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Something of interest because of what box it would classify compounds into.

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Estrogen-Like Properties of Perfluorooctanoic Acid (PFOA) as Revealed by Vitellogenin Expression in Rare Minnows (*Gobiocypris rarus*)

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Abstract

Perfluorooctanoic acid (PFOA) is one of important perfluorinated compounds (PFCs) with various applications and has been widely spread in the environment, wildlife and humans. This study investigated the effects of waterborne PFOA on liver estrogen-responsive gene, vitellogenin (Vtg), expression and gonadal development in a freshwater fish, rare minnow (*Gobiocypris rarus*). The expression of Vtg was determined using reverse transcription polymerase chain reaction (RT-PCR) techniques, and identified using enzyme-linked immunosorbent assay (ELISA). A significant increase of Vtg expression in the livers of both mature male and female rare minnows was observed after 14 and 28-day exposure to PFOA, indicating that PFOA could induce the synthesis of Vtg. The development of oocytes in testes exposed to PFOA also provided the evidence of estrogenic activity in males. The ovaries of exposed females underwent degeneration as reported in other species exposed to environmental estrogens. This preliminary study indicates that PFOA can be considered as a candidate of estrogenic compounds with the potential to induce hepatic estrogen-responsive genes in male rare minnow. These, together with other reported, data on rare minnow suggest that this fish provides a good model for assessing endocrine disruption by environmental estrogens.

Introduction

The commercial use of perfluorinated compounds (PFCs) for different applications during several decades has resulted in a broad distribution of stable precursors/metabolites in animals from terrestrial and aquatic environments (*1-10*). Despite the widespread of these compounds, relatively little is known of their fate and effects on animals (*11, 12*). The evidences that PFCs may disturb the endocrine system are increasing and deserve further investigation.

Perfluorooctanoic acid (PFOA) is a remarkably stable compound that does not undergo photolysis, hydrolysis and biodegradation. It has been shown to produce hepatomegaly, induce hepatic peroxisomes, increase β-oxidation and alter endocrine function in rodents (*13-18*). In teleosts, circulating sex steroid levels in a freshwater species, fathead minnow (*Pimephales promelas*), can be affected significantly by exposure to fluorinated chemicals (*19, 20*). However, the potential of PFOA for estrogen-like properties in teleosts has little been reported.

Natural estrogens, such as 17β-estradiol (E2), regulate the estrogen-responsive genes by binding to a specific estrogen receptor (ER). Then the estrogen-ER complex interacts with the estrogen responsive elements (EREs) of the target promoter genes and activates the transcription of estrogen-regulated gene (*21, 22*). Among the estrogen-responsive genes, vitellogenin (Vtg), the precursor of the egg yolk proteins, is synthesized in the liver of female teleosts in response to endogenous estrogens, transported to the ovary through the blood stream and incorporated into the vitellogenic stage oocytes (*23*). A high level of plasma and/or hepatic Vtg is observed in mature females, whereas Vtg levels in males and sexually immature individuals are relatively low or even undetectable. It is known to date that a

number of environmental estrogens, such as alkylphenolic compounds, the pesticides, can induce the Vtg synthesis in males. Therefore, the significant increase of Vtg in the plasma of a male is considered as a sensitive biomarker of exposure to an estrogenic chemical (*24*).

In the present study a freshwater teleost, rare minnow (*Gobiocypris rarus)*, was used. This gonochoristic species has many attractive features that make it a suitable organism in aquatic toxicity tests, including sensitivity to chemicals, small size, wide temperature range, easily being cultured in laboratory and short duration of embryonic development (*25-28*). The objective of the present study was to evaluate the *in vivo* effects of waterborne PFOA on both mature male and female rare minnows under continuous flow-through conditions. We examined integratedly the vitellogenin gene induction in the livers of male and female rare minnows using polymerase chain reaction (PCR) techniques, and measured the Vtg using enzyme-linked immunosorbent assay (ELISA). In addition, the histopathological changes in rare minnow gonads were examined under a light microscope at the end of the experiment, which provided insights into the effects of PFOA on reproductive health on this species.

Materials and Methods

Chemicals. Perfluorooctanoic acid (PFOA, 98%) was purchased from Acros Organics (Geel, Belgium). A polyclonal antiserum (anti-Vtg) that was raised in rabbits against carp was generously provided by Professor Xu. Anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) was purchased from Promega, USA.

Fish and exposed experiment. All rare minnows were obtained from laboratory hatchery. Two hundred and forty mature male and female rare minnows (about 9 months old with body

weight of 1.4 ± 0.4 g and total length of 47.7 ± 3.6 mm) were randomly assigned to eight 20-L glass tanks (30 individuals per tank) and acclimated for a week. Fish were supplied with de-chlorine tap water under continuous flow-through conditions at a temperature of 25 ± 2 °C and a photoperiod of 16:8 h (light:dark). Fish were generally fed with a commercial granule food (Trea, Germany) at a daily rate of 0.1% body weight. Waste and uneaten food were removed daily. Fish were randomly assigned to one of the 4 treatments after the one-week acclimation, the control or exposed to PFOA concentrations of 3, 10 or 30 mg/L. The flow rate of test solution was 8 L/h, and actual PFOA concentrations in tanks were not verified by chemical analysis. Experiment lasted for 28 d.

Gonadal Histology. To investigate the histopathological changes in rare minnow gonads, ten males and ten females were collected at the end of the 28 d exposure from each treatment. For each fish, gonadal tissue was excised from the middle portion of one of the two lobes, fixed in 10% formalin solution. Tissues were dehydrated in ethanol, embedded in paraffin wax, sectioned transversely at 4-5 μm, mounted on slides and stained with haematoxylin and eosin. Fish sexes and their maturation were determined on the basis of the gonadal structure and the degree of egg and sperm cell development, respectively (*29*, *30*).

RNA Extraction, Gene Expression Quantification Total RNA and first strand cDNA synthesis were performed as described in the manufacturer's protocol. Briefly, for each individual male or female analyzed, total RNA was extracted from 20 mg of liver tissue using RNeasy Mini Kit (Qiagen, Germany) and treated with RNase-free DNase I (Qiagen, Germany) to remove any remaining genomic DNA. The approximate 1 μg of total RNA for each sample was conducted reverse transcription using oligo $(dT)_{15}$ primer (Promega, USA) and M-MuLV

reverse transcriptase (New England Biolabs, USA) with incubation at 42 °C for 60 min, followed by at 95 °C for 5 min, according to the supplier's instructions.

The variations in Vtg mRNA expression in male livers were evaluated by semi-quantitative RT-PCR using the β-actin as an internal control. A cycle number within the exponential phase of the amplification curve was chosen for quantifying the expression of Vtg gene. Vtg primers were designed on the basis of sequence in our cDNA library available in GenBank (**EE392488**): forward: 5′- ACAAAGTCACTGCCAAGGTT -3′, reverse: 5′-AATGGTAAGAGTTCCGTCTG -3′. The product size was 364 bp. PCR was performed using PCR Master Mix (Promega, USA) in 25 μL containing 1× PCR buffer, 200 μM of dNTP and 0.5 μM of each primer set. PCR amplification of Vtg was carried out for 30 cycles with the following profile: denaturation at 94 °C for 5 min, primer annealing at 60 °C for 40 s, primer extension at 72 °C for 40 s, and with a final extension period of 72 °C for 5 min. The PCR amplification of β-actin was carried out for 27 cycles using specific β-actin primers (forward: 5′-GCCGTGACCTGACTGACTAC-3′, reverse: 5′-GCAAGATTCCATACCCAAGA-3′) with the following termocycler profile: 40 s at 94 °C, primer annealing at 60 °C for 40 s, and primer extension at 72 °C for 40 s. The product size was 272 bp. The band densities of the resulting products were measured using the Bandleader Software. Each analysis was repeated three times and normalized against β-actin.

Quantitative RT-PCR was performed to evaluate the Vtg expression of female livers using a QuantiTect SYBR Green PCR kit (Qiagen, Germany) and Stratagene Mx3000P qPCR system (Stratagene, USA) in a 20 μl total reaction volume including 10 μl of 2 × QuantiTect SYBR Green PCR master mix, 1 μl cDNA template, and 0.5 μM each of target specific primer. The

primers were the same as above. Thermal cycling conditions were as follows: 95 °C for 15 min, and 50 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, as displayed by a single peak (data not shown). Every sample was determined in triplicate. After verifying that the efficiencies of amplification of Vtg and internal control β-actin were approximately equal, fold differences in Vtg expression (compared to control) were calculated using the $2^{-DeltaDeltaCT}$ method (*31*).

Measurement of Liver Vtg Level. 30 mg of liver tissue from each individual male or female analyzed was sonicated in 500 μL of ice-cold PBS buffer (pH 7.6) on ice for 5 min with sonifier (Digital Sonifier, USA). Cellular debris was removed by centrifugation at 12000g for 20 min at 4 °C, and the supernatant was collected and stored at -80 °C until used for further analysis. Liver Vtg level was measured using the heterologous competitive ELISA method as described in reference (*32*, *33*) with a little adjustment. Briefly, the 96 micro-well plate was pre-coated with 750 ng/mL purified carp Vtg and then incubated at 4 °C for overnight. Standards of purified carp Vtg were diluted with 0.05 M Tris–HCl (pH 7.5) containing 0.05% Tween-20, 0.9% NaCl (TTBS) with 3% BSA in a twofold series from 2000 ng/mL to 7.8 ng/mL, then mixed (1:1, v/v) with diluted anti-Vtg (1:7000) in 96 micro-well plate and then incubated for 16 h at 4 °C. 100 μL of each pre-incubated solution was dispensed in triplicate into the coated plate, followed by incubation for 1 h for 32 °C after which it was washed four times with TTBS. A second antibody anti-rabbit IgG conjugated with HRP (prepared at a final dilution of 1:2500) was added to each well (100 μL per well). After 1 h at 32 °C, the second antibody was removed and the plate was washed five times. 0.1 M citrate buffer (pH

5.0) containing 0.4 mg/mL of *o*-phenylenediamine (Ameresco, USA) and 0.15% (v/v) hydrogen peroxide was added (100 μL/well). The plate was gently agitated and then incubated at room temperature for 15 min. The optical density was measured at 492 nm with a microtiter plate reader. The concentration of Vtg in liver was normalized to protein mass of the corresponding sample and expressed in ng/μg protein. Protein concentration was measured by the Bradford method (*34*) using BSA as standard.

Statistical analysis. All values are expressed as mean ± S.D. The statistical program SPSS (version 13.0) was used to collect and analyze all data. One-way analysis of variance (ANOVA) was used to determine the statistical difference between the test groups and control and $p < 0.05$ was considered as significant.

Results and Discussion

Histopathology. In the control, both male and female rare minnows were in mature with seminiferous tubules and sperm ducts were filled with sperm in the testes and vitellogenic stage oocytes dominant in the ovaries, respectively (Figure 1a, b). There was no evidence of degeneration in both testes and ovaries in the control. For mature females exposed to 3, 10 and 30 mg/L PFOA for 28 d, vitellogenic stage oocytes in ovaries were under degeneration (atresia) (Figure 2a, b, c). For mature males exposed to 3 mg/L PFOA, the testes were similar to those in the control. For males exposed to 10 mg/L PFOA, primary-growth stage oocytes (previtellogenic oocytes) developed in testes (three out of ten males) (Figure 3a, b). Such testes-ova were not in more advanced stage in males exposed to higher concentration of 30 mg/L PFOA (one out of ten males) (Figure 3c). In addition, in testes from control male rare

minnows and even in the PFOA concentration 3 mg/L, seminiferous tubules were filled with large numbers of fertile sperm. However, the number of sperms and various stages of germ cells within the spermatogenic cycle in the PFOA treatment at 10 and 30 mg/L were lower than in control males.

Similar testes-ova have also been observed in male rare minnows exposed to 17α-ethynylestradiol (EE2), 4-nonylphenol (NP) and diethylstilbestrol (DES) (*32, 35*). Gray and Metcalfe (*36*) reported that NP could induce testis-ova in male medaka (*Oryzias latipes*). Länge et al (*37*) also reported long term exposure to EE2 resulted in the formation of testis-ova and the appearance of sex reversals in male fathead minnows. However, not all studies showed that fish exposed to endocrine disruptors could be detected with testes-ova. For example, male fathead minnows exposed to E2 and EE2 for 2-3 weeks only exhibited the block of spermatogenesis and the hyperplasia of sertoli cells (*38*); similar changes were observed in males of zebrafish (*39*).

Ovarian changes of females exposed to estrogens have been observed through histological examination in several fishes. For example, oocytes in female fathead minnows (*Pimephales promelas*) exposed to waterborne E2 for 14 d exhibited degenerative changes (*38*). Mature females of zebrafish (*Danio rerio*) exposed to 17α-ethynylestradiol (EE2) for 24 d caused the degeneration of vitellogenic stage oocytes, the reduction of yolks in oocytes and the decrease of the gonadosomatic index (GSI) (*39*). Similar in this study, ovaries of mature females exposed to PFOA for 28 d underwent degeneration. The results of the present study indicate that rare minnow is a sensitive fish species to endocrine disruptors with the formation of

testes-ova in the testes and the degeneration of vitellogenic stage oocytes in ovaries after exposure to PFOA.

Vitellogenin mRNA Levels in Liver. Figure 4a and b showed an agarose gel displaying the vitellogenin and β-actin products generated in the RT-PCR reaction of total RNA isolated from the livers of individual male rare minnows exposed to PFOA for 14 and 28 d, respectively. The vitellogenin-specific cDNA band, which migrates at an effective predicted mass of 364 base pairs (bp), was generated with the Vtg primer pair. The β-actin amplification products observed in total RNA from livers have a mass of approximately 274 bp. Figure 4c and d showed the relative Vtg expression levels of individual rare minnows in controls and 3, 10 and 30 mg/L of PFOA from the same experiment after 14 and 28 d, respectively. An ANOVA was performed on digital values of pixel density ratios from those agarose gels that yielded graphical data, as shown in Figure 4c and d. The up-regulation of liver Vtg mRNA was observed in male rare minnows exposed to 10 and 30 mg/L PFOA for 14 d, but there was no significant difference in the expression level of liver Vtg in males exposed to 3 mg/L PFOA compared to the control. Treatments of all groups produced significant changes in Vtg gene expression relative to the control group after 28 d exposure.

Real-time quantitative PCR were performed on female rare minnow hepatic samples of exposed to PFOA. β-actin was used as endogenous control. The results indicated that the mRNA expression of Vtg was significantly higher in female treatment groups (10 and 30 mg/L) for 14 d and 28 d than those in female control, especially, a 37 fold increase in 30 mg/L PFOA treatment group for 28 d (Figure 4d).

Liver Vitellogenin Concentration. After 14 d of exposure to PFOA, liver Vtg concentrations of males ranged from 0.39 ng/μg protein in the control to 9.53 ng/μg protein in 30 mg/L PFOA exposure group (Figure 5a). There was an apparent increase in liver Vtg up to 0.69 ng/μg protein in males exposed to 3 mg/L PFOA, but this was not statistically significant. Liver Vtg concentrations of males exposed to 10 and 30 mg/L PFOA after 14 d were significantly increased compared to the control, i.e. the Vtg content was approximately 20 times higher than that in the control. After 28 d of exposure to PFOA, liver Vtg concentrations of males ranged from 0.21 ng/μg protein in the control to 4.85 ng/μg protein in 10 mg/L PFOA exposure group (Figure 5a). Maximum liver Vtg concentrations were between 1.87 ng/μg protein and 4.85 ng/μg protein at 3 and 10 mg/L PFOA exposures, respectively. Liver Vtg concentrations of males exposed to 3 and 10 mg/L PFOA after 28 d were significantly increased compared to controls. However, liver Vtg concentrations of males exposed to 30 mg/L PFOA after 28 d were significantly decreased compared to exposure to 3 and 10 mg/L PFOA treatments, which may be due to the damage of the livers in males. There was a subsequent decrease in liver Vtg concentrations of males exposed to 10 and 30 mg/L PFOA between 14 and 28 d exposure to PFOA (Figure 5a). Liver Vtg concentrations of females ranged from 1.17 ng/μg protein in the control to 4.75 ng/μg protein in 10 mg/L PFOA exposure group for 28 d. Liver Vtg concentrations of female from 10 and 30 mg/L treatments for 14 and 28 d were significantly increased compared to controls, however the liver Vtg concentrations of females exposed to 3 mg/L for 14 d were not significant (Figure 5b).

There were many evidences that estrogen compounds could induce the liver Vtg of males in fishes. Zha et al. (*35*) reported that plasma Vtg in male rare minnows exposed to different

concentrations of EE2 and NP were significantly higher than males in the control. A similar result was also obtained in a study that exposed rare minnow for 118 d to different diethylstilbestrol (DES), and the level of Vtg induction in whole body homogenates increased significantly, almost the same as that of the females in the reproductive phase (*32*). Harries et al. (*40*) reported that male fathead minnows exposed to 48.8-58.6 μg/L NP had plasma Vtg levels 4000-5000 times higher than males in control. Pawlowski et al. (*41*) reported that the level of Vtg in plasma of mature male and female fathead minnows exposed to 1 ng/L EE2 was significantly elevated. Using Vtg protein analysis, Kang et al. (*42*) reported that hepatic Vtg levels were significantly increased in male medaka exposed to 50.9 μg NP/L for 21 d. On the other hand, Tabata et al. (*43*) observed markedly increases in the serum Vtg concentrations of mature male medaka after 28 d of exposure to 1000 μg bisphenol A (BPA)/L. Kang et al (*44*) demonstrated that concentrations of liver Vtg increased significantly in male medaka in the reproductive phase treated with BPA at 3120 μg/L for 21 d. However, the Vtg concentrations in males did not always increase with the increase of exposure concentrations. For example, in this study, there was a subsequent decrease in liver Vtg concentrations of male rare minnows exposed to 10 and 30 mg/L PFOA between 14 and 28 d exposure to PFOA. It may be due to the damage of the livers of male rare minnows at higher exposure concentration of PFOA, or it is likely there is a maximum level of liver Vtg concentrations can be reached in livers of male rare minnows. In both male and female zebrafish exposed to higher EE2 concentrations with longer treatment time, plasma Vtg concentrations also showed a decrease (*39*).

To date, studies on endocrine disrupting capacities of fluorinated compounds are little. Lau et al. (*18*) highlighted that fluorinated compounds caused the disturbances of the thyroid gland. Maras et al. (*45*) found that 6:2 fluorotelomer alcohols 1H, 1H, 2H, 2H,-perfluorooctan-1-ol (FTOH) and 1H, 1H, 2H, 2H,-perfluoro-decan-1-ol (8:2 FTOH) behave like xenoestrogens *in vitro*, but PFOA seemed not to possess estrogen-like properties. While *in vitro* assays may not perfectly reflect the true *in vivo* response of fish to the same compound. Oakes et al. (*18*) reported that Vtg in immature rainbow trout was elevated in both sexes following exposure to 3 mg/L perfluorooctanesulfonate (PFOS), a structurally similar chemical of PFOA for 12 d, whereas no significant changes in plasma Vtg concentrations were observed in adults following PFOS exposure.

The structure of PFOA is not similar to that of estrogen; therefore, it can not act as estrogen mimics. However, our data have clearly confirmed the estrogenic action of PFOA, even though it is not clear how the endocrine system is disrupted by PFOA. PFOA may change the ratio of endogenous 17β-estradiol (E2):testosterone (T), which created an estrogenic milieu and have profound biochemical, physiological, reproductive and developmental consequences. First, an increase in the level or activity of aromatase, which catalyses the conversion of T to E2, could lead to elevated levels of endogenous estrogen and decreased T levels. Second, the impaired metabolism of E2 could also conceivably lead to unbalanced sex hormone ratios in key organs or tissues. Either of these mechanisms could lead to enhanced production of Vtg and a positive response shown in this study.

In mammals, PFOA exposure has been shown to induce aromatase cytochrome P450 activity, with the consequent reduction of T concomitant with increase in E2 (*46*). In teleosts,

although mature male fathead minnows exposed to 0.3 mg/L PFOS for 21 d exhibited decreased aromatase activity and elevated concentration of T, little evidences of increased aromatase were seen in fishes exposed to PFOA. Circulating T was significantly reduced in both male and female fathead minnows at PFOA concentrations above 1.0 mg/L, while circulating E2 was altered with PFOA exposure, but with significant reduction at concentrations above 30 mg/L in female fathead minnows (*19*). Testosterone was reduced equally in both sexes of fathead minnow, suggesting that PFOA affected oocytes in females in much the same way as Leydig cells in males. PFOA may also impair steroidogenesis by limiting cholesterol (precursor molecule for steroid production) availability, as has been shown in rats (*47*). Impairments in specific cholesterol-transporting proteins have been attributed to free radicals produced by enzyme systems, such as fatty acyl-CoA oxidase (FAO) (EC1.3.99.3).

In summary, PFOA has been demonstrated, for the first time, to increase significantly liver Vtg concentration and induce testis-ova gonad in male rare minnows. However, only relatively high exposure concentrations of PFOA (10 and 30 mg/L for 14 d; 3 and 10 mg/L for 28 d) induced testes-ova and caused severe lesions of livers and gills (data no shown). This laboratory studies suggests that PFOA should be considered as a candidate of estrogenic-like compounds with the potential to induced hepatic estrogen-responsive genes in male rare minnow, although the mechanism via which steroid profiles are altered is uncertain, and the mechanism of the development in the test-ova in rare minnow with PFOA exposure is unknown. Further research in this area would be desirable, with particular attention given to the status of enzymes involved in steroid metabolism. These data combined with other

reported data indicate that rare minnow provides a good model for assessing endocrine disruption by environmental estrogens.

Figure 1. Rare minnow gonads in the control. (a) Mature testis showing seminiferous tubules (ST) and sperm ducts (SD) were filled with sperm (× 40). (b) Mature ovary showing various stages of oocyte development (× 40). CO, cortical-alveolus stage oocyte; PO, primary-growth stage oocyte; VO, vitellogenic stage oocyte.

Figure 2. Ovaries of mature female rare minnows exposed to PFOA for 28 d, showing the degeneration of vitellogenic stage oocyte (atresia). (a) 3 mg/L PFOA (× 40). (b) 10 mg/L PFOA (× 100). (c) 30 mg/L PFOA (× 100). PO, primary-growth stage oocyte; VO, vitellogenic stage oocyte.

Figure 3. Testes-ova of male rare minnows exposed to PFOA for 28 d. (a) 10 mg/L PFOA, showing seminiferous tubules (ST) were filled with sperm and scattered primary-growth stage oocytes (PO and arrows) (× 200). (b) 10 mg/L PFOA, showing more developed PO with some earlier developmental oocytes (arrows) (× 400). (c) 30 mg/L PFOA, showing seminiferous tubules (ST) and sperm ducts (SD) were filled with sperm and scattered primary-growth stage oocytes (arrows) (× 100).

Figure 4. (a, b) A representative agarose (1.8%) gel image of amplification products of vitellogenin (Vtg; molecular size 364 base pairs) and β-actin internal standard (molecular size 272 base pairs) in individual male rare minnows exposed to PFOA. C♀: female control; C♂: male control; M: 1 kilobase DNA marker, (a) exposed for 14 d, (b) exposed for 28 d. (c) Graphical analysis of the relative band intensities (pixel densities) in male controls and males exposed to 3, 10 and 30 mg/L PFOA, digitized pixel density values were calculated as Vtg/(β-actin), expressed as means ± S.D. The asterisks denote significant difference from the control at $p < 0.05$. The error bars indicate standard

deviation of the mean. (d) Real-time quantitative PCR analysis of Vtg gene in the female liver of rare minnow. Expression levels in the liver of female rare minnows exposed to PFOA for 14 d and 28 d were quantified using real-time quantitative PCR. Bars represent the fold change of exposed versus control. Individual total liver RNA six fish was used, β-actin was used for normalization. Measurements were repeated three times, the asterisks denote significant difference from the control at $p < 0.05$. The error bars indicate standard deviation of the mean. For figure 4 (c, d) and figure 5, numbers of fish are given in parentheses.

Figure 5. Vitellogenin (Vtg) concentrations in mature adult rare minnows (*G. rarus*) exposed to PFOA. Values are means ± S.D. The asterisks denote significant difference from the control at $p < 0.05$. The error bars indicate standard deviation of the mean. (a) in males, (b) in females.

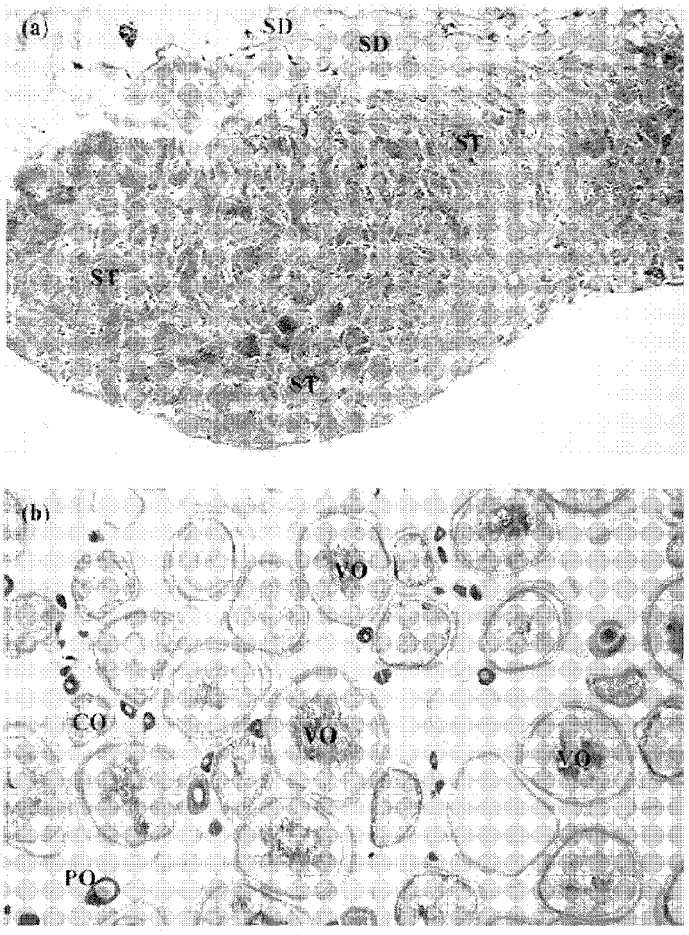
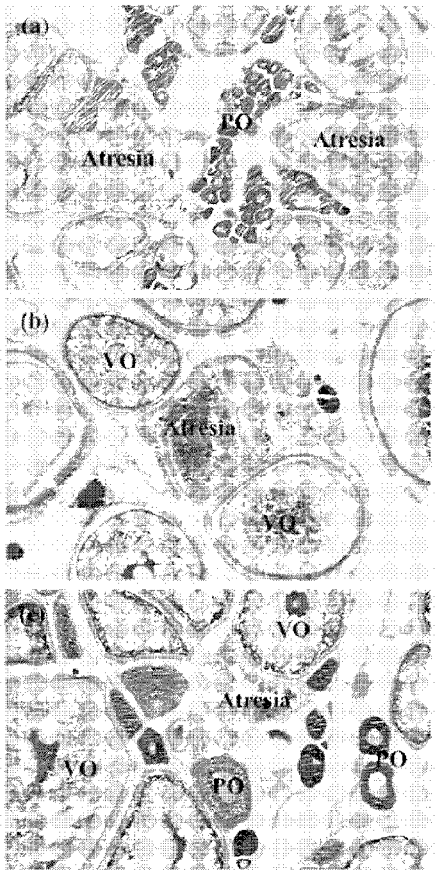


Figure 1



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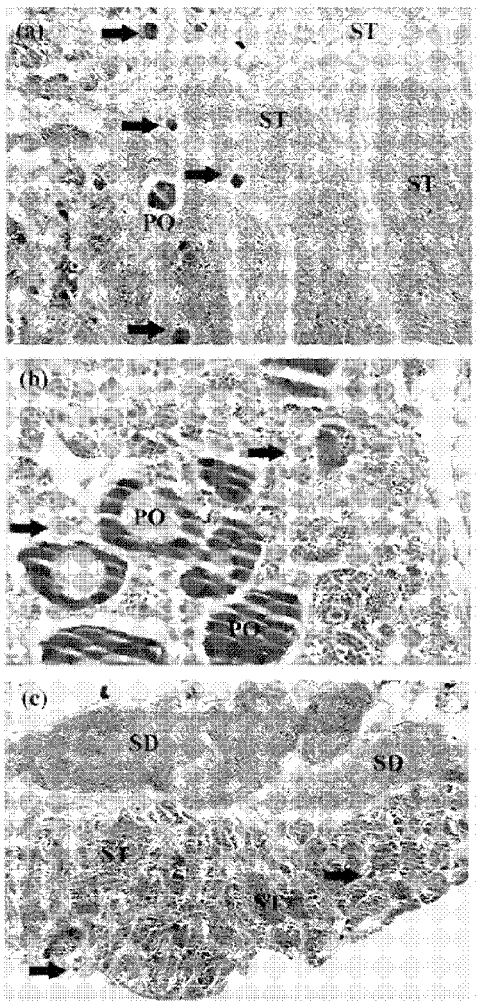
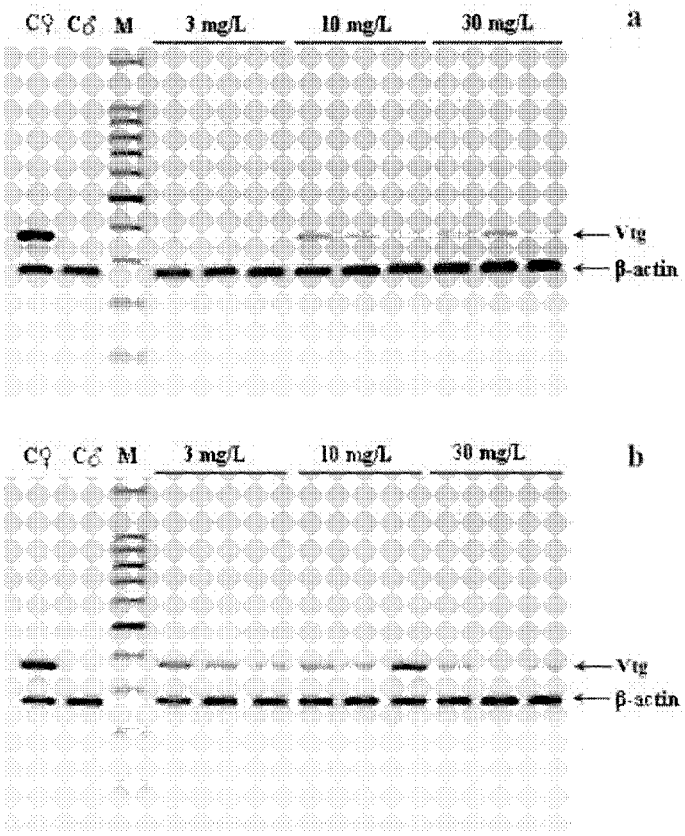


Figure 3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a
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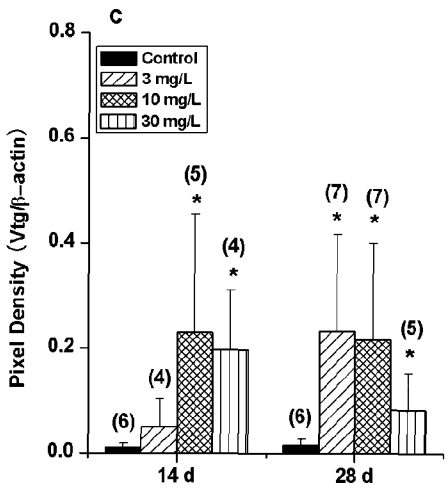
Figure 4 (a, b)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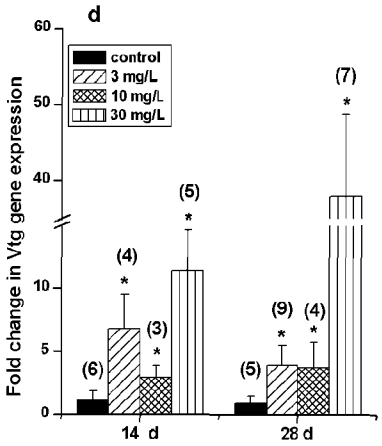
d
control
3 mg/L
10 mg/L
30 mg/L
Fold change in Vtg gene expression
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50
40
10
5
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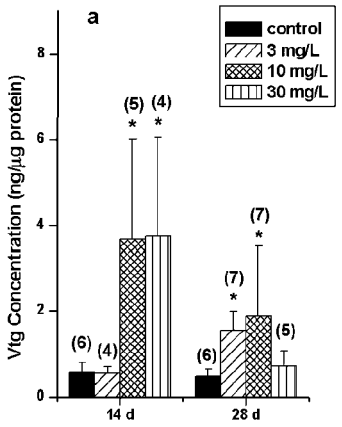
Figure 4 (c, d)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control
3 mg/L
10 mg/L
30 mg/L
Vtg Concentration (ng/μg protein)
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2
4
6
8
(6)
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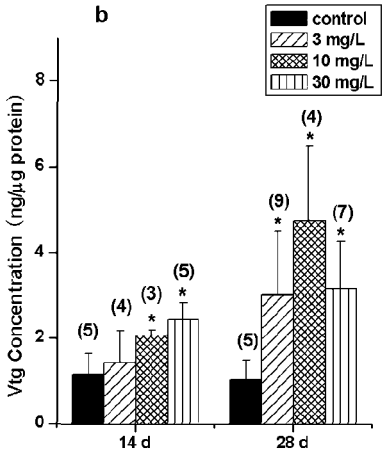
(6)
(7)
(7)
(5)
14 d
28 d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10 mg/L
30 mg/L
Vtg Concentration (ng/μg protein)
0
2
4
6
8
(5)
(4)
(3)
(5)
(5)
(9)
(4)
(7)
14 d
28 d

Figure 5

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