Determination of testicular estrogen receptor alpha expression of male chickens (Gallus domesticus) with age

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Abstract

Background and Aim: Estrogen activity, a central component of reproductive growth, is regulated by the receptor proteins, estrogen receptor alpha (ERα), and ER beta (ERβ) in chickens as in many other species. ERα expresses predominantly in gonads. Although the expression of ERα in embryonic gonads has been studied in detail, the expression of ERα in post-hatching male gonads has not been studied adequately. Therefore, the current research was conducted to determine the post-hatching changes in the expression of ERα in the left gonads of male chickens with age.

Materials and Methods: Shaver Brown male chickens were raised and cared for according to the management guide and sacrificed at the intervals of 1, 4, and 8 weeks of age. The total RNA was extracted from the left gonads using the Trizol method and reverse transcribed using a pair of gene-specific primers. Following polymerase chain reaction amplification, the expression of ERα was quantified relative to the expression of the reference gene GAPDH.

Results: The results showed that ERα expression significantly increases with age at p=0.0032. However, the increment of ERα expression from week 1 to week 4 was 2.04-fold and from week 4 to week 8 was 1.39-fold, with the later age reflecting a diminishing pattern in the increment.

Conclusion: These results differentiate the post-hatching ERα expression of the left gonads of male chickens increase with age but with a diminishing gradient that may support their reproductive functions in later stages of life.

Keywords: age, chicken, estrogen receptor alpha, gene expression, testicular.

Introduction

Estrogen is the primary female sex hormone which is responsible for the development and regulation of the female reproductive system and secondary sex characteristics [1]. Cellular estrogen activity is regulated by receptor proteins called estrogen receptors (ERs) which has two subtypes in vertebrates, ER alpha (ERα) and ER beta (ERβ) [2,3]. Research over the past two decades shows estrogen also plays a key role in the development and regulation of the male reproductive system [4]. Aromatase is the crucial enzyme responsible for the synthesis of estrogens by aromatization of the androgens [5]. Synthesis of estrogen and the relationship of estrogen and male reproductive performances have been previously studied in many species [6,7]. In the testes of adult roosters, estrogen is secreted by Leydig cells and immature germ cells where the aromatase gene is expressed [6]. In developing embryonic gonads, the expression of ERα has been observed differently in males and females (higher in females) while ERβ expressed indifferently in the two sexes demonstrating that α-type is more sex specific in chicken gonads [2]. The embryonic studies show that the early embryonic gonads (bipotential gonads) display ERα expression in the left but not in the right gonads of both sexes before gonadal differentiation [8]. Following gonadal differentiation (embryonic day 5), the expression of ERα gradually diminishes in males while expresses at higher concentrations in females [2,9-11]. These findings facilitate the previous argument that the ERα receptor is more sex specific in chickens. Further studies, therefore, are necessary to elucidate the actual role of ERα in the development of the reproductive system of male chickens.

Although the gonads show a diminishing trend of ERα during embryonic stage, the research involved in ERα expression of adult testes shows that the testicular and epididymal regions contain varying amounts of ERα reflecting its importance in the reproductive development of chickens during later stages of life [12]. This contradiction creates a research gap with the variation
PCR was performed with initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 97°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and final extension for 2 min at 72°C.

**PCR amplification of GAPDH**

GAPDH was used as the reference gene to quantify the relative expression of ERα. About 1 µL of the RT product from each sample was amplified. Initial denaturation was conducted at 94°C for 2 min, followed by 40 cycles of denaturation at 97°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min and a final extension for 2 min at 72°C.

**Agarose gel electrophoresis**

PCR amplicons were electrophoresed in 1.5% agarose gel, which was prestained with 30,000 times diluted diamond dye in 1× TBE buffer. Electrophoresis conditions were 60 V for 1.5 h, and the DNA was visualized directly on a blue light transilluminator.

**Relative quantification of ERα expression and statistical analysis**

ERα expression was semi-quantified using ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, USA), an image processing software, and the mean intensity of the bands was used for the expression analysis. ERα expression as a percentage of the GAPDH expression was calculated in each sample using the mean intensity values. Relative expressions were statistically analyzed using a one-way ANOVA procedure of Statistical Analysis Software (SAS version 9.0) (SAS Institute, USA) to test the effect of age on the ERα expression in the left gonads of the male chickens.

**Results and Discussion**

The relative expression of ERα showed a significant increase with age (Figures-1 and 2). Statistically it was significant with p-value of 0.0032. The least square mean comparison test specified that there is a significant difference between each age interval (Table-2). These results provide evidence of the rise in ERα expression in the left gonads of male chickens with age.

This finding contradicts the embryonic ERα expression results, which showed a diminishing expression of the ERα in male left gonads following gonadal differentiation. Therefore, this embryonic expression reduction can be interpreted as a sex-specific mechanism to support the differentiation of the bipotential gonads based on their genetic sex. Nevertheless, this finding is in compliance with the previous finding by the Gonzalen-Moran et al. [17], which displayed the highest ERα expression in testicles of mature chickens than in immature chickens showing an increment of ERα expression with age.

The post-hatching increase of the ERα expression in the gonads can be justified to support the increased activity of the estrogen in gonads for the reproductive development in male chicken with age, as per the latest finding of the role of estrogen in the male reproductive...
system observed in many species [4,18,19]. The stud-
ies involved in male infertility and ER knocked down
mice models revealed that estrogen activity which is
crucial in spermatogenesis and seminal fluid secretion
is mediated by the ERα and hence holds utmost impor-
tance in the mammalian male testes [19,20]. A similar
role of ERα expression could be suggested for avian
model too with the results of the current study.

However, a study on domestic goose pro-
vided contrasting evidence to this which showed an
inverse proportionate of ERα expression in testes
with the plasma estrogen concentration during annual
reproductive cycle [7]. Conversely, the sex reversal
trials of chicken model suggested an induced expres-
sion of ERα in the embryonic male left gonads with an
in ovo estradiol treatment [2,8]. This contradiction
creates a gap for further research to check the varia-
tion of expression and the role of ERα in the testes of
male birds with the age.

The experiment duration of the current study
was 8 weeks and the sexual maturity of male chick-
ens usually attains at 16 weeks of age [21]. Therefore,
the results of the current study give an idea about the
variation of the ERα expression during the presexual
maturity period of chickens. The results also revealed
that the post-hatching increase of ERα expression
shows a diminishing pattern, by increasing from week
1 to week 4 in 2.04-fold and from week 4 to week
8 in 1.39-fold. This result gives speculation that the
testicular ERα expression in chickens increases with
age and comes to a peak at a certain age where the
optimum reproductive development is facilitated and
then declines or remains constant thereafter.

However, the previous research has evidence
that the highest number of Sertoli cells and Leydig
cells was found in immature chicken testicles, and the
number drastically drops with the age [17]. Therefore,
with the previous evidence of ERα expression confin-
ing to Sertoli cells and Leydig cells, the diminishing
pattern of the increment of ERα expression can be
explained as a result of the diminishing of these cells
in the growing testicles. Although with a diminishing
gradient, the increase of ERα expression with age can
be suggested to support the other mechanisms behind
the reproductive development such as production
of seminal fluid and regulation of spermatogenesis.
Nevertheless, it was also found that ERα expression
was higher at the middle age than the aged chickens
while there can be low amount of germ cells in aged
chickens compared to middle-aged [17]. Combining
the evidence from all studies, it can be suggested
that the post-hatching ERα expression increases with
a diminishing gradient up to a peak at a certain age
and then it again declines with age corresponding
to the reduction of the reproductive performance of
chickens. However, further studies are essential to
determine the role of ERα in the male reproductive
development of the chicken model.

Conclusion

Considering the results obtained from this
research, it can be suggested that the ERα expres-
sion in the male left testes increases with age with

Table-1: Primer details of ERα expression analysis [2].

| Fragment name | Amplicon size (bp) | Primer sequence (5’ to 3’) | Temperature (°C) |
|---------------|--------------------|----------------------------|------------------|
| ERα forward   | 300                | GTGCCCTTAAGTCCATCATCCT      | 59.4             |
| ER reverse    |                    | GCGTCCAGCATCCTCCAGTAAG      | 63.3             |
| GAPDH forward | 348                | GTGGAGAGATGACAGAGGTG        | 60.5             |
| GAPDH reverse |                    | AACAAGCTTGACGAAATGGT        | 54.3             |

ERα=Estrogen receptor alpha

Table-2: p-values for least squares mean comparison for
male chicken.

| p-values       | Week 1 | Week 4 | Week 8 |
|----------------|--------|--------|--------|
| Week 1         | 0.0133 | 0.0011 |        |
| Week 4         | 0.0133 | 0.0220 |        |
| Week 8         | 0.0011 | 0.0220 |        |

Figure-1: Electrogram for estrogen receptor alpha (ERα)
expression in chicken gonads. L: 100 bp ladder, 1-3: ERα
expression of week 1 chickens, 4-6: ERα expression of
week 4 chickens, 7-9: ERα expression of week 8 chickens.

Figure-2: Variation of estrogen receptor expression with
age.
a diminishing pattern of the increment during the presexual maturity period of chickens.

Authors’ Contributions

WKRN contributed to conceptualization, investigation, data curation, and original manuscript draft. LW helped with the methodology, investigation, funding acquisition, data curation, and editing. RC and NSH helped with the methodology, investigation, analysis, and validation. MPSM contributed to conceptualization, funding acquisition, supervision, visualizations, editing, and review. All authors corrected the manuscript and read and read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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