Down regulation of male specific Cytochrome P450 by Profenofos

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ABSTRACT

The health hazards of individual OP insecticides have been characterized depending on the acute toxicity mainly by investigating their cholinesterase inhibition. However the chronic effect of most of these toxicant on the drug metabolizing enzymes have note been investigated.

Profenofos O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphoro thioate is an organophosphorus pesticide widely used in cotton fields. In the present study we investigated the effect of profenofos on male specific CYP450 enzymes. We administered 1/10 LD50 ; 35.6 mg/kg BW orally twice weekly for 65days to adult male Wistar rats. Profenofos down-regulate the hepatic CYP2C11 and CYP3A2 mRNA and protein expression levels. Testicular CYP2C11 and CYP3A2 mRNA and protein expression also were down-regulated by profenofos. Moreover profenofos treatment increased plasma testosterone concentaration and decreased testicular aromatase mRNA in the profenofos treated rats compared to control. Overall, the present study suggests that profenofos act as an endocrine disruptor affecting male specific cytochrome P450 enzymes and testosterone concentration which implicates its deleterious effects on male animal or human when they are chronically exposed to low dose.

INTRODUTION

Organophosphorous (OP) pesticides, widely used in agriculture, show several interesting features for environmental safety, such as limited persistence and selective toxicity to insects with respect to mammals *(1)*. However, in spite of their selectivity of action they are often highly toxic to humans and are responsible for most accidental intoxications in agriculture and the pesticide industry *(2)*. OP pesticides have been widely used in a variety of agricultural, commercial, and household applications *(3)*. Besides, the occupational exposure to relatively high doses, the general human and animal population may be chronically exposed to low levels of OPTs, essentially due to their presence as residues in food or drinking water *(4)*. Metabolic biotransformation plays a major role in modulating the toxicity of such chemicals. OPT pesticides require sulphoxidation, catalyzed by cytochromes P450CYPs) *(5)*. This reaction yields unstable phosphooxythiiran intermediates *(6)*, which are degraded upon desulphuration, to the corresponding phosphate triesters or oxons. These compounds are powerful inhibitors of brain acetylcholinesterase (AChE), actually exerting the toxic action *(7)*.

Profenofos (O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate) is a broad- spectrum organophosphorus(OP) insecticide, miticide and acaricide which is used heavily in cotton-growing areas *(8,9)* ranked its risk as about mid- range on the list of 12 priority cotton pesticides. Profenofos is reported to be highly toxic to some aquatic organisms, however, only limited eco-toxicology data are available *(8)*. OPT pesticides require sulphoxidation, catalyzed by cytochromes P450 (CYP) *(5)*. This reaction yields unstable phosphooxythiiran intermediates *(6)*, which are degraded upon desulphuration, to the corresponding phosphate triesters or oxons. These compounds are powerful inhibitors of brain acetylcholinesterase (AChE), actually exerting the toxic action *(7)*. Profenofos is also oxidized by monoxygenase system to form AChE inhibitors and its bioactivation varied with the pesticide substrate and the CYP isozymes *(10)*.

Evidence for a role of sulphoxidation in the differential toxicity of OPT in mammals is based on in vitro data and is not clear *(11)*. Parathion sulphoxidation in human liver has been traced mainly to the action of CYPs termed CYP3A4 and CYP2B6 and a wide variability in this activity has been assessed among human liver samples *(12)*. In human, CYP3A4 is predominantly active at high organophosphrous pesticide concentrations *(13,14)*.

Differently from human CYPs, rat liver contains at least a dozen sex-dependant isoforms of CYP that are regulated by gender-dependant profiles of circulating growth hormone (GH) *(15,16)*. CYP3A2 is expressed in the livers of both female and male neonatal rats, but levels drop below detectable limits at puberty in females, where they are preserved throughout life in males *(17)*. CYP2C11 accounts for approximately one third of the total CYPs in male rat liver and is essentially undetected in female rat liver *(18)*. Male specific CYP2C11 metabolizes a host of xenobitics such as benzphetamine, aminopyine, ethylemorphine, benzo[a]pyrene and warfarin *(19)*. Stereospecific metabolism of endogenous steroids, for example, 2 alpha- and 16 alpha-hydroxylation of testosterone (T) and 16 alpha- hydroxylation of estradiol are catalyzed by CYP2C11 *(20,21)* . The down-regulation of CYP2C11 by various agents occurs primarily by a decrease in its mRNA expression, which is followed by a similar decrease in protein levels *(21-24)*.

The aromatase is a product unique gene called CYP 19; *(25)*. The aromatase is the terminal enzyme involved in the irreversible transformation of androgens into estrogens, this microsomal enzymatic complex is composed of a specific heme-glycoprotein (P450-arom) that functions with ubiquitous reductase as an electron donor. The P450 aromatase plays a role in development, reproduction, sexual differentiation, and behavior, as well as in bone, lipid metabolism, brain functions, and diseases such as breast and testicular tumors . It is difficult to find a tissue completely devoid of aromatase gene expression *(25)*. In the mammalian testis it is well known that aromatase is mainly localized in leydig cells *(26)*. It was reported that aromatase inhibitors reduce spermatid maturation in rats and monkeys *(27)*. Furthermore, in the male mice deficient in aromatase, the disruption of spermatogenesis appears specifically to affect early round spermatid differentiation *(28)*.

Human and wild animal are in continuous exposure to OP through consumption of contaminated foods. It was reported that sub-lethal doses of pesticides lead to alterations in reproductive performance in birds and mammals *(29)*. However, the mechanism of organophosphate-induced gonadal-dysfunction is not fully elucidated. In this study we aimed to investigate the chronic effects of profenofos on male fertility by determination of its effect on the male specific CYPs in the liver and testis of male wister rats.

MATERIALS AND METHODS

Materials

Profenofes was purchased from Syngenta, Ciba-Geigy (Geiza EGYPT). Goat-anti rat CYP2C11, rabbit-anti rat CYP3A2 were obtained from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), rabbit horseradish peroxidase-labeled anti-goat IgG and diaminobenzidinetetrahydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Goat horseradish peroxidase-labeled anti-rabbit IgG from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals and treatments

Sixteen-week old Wistar rats (SLC., Hamamatsu, Japan) were divided into 2 groups, of 3 control and 3 profenofos treated, housed at 24 ± 1 °C with 12 h light and 12 h dark cycle, and given laboratory feed and water ad libitum. Treatments of all animals were performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University. Profenofos-treated rats were given 35.6 mg/kg profenofos, and the control rats

were given vehicle (distelled water) orally for 65 days (twice weekly). Twenty-four hours after the last dose, rats were sacrificed with carbon dioxide and livers and testis were removed. The microsomal fractions from livers and testis were prepared *(30)* with slight modifications. Livers and testis were minced and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution with a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000g at 4°C for 70 min to attain a mitochondrial free microsomal pellet. The washed microsomes were then suspended in 0.1M potassium phosphate buffer, pH 7.4 and divided into 1.5 ml tubes and snapped in liquid nitrogen and kept at -80 °C until use. Microsomal protein concentrations were determined *(31)* using BSA as a standard.

Western blot analysis

Aliquots of 12 µg protein liver microsomal protein for CYP2C11 and CYP3A2 measurement from treated and control rats were applied onto 10-12% sodium dodecylsulfate (SDS) polyacrylamide gels and separated by electrophoresis *(32)* using a Protean 2 mini 1-D cell (BioRad). Western blot analysis was performed *(33)*. The proteins were electrophoretically transferred to nitrocellulose membranes, blocked in 5% skim milk in phosphate suffer saline (PBS) containing 1% Tween 20 for 2 hours at room temperature. And probed with the polyclonal goat anti-rat CYP2C1 or polyclonal rabbit anti-rat CYP3A2 as solutions in PBS containing 1% Tween 20 on shaker for 2 hours at room temperature. Horsradish peroxidase-labeled anti-goat IgG or anti-rabbit IgG respectively were used as secondary antibodies. Immunoreactive protein bands were revealed colorimetrically by oxidation of 0.025% 3,3 diaminoben- zidine tetrahydrochloride with 0.0075% hydrogen peroxide and catalyzed by peroxidase in 50 mM Tris-HCL (pH 7.6). Intensities of immuneo- reactive bands were densitometrically analysed on a Macintosh computer using the Public domain NIH image program (U.S.National Institutes of Health), available on the internet at (http://rbs.info.nih.gov/nih-image/).

RNA extraction

Total RNA was isolated from 50 mg of liver and testis using TriReagent (Sigma-Aldrich). Briefly liver and testis tissue samples were homogenized in 1ml of TriReagent then 0.3ml of chloroform was added to the sample. The mixtures were then shaken for 30sec followed by centrifugation at 4°C and 15000g for 20min. The supernatant layers were transferred to a new set of tubes, and an equal volume of isopropanol was added and the samples were shaken for 15sec and centrifuged at 4°C and 15000g for 15min. The RNA pellets were washed with 70% ethanol. RNA was dissolved in DEPC water. The prepared RNA was checked by electerophoresis, and showed that the RNA integrity was fine then it was further checked by measuring the optic density on specterophotometer. The optic density of all RNA sample was 1.7 to 1.9 based on the 260/280 ratio.

RT-PCR

A mixture of 5µg of total RNA and 0.5 ng oligo dT primer in a total volume of 24 µl sterilized ultra-pure water, was incubated at 70 °C for 10 min and then removed from the thermal cycler and completed to 40µl with a mixture of 8 µl (5X) RT-buffer, 2µl 10 mM dNTP, 2µl DEPC water, and 2µl of reverse transcriptase (Toyobo CO., Ltd., Osaka, Japan) and incubated in the thermal cycler at 30 °C for 10 min, 42°C for 1h, and 90 °C for 10 min.

For semi-quantitative PCR, 1µl aliquots of the synthesized cDNA was added to 20 µl of a mixure containing sterilized ultra-pure water, 2µl of 10 X PCR buffer, 2µl of dNTP (2.5 mM), 0.3 µl of sense and anti-sense primers (10µM), and 0.1µl of Taq polymerase (Takara, Kyoto, Japan). Amplification was initiated by denaturation of 1 cycle at 95°C for 1 min followed by in each cycle, denaturation at 94°C for 1 min, and annealing at the proper temperature for 1 min then extension at 72°C for 1min for the proper number of cycles for each gene using a DNA thermal cycler (BioRad,

Hercules, CA, USA). The primer sequences are shown in table.1. The samples were finally incubated for 7 min at 72°C after the last cycle of amplification. The amplified PCR products were separated by electrophoresis through 1-1.5% agarose gel. Bands of DNA were stained with ethidium bromide and visualized by ultraviolet illumination. Photographic images were converted into computer files with an Epson color-image scanner in combination with Adobe Photoshop 6.0 software. The amounts of CYP mRNA were normalized to the corresponding bands of G3PDH and were expressed relative to control level.

Statistical analysis

All data are expressed as means± SD. An unpaired t -test was used to evaluate the comparisons between two means of two groups. P value less than 0.05 were considered statistically significant

Table 1. Primers used in the amplification of genes by semi-quantitative PCR

Primer	Sense	Anti-sense	bp
CYP2C11	TGCCCCTTTTTACGA GGCT	GGAACAGATGACTCTGAATTCT	36 8
CYP3A2	TTGATCCG TTGTTCTTGTCA	GGCCAGGAAATACAAGCAA	34 2
Aromatase	TGGAATCCATCAAGCAGCATT	GCGTGTTAGAAGTGTCCAGCAT	113
G3PDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGATGAGGTCCACC	

RESULTS

Effect of profenofos on CYP3A2protein and mRNA in rat's liver and testis

Treatment of Wistar rats with given 35.6 mg/kg profenofos which is the 1/10 LD50 of profenofos for 65 days produced about 30% decrease in the hepaticCYP3A2 protein and 60% down-regulates of the hepaticCYP3A2 mRNA (Figure 1). To further investigate the sites of profenofos effects, we measured CYP3A2 in the testis, which showed that testicular CYP3A2 following the pattern of the changes in the hepatic CYP3A2 being decreased to less than 40% of CYP3A2 protein and mRNA compared to control (Figure 2).

Effect of profenofos on CYP2C11 protein and mRNA in rat's liver and testis

In adult rats, CYP2C11 is expressed only in male. Profenofos treatment produced more than 50% decrease in the hepatic CYP2C11 protein and mRNA (Figure 3) which indicates the ability of profenofos to disturb the transcription of the hepatic CYP2C11. We further, measured the testicular CYP2C11 which also followed its pattern in the liver being down regulated by profenofos at the level of protein and mRNA expression as the expression of CYP2C11 protein was about 60% and mRNA was about 20% of the control level (Figure 4).

Effect of profenofos on testicular aromatase mRNA in rat

We further measured the expression level of testicular aromatase. The results demonstrate that profenofose treatment produced more than 80% decrease in the testicular aromatase mRNA expression compared to control (Figure 5).

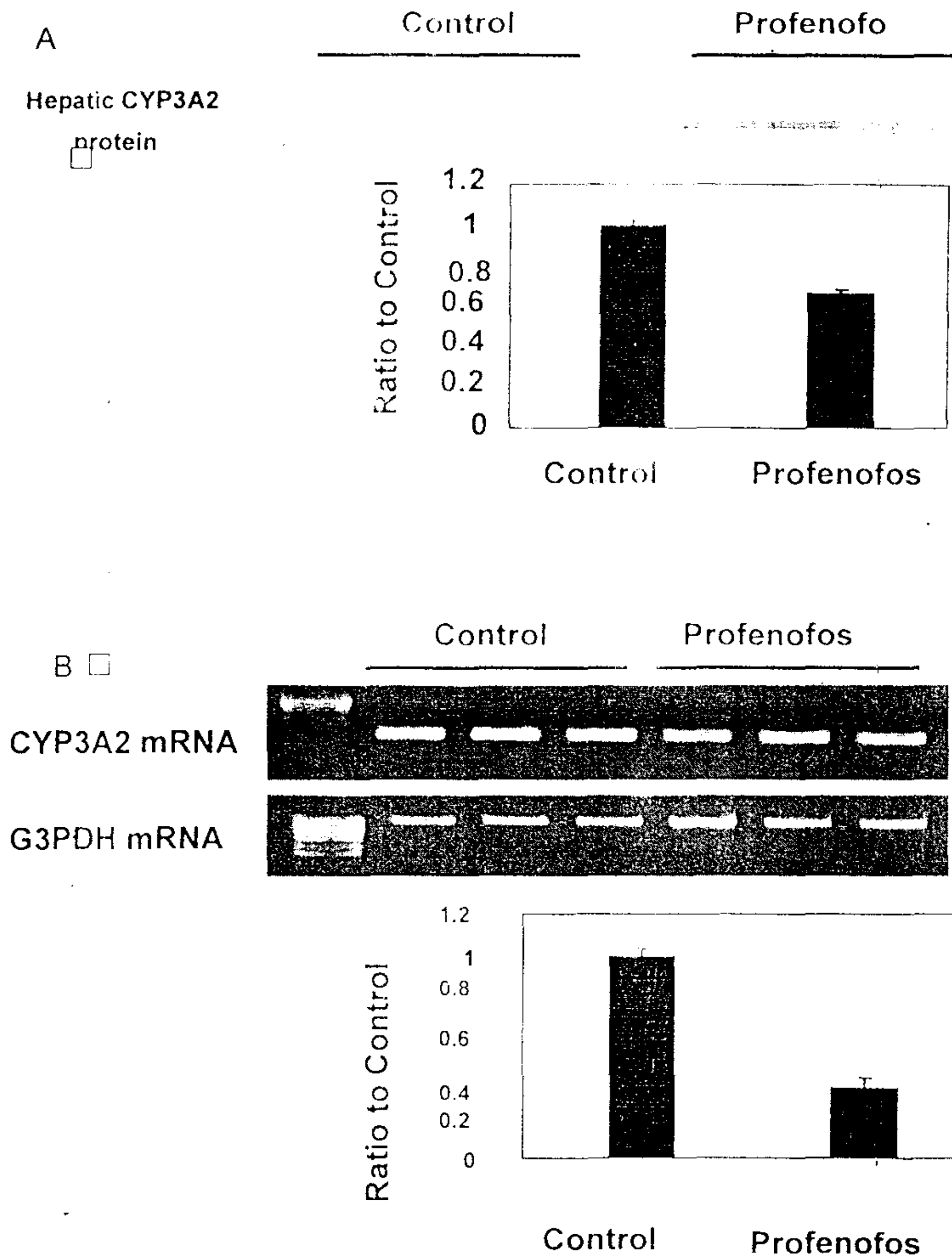

Fig.1. Down **regulation** of CYP3A2 expression levels by profenofos **treatment** (35.6mg/kg b.wt. **twice weekly** for 65 days) in liver of rats.

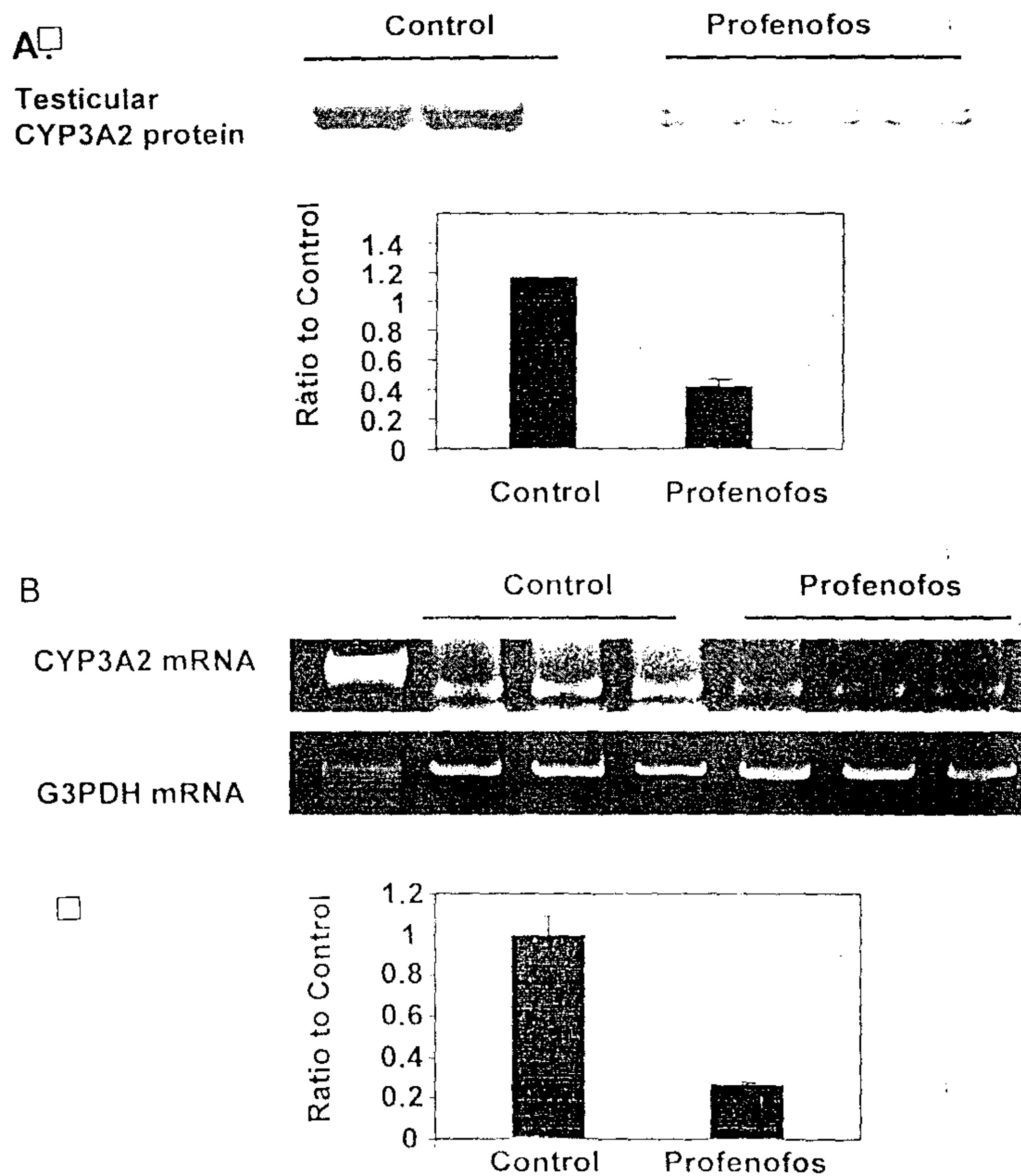

Fig. 2. Down regulation of CYP3A2 expression levels by profenofos treatment (35.6mg/kg b.wt. twice weekly for 65 days)in the testis of rats.

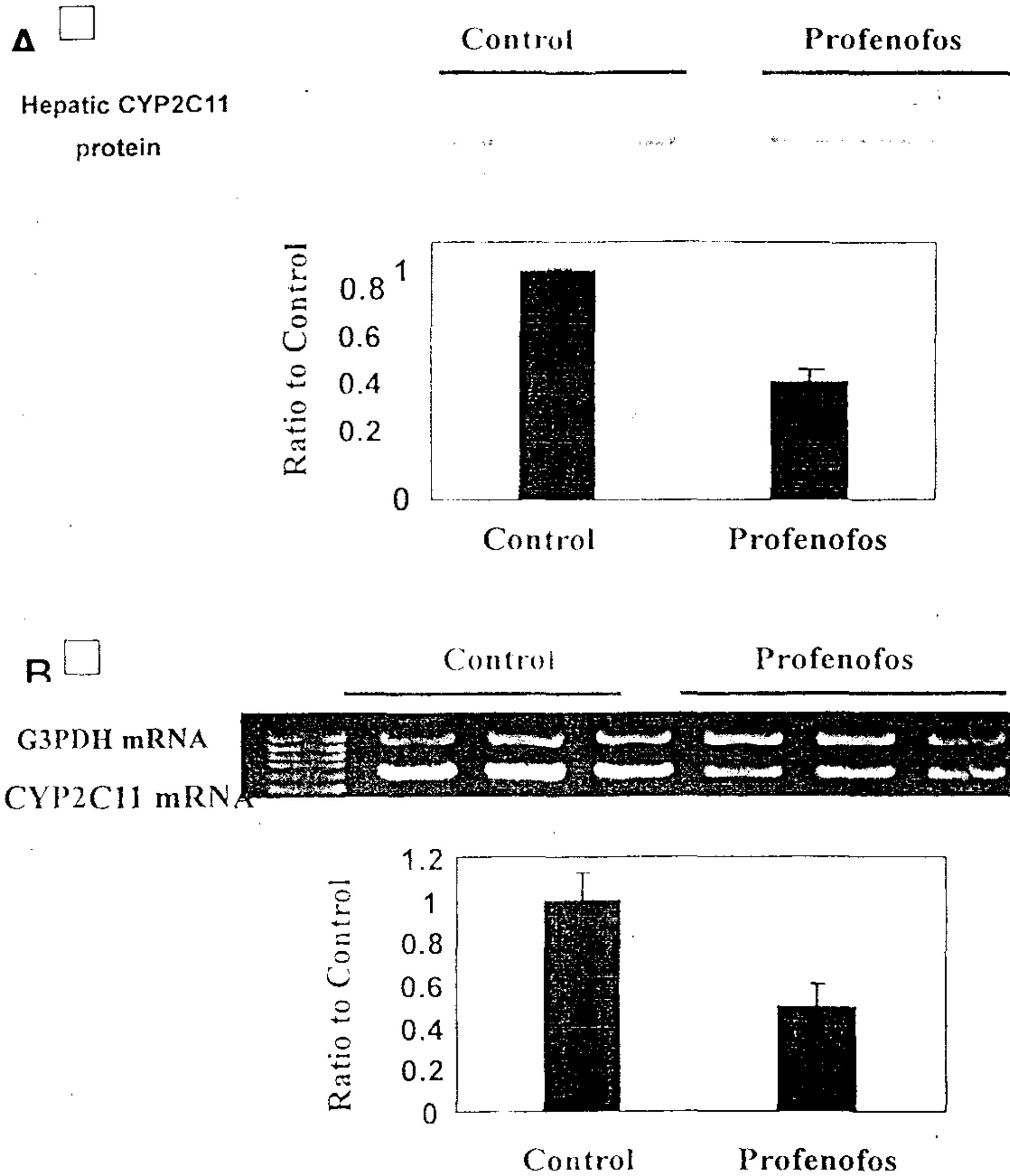

Fig. 3. Down regulation of CYP2C11 expression levels by profenofos treatment (35.6mg/kg b.wt. twice weekly for 65 days) in the liver of rats.

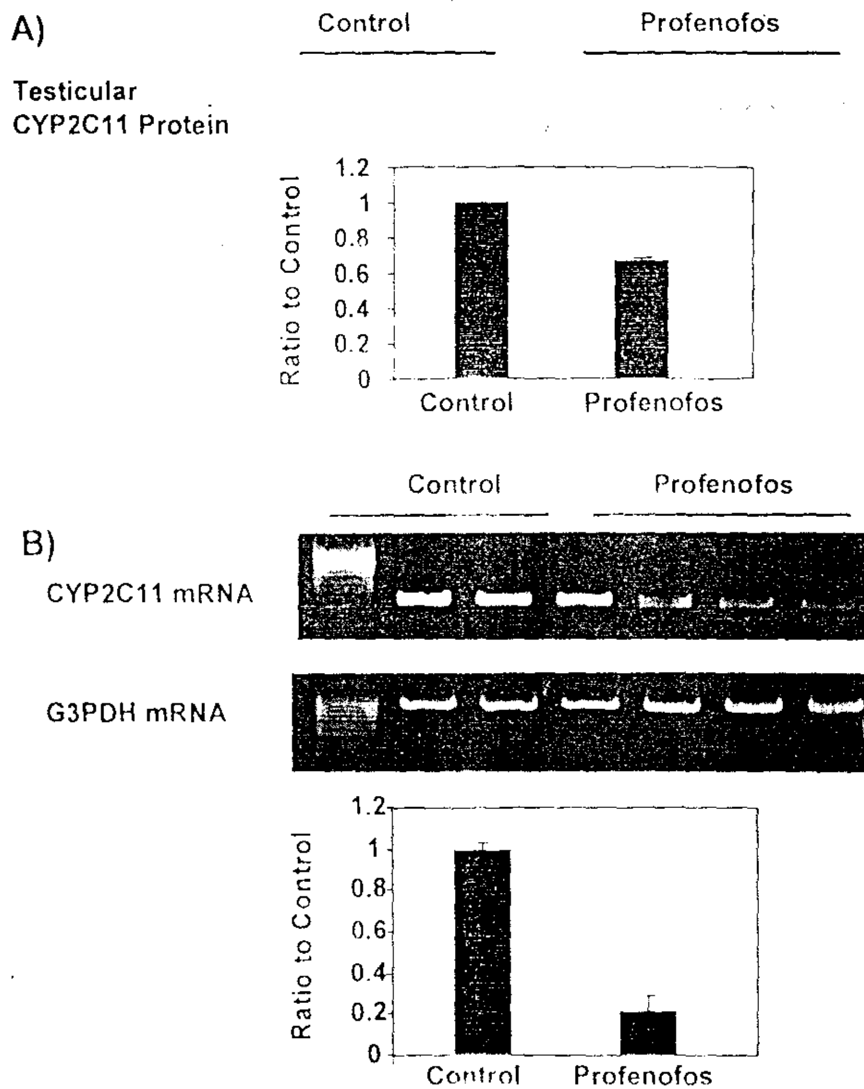

Fig. 4. Down regulation of CYP2C11 expression levels by profenofos treatment (35.6mg/kg b.wt. twice weekly for 65 days) in the testis of rats.

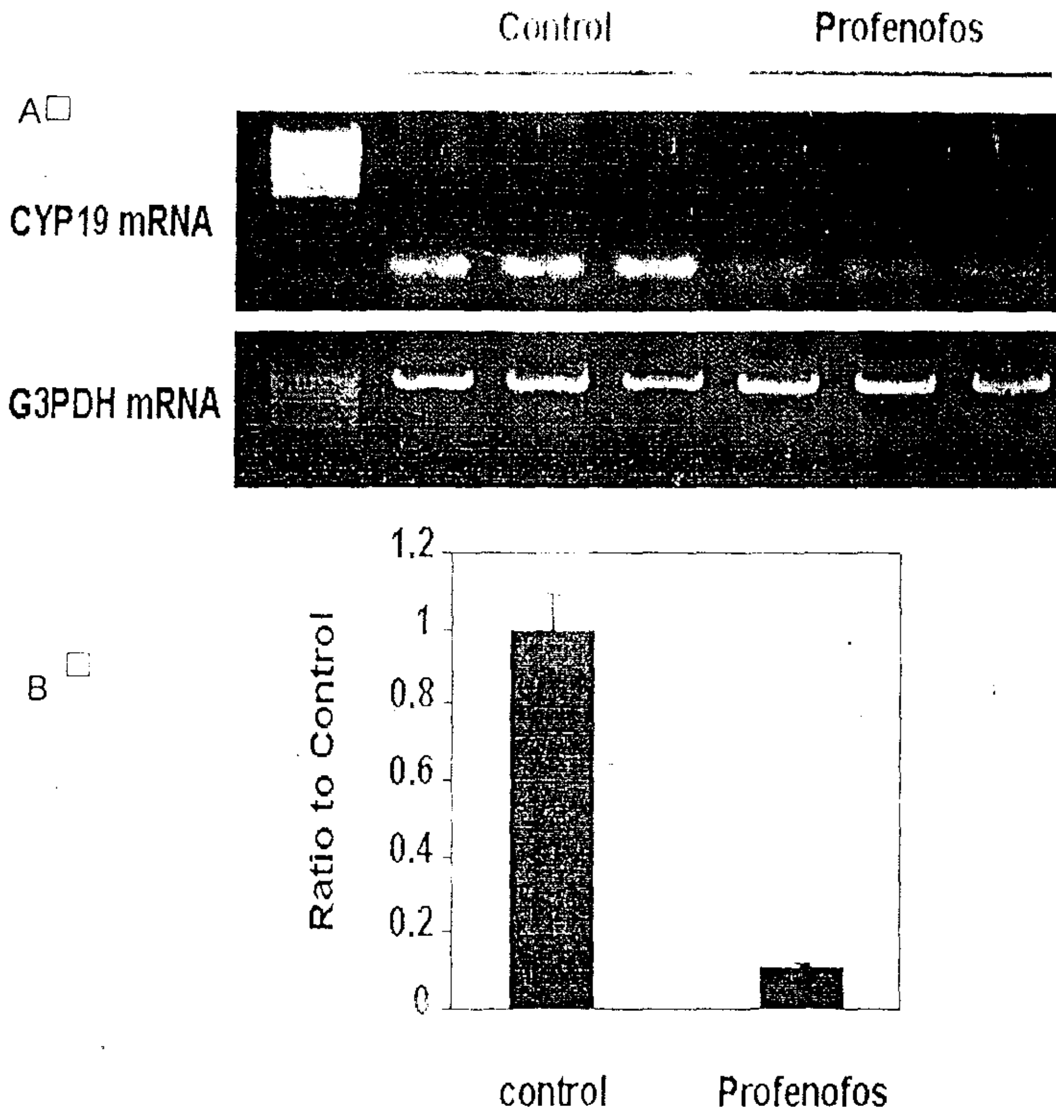

Fig. 5. Down regulation of testicular aromatase expression level by profenofos treatment (35.6mg/kg b.wt. twice weekly for 65 days) in rats.

DISCUSSION

Exposure to foreign compounds may exert changes in endocrine function both directly (hormone agonists or antagonists) or indirectly (altering circulating levels of hormones by influencing rates of hormone synthesis or metabolism) that can severely affect steroid hormone action *(34)*. Our study was designed to investigate the effect of profenofos (a phosphothoiate organophosphorous) on male fertility through exploring its effect on hepatic and testicular male specific CYPs.

In our study, treatment of male Wistar rats with the 1/10 of LD50 dose of profenofos for 65 days down regulated the hepatic CYP3A2 protein accompanied by reduction of its mRNA. The down regulation of CYP3A2 may indicates its role in the metabolism of profenofos as it was reported that CYP3A4 is predominantly active at high organophosphrous pesticide concentrations *(13,14)*. The long time (65 days) treatment of the rats in our experiment might lead to accumulation of the profenofos in the body. During OPT desulfuration, activated sulfur atoms are formed, binding irreversibly to the specific CYP isoforms that catalyze the reaction, resulting in time-dependent reduction of the related enzymatic activity *(12)*.

CYP3A is regulated mainly by the pregnane X receptor (PXR) which is now known to be the key factor in the oxidative metabolism of xenobiotics, through CYP3A and CYP2B induction, and also in their conjugative metabolism and transport *(35)*. The down regulation of CYP3A in the liver could implicate profenofos ability of interfering with PXR function. However, the result of a previous report *(36)* showed that PXR is not expressed in the testis of rats while CYP3A2 was demonstrated to be expressed in rat testis *(37)*. The ability of profenofos to down regulate CYP3A2 mRNA in the testis in addition to liver may indicate the ability of profenofos to affect CYP3A2 at the level of its transcription independently of PXR.

In our study treatment of male Wistar rats with the 1/10 of LD50 dose of profenofos for 65 days down regulated the hepatic CYP2C11 mRNA and protein levels compared to control (Figure 1). This results could indicate the involvement of CYP2C11 in profenofos metabolism and in consistent with the a reported study showed that CYP2C11 contribute in the metabolism of the organophosphorous diazinon *(38)*. Hepatic CYP2C11 expression is regulated by the male pattern of growth hormone (GH) secretion through the Janus-Kinase/ignal transducer and activators of transcription proteins (JAK/STAT5b) signal transduction pathway *(39)*. In our study, the down- regulation of CYP2C11 expression at the levels of mRNA in addition to protein may indicates the ability of profenofos to disturb the GH-JAK/STAT5b-CYP2C11 pathway through its effect on the pattern of GH secretion. Plasma pattern of growth hormone secretion directs the sex-specific expression of this enzyme and CYP2C11 is induced by a pulsatile pattern of growth hormone in the plasma *(38)*. The expression of CYP2C11 enzyme is down-regulated by the continuous pattern of growth hormone in the plasma *(40)*. The down regulation of CYP2C11 by the organophosphorus in our study could be deduced to disturbance of growth hormone level or pattern of secretion as it was reported that paraoxones disturb pituitary growth hormone and prolactin secretion *(41)*. The down regulation of CYP2C11 at the level of mRNA in addition to protein in both liver and testis could implicate a negative transcriptional mechanism of CYP2C11 by the phosphorothioate profenofos.

In our study treatment with profenofos led to down regulation of testicular aromatase (Figure 5) in treated rats compared to control. The irreversible conversion of androgens into estrogens is catalysed by aromatase, a product of a unique gene called Cyp19 *(42)*. Our result indicated a possibility of reduced estradiol production. It is well known that normal testicular development and maintenance of spermatogenesis are controlled by gonadotrophins and testosterones whose effects are modulated by locally produced factors, and

among them estrogens are obviously concerned *(26)*.In ArKO mice it has been shown that the infertility is consecutive to an impairment of spermogenesis associated with a decrease in sperm motility and an inability to fertilize oocytes *(28)*.

Phosphorothioate (parathion) was reported as a potent inhibitor of the male specific CYPs, CYP2C11 and CYP3A2 in rat liver *(43)*. In this study we presented the data which showed that profenofos (an other phosphorothioate) treatment of Wistar rats downregulated the hepatic and testicular male specific cytochrome P450 mainly CYP3A2, CYP2C11 and testicular aromatase. The overall results suggest that the phosphorothioate profenofos is an endocrine disruptor especially for male.

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المبيد الفسفورى البروفينوس يثبط جينات السيتوكروم الذكرية.

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أجريت هذه الدراسة على عدد سته من ذكور الفئران البالغة حيث قسموا إلى مجموعتين إحداهما اعتبرت كمجموعة ضابطة والأخرى تم تجريعها مرتين أسبوعيا بالمبيد الحشرى الفسفورى البروفينوفوس بجرعة ٣٥,٦ مليجرام لكل كيلو جرام من وزن الجسم لمدة خمسة وستون يوما. ثم ذبحت الفئران بعد المدة السابق ذكرها والتى تمثل دورة تكوين الحيوان المنوى. وتم أخذ عينات من خصى وأكباد تلك الفئران وحفظت فى درجة حرارة تحت ٨٠ درجة مئوية .

أثبتت الدراسة أن البروفينوفوس أدى الى تثبيط فى معدل الناقل الريبوسى الجينى وكذلك البروتينى الجينى للجينات الخاصة بالخصوبة وتشمل سيتوكروم ٢س١١ وسيتوكروم ٣ أ٢ فى أكباد وخصى الفئران التى تم معاملاتها بالمبيد مقارنة بالمجموعة الضابطة. كما أكدت الدراسة إحداث تثبيط فى الناقل الريبوسى الجينى لجين سيتوكروم ١٩ الخاص بالخصوبة.