Reproductive System & Sexual Disorders: Current Research

Feng et al., Reprod Syst Sex Disord 2017, 6:2
DOI: 10.4172/2161-038X.1000208

Research Article OMICS International

Toxic Effects of Atrazine on Reproductive and Immune Systems in Animal Models

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Received date: June 08, 2017; **Accepted date:** June 28, 2017; **Published date:** July 10, 2017

Abstract

Background: Each year massive amounts of over 70,000 chemicals are dumped into the sea. For many of the chemicals, the consequences of it coming into contact with marine life. For such reasons, there was a large die off of American lobsters in the Long Island Sound. Atrazine is one of the most widely used herbicides, and detected pesticide contaminant of ground water in the USA, which is linked to hormonal dysregulation of animal/human reproduction. However, Atrazine's effects on invertebrate reproduction and other systems are still unknown.

Objective: This study aimed to evaluate the effects of Atrazine on hemocytes, sperm motility and the androgen receptor, which are closely related to reproductive and immune systems.

Methods: In this study, we utilized marine mollusk and bovine sperm as animal model because the large die off of lobsters could be due to the use of large amounts of herbicides.

Results: Our results demonstrated that increasing level of atrazine significantly reduced sperm motility, and this chemical has the same effect on bull sperm as well. We firstly discovered that human androgen receptor like protein is present in both sperm and testicular tissue in mussels. Incubation of atrazine with mussel's testicular tissues significantly down regulated androgen receptor expression in target Leydig cells. Furthermore, to investigate its effect on immune tissues and observed that increasing concentration of atrazine increase the death of hemocytes (mussel immune cell) significantly, and it has similar effect on human immune cells. Thus this widely used herbicide has toxic effect on both reproductive and immune system of mollusk.

Conclusion: Atrazine significantly reduced mollusk sperm motility and bull sperm motility as well. Human androgen receptor like protein was firstly discovered in both sperm and gonad tissue of blue mussels. Incubation of atrazine with mussel's testicular tissues significantly down regulated androgen receptor expression in target Leydig cells. Furthermore, increasing concentration of atrazine increased the death of hemocytes (mussel immune cell) significantly, and it has similar effect on human immune cells. Thus this widely used herbicide has toxic effect on both reproductive and immune system of mollusk. However, the mechanism of endocrine disrupting chemicals (EDCs) impact on reproduction and immune systems needs to be further investigated.

Keywords: Atrazine; Mussel; Bovine; Testis; Sperm; Hemocytes

Introduction

Each year massive amounts of over 70,000 chemicals are dumped into the sea. For many of the chemicals, the consequences of it coming into contact with marine life may cause unforeseen consequences and may lead to the destruction of marine life. During the summer and fall of 1999 there was a large die off of American lobsters in the Long Island Sound. Lobster population in some parts of the Sound fell by almost 100%. The likely cause of the large die off of lobsters could be due to the use of large amounts of herbicides [1]. The herbicide's impact on the lobster population is still not fully clear, but the herbicide could have possibly damaged the reproductive system of the lobsters. Previous studies shown that a commonly used herbicide, Atrazine, disrupts and damages the reproductive system across vertebrate classes [2-6]. These previous studies also found that Atrazine causes a reduction in androgen and an induction of estrogen within vertebrates [7,8], but few researchers have tested the effects and toxic concentrations of Atrazine on invertebrates [9].

In this research project, the model organism *Mytilus edulis*, mussel, will be used to test the effects of Atrazine. Like lobsters, *Mytilus edulis* are invertebrates and serve as an excellent model organism for this study. Additionally, few experiments look at the effects of Atrazine on the androgen and estrogen receptors, which are very important in the reproductive system. Androgen receptors are linked to spermatogenesis, the process of creating sperm, and estrogen receptors are linked to oogenesis, the process of creating eggs, and also assist in spermatogenesis [10,11].

Atrazine has also been found to cause mitochondrial dysfunction within cells, and this could be due to the production of nitric oxide by Atrazine which then inhibits the production of ATP in dose-response

manner [12]. Because of this, it is possible that Atrazine may cause mitochondrial dysfunction within sperm cells and thus cause apoptosis which then causes infertility within animals. This study tests the effects of Atrazine on reproductive and immune tissues of both mollusk, and human cells. If there is a large decrease in the amount of living cells then it could also be inferred that Atrazine could be causing apoptosis within sperm cells, and causing infertility as well.

Materials and Methods

Atrazine preparation

Atrazine was purchased from Sigma Aldrich USA. About 1 mg of atrazine was mixed with 1000 microliters of ethanol. Then this was further diluted with PBS for experimentations described in the method.

Mussel's sperm preparation

To purchase mussels (Mytilus edulis) that were harvested from Long Island (LI) Sounds from LI bay. The mussels' testicular tissues were dissected by syringe-needle in culture medium (HTF plus 10% serum), the sperm was released into culture medium, soaked supernatant tissue liquid to glass slide and dried by air, and they were ready for use.

Mussels' testicular tissue preparation

The mussel's testicular tissues were obtained, and then soaked in 1 mL of PBS (Phosphate-buffered saline) without Atrazine (control group) and with Atrazine (stock solution, 4.6×10^{-3} M in ethanol) diluted at 10X, 100X and 1000X for one hour and 24 hrs at 4°C. The resulting concentrations of 10 ul of atrazine treatment in 1 ml of PBS solution was 4.6×10^{-5} M for the stock solution, 4.6×10^{-6} M for 10X, 4.6×10^{-7} for 100X, 4.6×10^{-8} for 1000X. All testicular tissues were cut by frozen section at 5 μm by using frozen-microtome.

Androgen receptor (AR) immunochemical staining

The sperm slides and the testicular tissues frozen sections were fixed in canoy's solution (methanol: glacial acid 3:1) for 15 minutes, then after washed three time via PBS. The slides' specimen were blocked using block solution either from kit or 10% serum in PBS for one hour, spill out blocking solution in experimental slides, then put on 1:50 androgen receptor antibody (Santa Cruz, CA, USA) for O/N at 4°C, respectively. Control slides were kept in block solution without primary antibody. After that, slides were dropping three times by dropping PBS. The slides were stained by Zymed kit (Life Corp, CA, USA) following manufacture instruction. The slides were observed under computerized compound microscope for androgen receptor localization and expression in sperm and testicular tissues. Experiment was repeated for 3 times.

Atrazine toxic assay in bull sperm

Frozen bull semen was purchased from Applied Reproductive Technology Company (Madison, WI,USA), then thawed and centrifuged over gradient medium (90% Enhance-S-Plus; Conception Technology, Nashville, CA, USA) for 10 min at 500 g. The supernatant was then discarded and the pellet washed with 3 ml fertilization medium. Following centrifugation for 5 min at 500 g, the supernatant was discarded. The pellet was re-suspended in 0.5 ml fresh fertilization medium and incubated for 15 min in a humidified 5% CO_2 air atmosphere at 38.5°C, and then it was ready for use. The experiments have been divided into four groups depended upon whether containing Atrazine, they were: 1). Control group, organ culture dish contained 0.5 ml fertilization medium; 2). 10X group organ culture dish contained 0.5 ml fertilization medium with 10X dilution of Atrazine stock solution (10^{-3}M in ethanol); 3). 100X group organ culture dish contained 0.5 ml fertilization medium with 100X dilution of Atrazine stock solution; 4). 100X group organ culture dish contained 0.5 ml fertilization medium with 100X dilution of Atrazine stock solution. Put 200 μl sperm into different group's culture dishes, respectively. The sperm motility and progression were evaluated by using sperm Makler Counting Chamber under inverted microscope after incubation at 38.5°C, 6% CO_2 and 5% O_2 for 1 hr and 24 hrs. Experiment was repeated for 3 times.

Mussels hemocytes preparation and treatments

Hemocytes were extracted from the duct muscles of mussels. Three groups of 50 microliters of hemocytes, 150 microliters of PBS and 10 microliters atrazine treatment were made. Then the samples were incubated at room temperature for 1 hour and 2 hours. After incubation, 5 microliters of trypan blue were added to 45 microliters of hemocytes. Then, 10 microliters of the sample was loaded onto a hemocytometer for a cell viability test. This experiment was further repeated twice with 5 groups of treatment including the 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M and the control, and was also loaded onto a hemocytometer.

Treatment of human U937 cells

200 microliters of U937 cells (human immune cells line) with medium were pipetted into 3 test tubes each. 10 microliters of 10X (0.46 mM) and 100X (0.046 mM) concentration atrazine were pipetted into 2 test tubes. The samples were incubated at 32 degrees Celsius for 1 hr. After incubation, 5 microliters of trypan blue were added to 45 microliters of U937 cells. Then, 10 microliters of this mixture was loaded onto a hemocytometer for a cell viability test. Experimentation repeated for 3 times.

Mussels hemocyte viability test

Mussel hemolymph was extracted from the adductor muscles of the mussels. The mussel hemolymph was aliquoted into 8 groups. The 8 groups were control, 4.6×10^{-5}M, 4.6×10^{-6}M, 4.6×10^{-7}M, 4.6×10^{-8}M pure atrazine dilution, and atrazine product purchase in Homedepot $4.6 \times$ 10-5M, 4.6×10^{-6}M, 4.6×10^{-7}M, 4.6×10^{-8}M. ATR stands for the Southern AG brand of weed killer with atrazine and additional ingredients where as AZ stands for the pure substance of Atrazine. 100 microliters of the hemolymph was put in each tube and 10 microliters of treatment were put in with the hemolymph. The eight tubes were put in the refrigerator for one hour incubation. Then, a cell viability test was performed with trypan blue and a hemocytometer. The trypan blue indicated which cells were dead and alive. The dead cells were colored blue since their cell membranes could not keep the dye out of the cells while the alive ones were not colored. Using a hemocytometer, the numbers of alive and dead cells were counted and then a mortality rate was calculated. Experimentation repeated for 3 times.

Results

Androgen-receptor expression and localization in mussel sperm

In this study, it demonstrated that presence of a functional AR in sperm. Preliminary data suggested that the expression and immunolocalization of AR in mussel sperm using an immunochemical staining technique, the results showed a positive signal for AR in mussel sperm (Figure 1A). No immunoreaction was detected by adding anti-AR antibody peptide by immuno precipitation (Figure 1B) or when the primary antibody was omitted (data not shown). AR immunoreactivity was specifically located at the sperm head (Figure 1A).

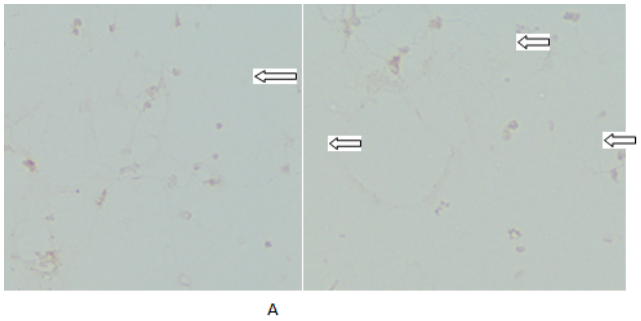

Figure 1A: Androgen receptor expression and localization in mussel sperm. Arrow indicated AR positive staining in sperm head.

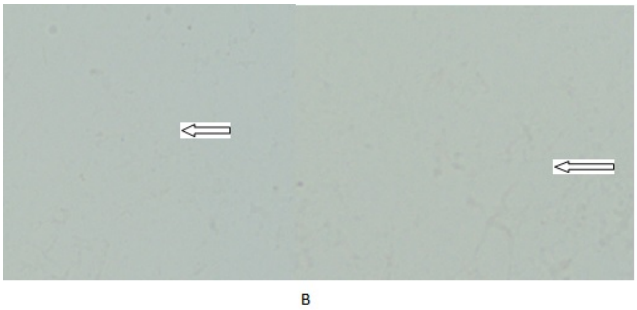

Figure 1B: Androgen receptor expression and localization in mussel sperm. Arrow indicating negative staining in sperm.

Androgen receptor (AR) expression and alteration in mussel testicular tissues

The immune-chemistry staining results demonstrated that only highly concentration Atrazine at (10^{-5}M) significantly decrease AR activity and decline its expression with less staining in the Leydig cells of mussel testicular tissues after incubation for 24 hours at 8°C temperature (Figure 2A) as comparing with testicular tissues from control group, while other diluted groups did not see significant difference in AR expression after soaking for 1hr and 24 hours, which were similar to control group with strong AR staining (Figure 2B). The staining control slides did not show positive staining (Figure 2C).

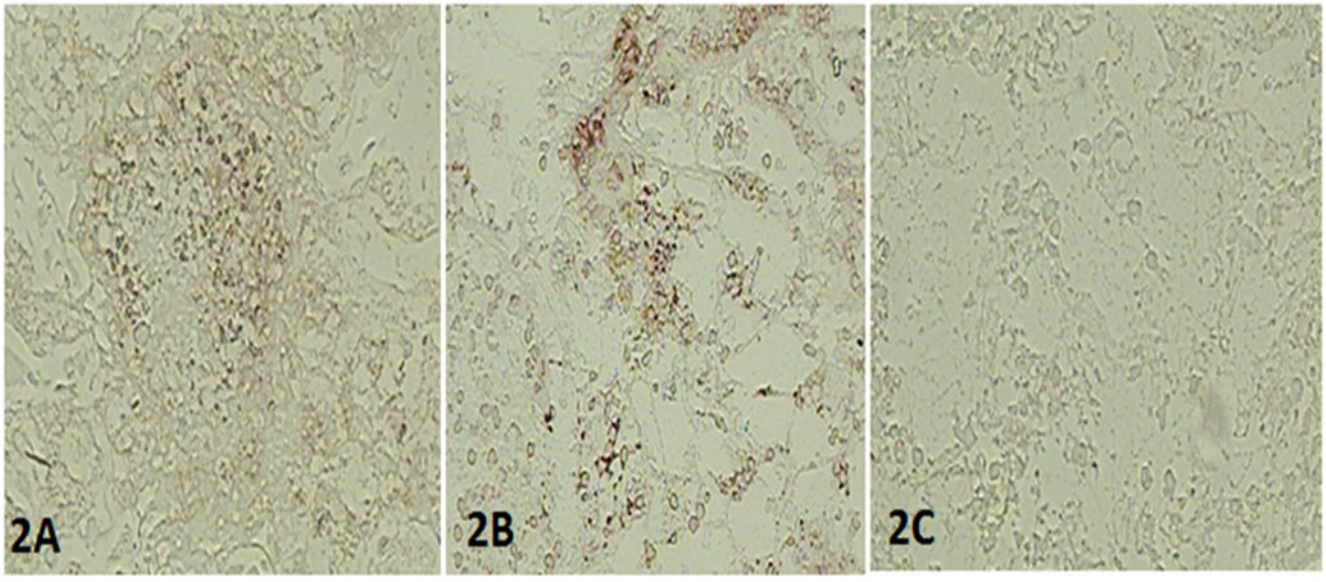

Figure 2: Androgen receptor staining in the Leydig cells of mussel testicular tissues that affected by 24 hour incubation with Atrazine (2A) comparing with control group (2B). The staining control slides did not show positive staining (2C).

Atrazine toxic assay in mussel sperm

The data suggested that the Mussel sperm was significantly killed after culture for 24 hours at fertilization culture medium with 10^{-5}M and 10^{-5}M Atrazine dilutions, which was significant difference as compared with control group ($P<0.01$). The 10^{-7}M dilution group had lower sperm motility and slow progression as comparing with control group after culture for 24 hours (Figure 3).

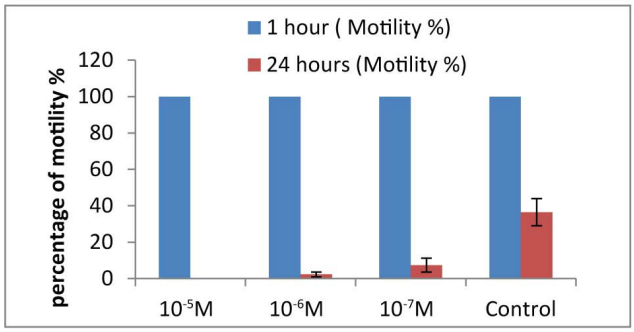

Figure 3: Mussel sperm motility effect by various concentration of Atrazine.

Atrazine toxic assay in bull sperm

Very similar to the mussel sperm, bull sperm was significantly killed after culture for 24 hours at fertilization culture medium with various concentration of Atrazine, which was significant difference as compared with control group ($P<0.01$). The 10^{-7}M dilution group had lower sperm motility and slow progression as comparing with control group after culture for 24 hours (Figure 4).

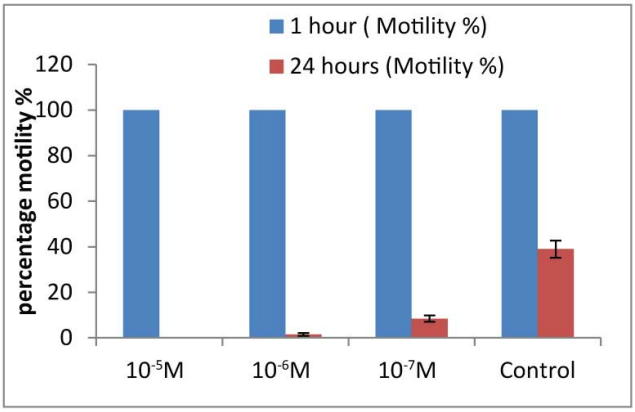

Figure 4: The motility of bull sperm after culture with and without Atrazine.

Atrazine toxic assay in mussels hemocytes

The control after 1 hour had a mortality percentage of 18.81%, the 10^{-8}M ATR group had 23.12% dead, the 1,000X ATR had 29.31% dead, the 100x ATR had 31.63% dead, the 10X ATR had 38.36% dead, the 0.0046 mM AZ had 26.67% dead, the 0.046 mM AZ had 41.48% dead, the 0.46 mM AZ had 37.41% dead. As shown in the graph, the mortality rate significantly increased ($P<0.05$) as the concentration increased for both ATR (at 1000X, 100X, 10X) and AZ (at 0.046 mM and 0.46 mM) as compared with control group. The only exception to this was with 0.46 mM AZ which had a lower mortality rate than 0.046 mM AZ, but there was still significant difference as compared with control ($P<0.05$) (Figure 5).

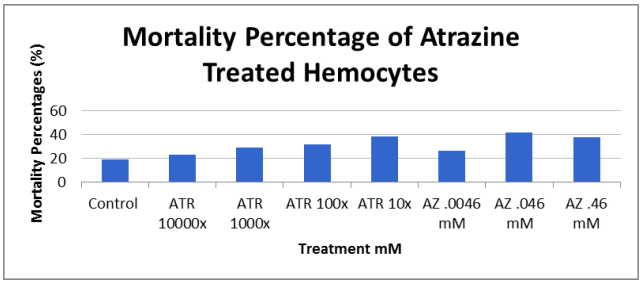

Figure 5: Increasing concentration of Atrazine and Atrazine commercial products significantly kill the hemocytes.

Atrazine toxic assay in human U937 cells

Increasing concentration of Atrazine significantly kill the human immune cells. The control group after 1 hour of treatment had a mortality percentage of 42.38%, the 0.046 mM AZ had group had a mortality percentage of 59.13%, and the 0.46 mM AZ group had a mortality percentage of 72.55%. As shown in the graph, the mortality percentage of the U937 monocyte cells significantly increased as the AZ concentration increased at 0.46mM ($p<0.05$) (Figure 6).

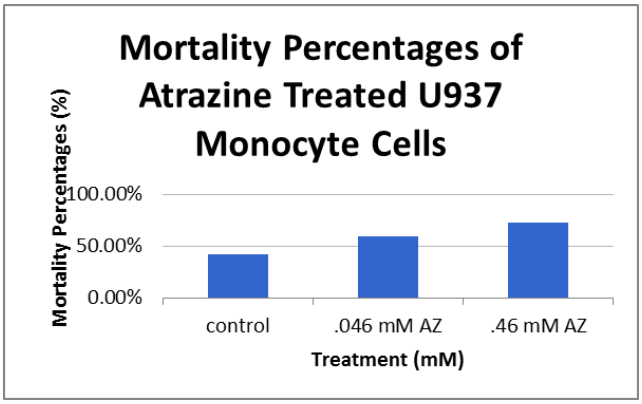

Figure 6: Increasing concentration of Atrazine effect on human U937 monocyte cells.

Discussion

In vertebrate reproductive endocrinology sex steroids play a pivotal role via binding to receptors. However, information on the origin and relevance of sex steroids and receptor in invertebrates is limited.

To our knowledge, this is the first report that indicates that mollusk sperm and gonad tissue express androgen receptors. Previous studies show that 17-beta-estradiol and an estradiol isoform is present in *M. edulis* gonadal tissues [13], suggesting that they have functions related to reproduction. RT-PCR and sequence analysis of *Mytilus edulis* tissue was found to express a 266 bp fragment of the estrogen receptor-beta gene, which exhibits 100% sequence identity with the human counterpart [14]. This further suggests that estrogen's association with reproductive activities has a long evolutionary history and that this

association began in invertebrates. Our results demonstrated that androgen receptor signaling mechanism is also present in the mollusk.

Importantly, our results show that atrazine significantly inhibited motility of sperm from both mussels and bull. This indicates the molecular target for the atrazine would be very similar, and control of sperm motility has been conserved during evolution. Atrazine incubation with mussel gonad tissue also significantly down regulates androgen receptor staining by comparing with control. Therefore, this chemical can also disrupt androgen receptor signaling mechanism, and consequently impact on sperm motility, affect reproductive function. Also, atrazine may decline sperm motility and kill sperm via damage spermatozoa mitochondria, because sperm mitochondria are the ATP source for sperm movement [12].

In vertebrates, androgen and androgen receptors play a key role in male fertility. The androgen receptor (AR), also known as NR3C4(nuclear receptor subfamily 3, group C, member 4), is a type of nuclear receptor [13] that is activated by binding either of the androgenic hormones, testosterone, or dihydrotestosterone [14] in the cytoplasm and then translocating into the nucleus. The main function of the androgen receptor is as a DNA-binding transcription factor that regulates gene expression [15]. However, the androgen receptor has other functions as well [12]. Androgen regulated genes are critical for the development and maintenance of the male sexual phenotype [13]. From our results and others, we surmise that atrazine may interfere with the ligand-dependent transcriptional function for the biosynthesis, metabolism or action of endogenous androgens resulting in a deflection from normal male developmental programming and reproductive tract growth and function. Since male sexual differentiation is entirely androgen-dependent, it is highly susceptible to androgen-disruptors. Animal models and epidemiological evidence link exposure to androgen disrupting chemicals with reduced sperm counts, increased infertility, testicular dysgenesis syndrome, and testicular and prostate cancers [16]. Studies have raised the possibility that endocrine disrupting chemicals (EDCs) may be contributing to a decline in the human sperm count that has been observed over the last 50~60 years [17,18]. Furthermore, it has been shown that some pesticides can act by reducing androgen receptor expression [19,20].

Previous studies suggested that Atrazine affected testicular Leydig cell androgenesis via the inhibition of steroidogenesis gene expression, leading to change in spermatogenesis [21]. In human study, the result suggested that AR expression in human sperm and localized in sperm middle piece reveals distinct roles of this receptor in the physiology of sperm and, perhaps, also in the process of fertilization [22]. Furthermore, this indicates that AR could be related to mitochondria of sperm, in where the glycolytic production of ATP is required for sperm motility and hyperactivation. Therefore, AR may be indirectly involved in the regulation of their motility [23]. Human sperm express a functional AR that has the ability to modulate the PI3K/AKT pathway, on the basis of androgen concentration [24].

Since Atrazine reduced viability of both invertebrate hemocyte and human immune cells, we speculate that this chemical may target mitochondria of these cells. Further experiments are needed to investigate the chemical's effect on which passways cause mitochondria dysfunction. Recent study shown that Atrazine may interrupt nitric oxide signaling system, which plays very important role in maintain mitochondria energy metabolism [12].

In summary, atrazine is one of the most widely used herbicides in the U.S., and is the most commonly detected pesticide contaminant of ground water. Many studies have demonstrated exposure to atrazine is linked to hormonal dysregulation of animal/human reproduction, especially on female and more recently on male. However, few study has investigate its effect on invertebrate reproduction. In this research project, we utilize marine mollusk as animal model because previous study shown the presence of estrogen signaling mechanism. From the experiment with mussel sperm, we observed that increasing level of atrazine significantly reduced sperm motility, and this chemical has the same effect on bull sperm as well. We further discovered that human androgen receptor like protein is present in both sperm and gonad tissue of blue mussels. Incubation of atrazine with mussel's testicular tissues significantly down regulated androgen receptor expression in target Leydig cells. We further investigated its effect on immune tissues and observed that increasing concentration of atrazine increase the death of hemocytes (mussel immune cell) significantly, and it has similar effect on human immune cells. Thus this widely used herbicide has toxic effect on both reproductive and immune system of mollusk. Since both reproductive and immune system are evolutionary conserved in mussel and human, the use of atrazine and other chemicals with hormonal dysregulation effect has to be strictly regulated for the sake of animal and human health. The mechanism of endocrine disrupting chemicals (EDCs) impact on reproduction and immune systems needs to be further investigated.

Acknowledge

We want to thank Dr. Song and Dr. Tony Tsai's technical supports in their research laboratories.

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