Sex reversed chicks (*Gallus domesticus*) hatched from eggs treated with aromatase inhibitor

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Abstract: The objectives of this work were to assess post-hatch development of chickens treated in ovo with the aromatase inhibitor. A total of 137 eggs coming from artificially inseminated hens were at first injected in the albumen with either DMSO alone (54 eggs injected, control group) or with DMSO + aromatase inhibitor (aromatase inhibitor 1 mg/egg, 83 eggs injected, treated group) and then incubated under standard conditions. Out of the 24 chicks hatched in the treated group, 16 were genetic males (ZZ) and 8 were genetic females (ZW). By 26 weeks of age, secondary sex characteristics of 8 females (cloaca, comb, wattles, song, feathers of hackle and tail) progressively transformed into a male phenotype.

Keywords: aromatase; inhibitor; sex reversal; chicken

INTRODUCTION

Birds exhibit a ZW/ZZ mechanism of genetic sex determination in which the female is heterogametic (ZW) and the male homogametic (ZZ). The production of estrogens, along with the presence of oestrogen receptors during the early stages of embryo development (≤ 5.5 days of incubation in chickens) play a crucial role in the phenotypic sex differentiation of females. Previous studies in the chicken indicated that genetic females can be transformed into neo-males after in ovo injection of steroidal and non-steroidal aromatase inhibitors (Abinawanto *et al.*, 1996). Based on data obtained following the injection of various types of aromatase inhibitors (Wartenberg *et al.*, 1992, Dewil *et al.*, 1998), evidence exists that the loss of aromatase function in female embryos between 7 and 14 days old leads to partial phenotypic sex reversal of adult females accompanied by the development of secondary male sex characteristics along with the occurrence of testes containing some spermatozoa (Abinawanto *et al.*, 1997). Observations on the effects of the cytochrome P450 aromatase inhibitor (P-450arom) in chicken embryos indicated that an early exposure to estrogen is crucial for the sexual differentiation of gonads in avian species (Nomura *et al.*, 1999, Elbrecht and Smith, 1992). The cytochrome P450 aromatase is responsible for the transformation of androgens into estrogens (mainly the conversion of testosterone into estradiol-17β). More recently, it was also demonstrated that genetically female embryos treated in ovo with Fadrazole (a nonsteroidian aromatase inhibitor) on day 4.5 of incubation resulted in the development of male phenotypes including the presence of more or less achieved male gonads (Vaillant *et al.*, 2001).

The purpose of this experiment was to assess neither or not inhibitor of aromatase can convert genetic females into phenotypic males.
Materials and methods

**Egg treatment.** Chicken eggs were obtained from a line of Barred Leghorn mature hens (ii, ee, B/ -). Hens were inseminated with pooled semen (100 x 10^6 spz/hen) from Minor Black strain males (-ii, EE, b/b). Both of them are homozygous recessive (ii) at the dominant white locus (I). Barred gene B is a sex-linked gene located on the non-homologous segment of Z chromosome. Therefore, genetic females issued from the above cited sex genotypes can only have a black phenotype (b-) while males can only have a barred phenotype (Bb).

From a number of 137 treated eggs, 83 were at first disinfected with ethanol (70%) and then injected (0.6x25 mm needle) with a single dose (1 mg/egg) of aromatase inhibitor YM 511 (Yamanouchi Pharmaceutical Co. LTD, Japan) diluted in 50 µl dimethyl sulfoxide (DMSO) and the remaining 54 eggs (control group) were treated with DMSO alone. Second control group was consisted from 120 non-treated eggs. All eggs were incubated under standard conditions. Egg candling was performed on Days 6 and 18 of incubation to eliminate infertile eggs or those containing dead embryos.

**Animal husbandry.** During the first 6 weeks post-hatch, all birds were fed daily with a standard starter diet K1 (ME: 2850 kCal/kg, N 19-20%) provided *ad libitum*. During the next 10 weeks all hatchlings were fed *ad libitum* with standard diet K2 (ME: 2750 kCal/kg, N 16-17%) and then with standard diet KZK (ME: 2650 kCal/kg, N 13-14%). Chicks were subjected to permanent light (100 Lux) from hatch to 6 weeks of age. They were then placed under a14L:10D (L = light; D = dark) photoperiod up to 16 weeks and finally under a 16L:8D photoperiod up to the end of the experiment.

**Determination of sex phenotype and genotype.** Phenotypical sex was determined by visual examination of secondary sex characteristics (cloaca, comb, wattles, song, feather colour and shape of hackle and tail) during the whole experiment. At 26 week of age, photographs of the comb along with a non invasive observation of the body cavity (using a CT- SIM/0600 scanner manufactured by MHTI) were also performed.

Individual blood samples were collected at 6, 16 and 20 weeks of age to perform molecular sexing. DNA extraction for PCR analysis from blood cells was performed as previously described by Trefil et al. (1999). Briefly, pellet of blood cells was at first washed twice in cell lysis solution (320 mM sucrose, 5 mM MgCl₂, 10 mMTris-HCl, pH 7.6 and 1 % of 100xTriton), then centrifuged and then resuspended in a digestion buffer (10mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂ 50 mM KCl, 0.1% gelatine, 0.45% (w/v) Nonidet P40 and 0.45% (w/v) Tween 20) added with proteinase K (final concentration 0.1 mg/ml). Following incubation overnight at 60 °C, proteinase K was inactivated by heating preparations at 95 °C for 15 min.

PCR were performed using W chromosome specific primers able to amplify a 447 bp fragment of the EcoR1 1.2 kb repeat (EMBL/P GenBank No. X57344) as originally described by Simkiss et al. (1996). Molecular sizing was determined by electrophoresis using 1.5% agarose-TBE gel.

**Statistical analyses.** For the comparison of the influence of YM 511 on hatchability, mortality of treated eggs and sex ratio of hatched chicken the χ² was used (Likeš and Machek, 1983).

Results and discussion

From a total number of 83 YM 511 treated and incubated eggs, only 24 were hatched. Among the remaining 24 hatched chickens, 16 were genetic males and 8 were genetic females (see Figure 1, Table 1). In comparison of YM 511 treated incubated eggs with results from the hatching together with the control group, there were not found statistically significant differences in the number of hatched chickens, hatched males and non-hatched chickens. There were statistically significant differences in the group of hatched females (P < 0.05) and in the number of sex converted females (P < 0.05) - see
Table 2. In the second control group hatchability was 79.1 % (95 hatched chickens from 120 incubated eggs) and this result showed on standard incubation conditions in the incubator.

In comparison of hatched chickens with results from the hatching and sex converted females between the control and YM 511 treated group, there were not found statistically significant differences in the number of hatched males. There were statistically significant differences in the group of non-hatched chickens (P < 0.01) and in the number of sex converted females (P < 0.01) - see Table 3. In comparison of sex ratio with hatched chickens and converted females, there were not found statistically significant differences in the sex ratio of hatched chickens where YM511 was used. There were statistically significant differences in the number of sex converted females (P < 0.01) – see Table 4.

From hatch up to 20 weeks, all genetic males exhibited a normal male phenotype (including barred feathers) while the 8 genetic females exhibited a normal female phenotype (including black feathers). Between 20 and 26 weeks of age, the 8 birds primarily identified as females progressively converted into a male phenotype with regards to hackle, comb, wattles, pointed feathers of the tail and cloaca . From 24 weeks of age, their comb grew dramatically in size up to reaching at 26 weeks about 3 times the size observed at 24 weeks. At 26 weeks of age, the averted cloaca of these birds appeared very similar to that observed in a “normal” genetic male.

By contrast with pre-cited birds, all chicks (n =29) hatched from eggs injected with DMSO alone (control group) had a sexual phenotype in accordance with their genetic sex.

Table 1 Percentage of sex converted individuals hatched from aromatase inhibitor treated eggs.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aromatase inhibitor</th>
<th>Control (DMSO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ eggs</td>
<td>83</td>
<td>54</td>
</tr>
<tr>
<td>Hatched males (%)</td>
<td>16 (19.2)</td>
<td>15 (27.8)</td>
</tr>
<tr>
<td>Hatched females (%)</td>
<td>8 (9.6)</td>
<td>14 (25.9)</td>
</tr>
<tr>
<td>Number of sex converted females (%)</td>
<td>8 (100)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1  PCR (25 cycles) of DNA isolated from blood in chickens treated with YM 511.
### Table 2 Comparison of incubated eggs with results from the hatching and sex converted females.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Aromatase inhibitor</th>
<th>Control (DMSO)</th>
<th>Statistical significance</th>
<th>$\chi^2$ value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated eggs</td>
<td>83</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched chicken</td>
<td>24</td>
<td>29</td>
<td></td>
<td>3.64</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Non-hatched</td>
<td>59</td>
<td>25</td>
<td></td>
<td>2.11</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Hatched males</td>
<td>16</td>
<td>15</td>
<td></td>
<td>0.84</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Hatched females</td>
<td>8</td>
<td>14</td>
<td></td>
<td>4.54</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Number of sex converted females</td>
<td>8</td>
<td>0</td>
<td></td>
<td>5.02</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

N.S. = not significant

### Table 3 Comparison of hatched chicken with results from their hatching and sex converted females.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Aromatase inhibitor</th>
<th>Control (DMSO)</th>
<th>Statistical significance</th>
<th>$\chi^2$ value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatched chicken</td>
<td>24</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched males</td>
<td>16</td>
<td>15</td>
<td></td>
<td>0.31</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Hatched females</td>
<td>8</td>
<td>14</td>
<td></td>
<td>0.51</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Number of sex converted females</td>
<td>8</td>
<td>0</td>
<td></td>
<td>8.34</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

N.S. = not significant

### Table 4 Sex ratio of hatched chickens and converted female.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Aromatase inhibitor</th>
<th>Control (DMSO)</th>
<th>Statistical significance</th>
<th>$\chi^2$ value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatched females</td>
<td>8</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched males</td>
<td>16</td>
<td>15</td>
<td></td>
<td>1.21</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Hatched converted females</td>
<td>8</td>
<td>0</td>
<td></td>
<td>9.54</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

N.S. = not significant
Experiments conducted in purpose of studying the consequences of hormonal changes on subsequent sex differentiation in female avian embryos have demonstrated that in ovo injection of steroidal and non-steroidal aromatase inhibitors may result in more or less marked changes of phenotypical sex including the evolution of female gonads into testis-like structures (Abinawanto et al., 1996). From the present observations performed in chickens, we demonstrate that the injection of aromatase inhibitor into the albumen prior to incubation, despite being highly toxic to chicken embryos (only 8/32 hatched eggs), may result in the transformation of genetic females into neo-males (development of testicle-like structures) expressing male secondary sex characteristics. However, in the present work, it is noteworthy that genetic females treated with aromatase inhibitor developed male characteristics well after hatch (22-26 wks of age), an indication that this product also exerts its aromatase-inhibiting action for prolonged periods. Previous studies indicated that the lack of estrogen synthesis in male embryos appears to be due to the extremely low levels of 17β-hydroxysteroid dehydrogenase accompanied by low P450 aromatase expression. In female chickens, the intense expression of the aromatase gene (by Day 5-6 of incubation) leads to estrogen synthesis. This, coupled with the expression of the m-RNA estrogen receptor in the left gonad, result in the development of a functional left ovary (Bruggeman et al., 2002). Estrogen receptor transcripts (cER) have already been detected in female urogenital tissues on Day 3.5 of incubation and in male and female gonads on Days 4, 5, 5.5 and 6.5 of incubation. As aromatase (cAROM) transcripts were also detected in female (but not male) gonads on Day 6.5 of incubation, and in gonads from both sexes at the adult stage (Smith C.A. et al., 1997), it is therefore apparent that, in the early chicken embryo, female gonads maintain a bi-potential status which, depending on the hormonal environment, may result in their evolution into male or female gonads (Vaillant et al., 2003). In the present work, attempts to collect semen by massage from neo-males were unsuccessful (only some drops of azoospermic fluid collected). In our view, this may not mean that, by contrast with Vaillant et al (2003) work, neo-males obtained from females embryos treated with aromatase inhibitor cannot develop full spermatogenesis.

In our experiments 8 females at 26 weeks of age progressively transformed into a male phenotype and further experiments are necessitated to verify structures of testes, various stadium of spermatozoa development.

REFERENCES


