Adverse Effects of Organophosphorus Insecticides on Macrophage Activity in Persons at High Risk for Parasitic Infection

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Abstract

Background: It has been suggested that acute exposure to pesticides altered the ability of macrophages to overcome the pathogen invasion.

Aim: To evaluate the levels of NO, IFN-gamma and arginase in both rats and patients with cutaneous leishmaniasis (CL) exposed daily to the chlorpyrifos (CPF) pesticide. Also, to evaluate the benefits of curcumin and/or arginine as a prophylactic agents.

Materials and Methods: Plasma samples from 25 CL patients, 10 healthy control and CPF-treated rats, divided into five groups, were extracted. Also, PBMNCs recruited only from CL patients.

Results: A significant increase in the level of arginase coupled with a significant decrease in the levels of NO and INF-gamma were observed in CPF-treated animals. Restoration of the previous biochemical markers were significantly observed when a mixture of curcumin and arginine was used as a prophylactic remedy. In CL patients, a significant increase in the level of arginase activity was noticed. However, when curcumin is added to the cultural media containing active PBMNCs recruited from CL patients, a significant decrease in the level of NO associated with an increase in the arginase levels were noticed.

Conclusion: The negative effect of CPF on macrophage activity can be attenuated by oral intake a mixture of curcumin and arginine.

Introduction

Organophosphorus (OP) compounds are one of the pesticides most widely used for a variety of purposes in agriculture and in human and veterinary medicine. Acute poisoning with OP in human is frequently seen in many countries and it is estimated to be the cause of more than 200,000 deaths around the world [1]. OP compounds used to fight pests include, parathion, chlorpyrifos (CPF) and malathion. In the Kingdom of Saudi Arabia (KSA), chlorpyrifos (CPF) (O, O'-diethyl3, 5, 6-trichloro-2-pyridinyl-phosphoro-thioate), a broad-spectrum organo-phosphorous (OP) insecticide, has been widely used for the control of agricultural pests [2]. It has been reported that OP compounds are highly toxic to vertebrates [3] and CPF is particularly considered to be neurotoxic [4], cardiotoxic, hepatotoxic [5] and immunotoxic [6].

Macrophage cells, member of the innate immune response, are mainly responsible for destroying the foreign compounds by phagocytosis and inflammation.
Macrophages use endogenous NO as one of the key weapons for fighting various pathogens, such as leishmania parasites [7]. Nitric oxide synthase (NOS) is an enzyme catalyzing the production of NO from L-arginine. There are three isofoms of NOS which are encoded by distinct genes. In addition, these three known isofoms of NOS may be also classified into two categories; (1) Ca$^{2+}$-dependent nNOS [8] and constitutive, eNOS [9] (2) Ca$^{2+}$-independent, iNOS [10].

Macrophages also posses arginase (ARG) enzyme. Unlike iNOS, arginase hydrolyses arginine into urea and ornithine. Both arginase and NO synthase use arginine as a common substrate [11]. Furthermore, Arginase activity is induced by cytokines secreted by T helper-2 lymphocyte, while iNOS is activated by gamma interferon (IFN-gamma) secreted by T helper-1 lymphocyte [7]. Recent studies reported that chronic exposure to low levels of the pesticide aggravates and delays the healing of cutaneous leishmaniasis lesions [12]. In Tabouk region, North of KSA, farmers are inevitably exposed to high amount of chlorpyrifos (CPF) insecticide. Therefore, the susceptibility of these farmers to be infected by leishmania is highly expected.

So far, no literature pointed to the levels of NO, IFN-gamma and arginase following pesticide exposure, specifically CPF. Therefore, the present study aiming to investigate the effect of CPF exposure on the levels of NO, IFN-gamma and arginase activity in plasma and monocyte cells of human. In addition, the role of arginine and curcumin as prophylactic agents against CPF toxicity will investigate.

Subjects and Methods

Chemicals and Reagents

Kits and reagents used in the present study were purchased from the International Development and Construction (IDCO)Co., Jeddah, and Chlorpyrifos (CPF) was purchased from the Arabic Company of Chemical Products (APCO, Kingdom of Saudi Arabia).

Experimental Animals

Fifty adult male albino rats weighing between 200 – 250 g were housed in wire mesh cages at room temperature. Veterinary care was provided by the Laboratory Animal House Unit of the Faculty of Medicine, Cairo University. Rats were housed with normal light dark cycle, and were allowed to acclimatize to their environment for five days before the start of the experiment. All animals were kept under the same environmental conditions and had free access to water and food. The animals were divided into the following groups (each group consists of 10 rats):

- **Group I (Gr-I):** Control group included normal male albino rats (fed on normal diet, water and vehicle);
- **Group II (Gr-II):** CPF supplemented male rats;
- **Group III (Gr-III):** Curcumin supplemented male rats exposed to CPF;
- **Group IV (Gr-IV):** Arginine supplemented male rats exposed to CPF; and
- **Group V (Gr-V):** Curcumin and arginine supplemented male rats exposed to CPF

**Pesticide Treatment**

Chronically exposed animals were randomly divided into four groups with ten animals included in each. The first group served as a control group, while second, third, fourth and fifth groups received 0.6 mg CPF/kg body weight for 1 month. Only animals of the third, fourth and fifth groups received curcumin, arginine and a mixture of (curcumin and arginine) concomitantly for one month, respectively.

**Organophosphorus Administration Protocol**

The CPF pesticide (0.6 mg/kg body weight) was dissolved in corn oil and given daily to the rats for one month orally as described by Al-Dawood et al., 2008 [13].

**Prophylactic treatment Curcumin Supplementation**

Curcumin was given to rats 300 mg/kg by oral injection once daily for one month as described by Brouet and Ohshima, 1995 [2].

**Arginine Supplementation**

Arginine was given to the rats by dissolving 3% of this amino acid in their drinking water for one month as described by Morris, 2000 [14].

**Sample Collection**

Animals blood samples were withdrawn from all rats through the retro-orbital route using heparinized
capillary tubes. The blood samples were delivered into centrifuge tubes and then centrifuged at 2000 rpm for 20 minutes and plasma was separated and stored at -80°C until used. The plasma was divided into 3 tubes for further determination of plasma level of nitric oxide, arginase, and IFN-gamma.

**Peripheral Blood Mononuclear Cells (PBMCs) Isolation**

Peripheral blood mononuclear cells were separated from heparinized blood by the Ficoll-Hypaque density gradient centrifugation method and after two washes, the peripheral blood mononuclear pellets were frozen at -80°C.

**Cell Culture**

The PBMCs extracted from patients infected with cutaneous leishmania and working as a farmers in Tabuk region, and isolated by Ficoll-Paque centrifugation at room temperature were washed three times in RPMI medium (Gibco) and resuspended in Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 50 μmol/l 2-mercaptoethanol, 2 mmol/l L-glutamine (Gibco), 40 μg/ml gentamicin, and 5% fetal calf serum as described by Iniestat et al., 2005 [15]. Then cells from humans were treated with lipopolysaccharide (LPS) and interferon-gamma (IFN-gamma) to measure the rate of nitric oxide production, as well as arginase activity in the presence or absence of curcumin at a concentration of 10 μM.

**Human Samples**

The study subjects recruited in the present study were divided into two groups; (I) The first group consisted of 25 patients suffering from CL and using curcumin as a house hold remedy for insect bites. All patients had a confirmed diagnosis of leishmaniasis based on visualization of Leishmania amastigotes in Giemsa-stained smears. All cases recruited in this study were living in the rural areas of Tabouk region (Fig. 1), Saudi Arabia. (II) The second group consisted of 10 individuals, working also as farmers from the same region, but without a history of leishmaniasis. After a consent was obtained from the subjects, 10 ml of anticoagulated (e.g. heparinized and EDTA) venous blood was extracted from each study subject. Each extracted sample was divided into two tubes, an EDTA coated tube for the complete blood count and a heparin-coated tube for measuring the biochemical parameters. Samples in heparin-coated tubes are centrifuged to separate the plasma. The plasma samples were decanted into clean eppendorf tubes and stored at -80°C.

**Preparation of blood plasma samples and CPF extraction procedure**

In Eppendorf tubes, aliquots each of 0.35 ml plasma sample were mixed with 0.5 ml toluene for 2 min and then centrifuged at 14,000 rpm for 5 min at 4 °C (to avoid emulsion formation). An amount of 1 μl of the supernatant was analyzed directly for the presence CPF by gas chromatography/mass spectrometry (GC/MS) system as described by Tarbah et al., 2006 [26].

**Biochemical Measurements**

**Nitric oxide (NO) Assay:** In acid medium and in the presence of nitrite, the formed nitrous acid diazotise sulphanilamide and the product is coupled with N-(1–naphthyl) ethylenediamine. The resulting azo dye has a bright reddish – purple color which can be measured at 540 nm as described by Erel et al., 1999 [7].

**Arginase Activity Assay:** The method used is based upon the colorimetric determination of urea by condensation with diacetyl monoxime in an acid medium in the presence of ferric chloride (oxidant) and carbazide (accelerator) [15]. Arginase was assayed in cells and supernatant by a modification of the method of Morris, 2000 [14].

**Cytokine Measurement**

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat IFN-gamma has been pre-coated onto a microplate. Standards, controls, and samples are pipetted into the wells and any rat IFN-gamma present is bound by the immobilized antibody. The intensity of the color
measured is proportional to the amount of rat IFN-gamma bound in the initial step. The sample values are then read from the standard curve as described by Ajdary et al., 2000 [16].

**Statistical Analysis**

The data were analyzed by using SPSS (version 14.0) for Windows and expressed as means ± SD. Paired samples t-test was used to compare between the data of the control and those of treatments. ANOVA was used to compare between more than two groups. P value < 0.05 is considered significant.

**Results**

Results are represented in Tables 1-5. Table 1 represents the mean levels of GPT, GOT and albumin in experimental rats treated with CPF pesticides. CPF-treated rats in groups II, III, and IV demonstrated a significant increase in liver enzymes (GPT & GOT) and a significant decrease in plasma albumin than their control counterparts in group I. The chemo-preventative agents (curcumin, arginine and their combination) had beneficial effects on the activities of liver enzymes and albumin concentration. Table 2 and Figures 2, 3 and 4 showed that the mean plasma levels of NO, IFN-gamma and arginase activity in rats exposed to CPF pesticide (Gr-II, III, IV and V) and the effect of using curcumin, arginine and a mixture of them as a prophylactic agents against the toxicity of CPF. In this regard, rats in group II revealed a significant reduction in the levels of NO and IFN-gamma coupled with a significant induction in the arginase activity as compared to their control counterparts (Gr-II). However, when arginine associated with a significant decrement in the arginase activity were noticed as compared to their control counterparts (Gr-II). When CPF-treated rats treated by curcumin (Gr-III) or by a mixture of curcumin and arginine (Gr-V), a significant increment in the levels of NO and IFN-

![Figure 2: Mean plasma levels of nitric oxide (NO) among different groups of rats.](image)

![Figure 3: Mean plasma levels of arginase activity among different groups of rats.](image)

![Figure 4: Mean plasma concentration of interferon gamma among different groups of rats.](image)

**Table 1:** Profile of liver enzymes and plasma albumin concentrations in experimental rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gr-I</th>
<th>Experimental animals (mean ± SD)</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPT (U/L)</td>
<td>33.6 ± 3.5^a</td>
<td>56.2 ± 2.14^a</td>
<td>40.2 ± 1.13^a</td>
</tr>
<tr>
<td>GOT (U/L)</td>
<td>34.3 ± 6.2^a</td>
<td>65.5 ± 1.66^a</td>
<td>37.5 ± 1.35^a</td>
</tr>
<tr>
<td>Albumin (g/d)</td>
<td>4.2 ± 0.8</td>
<td>3.1 ± 0.25</td>
<td>3.5 ± 0.25</td>
</tr>
</tbody>
</table>

*P*-value < 0.05 is considered significant; N.S. = Not significant; *For each significant test, mean groups sharing subscript are not different from each other, p > 0.05.

**Table 2:** The mean plasma levels of nitric oxide, arginine and interferon-gamma (IFN-gamma) in the CPF-treated rats before and after addition of the chemo-prophylactic agents.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gr-I</th>
<th>Gr-II</th>
<th>Gr-III</th>
<th>Gr-IV</th>
<th>Gr-V</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide (muM)</td>
<td>12.4 ± 2.2</td>
<td>4.2 ± 1.4</td>
<td>7.1 ± 1.2</td>
<td>9.2 ± 1.5</td>
<td>9.1 ± 1.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Arginine (g/d)</td>
<td>37.4 ± 5.2</td>
<td>16.8 ± 6.0</td>
<td>13.6 ± 1.5</td>
<td>17.5 ± 7.2</td>
<td>12.3 ± 1.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IFN-gamma (pg/ml)</td>
<td>60.4 ± 11.6</td>
<td>29.3 ± 8.9</td>
<td>37.5 ± 7.2</td>
<td>28.3 ± 7.3</td>
<td>30.3 ± 11.6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*P*-value < 0.05 is considered significant; N.S. = Not significant; *For each significant test, mean groups sharing subscript are not different from each other, p > 0.05.
added solely to the CPF-treated rats (Gr-IV), no changes in the levels of nitric oxide and INF- gamma and arginase activity were noticed as compared to their control counterparts (GrII). However, the degree of reduction in the plasma level of arginase activity in Gr-V is more obvious when a mixture of curcumin and arginine is used as a prophylactic agent than using curcumin (Gr-III) or arginine (Gr-IV) solely.

In CPF-treated rats (40 rats), a general significant positive correlation coefficient is noticed between NO and IFN-gamma (r=0.75, p<0.01, Figure 5), while a significant negative correlation coefficient are observed between arginase and each of INF-gamma and NO, where r = -0.65 and -0.73, p<0.01 and 0.01 respectively, (Figure 6 and 7). In control group (Gr-I), a significant positive correlation was also noticed between NO and IFN-gamma (r=0.7, p=0.026, Figure 5), while no significant correlations are noticed between arginase and each of IFN-gamma and NO, where r = 0.22 and 0.5, p=0.5 and 0.14 respectively (Figure 6 and 7).

Table 3 shows that CL patients were contaminated with CPF pesticide and had a disease duration ranging from 2 to 12 months. The mean levels of WBCs and liver enzymes (GPT and GOT) were significantly increased in CL patients than the control group. On the other hand, the mean values of RBCs, Hb and Hct% were significantly decreased in CL patients than their control counterparts.

Table 4 revealed a significant increase in the arginase activity. In contrast, no significant difference is observed in the mean plasma levels of NO and IFN-

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patients group (n=15)</th>
<th>Control Subjects (n=10)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide (µmol/L)</td>
<td>21.4 ± 9.5^*</td>
<td>20.0 ± 5.14^*</td>
<td>N.S.</td>
</tr>
<tr>
<td>Arginase (U/L)</td>
<td>7.2 ± 1.6^*</td>
<td>1.2 ± 0.2^*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IFN-gamma (Pgf/mi)</td>
<td>139 ± 26.7^*</td>
<td>133.3 ± 22.1^*</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

*P*-value < 0.05 is considered significant; N.S. = Not significant. For each significant test, mean groups sharing subscript are not different from each other, p > 0.05.
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gamma between leishmaniasis patients and their control counterparts.

Table 5 shows the mean level of NO and arginase activity in two cell cultures containing human monocytes activated by LPS and IFN-gamma. One of these culture was treated by curcumin, (curcumin added to the culture, three hours after monocyte activation), while the other was not. In this regard, a significant decrease in the NO concentration, associated with a significant increase in arginase activity was detected in the cell cultures treated by curcumin than untreated ones.

Table 5: Effect of curcumin on the levels of nitric oxide, arginase and interferon-gamma (IFN-gamma) in culture supernatant of stimulated cells from patients with cutaneous leishmaniasis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Culture of stimulated cells</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO (μmol/L)</td>
<td>Curcumin (10 μM)</td>
<td>15.4 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arginase (U/L)</td>
<td>Absent of curcumin</td>
<td>30.4 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*P*-value < 0.05 is considered significant. For each significant test, mean groups sharing subscript are not different from each other; p > 0.05.

Discussion

Organophosphate (OP) pesticides are among the leading chemicals used extensively for agricultural pests control throughout the world. CPF is one of the most widely used OP pesticides in Saudi Arabia due to its greater stability, persistence and less toxicity. CPF elicits a number of effects, which include immunological abnormalities [6] and hepatic dysfunction [17].

The present study focused on the effect of CPF exposure on the levels of NO, IFN-gamma and arginase activity in humans and rats. So far, no data is available demonstrating any relationship between these biochemical parameters during CPF exposure. The liver plays a central role in the detoxification process of the xenobiotics and their metabolic byproducts [18, 19]. In the present study, liver function tests were adversely affected by CPF exposure. These results are in agreement that obtained by Khan et al., 2005 [18] and Mansour and Abdel-Tawab, 2009 [19].

NO has a very short half-life. Within seconds of its production, NO is converted to nitrite and nitrate, sequentially, in the presence of molecular oxygen. Therefore, determination of plasma nitrite and/or nitrate concentrations mainly reflects NO produced by iNOS. In addition, NO produced by means of eNOS and nNOS is nearly a thousand times lower than that produced by iNOS [7, 8]. Rats in group II treated with CPF alone, revealed a significant decrease in plasma level of NO and IFN-gamma associated with an increase in arginase activity as compared to their control counterparts (Group I). It has been reported that Th-1 cytokines such as IFN-gamma, tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-2 (IL-2) and lipopolysaccharides (LPS) induce iNOS enzyme, which enhances nitric oxide synthesis [20]. Therefore, the reduction in the plasma level of IFN-gamma in rats of group II may explain their reduced NO synthesis.

In Tabuk region, KSA, where leishmaniasis is endemic and curcumin is used by individuals working in the agricultural field as a household remedy for insect bites. It has been reported that curcumin offers an anti-inflammatory effect through inhibition of nuclear factor-kappa (NFkB) activation [21, 22]. A significant improvement in the levels of NO and IFN-gamma associated with a significant decrease in arginase activity was observed in group III as compared to group II. For this reason, we suggest that oral intake of curcumin may act as an antioxidant agent, as well as inducer of Th-1 related cytokines, rather than NO scavenger (anti-inflammatory agent), which may explain the increment in NO level. Furthermore, when CPF-treated rats get an oral intake of a mixture of curcumin and arginine as in group V, a progressive increase in NO and IFN-gamma activity and a progressive decrease in arginase activity were observed. On the other hand, when curcumin was added directly to the culture media, a significant decrease in NO associated with a significant increase in arginase activity, when compared to the culture media not treated by curcumin. Our results are in agreement with those obtained by Brouet and Ohshima, 1995 [13]. These authors observed a gradual decrease in NOS activity in the cultural media concomitant with increased curcumin concentration. Also, Chan et al., 2005 [23] reported that curcumin rescued the leishmanial parasites from growth inhibition mediated by NO. The decrease in NO concentration and induction of arginase activity may explain the inability of macrophages to overcome the parasite of leishmania. Further investigations are required to clear this point.

Arginine is the sole substrate for both NOS and arginase, so its bio-availability may affect the rate of NO production. For this reason, we also hypothesized that oral intake of arginine in parallel with CPF may alleviate its toxicity on the cytokines activating iNOS. In this regard, daily intake of arginine alone in CPF-treated rats (Group IV) revealed no significant changes in the levels

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of NO, IFN-gamma and arginase activity as compared to group II. Consequently, we can assume that, the hazardous effect of CPF exposure on the immune system may arise from down-regulation of Th-1 cytokines and up-regulation of Th-2 cytokines rather than arginine deficiency.

Arginase, is now known to exist in cells of the immune system. This enzyme is known to convert arginine into urea and ornithine. Also, it has been reported that Th-2 related cytokines, such as interleukin IL-4, IL-10 and transforming growth factor-beta (TGF-beta), can induce arginase activity but down-regulate iNOS [14]. On the other hand, IFN-gamma (Th-1 lymphocyte) can down-regulate arginase activity [24, 25]. Therefore cytokines of both Th-1 and Th-2 lymphocytes govern the induction of both iNOS and arginase. In this regard, rats in group-II showed a significant increase in the plasma level of arginase as compared to group I. The high level of arginase owing to CPF exposure may be due to down-regulation of Th-1 related cytokines and up-regulation of Th-2 related cytokines.

On the human level, leishmaniasis patients exhibited a significant decrease in hematological values such as RBCs, Hb and Hct and a significant increase in WBCs values as compared to the control group. So far, no data is available concerning the effect of CPF on haematological parameters in humans. The decrement in some of the blood parameters in our study may be due to the CPF-induced oxidative stress associated with deficient in the antioxidant intake. The CPF-toxicity is related to the generation of ROS, which causes damage to the various membrane components of the cell. In this regard, Mansour and Abdel-Tawab, 2009 [19] reported a significant reduction in the enzymatic antioxidants such as superoxide dismutase (SOD), catalase and glutathione-S-transferase (GST) in the erythrocytes extracted from rats treated by CPF, than their control counterparts. Also, on the human level, Lypez O et al., 2007 [27] reported that OP pesticides can induce an oxidative stress due to generation of free radicals and alteration in antioxidant defense mechanisms.

No data was reported on arginase activity in human subjects infected with leishmania. The present study revealed a significant increase in the mean plasma levels of arginase activity in leishmaniasis patients as well in CPF-treated rats in group II as compared to their control counterparts. It has been revealed that induction of arginase enzyme favored the replication of leishmania inside the macrophages, but inhibition of arginase reduces the number of parasites found in macrophages [15]. Furthermore, Al-Dawood et al., 2009 [12] reported that leishmaniasis persons exposed to CPF exhibited a delay in the healing of their skin lesions as compared to leishmaniasis persons not exposed to CPF. Therefore, high level of arginase activity as a result of CPF exposure associated with adding curcumin directly on the insect bites may explain the delayed healing of skin lesions in our patients. Also, the present study revealed an insignificant increase in the levels of NO and IFN-gamma in CL patients. These results are in contrast with that obtained by Ajdary et al., 2000 [16] and Castellano et al., 2009 [24]. The discrepancy in the results may be related to exposure to CPF pesticides.

### Conclusion

The negative effect of CPF on the defense system can be attenuated by oral intake a mixture of curcumin and arginine, specifically in individuals devoid of parasitic infestation. On the other hand, direct contact between curcumin and skin lesions is not recommended, since the direct addition of curcumin solely may down-regulate their immune system. The latter observations need more investigations to study the mechanism of curcumin metabolism in our body and to find the optimal concentration for using curcumin as a household remedy against insect bites.

### References

5. Meyer A, Seidler FJ, Slotkin TA. Developmental effects of chlorpyrifos extend beyond neurotoxicity: critical


