrane lipid peroxidation resulting from the interaction of ROS and the cellular membrane. The final membrane damage can lead to a loss of cellular homeostasis by changing the membrane characteristics [34]. Data in this study show that MDA content significantly increases in the heart tissues of rats in chlorpyrifos treated group. This increase in MDA content in the heart tissues may be due to an increase in free radicals resulting from the induction of oxidative stress in rats treated with chlorpyrifos.

The cells have different mechanisms to alleviate oxidative stress and repair damaged macromolecules. The primary defense is offered by enzymatic and nonenzymatic antioxidants which have been shown to scavenge free radicals and reactive oxygen species. The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) have been shown to be significantly affected by pesticides including chlorpyrifos [35,36]. We can give vitamin E and C, urate, melatonin and flavonoids for nonenzymatic antioxidant examples [8].

SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, while CAT converts hydrogen peroxide into water. These antioxidant enzymes can, therefore, alleviate the toxic effects of ROS [37]. CAT is a heme enzyme found in all cells that regulates intracellular hydrogen peroxide levels and is essential for the conversion of toxic hydrogen peroxide into water [3]. Increased SOD activity is known to serve as a protective response that helps eliminate reactive free radicals [33]. Organophosphate pesticides have been reported to increase CAT activities in different tissues [38]. In this study, SOD and CAT activities significantly increased in the heart tissues of rats in chlorpyrifos treated group. This increase in SOD and CAT activities in the heart tissues may be due to eliminate reactive free radicals’ adverse effects.

GST is detoxifying enzyme that catalyze the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms [37]. In this study, GST activity decreased significantly in heart tissues of rats treated with chlorpyrifos. Similarly, GST inhibition has been documented to occur under other oxidative stress conditions [37]. The major function of GPx, which uses glutathione as a substrate, is to reduce soluble hydrogen peroxide and alkyl peroxides [3]. In our study, GPx activity decreased in heart tissues of rats treated with chlorpyrifos. Similarly, GPx inhibition has been documented in liver [39] and lung tissues [8] of rats treated with chlorpyrifos-ethyl.

Antioxidants have been shown to inhibit free radical formation [40]. The antioxidant properties of flavonoids are due to their ability to directly scavenge some radical species. Flavonoids may also act as chain-breaking antioxidants and/or may recycle other chain-breaking antioxidants, such as α-tocopherol, by donating a hydrogen atom to the tocopherol radical [16]. Catechins have been reported to have many pharmacological properties such as effects of antioxidative, antimutagenic, antitumor, hypotensive and antiulcer [41]. Catechin has iron chelating and antioxidant properties [18]. Flavonoids, among them quercetin, scavenge free radicals [42], prevents tumour development and carcinogen activation [43]. Quercetin prevents oxidant injury and cell death by several mechanisms, such as scavenging oxygen radicals [44], protecting against lipid peroxidation and chelating metal ions [45]. The cytoprotective effect of quercetin may also be due to its ability to interact with and penetrate the lipid bilayer [46]. Several previous studies have reported that catechin and/or quercetin have protective effects on antioxidant enzyme metabolism [18].

It is known that the subchronic and chronic toxicity of organophosphate insecticides may cause histopathological changes in different tissues [47]. We can give example liver [14], lung [8], kidney [48], testes [11] for target tissues of pesticides. Many studies show that pesticides have adverse effects also in heart tissue [47,49]. In this study, we observed that chlorpyrifos treatment led to disorganization and degeneration in myocardial fibers, cytoplasmic vacuolization in cardiac muscle cells, edema in connective tissue, degenerative changes in cardiac muscle cells were detected in heart tissues. These changes may result from an increase in reactive oxygen species in heart tissues. Moreover, we observed that the histopathological changes in the antioxidant-treated groups were more mild.

In summary, these data indicate that a low dose of chlorpyrifos causes heart toxicity, chlorpyrifos caused significant changes in the activities of antioxidant enzymes and the antioxidants catechin and quercetin can ameliorate this toxicity, but are not completely protective.

REFERENCES


