Effects of in ovo Exposure of Imazalil and Atrazine on Sexual Differentiation in Chick Gonads

Junko Yamashita[1], Sachiro Matsushita[2], Toshiyuki Iwasawa[2], Moriji Ikeya[2], Masahiko Ikeda[1]

[1]University of Shizuoka, Shizuoka
[2]Shizuoka Swine & Poultry Experiment Station, Kikugawa

Introduction

In contrast to mammals, the heterogametic sex (sex chromosome: ZW) in avian species is the genetic female whereas the homogametic (sex chromosome: ZZ) is the genetic male. The W chromosome positively controls early aromatase synthesis and consequently estrogen production. The presence of estrogens and their receptors plays a crucial role in female sexual differentiation. Chicken embryonic gonads are bipotential at an early stage. During development of the female, the left gonad differentiates to a single ovary/oviduct, and the right gonad regresses, developing a permanent female phenotype. This sexual differentiation occurs as a result of aromatase expression in the left gonad at day 6.5 and the production of estrogen from testosterone. In the male genotype, both gonads develop into two testes [1]. The time- and sex-dependent expression of enzymes involved in steroidogenesis, which determine the ratio of androgens/estrogens produced by the gonads, has been extensively investigated during the last 5-6 year. These results show that the lack of estrogen synthesis in the male appears to be due to the extremely low levels of P450 aromatase expression. In female, extensive expression of the aromatase gene (around day 5-6 incubation), leading to estrogen synthesis, and specific expression of the estrogen receptor-mRNA in the left gonad result in the development of a functional left ovary. Experimental sex reversal has been performed using anti-estrogens, androgens, aromatase inhibitors and synthetic steroid. Differences between male and female gonadal differentiation and development are depended on the absence of aromatase and estrogen. On the one hand, differences between left and right ovarian development are depended on the specific expression of the estrogen receptor in the left gonad [2].

Persistent chlorine-containing pesticide, imazalil is structurally similar to various imidazole-containing chemicals used clinically such as the potent aromatase inhibitor, fadrozole and numerous anti-fungal chemicals. These chemicals have shown to reversibly (although not necessarily competitively) inhibit aromatase activity in human placental microsomes. It is reported that imazalil and difenoconazole inhibit aromatase activity in human adrenocortical carcinoma cell line H295R [3].

Atrazine is the most commonly used herbicide in the word. There are several reports about the adverse effects of atrazine exposure. Atrazine induced hermaphroditism in African clawed frogs and demasculinized the larynx in male frogs [4]. Plasma testosterone concentration in male frogs was decreased by atrazine exposure, and plasma estradiol concentration in rats was increased by

atrazine exposure. Atrazine also increased aromatase activity in human adrenocortical carcinoma cell line H295R by inducing aromatase mRNA [3].

In this study, the effects of *in ovo* exposure to an aromatase-inhibiting chemical (imazalil) and an aromatase-activating chemical (atrazine) on the sexual differentiation of chick gonad were investigated.

Methods

***In ovo* exposure and animal treatments:**

Rhode Island Red and Plymouth Rock were obtained from Hoshino Poultry Breeding Farm (Shizuoka, Japan) and maintained in Shizuoka Swine & Poultry Experimental Station under natural sun light (approximately 11 hr light-13 hr dark). Rhode Island Red males were crossed with Plymouth Rock females to obtain fertile eggs of autosexing chicks. Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1-imidazole) and atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine) were dissolved in 50 µl of propylene glycol and injected into the egg white on day 0 using 1ml syringe with a 23G needle. The eggs were incubated at 37.6°C in a relative humidity of 53% in a SHYOWA FURANKI incubator (model AH3). The eggs were automatically turned once per hour. At hatching, genotype sex was determined by the color of determined by the color of the head feathers, legs and mandible. The chicks were anesthetized with diethyl ether and blood was collected from heart. Gonads were stereomicroscopically observed to determine phenotype sex and taken picture. The gonads were dissected under a stereomicroscope and fixed in 10% formalin neutral buffer solution for two weeks. The gonads were embedded in paraffin and 5 µm of paraffin sections were prepared. Each section was stained with hematoxylin and eosin for histological analysis.

Assay for aromatase activity:

Chick ovary from control female was homogenized in phosphate buffer and centrifuged for 10 min at 1,900 g. Supernatant was collected and used for enzyme solution. Enzyme solution was incubated with imazalil for 10 min at 37°C and then aromatase activity was measured in terms of released ^3H_2O from [^{3}H] androstenedione as reported previously [5]. Briefly, enzyme solution was incubated with potassium phosphate buffer containing 1β-[^{3}H]-androstendione, NADPH, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase for 60 min. Reaction was terminated by an addition of $CHCl_3$. Aqueous layer was treated with charcoal in Dextran T-70 and radioactivity was measured using liquid scintillation counter.

Imazalil or atrazine exposed chick ovary was homogenized in phosphate buffer and centrifuged for 10 min at 1,900 g. Aromatase activity of the supernatant was measured as described above.

Results and Discussion

Inhibition aromatase activity by imazalil *in vitro*

Inhibitory effect of imazalil on aromatase activity *in vitro* was confirmed. Aromatase activity in chick ovary homogenate was significantly inhibited by the incubation with 10 μM of imazalil for 10 min (Figure 1). Vinggaard et al. reported that imazalil inhibited aromatase activity in human placental microsome at IC_{50}=0.04 μM [6]. Weak inhibitory effect of imazalil in our experiment will depend on the enzyme purity.

Hatchability and the size of gonad

In ovo exposure of imazalil dosed at less than 1 mg/egg did not influence the hatchability, whereas that of more than 2 mg/egg inhibited the hatchability. *In ovo* exposure of atrazine (0.1, 1, 3 mg/egg) did not influence the hatchability. The genotype sex was completely coincided with the phenotype sex in control, imazalil and atrazine exposed groups. In control females, the right gonad was completely regressed at hatching. However, the right gonad was remained by imazalil (0.01, 0.1, 1 mg/egg) and atrazine (0.1, 1, 3 mg/egg) exposed females. The size of the right gonad was increased by imazalil exposure in a dose-dependent manner. In contrast, the size of the right gonad was about one fifth of the left gonad without dose-dependent relationship by atrazine exposure. The size of the left gonad from female chicks was not changed by both imazalil and atrazine exposure. The size of testes was not also changed by both imazalil and atrazine exposure.

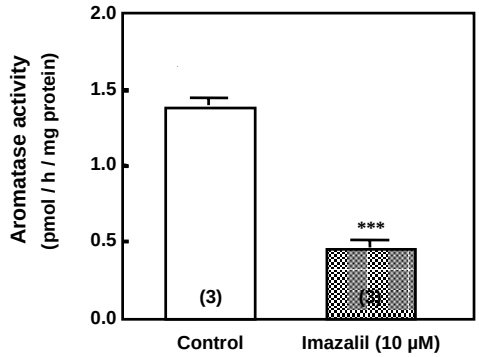

Figure 1. Inhibitory effect of imazalil on chick ovary aromatase activity.
Data represent Mean±S.D. for three determinations.
Significance: ***p<0.001 vs Control by Student's t-test.

Histochemical changes in gonads

In imazalil exposed female chicks, some remaining right gonads had testis-like structures without cortex, but its left gonad had. The other remaining right gonad had testis-like structure and its left gonad had ovary medulla structure without cortex and tubules (like seminiferous tubules). One left gonad had cortex that contained degraded cells and ovary-like medulla structures. These results suggest that imazalil inhibit female gonadal aromatase activity and partially inhibit the

differentiation of female gonads. These adverse effects of imazalil were observed in a dose-dependent manner. Aromatase activity in female left gonad was not changed by imazalil exposure, significantly. These results suggest that the expression of aromatase mRNA and the degradation of aromatase protein are not changed by imazalil. There was no change in male gonads (both left and right testis) at hatching by imazalil exposure in a stereomicroscopic observation.

In atrazine exposed female chicks, the cortex of remained right gonad was regressed completely and the medulla had ovary-like structures. The left gonad had normal ovary structures. Aromatase activity of left gonad from female chicks was not changed by any concentrations of atrazine exposure. Although the induction of aromatase activity by atrazine has reported in H295R cells [7], atrazine did not induce aromatase activity in chick ovary. These results suggest that the atrazine inhibit the regression of right gonad in female chicks. Although it is not unclear whether remaining right gonad has aromatase activity or not, right gonad will differentiate to ovary. There was no change in male gonads (both left and right testis) at hatching by atrazine exposure in a stereomicroscopic observation. Histological observation of testes is proceeding.

Conclusion

In ovo exposure to imazalil inhibits the sexual differentiation of ovary by inhibiting aromatase activity. *In ovo* exposure to atrazine influences the sexual differentiation of ovary by different mechanisms, possibly induction of aromatase in right gonad.

References

1. Shimada K. (1998) J Exp Zool. 281, 450.
2. Bruggeman V., Van As P., Decuypere E. (2002) Comp Biochem Physiol A Mo. Intergr Physiol. 131, 839.
3. Sanderson J.T., Boerma J., Lansbergen G.W.A., van den Berg M. (2002) Toxicol. Appl. Pharmacol. 182, 44.
4. Hayes T.B., Collins A., Lee M., Mendoza M., Noriega N., Stuart A.A., Vonk A. (2002) Proc. Natl. Acad. Sci. USA 99, 5476.
5. Ikeda M, Inukai N., Mitsui T., Sone H., Yonemoto J., Tohyama C., Tomita T. (2002) Environ Toxicol Pharmacol. 11, 1.
6. Vinggaard A.M., Hnida C., Breinholt V., Larsen J.C. (2000) Toxicol in Vitro. 14, 227.
7. Sanderson J.T., Seinen W., Giesy J.P., van den Berg M. (2000) Toxicol. Sci. 54, 121.