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Experimental Sex Inversion of Chicken Embryos at Aromatase Inhibition, Estrogen Receptor Modulation, DNA Demethylation and Progesterone Treatment

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Abstract

The work was carried out by microinjections of an aromatase inhibitor letrozole, an estrogen receptor modulator tamoxifen in incubated eggs *Gallus g. domesticus*, the first day of incubation. It also used 5-azacytidine (5-AC) at the same time. Injection of progesterone was carried out before the onset of meiosis prophase 1 on 16 h of incubation. Morphologically and histologically and by PCR, sex of the 17-day embryos was controlled. According to information received microinjection of letrozole caused almost a 100% inversion of genetic males to females, which is manifested in the morphology of the gonads. In other experiments, sex reversal is not revealed. The results are obtained in this study, and the data suggest that the presence of gonadogenesis in female chickens makes earlier emergence of aromatase in the beginning of incubation, than that according to the classical scheme of sex determination in birds. Presumably this kind of synthesis is triggered by some W-chromosomal factors. It failed to detect the phenomenon of gender inversion—the transformation of males into females after exposure to demethylating agent 5-AC that casts doubt on the participation of male hypermethylated (MHM)-RNA-segment in regulating the activity of sex determining genes.

Keywords

Gallus gallus, Sex Determination, Aromatase, Gonad, *DMRT*1

1. Introduction

At the moment, general ideas were accumulated of sex determination in a number of

organisms and in particular vertebrates—fish [1], amphibians [2], reptiles [3] [4] [5], mammals [6] [7]. A general scheme of its determination in birds is largely based on knowledge accumulated from chicken *Gallus gallus domesticus* [8]. For animals mentioned above two different mechanisms of sex determination were described as GSD (genetical sex determination) and ESD (external sex determination) [9], as well as a number of fundamentally different sex determining genes [10] and series of epigenetic mechanisms [11]. For birds heterogametism of females is typical, which is characterized by the presence of specific non-mammalian sex determining gene *DMRT*1 localized on the Z chromosome. Expression of this gene is switched off in females in their single Z chromosomal allele by synthesis of MHM lnc RNA [8]. In birds, as in reptiles and marsupials the influence of estrogen on early ontogenesis was noted. Within 3 - 4 days of embryonic development in birds this hormone controls the development of females' gonads as it has been demonstrated by inhibition of aromatase—an enzyme of the biosynthesis of estrogens from androgens. This is the fundamental difference of birds from mammals, whose sex hormones do not affect sex determination. In addition, birds have a marked cell-specific mechanism of sex development [12]. Also the ability of females to control the heterogametic sex offspring due to meiotic drive attracts attention [13].

This study is focused on the more accurate definition of the effect period by aromatase inhibitor (letrozole) and estrogene receptor modulator (tamoxifen) on sex inversion during its influence at an earlier period of incubation (from the moment of laying the egg to the time of formation of the vascular system of the embryo, *i.e.*, 0 - 3 days of incubation), as well as experimental verification of the ability to reverse sex by the demethylation of DNA in MHM-segment of the Z chromosome in females with 5-AC. This study was also intended to show the role of progesterone as a possible trigger mechanism of meiotic drive in females.

2. Material and Methods

We have performed a number of experiments with letrozole (Novartis Pharma, Switzerland) and tamoxifen (Ebewe, Pharma, Austria), progesterone (Dal'chimform, Russia) and 5-AC (Sigma-Aldrich, Canada). All experiments have been carryed out from three (tamoxifen, 5-AC, progesterone) to five times (letrozole). In each experiment, 20 fertilized chicken eggs of Highsex White cross (Tosno, Lisii Nos, Russia) were used. Injections have been done into air pocket of each egg once (at the 1st day of egg incubation, 100 μL per egg, concentration 1 mg/mL for letrozole, tamoxifen and progesterone, 10 μm/ml for 5-AC). Also one hundred microliters of 0.9% NaCl were injected into other 20 fertilized eggs as control. Eggs have been incubated for 17 days at 37.8˚C and humidity 28%. Moreover the same experiments have been carried out at the 4th day of egg incubation.

Gonads isolated from 17 day old embryos after determination of their type (testicles or ovaries) were fixed with Clark mixture (ethanol mixed with glacial acetic acid in ratio 3:1). Gonads were then washed from the fixator, treated with alcohols of increasing concentration (ethanol-isobutanol-O-xylol), and embedded into paraffin according to

the routine technique to prepare sections on the microtome. Sections were purified from paraffin, stained with Mayer's hematoxylin, dehydrated by alcohols with increasing concentration and embedded into Canadian balsam (DiaM, Russia). Gonadal histology was analyzed under a Leica DM6000B microscope (Leica Microsystems GmbH, Germany) equipped with a Leica DC500 CCD camera and Leica QWin v.1.2 software. Genetic sex of each embryo was defined by polymerase chain reaction (PCR) with DNA isolated by the routine procedure [14].

Primers for the chicken *CHD*1 gene were CHD1F 5' GTTACTGATTCGTCTACGAGA3' and CHD1R 5'ATTGAAATGATCCAGTGCTTG3' [14] [15]. The PCR conditions were denaturation, 94˚C 5 min; gradual temperature decline from 60 to 50˚C, 1˚C, 1 min; 35 cycles, 94˚C for 30 s, 50˚C for 30 s, 72˚C for 60 s. Last PCR step was performed at 72˚C, 10 min. Amplified DNA was assayed in 2% agarose gel. Single fragment (380 bp) was identified in males and two fragments (500 and 380 bp) were observed in females.

3. Results

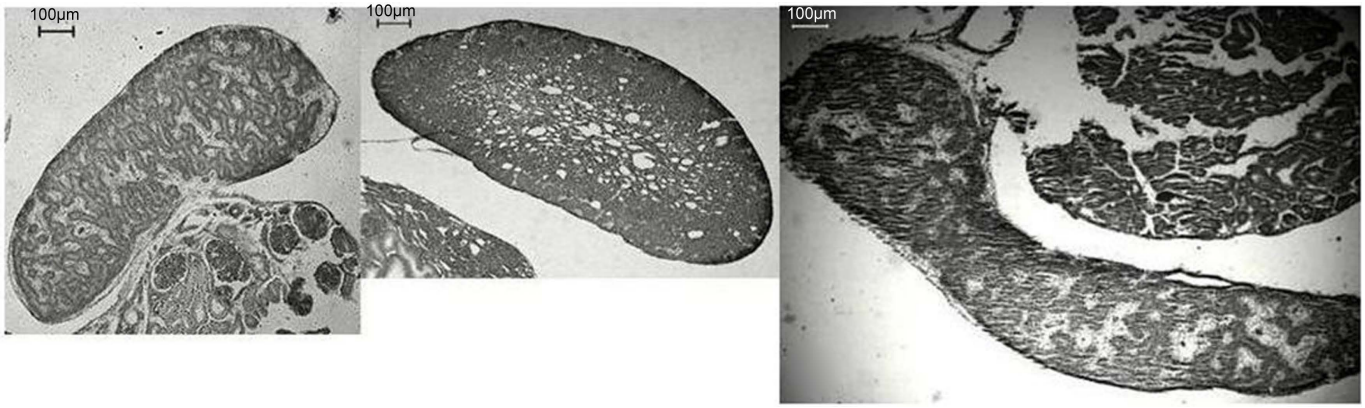
According to the received data, microinjections of letrozole on the first day of embryonic development causes almost 100% sex inversion from genetic females to males, which is manifested by the morphology of the gonads (**Table 1**, **Figure 1**). This means that the control females (injected only with 0.9% NaCL solution) had a typical left gonads with medulla and cortex and reduced the right gonad. In the cortex sex cells undergoing prophase I were discovered. Control males of the same age had smaller testicles and a well developed system of seminiferous tubules with spermatogonia that mostly did not enter the meiotic pathway. Genetic sex of control males and females fully corresponded to the phenotypic type (**Figure 1(a)**, **Figure 1(b)**). After injection of letrozole on the first day of egg incubation genetic females of chicken had alterations into gonads. Experimental females had pairs of gonads with poorly developed system of seminiferous tubules (**Figure 1(c)**). Injections of letrozole on the fourth day of egg incubation gave lesser percentage of sex alterations.

Injection of tamoxifen on the first day of egg incubation led to the results described

Table 1. Sex ratio in the domestic chicken in experiments with letrozole.

The experiment (number of eggs)	a number of experiments	Sex	Time injection of letrozole	
			in the first day of incubation	in the fourth day of incubation
control (20 eggs)	1	males	9/9/0	10/10/0
		females	11/11/0	10/10/0
experiment (20 eggs)	5	males	97/50/47	67/35/32
		females	3/3/0	33/33/0

Note: the criterion for sex determination: the presence of paired/unpaired gonad in the embryo; changes in the structure of the ovaries, the pairing of the gonads in females; sex was confirmed by PCR and primers for gene *CHD*1. **n/n/n**—morphological gender/confirmed genetic gender/altered histology in females with sex inversion.



(a) (b) (c)

Figure 1. Morphology and histology of chicken gonads: (a) (b) in control (a—male, b—female) and (c) after injection of letrozole (genetic female).

for the later periods of egg incubation (on the fourth day) and less significant than with letrozole [16]. There was a slight (1.5 times) hypertrophy of both testes in males. In treated females the ovary was also larger than in the controls. Female gonads had an increased number of lacunae in medulla, thickened cortex, and rare structures resembling seminiferous tubules. The number of germ cells at the stage of meiotic prophase I in the left gonad increased. However, in the right gonad, germ cells did not enter the meiotic prophase.

Using 5-AC has not led to any significant change in the number of phenotypic males and females (**Table 2**). It does not reveal any significant change in the phenotypic sex ratio of progesterone injection.

4. Discussion

According to our data, the effect of letrozole as an aromatase inhibitor is clearly evident when its injections are carried out into the eggs at the first day of incubation. There is inversion of sex (female to male) in almost 100% of cases (**Table 1**), whereas for the usual way of microinjection at 3 - 4 day of egg incubation phenomenon is less pronounced [16] [17]. Earlier it was noted that in birds the expression of aromatase is observed only on day 5 of embryonic development; on day 2 there are other enzymes involved in the synthesis of precursors of sex hormones [18]. There is evidence that injection of fadrozole (aromatase inhibitor) into chicken and turkey eggs prior to incubation led to sex inversion in 99% of cases [19]. In the most resonant work of this kind, the possibility of supplementing of eggs in 3 - 4-day period with fadrozole was shown [17]. It was suggested that an extra early estrogen synthesis is possible and that it is controlled by a still unknown W-chromosome factor [12]. Possible abnormal development of ovaries in genetic females may be due to violations of terms of the effects of estrogen on normal and inverted by aromatase inhibitors females. In this regard, it is worth mentioning the results of two studies on the production of transgenic chickens' aromatase gene, which has been demonstrated as almost complete inversion of the roosters

Table 2. The sex ratio in domestic chicken in the experiments with 5-AC.

The experiment (number of eggs)	A number of experiments	Sex	Injection time of 5-AC	
			in the first days of incubation (10 μm)	in the fourth day of incubation (10 μm)
Control (20 eggs)	1	males	8/8	11/11
		females	12/12	9/9
Experiment (20 eggs)	3	males	24/24	27/27
		females	26/26	23/23

Note: the criteria for sex determination: paired/unpaired gonads in the embryo; gender was confirmed using PCR and primers for the gene *CHD*1; changes in the ratio goniums, passed over in the prophase I of meiosis in males and females. **n/n**—morphological gender/confirmed genetic gender.

into the females [18] or only partial without affecting the gonads in the proper degree [19].

It is necessary noted that the change of time of injection of other agent—tamoxifen, which is not an aromatase inhibitor and is carrying out a modifying action of estrogen and the estrogen receptor, as well as in the case of injection on the 4th day of incubation did not lead to inversion of sex in genetic females, but contributed to the increase in size of the gonads of genetic males by 1.5 folds.

In the modern scheme of genes controlling sex determination in birds, epigenetic mechanism for turning off the expression of the only allele of the main sex determination gene *DMRT*1 with non-coding MHM-RNA now takes an important place [8]. In 2001 in *Gallus gallus* during the study of the mechanism of dose compensation genes in birds, MHM-area on the Z-chromosome was described, consisting of 210 tandem repeats, and ncRNA was synthesized in females. This molecular is about 2000 bp and it initially accumulates in the MHM-area of the Z-chromosome and then spreads to its neighboring region already in the first day of incubation. It turned out that such a process is characteristic only for heterogametic sex—females and it was suggested that macromolecular RNA covers nearby area where the main sex determination gene *DMRT*1 is located. In homogametic sex (males) MHM-area is hypermethylated and high molecular weight RNA is lacking there, and such status is set on the first day after fertilization [20].

Study of the induction of sex inversion in chicken embryo with 5-AC (agent that leads to DNA demethylation) confirmed that the lowest level of methylation in the MHM-area Z chromosomes is characteristic for the cells in the ovaries of females and the highest in the testes of males. Methylation was higher in the left gonad at significantly reversed females than in the left ovotestis at weakly inverted females [21]. In the work by the Brazilian geneticists the highest level of transcription of the MHM-segment at the 8th and 14th day was demonstrated by Real-Time-PCR. It was shown that the lack of transcription in this segment coincides with high activity of *DMRT*1 [22] [23] [24] [25] [26]. Some inconsistency was noted regarding the time of maximum expression of MHM-segment in embryogenesis from the first day of incubation to 8 and even 14. In a

recent study was found that MHM-amplicon shows maximum demethylation at day 8 of incubation in the developing testes that is coincident with both the lowest methylation levels found in the developing ovary and with a dramatic increase in expression of key genes required for gonadal differentiation such as *DMRT*1, *SOX*9, *AMH*, *P*450*arom*, *FOXL*2 and *ERα* [27].

It was possible to believe that the demethylation of this allele using a 5-AC will lead to its activation in males and initiation of synthesis of a chain of factors controlling the appearance of the ovaries, and development of females instead of males. However, no inversion of gender was found in our experiments (**Table 2**). There is only one article for using demethylation of this segment with 5-AC made on the culture of embryonic fibroblasts, which demonstrated cytologically the phenomenon of different methylation of the MHM-segment of females and males. Demethylation with 5-AC in fact did not affect the corresponding status of this segment in females, but affected only one of the two phase Z-chromosome in males. The authors suggested the presence of some conformational differences between these sites [28]. Sex inversion by using this agent at egg incubation can be expected by analogy with the turning off of one of *DMRT*1 gene by RNA interference and produce females from males [29]. Additional experiments are required to verify the relationship between MHM-segment and *DMRT*1-gene and in particular the time period of the first transcriptional activity of these effects on the specificity on each of the three alleles.

Our experiments for study of the progesterone role on sex ratio were a preliminary nature and were aimed at finding the mechanism of meiotic drive, which is used in heterogametic females. Now several possible mechanisms are under consideration. One is the effect of the concentration of the steroid hormones on the first division of meiosis, occurring in 16th day of incubation [13]. There is a study according to which intramuscular injection of progesterone to chickens really shifts the sex ratio towards females (75%) [30]. There are other more complex assumptions about meiotic drive in chickens [31].

Data obtained in our study suggest the earlier presence of aromatase during gonadogenesis of female chickens, than can be assumed from the scheme of genetic control of sex determination in *Gallus gallus* [8], somewhere during the first day of incubation. Perhaps her synthesis is triggered by is still unknown W-chromosomal factor. Moreover this factor may be provided in yolk at its mature. In this connection effect on sex determination of bird offspring by injections into eggs will be impossible after one day of egg incubation. We failed to detect the phenomenon of gender inversion, the transformation of males into females, after exposure with 5-AC, which warrants further study of the true involvement of MHM-RNA-segment in regulating the activity of sex determination genes. It is possible 5-AC have different influence on various types of cells and processes of methylation/demethylation have significance on definite stage of embryonic development.

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