Testicular toxicity of profenofos in matured male rats

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Abstract

To investigate the effect of the phosphorothoate insecticide profenofos on male specific gene expression on rat testis, 16-week-old Wistar rats were orally administered at dose of 17.8 mg/kg twice weekly for 65 days. Gene expression in the testes was monitored by DNA microarray analysis and real time RT-PCR which revealed that genes related to steroidogenesis including cytochrome P450 17A1 (CYP17A1), steroidogenic acute regulatory protein (StAR) and CYP11A1 were significantly increased. Besides the testes were histopathologicaly examined which revealed testicular destruction and degeneration represented by a layer of columner epithelium, oedematous changes surrounding the siminiferous tubules besides vacuolated spermatogonial calls and more elongated Leydig cells. These data suggest that profenofos considered as one of the male reproductive toxicants. Furthermore, we propose that the above 3 steroidogenic–related genes and the gene of acrosomal reaction as potential biomarkers of testicular toxicity.

Key Words: profenofos, cytochrome P450, testosterone, testis

Introduction

Currently used pesticides, however are broad–spectrum biocides that are toxic not only to target arthropods but also to vertebrates and mammals. The organophosphorus, profenofos [(O-4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate] is a broad-spectrum organophosphate insecticide and acaricide used widely for agricultural and household purposes (Prabhavathy et al., 2006). The toxicity by profenofos appeared fatal even at a relatively low plasma concentration as recorded in a case of fatal human poisoning where high concentrations of metabolites were detected suggests profenofos is rapidly metabolized (Gotoh et al., 2001). Profenofos is bioactivated, probably by phosphorothioate oxidation with microsomal enzyme and NADPH (Wing et al., 1983; Glickman et al, 1984). The toxicity profile of profenofos in vitro, using lymphocytes from peripheral blood samples of healthy human donors via comet assay revealed single strand breaks in DNA as comet tail lengths which indicate genetic damage (Prabhavathy et al., 2006). Previous studies have revealed that at low doses, organophosphorus pesticides not only act as genotoxic agents but also, affect several other biochemical pathways where profenofos induced apoptosis and necrosis in cultured human peripheral blood lymphocytes under in vitro conditions using the DNA diffusion assay (Das et al., 2006).

Concerning on reproductive toxicities of organophosphorus pesticide, the methyl parathion caused increasing in the levels of abnormal sperm and testosterone, whereas the luteinizing hormone level and total number of seminiferous tubules decreased in the testis besides a few tubules showed exfoliation of epithelium and vacules in the testis of the treated new born Wistar rat (Narayana et al., 2006). The pesticide vinclozolin which

is known as an endocrine disrupting chemical regarding spermatogenesis and gene expression revealed significant increase in the expression level of mRNAs of the testicular steroidogenic enzyme genes; cytochrome P450 (CYP) side chain cleavage (P450scc or CYP11A) and CYP17A (Kubota et al.,2003). The testicular histomorphological studies of the organophosphorus insecticide pollutant quinalphos was detected in albino rats showing shrinkage of the tubular diameter and testicular atrophy leading to degenerative changes in the germinal epithelium (Debanth and Mandal, 2000).

The reproductive toxicity of profenofos in mammalian germ cells was studied by El Nahas et al. (1989). They reported the chromosomal aberrations in spermatogonial cells and sperm abnormalities in mice after profenofos treatment, and showed damaging effect on spermatogonial cells via significant increase in structural chromosomal aberrations and on sperm morphology via significant increase in sperm abnormalities and significant decrease in sperm count and motility.

In current study, we evaluated the relationship between potential reproductive toxicity and differential gene expression profiles of rat testis through DNA microarray analysis. Furthermore, we pathologically examined the effect of profenofos on rat testis by electron microscopy technique.

Materials and Methods

Animals and treatments

Sixteen-week old Wistar rats (SLC. Hamamatsu, Japan) were divided into 2 groups, of 3 rats each, 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. Rats were given 17.8 mg/kg profenofos (1/20 of LD50), vehicle (distilled water) orally for 65 days (twice weekly) (Kniewald et al. 2000). Twenty-four hours after the last dose, rats were sacrificed with carbon dioxide and testis were removed and kept in 1.5 ml tubes and snapped in liquid nitrogen and kept at –80 ºC until use.

RNA extraction

Total RNA was isolated from 50 mg of testis using TriReagent (Sigma-Aldrich, MO, USA). Briefly 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 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 electrophoresis, and showed that the RNA integrity was fine then it was further checked by measuring the optic density on spectrophotometer. 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 ºC 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 ºC for 10 min, 42ºC for 1h, and 90 ºC for 10 min.

DNA microarray analysis

Standard Affymetrix (Santa Clara, CA) protocol required 10μg of total RNA with a minimum 260:280 ratio of 1.80. Double-stranded cDNA was synthesized from the pooled total RNA from testes of three rats using reverse transcription with an oligo-dT primer followed by RNase A treatment and DNA polymerase synthesis of the second strand. The double-strand cDNA was used as a template for an in vitro transcription reaction using biotinylated cytosine 59-triphosphate and uridine 59-triphosphate to produce labeled cRNA (MessageAmp II Biotin Enhanced, Ambion, Austin, TX). The biotinylated cRNA was then fragmented and hybridized to the GeneChip Rat Expression Array 230A (Affymetrix). The microarrays were processed with Affymetrix GeneChip Fluidics Workstation 400, stained with phycoerythrin-coupled streptavidin, and scanned on a GeneArray Scanner. Microarray data were scaled to a target signal of 125 and analyzed with Microarray Suite 5.0 software (Affymetrix). Raw data were processed with the statistical algorithm according to the Affymetrix instruction (http://www.affymetrix.com/support/technical/whitepapers.affx) and a global scaling adjustment was applied. The GeneChip software provided a detection p-value, which is calculated using the One-Sided Wilcoxon's Signed Rank test from the probe set of each

gene. We picked up the data which showed the p-value smaller than 0.04 as “present” signals. We also decided the alteration of gene expression, referring the data which provided by Affymetrix Data Mining Tool using the One-Step Tukey's Biweight Estimate. The qualitative assessment "increase" and "decrease" were considered as alteration data of RNA expression in testis, but we selected out the data which were called as "marginal decrease", "marginal increase" or "no change". Then, we picked up the gene which showed more than 2-fold or 0.5-fold difference between the control and treated groups.

Real-time PCR

For quantification of the steroidogenic enzyme (CYP11A1,CYP17A1, StAR) mRNAs expression in testes, a real-time PCR approach was used, we used the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The amplification reactions were performed in 20 μL final volume containing 10 μL of 2 TaqMan Universal PCR Master Mix (ABI), 1 μL of TaqMan® Gene Expression Assays (ABI), and 9 μL of cDNA. The conditions of PCR were 2 min at 50 °C followed by 10 min at 95 °C and then 40 cycles of 15 sec at 95 °C and 1 min at 60 °C.

Plasma testosterone measurement

Plasma testosterone was measured by ELISA using the Testosterone EIA kit (Cayman Chemical Co. Ann Arbort U.S.A) and essentially following the protocol in the manual of the kits (No. 582701).

Histopathology

To investigate the relationship between histopathological alterations and testicular male specific gene expression changes, we evaluated the testis histopathologically for light and electrone microscope methods, where the data of the testis stained with hematoxylin eosin stain (HE) considered as a guide for more detailed histopathological lesions by electron microscope.

Samples of rat testicular tissues of the control and treated groups were fixed by immersion in 3% glutaraldehyde solution in 0.1M phosphate buffer solution (pH 7.4) for 2 hrs followed by post-fixation in 1% osmium tetraoxide (OsO_4) in. 0.1 M phosphate buffer (pH 7.3) for 2 hrs at 4 °C. Then the tissues were dehydrated in up-graded ethanol and finally embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Semi-thin sections (1μm) were stained with 1% methylene blue. Ultra – thin sections were double stained with uranium acetate and lead citrate, and photographed with a transmission electron microscope (JEM 1210, JOEL, Japan).

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.

Results

DNA microarray analysis of gene expression in the testes.

Regulation of the rat testis genes by profenofos was analyzed using DNA microarray. Testes isolated from profenofos-treared rats showed up/down-regulated genes related to steroidogenesis and acrosomal reaction (Table 1 and 2). In particular, we identified induction in steroidgenesis-cascade in the testis of profenofos treated groups. Genes related to steroidogenesis such as CYP17A1, StAR and CYP11A1 were up-regulated (2.83, 2.83 and 4.29 fold changes, respectively, compared to control). Profenofos also down-regulated the expression of glycine receptor gene (the gene related to acrosomal reaction) which deceased by 0.54 fold change compared to control.

Real-time RT-PCR analysis of gene expression in the testes.

The data of DNA microarray was verified by quantitative real-time RT-PCR where the expression of the mRNAs of the steroidogenic enzyme genes showed significant increase as following; CYP11A1 increase about 2.5 fold change (Figure 1A), CYP17A1 increase by 2.5 fold change (Figure 1B), StAR increase about 5 fold change (Figure 1C).

Testosterone concentration in plasma

Testosterone concentrations in plasma were shown in Figure 2. Profenofos treatment significantly increased the testosterone levels in male rats. The testosterone levels in profenofos treated rats were 2-fold higher than that in control animals.

Histopathological findings of testes.

We found the testicular degeneration, congested blood vessels with edema among somniferous tubules in HE photographs (data not shown). The characteristics of the pathological lesions showed testicular destruction and degeneration represented by a layer of tall columner epithelium with edematous changes surrounding the simininferous tubules leading to displacing the interstitial cells away from the simininferous tubules and basement membrane (Figure 3 B and C). Besides that the nuclei of the spermatogonia appeared vacuolated and these vacuoles including dense and coalesced materials might be resulted from destruction and degeneration of some organelle (Figure D). Some sections showed azospermia and spermatid abnormalities, besides strange cytoplasmic inclusion bodies in some spermatogonial cells and also, there is elongation of Leydig cells may indicate more activity (Figure E).

Discussion

Organophosphorus (OP) pesticides have been widely used in a variety of agricultural, commercial, and household applications. Organphosphates make up the major class of pesticides (U.S. EPAhttp://www.epa.gov/oppbead1/pestsales/99pestsales/usage1999_3.html), yet little is known about their potential genomic effects. The goal of the present study was carried to evaluate the testis toxicity in mature male rats exposed to the phosphorothioate OP profenofos, where this experiment was made to detect the relationship among the toxicological effects of profenofos, the alteration in the gene expression profiles and histopathological changes for the testes.

The results of semi-quantitative DNA microarray analysis revealed that the steroidogenic genes (CYP17A1, CYP11A1, StAR) were up-regulated in profenofos–treated rats. The previous literature referred that CYP17A1 is a key enzyme in hormonal steroidogenesis mediating androgen biosynthesis where it catalysis two reactions. The CYP17A acts in 17 alpha hydroxylation and the cleavage of the C-17,20 bond C-21 steroids (Valle et al.,2002; Voutilainen et al.,1986). The rate limiting step in steroid biosynthesis is regulated by acute regulatory protein (StAR) (Stocco and Clark,1996; Stocco 2001), where StAR mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane, where cholesterol is then converted by CYP11A1 to pregnenolone, the precursor for all steroids (King et al.,2002), where it well accepted that StAR and other steroidogenic genes expressed in the gonads (Simpson and Waterman, 1988 ; Waterman, 1994). The similarities in the known biologic functions of these genes and their relation to steroidogenesis suggest that exposure to profenofos may increase the possibility of steroid genesis. Regardless, these

three steroidogenic genes are a starting point in the search for a genetic biomarker of the testis toxicity of exposure to profenofos and perhaps other pesticides. The up-regulation of CYP17A1, CYP11A1 and StAR genes, which firstly screened by DNA microarray and verified by quantitative real-time RT-PCR, appeared to cause extraordinary increase in testosterone level. Recently, it has been indicated that an abnormally high level of intratesticular testosterone inhibits spermatogenesis (Meistrich and Kangasniemi,1997; Tohda et al.,2001), where these consecutive studies conducted by Meistrich and coworkers, strongly suggest that a supra-higher level of testosterone directly inhibits spermatogenesis and this inhibition is due to apoptosis of spermatogonia caused by the high level of testosterone (Shetty et al.,2001; Tohda et al.,2001).

The EPA reported that profenofos did not show any reproductive toxicity in male rats at the dose of 20 mg/kg/day for 90 day (http://www.epa.gov/pesticides/op/profenofos/prof_hed.pdf). In our preliminary experiment, we observed suppressive effect of profenofos on testosterone levels before 65 days (data not shown). We suggested the toxicological effects of profenofos might be reversible and it was possible that feed back phenomenon caused the induction of testosterone production and testis enlargement at the administration day of 65. Further study may be needed to clarify this point.

We suggested that the increase in the level of testosterone in organophosphate treated rats may be due to following mechanisms. Firstly, increase in serum testosterone concentrations may be explained by the fact that the treatment causes an increase in serum LH concentrations in male rats, circulating LH is responsible for maintaining normal increased serum testosterone concentrations (Ellis and Desjardins, 1982). The high LH concentrations stimulate the Leydig cells to release large amounts of

testosterone. Secondly, organophosphates pesticides may act directly on Leydig cells to stimulate testosterone synthesis (Morris and Saxena, 1980). The second speculation is virtually consistent with the present study of histopathologicaly, which included testicular destruction and degeneration represented by a single layer of tall columner epithelium, oedematous changes surrounding the seminiferous tubules, the cytoplasm of spermatogonia vaculated and these vacules contain dense and coallesed materials from degeneration and destruction of other organells, intracytoplasmic inclusion bodies in spermatogonia and abnormalities in spermatids besides elongation in Leydig cells. All these histological lesions confirmed our speculation that profenofos doing its testicular toxic effect by acting directly on the testis tissues as previously mentioned. The stimulation of gonadotrophins releasing hormones and increase of LH secretion which confirmed by the ultrastructure of Leydig cells leaded to increase in the level of testosterone via increasing the levels of steroidogenic enzyme genes, which recorded in this study. The concentration of plasma GnRH in profenofos treated rats were 1.5-fold higher than those of control animals (data not shown) suggesting the possibility of acceleration of testosterone productive cascade. The plasma LH and hypothalamic GnRH concentration or synthesis of mRNA of these peptide hormones should be measured to elucidate the effect of profenofos on testicular function.

In our conclusion, the present investigation of the relationship between histopathological alteration and testicular gene expression of steroidogenesis and fertilization suggest that the phosphorothioate profenofos considered one of the chemicals that may lead to testicular toxicity and affecting on male fertility.

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1 Table 1. Decreased gene in testis of profenofos treated rat.

Gene ID	Gene Title	Function or annotation	Gene Symbol	Fold
AA819658	Kohjirin (Chordin-like protein 1 precursor, Neurogenesin-1)	plays a role in neuronal differentiation; inhibits glial cell fate determination in neural stem cells.	Chrdl1	0.10
NM_024356	GTP cyclohydrolase 1	rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis	Gch	0.11
AI236283	Similar to Serologically defined colon cancer antigen 13		---	0.20
U39555	solute carrier family 1	epithelial high affinity glutamate transporter, system Xag	Slc1a1	0.23
NM_031069	NELL1	protein kinase C-binding protein	Nell1	0.25
AF001423	glutamate receptor, ionotropic, N-methyl D-aspartate 2A		Grin2a	0.31
AA997129	laminin, gamma 1	heterotrimeric extracellular matrix protein	Lamc1	0.31

BI296516	Transcribed locus, moderately similar to NP_989660.1	general control of amino-acid synthesis 5-like 2	---	0.44
AB035507	melanoma cell adhesion molecule		Mcam	0.47
BI279786	myosin IC	molecular motors that, upon interaction with actin filaments	Myo1c	0.47
AA900926	AT rich interactive domain 1A	retinoblastoma-binding protein that interacts with viral oncoproteins	---	0.50
NM_022186	nuclear receptor binding factor 2		Nrbf2	0.50
AW531716	Similar to Msx-2 interacting nuclear target protein	endogenous inhibitor of Notch regulation	---	0.50
AI547447	potassium voltage gated channel, Shaw-related subfamily, member 3		Kcnc3	0.54
U06434	small inducible cytokine A4		Ccl4	0.54
AJ310836	glycine receptor, alpha 1 subunit		Glra1	0.54
NM_031826	fibrillin 2		Fbn2	0.54

AI407169	Similar to IL-17D		---	0.57
NM_022284	guanylate cyclase activator 2b	peptide homologs of the bacterial heat-stable enterotoxins and endogenous activators of the guanylate cyclase-2C receptor	Guca2b	0.57
AW525342	ubiquitin specific protease 1		Usp1	0.57
BF398368	bromodomain adjacent to zinc finger domain, 2B	structural motif characteristic of proteins involved in chromatin-dependent regulation of transcription	Baz2b	0.57

1 The semi-quantitative DNA microarray analysis showed the down-regulated genes of rat testis after oral treatment with 17.8 mg/kg of
2 profenofos twice weekly for 65 days.

Table 2. Increased Genes in testis of profenofos treated male rats.

Gene ID	Gene Title	Function or annotation	Gene Symbol	Change
AI104234	insulin-like growth factor binding protein 2		Igfbp2	1.52
AI137605	similar to chemokine-like factor super family 7		LOC501065	1.52
AI232643	similar to citrin	containing a mitochondrial carrier motif and 4 EF-hand domains	LOC362322	1.62
NM_053445	fatty acid desaturase 1	introduce double bonds between defined carbons of the fatty acyl chain	Fads1	1.87
NM_032082	hydroxyacid oxidase 2	oxidize a broad range of 2-hydroxyacids, ranging from glycolate to long-chain 2-hydroxy fatty acids such as 2-hydroxypalmitate	Hao2	2.00
AW532566	PDZ domain containing RING finger 3	(KIAA1095, contains a C3HC4 zinc finger motif)	Pdzrn3	2.64

BI295567	similar to open reading frame 5		RGD1305062	2.83
AB006007	steroidogenic acute regulatory protein		Star	2.83
NM_012753	cytochrome P450, family 17		Cyp17a1	2.83
AB006007	steroidogenic acute regulatory protein		Star	2.83
NM_012582	haptoglobin		Hp	3.48
BF395317	membrane-spanning-4-domains, subfamily A, member 11	protein with at least 4 potential transmembrane domains and N- and C-terminal cytoplasmic domains.	Ms4a11	3.73
NM_017286	cytochrome P450, family 11		Cyp11a1	4.29
AY043246	regulator of G-protein signaling 2	basic helix-loop-helix phosphoprotein G0S8, G0/G1 switch regulatory gene	Rgs2	4.59

The semi-quantitative DNA microarray analysis showed the up-regulated genes of rat testis after oral treatment with 17.8 mg/kg of profenofos twice weekly for 65 days.

Figure legends

Figure 1. Effects of profenofos on gene expressions in testis. Male Wistar rats orally administered 17.8 mg/kg, The expression of mRNA were measured by quantitative real-time RT-PCR as described in materials and methods. The results were analyzed relative to control level. A) CYP17A1, B) CYP11A1, C) StAR. N= 3 rats for each group. Data are means ± SD; * Higher than control, $p<0.05$.

Figure 2. Plasma testosterone concentration. Rats were orally treated with 17.8 mg/kg of profenofos twice weekly for 65 days. Plasma testosterone in control and profenofos-treated rats were measured by ELISA. * Higher than control, $p<0.05$.

Figure 3. Transmission electron microscopic observation (semi-thin section). A) Germ cells in control rat testis showed normal ultra-structure features. B) Germ cells in rat testis treated with profenofos showing spermatogonial cell with large vacuoles and strange inclusion bodies. C) Germ cells in rat testis treated with profenofos showing oedematous changes surrounding the simininferous tubules, and spermatid abnormalities besides vacuolated spermatogonial nuclei. D) Cytoplasm of spermatogonia cell in rat testis treated with profenofos showing large vacuoles containing electronic dense materials from destruction of other organelles. E) Leydig cell in rat testis treated with profenofos showing more enlargement and elongation indicating more biological activation.

Figure 1

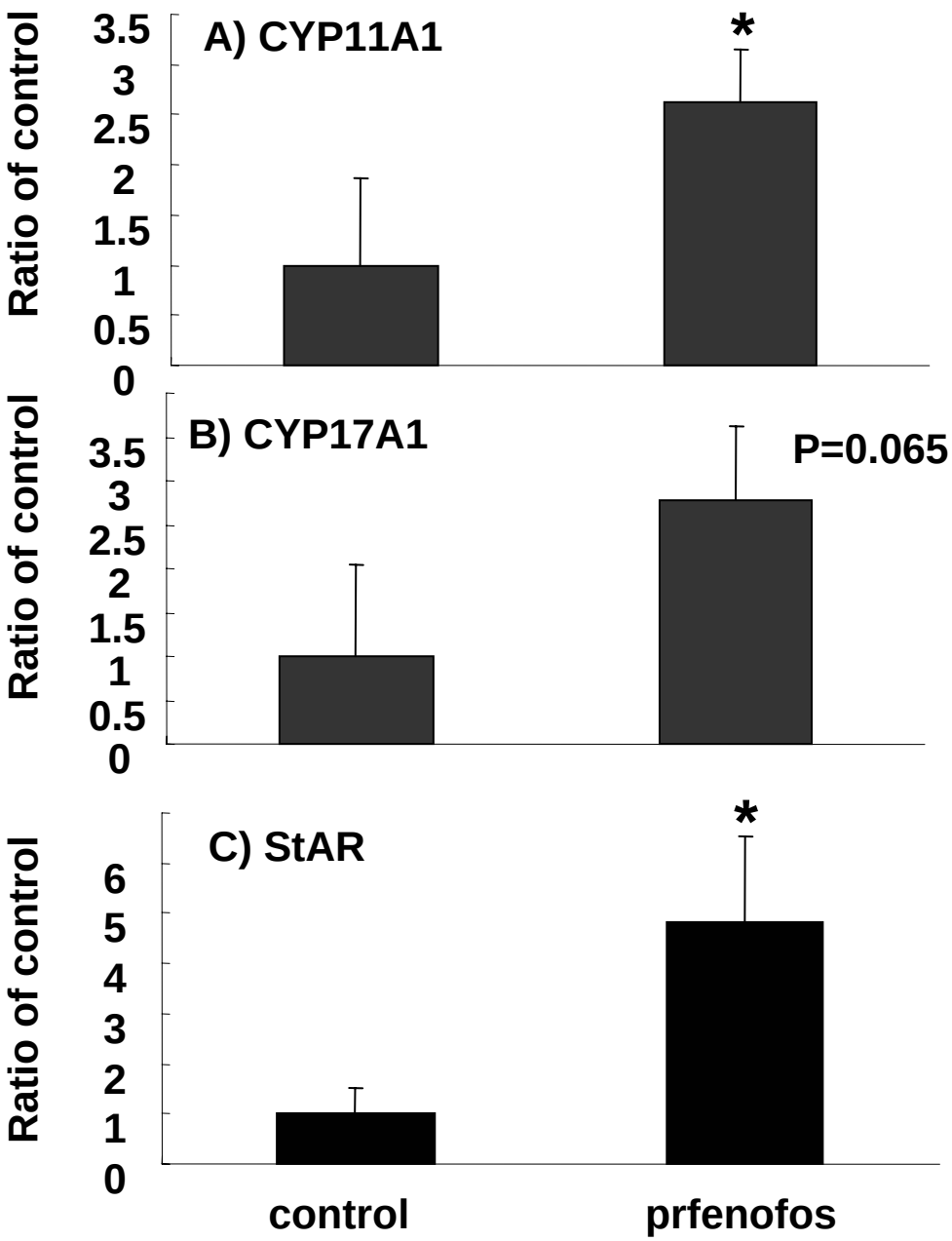
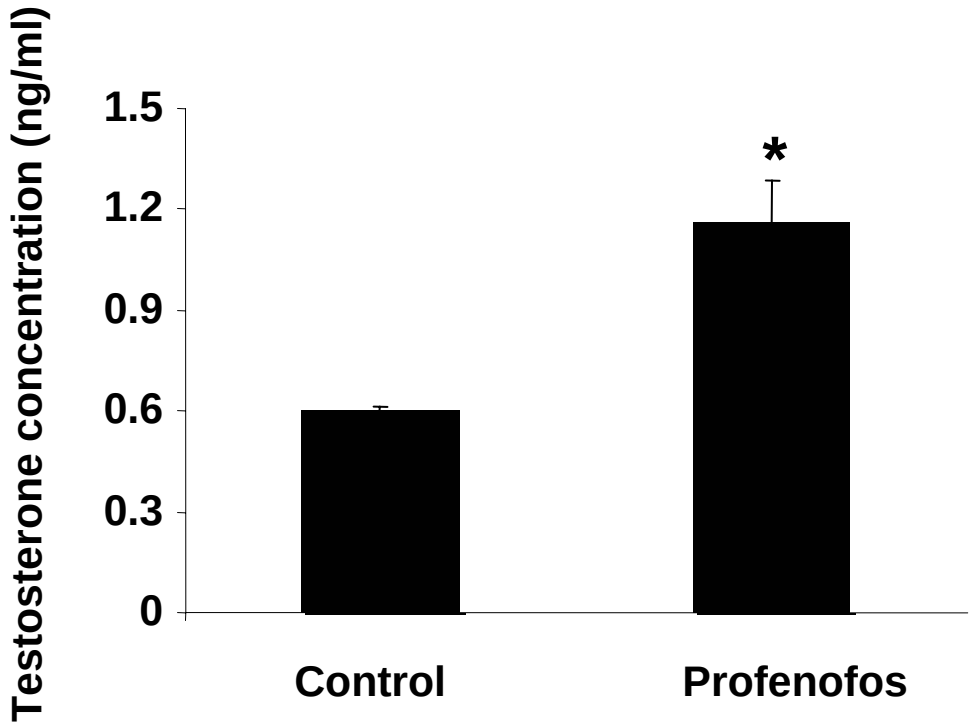

A) CYP11A1
Ratio of control
3.5
3
2.5
2
1.5
1
0.5
0
*
B) CYP17A1
Ratio of control
P=0.065
C) StAR
Ratio of control
6
5
4
3
2
1
0
*
control
prfenofos

Figure 2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1.5
1.2
0.9
0.6
0.3
0
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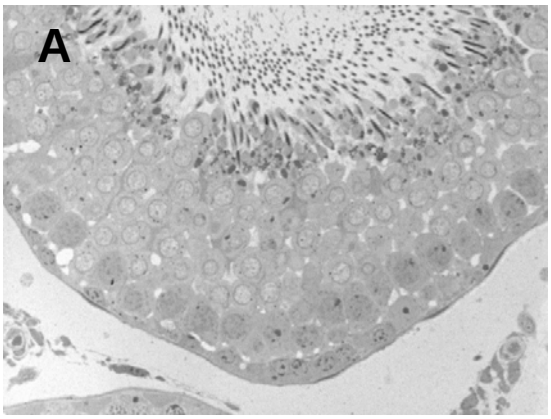
Control
Profenofos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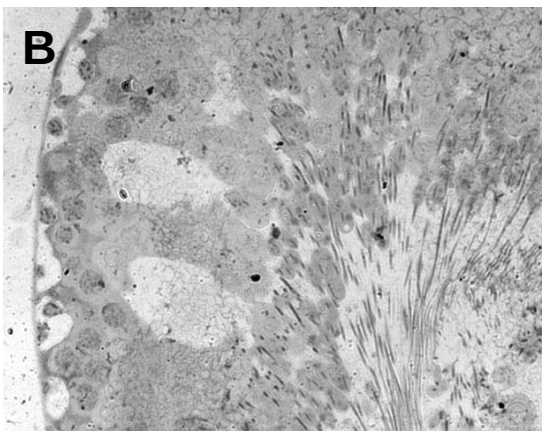
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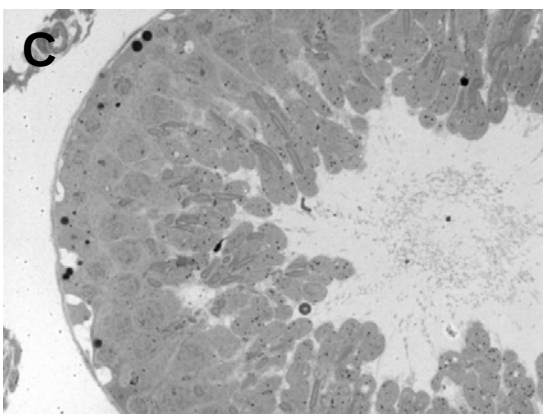
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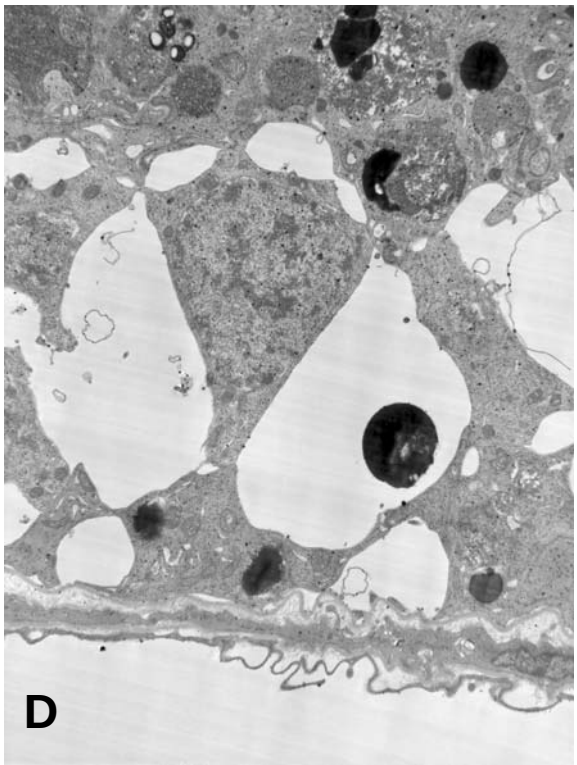
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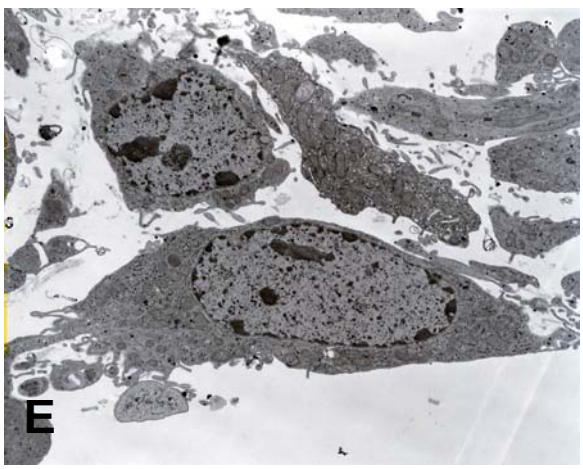
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