Effect of exogenous estrogen treatment on gonadal morphology, morphometry and estrogen receptor alpha mRNA expression in gonads of male chickens (*Gallus domesticus*)

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Abstract — The current study was designed to investigate the post-hatching ERα expression in left gonads of male chickens along with gonadal morphology when the exogenous estradiol supply is continuous, assuming the gonadal ERα is the limiting factor to maintain the feminizing effects of the male to female sex reversal trials in chickens. Shaver brown chicken eggs were incubated with two in-ovo injections (0.1mg/egg) of estradiol cypionate. Males hatched from treatment eggs were raised with (T2) and without (T1) weekly post-hatching injection of 0.1mg/chick estradiol cypionate intra muscularly. At 1, 4 and 8 weeks of age morphology and ERα mRNA expression of gonads were determined. ERα expression increased significantly with age (P<0.0001) in all groups and differed significantly among treatments (P<0.0001) where the highest expression observed at T2 followed by T1. However the interaction effect was insignificant (P=0.1203). All treated chicks showed left gonad similar to control female. However, T1 chicks showed a poorly developed right gonad while T2 showed a prominent right gonad which was similar to C male gonads. In conclusion, the exogenous estrogen supply causes an increase in ERα expression in male left gonads with age in a diminishing pattern along with morphological and morphometric changes of gonads.

Index Terms— Estradiol Cypionate, Estrogen Receptor α, Feminization, *Gallus Domesticus*, Gonadal differentiation, Gonadal morphology, Sex Reversal

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1 INTRODUCTION

The sexual differentiation of chickens, which primarily involves the gonadal differentiation takes place during the embryonic development, is regulated by both direct genetic factors and well-characterized hormonal pathways [1]. The bipotential gonads at the early embryonic stage of chickens differentiate into either testes or ovary around the day 6 of embryonic development [2].

Estrogens are a group of hormones that promote the sexual differentiation of gonads and external traits in birds [3]. It plays a key role in ovarian differentiation and responsible for the development and regulation of the female reproductive system and secondary sex characteristics (Figure 1). Therefore, various studies have been conducted to see the possibility of manipulation of the gonadal differentiation by manipulating estrogens in early embryonic chickens.

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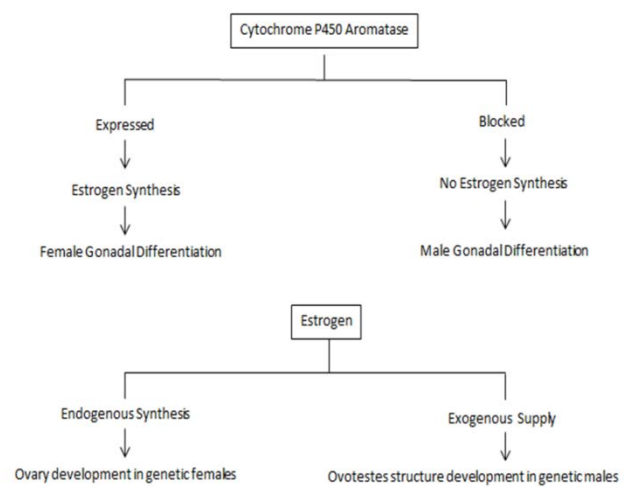
Figure 1: Role of estrogens on sex differentiation.

The cellular functions of estrogen are mediated by its re-

ceptors which bind estrogen and can act as transcription factors [3]. Chickens have two major types of estrogen receptors, encoded by separate genes namely, estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ). ERα is the predominant type of estrogen receptor found in chicken ovaries and functions in the development of ovary and female reproductive system. The ERβ does not show any sex dependent difference within the embryonic gonads [4].

Gonads of both sexes express ERα prior to their differentiation. However, the expression of ERαin genetic male disappears after the gonadal differentiation. This suggests that the gonads of both presumptive sexes are sensitive to estrogens during the early embryonic stage while only embryonic females retain the sensitivity reflecting the role of estrogens in the ovarian development [3],[5]. ERα is found to be expressed only in left gonads of both sexes but not in the right gonads [6]. The natural inactivation of the ERα gene expression after the gonadal differentiation in embryonic male chicken gonads remains unclear.

Sex reversal trials confirmed that the female-to-male reversal is permanent where genetic females (ZW) show both anatomical and physiological male characters. However, male-to-female sex reversal trials show either partial or temporary reversal where sex reversed genetic males (ZZ) transform back to males with the age. The factors underlying this phenomenon *i.e.* why the exogenous hormonal intervention only override the direct genetic factors in female-to-male sex reversal but not in male-to-female, still remains a question.

Previous studies show that ERα expression in embryonic male gonads is increased in several folds when they are treated with Estradiol 17β *in-ovo*. This suggests the potential of expressing ERα under the stimulation of exogenous estrogens. The expression of Aromatase gene in the gonads also significantly increases with estradiol treatment. However, ERβ expression remains unchanged [4]. This shows that the exogenous estradiol injection induced the factors necessary for the ovary development in the genetic male chickens. But the reason for reverting of the ovotestes structure again in to the testes is not elucidated.

If the direct genetic factors could be overridden by the hormonal regulation of the gonadal differentiation in female to male sex reversal, there should be another specific genetic or hormonal regulatory mechanism for the non-permanent nature of the male to female sex reversal. Previous observations on transgenic chicken experiments show that overexpression of aromatase gene results in high serum levels of estradiol. The data also shows that high levels of circulating estrogen are insufficient to maintain female phenotype in gonadal tissues and external anatomy of sex-reversed male chickens [7]. This data therefore support the fact that estrogen or aromatase expression cannot be the limiting factor which maintains the male to female sex reversal.

Beside the hormonal regulatory factors favoring the ovotestes development in genetic male chickens, ERα receptor expression also plays an important role. Although the ERα was found to be increased in the embryonic gonads with the estradiol treatment, the post hatching fate of that ERα with age

has not been studied adequately. Therefore, we hypothesized that there is an inactivation of the ERα in the gonads of genetic male chickens with age and that would be the main limiting factor to trigger the subsequent role of estrogens to regulate the ovarian function.

2 OBJECTIVES

The objective of the current study was to analyze the ERα expression in the left gonads and the gonadal morphology of *in-ovo* estrogen treated ZZ male domestic chickens with and without continuous post hatching estrogen treatment to check whether the ERα expression is maintained with age under above experimental conditions

3 MATERIALS AND METHODS

3.1 Statement on animal use
All animal procedures were performed in accordance with guidelines and approval of the Ethical Review Committee (ERC) and Institutional Animal Care and Use Committee (IACUC) of Sabaragamuwa University of Sri Lanka.

3.2 Experiment groups
Two groups including a treatment group, which was subsequently divided into Treatment 1 (T1) and Treatment 2 (T2), where T1 received only *in-ovo* estradiol injection while T2 received both *in-ovo* and post-hatching estradiol injections and a control group (C) were included in this study. Eggs (n=120) were randomly allocated to treatment and control groups.

3.3 Incubation of eggs and *in-ovo* injections
Eggs of Shaver Brown chicks (n=120) were used for this study. Eggs were incubated under standard conditions (37.5⁰C temperature and 65% and 75% RH during the 1st -18th day and 19th to 20th day of incubation respectively).

Treatment eggs (n=80) were injected with two doses, each of 01mg/egg, of Estradiol Cypionate (Vemedim Corporation, Vietnam)on the 2nd and the 14th day of the incubation via the air cell and were sealed with sterilized paraffin. The control eggs (n=40) were injected in the same way, with similar volume of sterile Phosphate Buffered Saline (PBS).

3.4 Genetic sexing
The genetic sexing of chicks was performed by Polymerase Chain reaction (PCR) using a previously described and revalidated, W chromosome linked HUR0424 dominant marker [8] (Table 1).

Table 1. Primer details for the genetic sexing of chickens

Primer	Amplicon Size	Sequence details (5'-3')	T_m (°C)
HUR0424 –Forward	315bp	GGTCGGGGAGAGGAATAAAA	58.4
HUR0424 –Reverse		GCACCACAGGCTTACGCTAT	60.5

Blood samples for sexing were obtained on the 2nd post-hatching day of chicks and the DNA extraction was carried out using commercial DNA purification kit (Qiagen Flexi-

Gene, Germany). The total PCR reaction mixture was made up to 20 µl by addition of 100 ng of genomic DNA, 10 pM of forward and reverse primers,2x HOT FIREPol® Blend Master Mix with 7.5 mM MgCl₂ (Solis Biodyne, Estonia) and PCR grade water (Solis Biodyne, Estonia). Each PCR reaction was standardized as follows: an initial denaturation at 94°C for 2 min, denaturation at 95°C for 5 min, annealing at 54°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 7 min. 35 cycles were used. DNA from mature animals (male and female) and PCR grade water were used in place of the template for positive and negative controls respectively. PCR products were electrophoresed in a 2% Agarose gel, which was pre stained with Diamond dye (Promega, USA), at 80V for 1 hour and was observed under the blue light transilluminator (Safe Image 2.0, Invitrogen USA). The presence and absence of 315 bp band in gel electro-gram was interpreted as female and male respectively.

3.5 Post-hatching injection
Following the genetic sexing, only male chicks were selected for the subsequent experimental procedures. The *in-ovo* Estradiol treated male chicks were randomly selected for T1 and T2 treatment groups. PBS treated male chicks were selected for the control group. TheT2 chicks were injected with Estradiol Cypionate (Vemedim Corporation, Vietnam) (0.1mg/chick) intra muscularly to the breast muscle weekly starting from 5th day till the 8th week of post-hatching.

3.6 Sacrificing of Chicks
Three Chicks from each group (T1, T2 and C) were sacrificed at the age of 1 week, 4 weeks and 8 weeks by sending an air bubble via the brachial vein using a 1mL Syringe with a 31 gauge needle.

3.7 Data and sample collection
Weight, maximum length and the average diameter of two perpendicular places of each egg were measured and recorded at the time of placing them for incubation. Weights of chicks were recorded before performing the sacrifice at each time interval. After dissection, weights of heart, liver and the gizzard of each chicken were measured and recorded. The gonadal morphology was visually assessed comparative to morphology described in Vaillant *et al.,* (2001) [9] and the maximum length and the diameter of each gonad were measured using a Vernier calliper (± 0.01mm) (Mitutoyo Dial Calliper, Japan).Weights of each gonad were recorded. After taking measurements gonads were transferred into micro centrifuge tubes containing sterilized PBS solution. Tubes were transported to the laboratory in chilled condition (in ice) and were stored in -80°C ultra low temperature freezer until analysis.

3.8 Estrogen Receptor α (ERα) Analysis

3.8.1 Total RNA extraction and quantification
Total RNA from each left gonad was extracted using Trizol reagent (FAVORGEN biotech Corp., Taiwan) following the manufacture's protocol. Total RNA was quantified at wavelength of 260nm using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Purity of samples was determined by 260/280 and 260/230 ratios.

3.8.2 Reverse Transcription and cDNA synthesis
Reverse Transcription of ERα transcripts was performed by gene specific primer set [4] and FireScript Reverse Transcription Kit (Solis BioDyne, Estonia) following manufacturer's instructions. 1ug of the template RNA, 1uM gene specific primer and PCR grade water were added to form a 17uL of final reaction mixture. It was placed at 65°C for 5 minutes for annealing of the primer and then immediately transferred to ice. After a short spin, 1X RT reaction buffer with DTT (Solis Bio-Dyne, Estonia),500uM dNTP mix (Solis BioDyne, Estonia) and200U Reverse transcriptase enzyme (Solis BioDyne FIREScript RT) were added to the same reaction mixture. Reverse transcription was done at 50°C for 30 minutes followed by an enzyme inactivation at 85°C for 5 minutes. Resulted cDNA were stored at -20°C.

3.8.3 PCR amplification of ERα
Amplification of the ERα sequence was carried out in a total reaction volume of 20uL containing 1X Hot FirePol Blend Master Mix (Solis Biodyne, Estonia), 0.5uM forward and reverse primer [4] (Table 2), 1uL of the RT product and PCR grade water to top up to 20uL. A negative control was also carried out with PCR grade water in place of cDNA. 25 cycles of PCR (97°C for 10 seconds, 55°C for 30 seconds and 72°C for 1 minute) were carried out with an initial denaturation and final extension step at 94°C for 2 minutes and 72°C for 2 minutes respectively.

Table 2. Primer details of ERα expression analysis [4].

Fragment name	Amplicon Size (bp)	Sequence details (5'-3')	Tₘ (°C)
ERα Forward	300	GTGCCTTAAGTCCATCATCCT	59.4
ER Reverse		GCGTCCAGCATCTCCAGTAAG	63.3
GAPDH Forward	348	GTGGAGAGATGACAGAGGTG	60.5
GAPDH Reverse		AACAAGCTTGACGAAATGGT	54.3

3.8.4 PCR amplification of GAPDH
Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) gene was used as the internal control. Expression of GAPDH and treatment combination was used to normalize the ERα expression of the samples in the same combination. RNA extracted from samples was reverse transcribed using a gene specific primer [4] (Table 2). Amplification was carried out in a total reaction volume of 20uL containing 1X Hot FirePol Blend Master Mix (Solis Biodyne, Estonia), 0.5uM forward and reverse primer [4] (Table 2), 1uL of the RT product and PCR grade water to top up to 20uL. A negative control was also carried out with PCR grade water in place of cDNA.. 25 cycles of PCR (97°C for 10 seconds, 50°C for 30 seconds and 72°C for 1 minute) were carried out with initial denaturation and final extension steps at 94°C for 2 minutes and 72°C for 2 minutes

respectively.

3.8.5 Gel Electrophoresis

10uL of ERα PCR amplicons were run for 1 hour at 80V in a 1% Agarose gel which was pre stained with Diamond dye (Promega, WI, USA). 5uL of GAPDH amplicons were run in a similar gel at 60V for 1.5 hours. The gels were visualized through the blue light trans-illuminator (Safe Image 2.0, Invitrogen USA).

3.8.6 Relative quantification of ERα expression

The ERα expression was semi-quantified using Image J image processing program (Image J, Fiji.) and the mean intensity of the bands were taken for the expression analysis. The relative expression of each sample was obtained as a percentage of the mean intensity of the GAPDH gene expression of the relevant age and treatment combination.

3.8.7 Data Analysis

Data were analyzed using the Statistical Analysis Software (SAS) version 9.0. Two sample t-test was used to analyze egg parameters (weight and shape-index) to check the uniformity of the two sets of eggs randomly assigned for treatment and control groups. The weights of chickens and organs and dimensions of the gonads were analyzed using one way ANOVA to check the effect of the treatment at each age interval. Relative expressions of ERα were analyzed by two-way ANOVA to test the treatment effect and the effect of age on the ERα expression in the left gonads among the treatments. The interaction effect was also tested.

4 RESULTS

4.1 Analysis of body weights at sacrifice

The statistical analysis of body weights displayed a significant difference among the treatments only at the 4th week where T2 was greater than T1 and C (T1 = C). However the results were not significant among treatments at 1 and 8 weeks age (Table 3).

Table 3. Analysis of body weight.

Age	p Value	Significance	Mean comparison
1 week	0.0812	Not significant	T1=T2=C
4 weeks	0.0053	Significant	T2>T1=C
8 weeks	0.0578	Not significant	T1=T2=C

4.2 Analysis of organ weights

Organ weights did not show a significant difference among treatments, but only the gizzard weight of the week 1 chickens showed a random significance (Table 4).

Table 4. Analysis of Organ weights.

Age	Organ	p Value	Significance	Mean Comparison
1 week	Liver	0.2684	Not Significant	T1=T2=C
	Heart	0.2414	Not Significant	T1=T2=C
	Gizzard	0.0120	Significant	T2>C>T1
4 weeks	Liver	0.7761	Not Significant	T1=T2=C
	Heart	0.3964	Not Significant	T1=T2=C
	Gizzard	0.8942	Not Significant	T1=T2=C
8 weeks	Liver	0.8742	Not Significant	T1=T2=C
	Heart	0.4904	Not Significant	T1=T2=C
	Gizzard	0.8910	Not Significant	T1=T2=C

4.3 Visual Assessment of Gonadal Morphology

4.3.1 Week 1

Control males had two testes with prominent whitish colour and hard texture. Both testes were similar in size (Figure 2a). T1 males showed slightly developed fragile right gonads and fully developed left gonads. Texture and the colour of the left gonad was pinkish and lobulated similar to female left ovary (Figure 2b). T2 males showed right gonads which were less developed than control males but more developed than T1. Left gonads of T2 were well developed and showed female like morphology (Figure 2c).
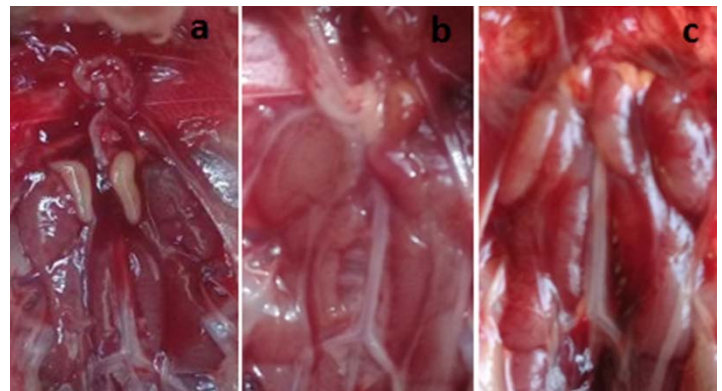

Figure 2. Morphology of gonads at week 1. Control (a). T1 (b). T2 (c).

4.3.2 Week 4

Control males had two testes with prominent whitish colour and hard texture. Both testes were similar in size (Figure 3a). T1 males showed slightly developed fragile right gonads and fully developed left gonads. The texture and morphology of left gonad was similar to female gonads (Figure 3b). Right gonad was developed than T1 but

not up to the size of control. The left gonad showed the morphological similarity to female ovary (colour and form) (Figure 3c).
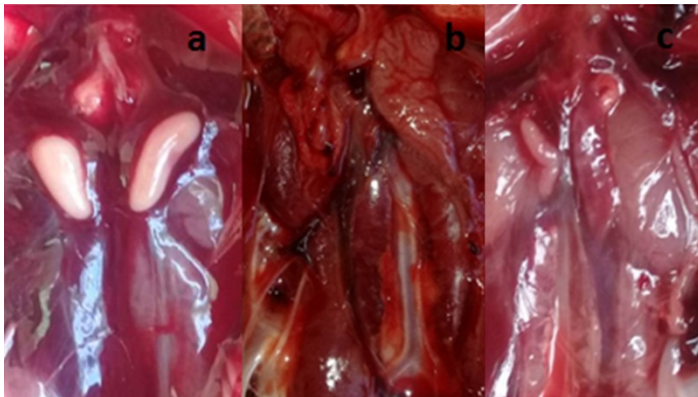

Figure 3. Morphology of gonads at week 4. Control (a). T1 (b). T2 (c).

4.3.3 Week 8

Both gonads of controls were similar in size. Cream colour and bean shaped (Figure 4a). T1 right gonads looked like a fragile little piece of tissue and not well developed. The left gonads were lobulated and well grown with a pinkish colour and elastic nature (Figure 4b). T2 left gonads were similar to female morphology while right gonads showed male morphology. Left gonads had typical lobulated nature of ovaries and were well developed (Figure 4c).
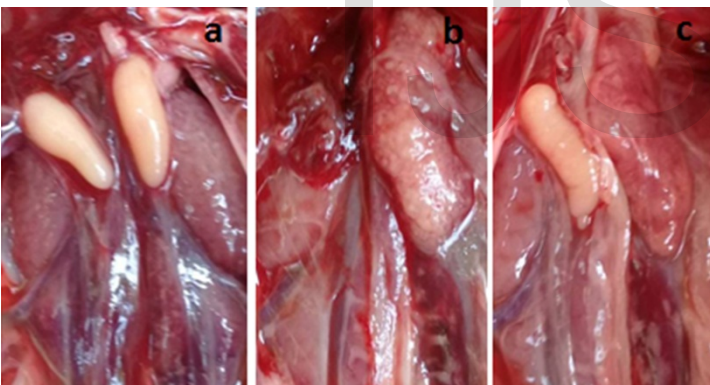

Figure 4. Morphology of gonads at week 8. Control (a). T1 (b). T2 (c).

4.4 Analysis of gonadal morphometry

The weights of both right and left gonads showed significant difference only at the 8th week while the width of the left gonad at 1st and 8th weeks showed a significant difference. All the other gonadal morphometry results were statistically insignificant (Table 5).

Table 5. Analysis of gonadal morphometry.

Parameter	Gonad	Age	p Value	Significance	Mean Comparison
Weight	Left	1 week	0.2195	Not Significant	T1=T2=C
		4 weeks	0.4912	Not Significant	T1=T2=C
		8 weeks	0.0199	Significant	T1>T2=C
	Right	4 weeks	0.6774	Not Significant	T1=T2=C
		8 weeks	0.0257	Significant	C>T2
Length	Left	1 week	0.1067	Not Significant	T1=T2=C
		4 weeks	0.3191	Not Significant	T1=T2=C
		8 weeks	0.3202	Not Significant	T1=T2=C
	Right	4 weeks	0.1421	Not Significant	T1=T2=C
		8 weeks	0.1396	Not Significant	T1=T2=C
Width	Left	1 week	0.0318	Significant	T1=T2>C
		4 weeks	0.9050	Not Significant	T1=T2=C
		8 weeks	0.049	Significant	T1>T2=C
	Right	4 weeks	0.6744	Not Significant	T1=T2=C
		8 weeks	0.2450	Not Significant	T1=T2=C

### 4.5 Analysis of parameters of left gonad relative to right gonad

Parameters of left gonads were analyzed compared to the relevant parameter of the right gonads No parameter showed a significant difference among treatment (Table 6).

Table 6. Analysis of relative gonadal parameters

Parameter	Age	p Value	Significance	Mean Comparison
Weight	4 weeks	0.1295	Not Significant	T1=T2=C
	8 weeks	0.6239	Not Significant	T1=T2=C
Length	4 weeks	0.5691	Not Significant	T1=T2=C
	8 weeks	0.4504	Not Significant	T1=T2=C
Width	4 weeks	0.6407	Not Significant	T1=T2=C
	8 weeks	0.5335	Not Significant	T1=T2=C

4.6 Analysis of Estrogen Receptor α expression in left gonads

4.6.1 Electrogram for GAPDH expression
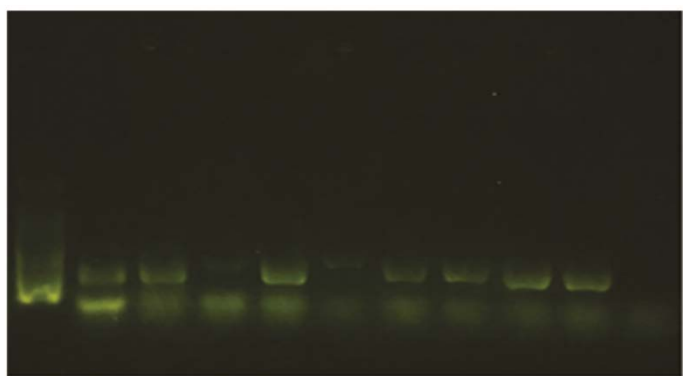
Electrogram for GAPDH expression

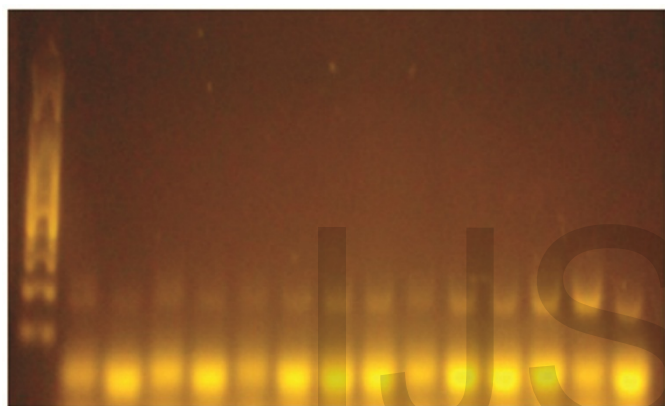## 4.6.2 Electrograms for ERα expression

Figure 6. ERα expression of samples. L = 100bp ladder, 1 = 1 week C, 2 = 1 week T1, 3 = 1 week T2, 4 = 4 weeks C, 5 = 4 weeks T1, 6 = 4 weeks T2
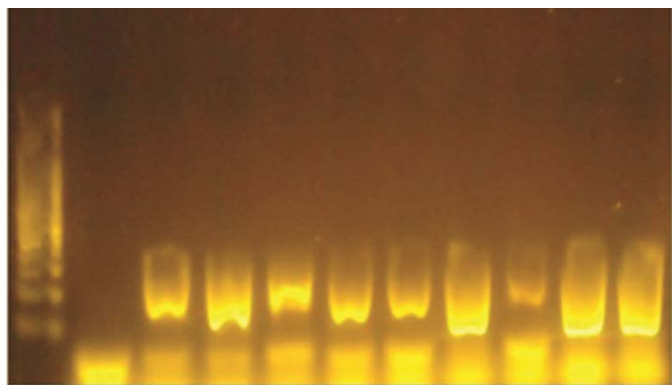

Figure 7. ERα expression of samples. L = 100bp ladder, NC = Negative Control, 7 = 8 weeks C, 8 = 8 weeks T1, 9 = 8 weeks T2.

4.6.3 Variation of ERα expression among treatments

The ERα expression of gonads on the 1st week did not show any statistically significant difference among the groups. However results of 4th and 8th week analysis, showed a significant difference among the treatment groups (Figure 8, Table 7).
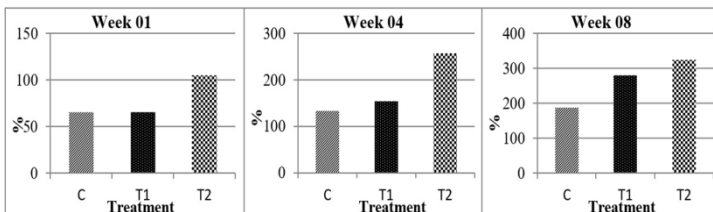

Figure 8. Variation of ERα expression among treatments.

Table 7. Variation of ERα expression among treatments.

Age	p Value	Significance among treatments	Mean Comparison
Week 01	0.0526	Not Significant	T2 = T1 = C
Week 04	0.0021	Significant	T2 > T1 = C
Week 08	0.0329	Significant	T2 > T1 & C

4.6.4 Variation of ERα expression among age intervals

All the treatment groups including the control, showed a statistically significant increment with the age (Figure 9, Table 8). However the increment showed a diminishing pattern. The highest diminishing gradient was observed in the T2 group where similar gradient was observed in C and T1.
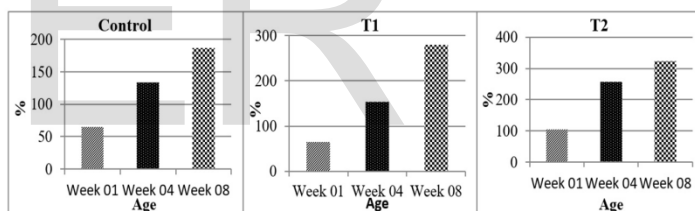

Figure 9. Variation of ERα expression among age intervals.

Table 8. Variation of ERα expression among age intervals.

Treatment	p Value	Significance among age intervals	Mean Comparison
T1	0.0236	Significant	
T2	0.0010	Significant	Week 08 > Week 04 > Week 01
C	0.0032	Significant	

4.6.3 Effect of treatment and age on ERα expression

Over all, the treatment effect (p<0.0001) and the effect of age (p<0.0001) on the ERα expression in the left gonads were statistically significant. However the treatment and age interaction effect was not statistically significant (p=0.1203).

5 DISCUSSION

Estrogen plays a central role in embryonic gonadal sex differentiation. Previous studies have proven this by experimentally through exogenous hormone treatments and gene knockdown experiments. These studies lead us to hypothesize that change

in ERα expression with age would be a limiting factor to trigger the subsequent role of estrogens to regulate the ovarian function which cause sex reversed male chickens to lose the ovarian phenotype. The current study addresses this idea through analyzing the ERα expression in the left gonads and gonadal morphology of *in-ovo* estrogen treated ZZ male chickens with and without continuous post hatching estrogen treatment to test whether the ERα expression is maintained with age. The results of the current study showed a regular pattern of ER α expression in the left gonads of male chickens in the post-hatching period.ER α expression in the male left gonads increased significantly with age. Although there were evidence s of ER α expression in embryonic gonads of male chickens, this is the first report which demonstrates the ER α expression of male gonads until the age of eight weeks in male chickens.

Estradiol treatment has caused an increase in the expression of ERα in male left gonads. This result is in agreement with the previous literature showing the increment of ER α in embryonic gonads of male chickens with *in-ovo* estradiol treatment [4],[6]. Continuous post hatching estradiol treatment caused the increase in ER α expression confirming that exogenous estrogen triggers the ER α expression in gonads.

Interestingly, the treatment and age interaction effect on the ER α expression was not significant although both individual effects were significant. This indicates that the increment of the ER α expression due to the treatment is decreased with age while natural ER α expression increment becomes greater than treatment effect with age. Because of that, with the age, the ER α expression of the control chickens increases, making it close to the expression of treatment chickens. Therefore when the two effects are interacted, we could say that the interaction effect makes no significant difference among the treatments. From this evidence we can suggest that there might be a male specific mechanism to decrease the ER α expression triggered by the exogenous estradiol. This phenomenon can be supported by a previous observation on transgenic chickens over-expressing aromatase which shows that high levels of circulating estrogen cannot maintain female phenotype with age in gonadal tissues of sex-reversed male chicks [7]. Therefore, it can be suggested despite the ER α expression was increased naturally with age in the control males, that level is not adequate for the ovarian maintenance in sex reversed males. Even if exogenous estrogen supply was continuous, there might be a male specific mechanism to reduce or control the ER α expression to normal levels in treated males making it unable to maintain the ovotestes structure. This can be hypothesized as the mechanism behind non-permanent nature of the male-to-female sex reversal.

Physical observations showed that the morphology of the left gonads of treated chicks was deviated from the controls towards the female morphology with the colour and lobulated nature. This was observed in all age intervals analyzed. This same result could be observed in the previous studies by over-expressing the aromatase gene in male chickens having enlarged left gonad with similar histology to females [9],[10]. These feminized left ovaries were termed as ovotestes and

were transient. This was also reported previously in the ZZW triploid chickens studies. They also had these left ovotestes and showed inter sex features [11]. But according to Tienhoven, this left gonadal feminization was reverted within 7 weeks of age [12]. Since we could observe the feminized left gonads even at 8th week it is important to study further about this up to sexual maturity.

Comparison of right gonads of treated chicken showed a significant difference between T1 and T2, where T2 with continuous estrogen treatment showed a developed right gonad than the T1 to which only *in-ovo* treatment was given. It has found that estrogen is important in male sexual development as well in many animal models not only in the functional aspects but also in the differentiated epithelial morphology [13]. Accordingly, we could assume that the continuous higher estrogen levels caused the development of right gonad along with the left ovotestes. The poorly developed right gonads in T1 may indicate the inadequate estrogen for the right gonadal development along with left ovotestes.

Gonadal morphometry results were not statistically significant. However, there were few changes with the left gonad indicating the weight and the width of the treatment groups were higher than control group. This also provides evidence for the development of left gonad with the estradiol treatment. But when the left gonadal parameters were compared relative to the right gonadal parameters, it did not show a significant treatment effect between both T2 and C. This shows that the treatment has no significant effect on increase of left gonad relative to right gonad and the both gonads has developed simultaneously in similar proportion to the control males although the morphology of left gonad has been affected by the estradiol treatment.

When the ER α expression and the gonadal morphology results are considered, it was evident similar pattern in the gonadal development and the ER α expression in the treatment groups. The highest expression was observed in T2 group and the feminization of the left gonads was observed with the development of the right gonads of T2 chickens. Among theT1 chickens which showed the second highest ER α expression, well-developed left gonad was seen but the right gonad was poorly developed. Therefore, hypothesis was agreement with the results that the continuous estrogen treatment facilitated the right gonadal development along with the feminization of the left gonad giving it the highest ER α expression while the estrogen supply was inadequate to develop the right gonad of the T1 chickens along with the feminization of the left gonad making it the second highest in ER α expression. But this should only be confirmed by future research based on the serum estrogen level with the gonadal development.

The body weight results showed that there is no effect of exogenous estradiol on bodyweight of chicks at week 1 age interval. Since the post hatching injection was started on day 5 and sacrifice was done on day 7, we could assume that there was no time to express the effect of the post hatching treatment on body weights. This is also confirmed by the pharmacological properties of Estradiol Cypionate which is the slow-

est to come to the peak among the available estrodiol esters [14]. However, a significant effect of the treatment on the body weights at the age of 4 weeks was observed. At week 8, the weights of the chickens were not significantly different. Similarly in transgenic chickens over-expressing aromatase gene has shown that the body weights were similar to control males even with the high serum estrogen level [7]. Therefore, male weights presumably cannot be affected by endogenous estrogen level. However, this needs further studies with a higher sample number for the validation of the results. When organ weights are considered, there were no significant differences among the treatments except for week 1 gizzard weights. In contrast, it has been shown that the relative liver weight was increasing with the estrogen treatment in broiler chickens [15]. However, this also should be confirmed using a large sample number in a future research.

6 CONCLUSION

The current study provides new evidence for the presumed mechanism of ER α for maintaining the female phenotype in the male-to-female sex reversal. Being either regulated through genetically, epigenetically or neuro-endocrinologically, this mechanism is seemed to affect the ER α expression in gonads with age. This could lead to poor modulation of the estrogen in the gonadal tissues for maintaining the ovotestes structure with age dependent manner even at high serum levels of estrogen. Since control chickens showed a clear increase in the ER α expression in gonads, we could suggest that this mechanism is only activated with the elevated estrogen levels or elevated ER α mRNA expression in the male chickens as a negative feedback mechanism. This could be further investigated in future studies to identify the key cause. Further it could be hypothesized that a way for permanent sex reversal in the genetic male chickens will be achieved by regulating or eliminating underline mechanism.

ACKNOWLEDGMENT

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