Occurrence and cellular distribution of estrogen receptors ER\(\alpha\) and ER\(\beta\) in the testis and epididymal region of roosters

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Estrogen signaling is required for the maintenance of male reproductive function and is mediated by the estrogen receptors ER\(\alpha\) and ER\(\beta\). These receptors are widely distributed in mammalian reproductive tissues, but information is limited in non-mammalian species including birds. The aim of this study was to investigate the occurrence and cellular distribution of ER\(\alpha\) and ER\(\beta\) in the testis and epididymal region of roosters. The results showed for the first time that ER\(\beta\) was the predominant receptor detected in the testis, being expressed in the somatic and some germ cells. Within the epididymal region, ER\(\beta\) was strongly expressed in all segments, whereas the most intense reaction for ER\(\alpha\) was found in the distal efferent ductules. The differential expression of ER\(\alpha\) and ER\(\beta\) within the rooster testis and epididymal region suggests that these organs may be a target for different actions of estrogen.

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1. Introduction

Estrogen signaling mediated by the estrogen receptors ER\(\alpha\) and ER\(\beta\) is important for the maintenance and regulation of several biological functions, including male reproduction. In mammals, it has been demonstrated that the estrogen responsive system is crucial for the maintenance of male fertility since the inactivation of ER\(\beta\) and/or ER\(\alpha\), as well as the enzyme P450 aromatase, results in infertile males [7,27,36,52,59,60]. Estrogen receptor localization have been described in the male genital system of several mammalian species, including primates (humans and non-humans), rodents (mice and rats), domestic animals (dogs, cats, porcine, primate species, including primates (humans and non-humans), rodents (mice and rats), domestic animals (dogs, cats, porcine, sheep and goat) as well as wild species, such as bats, roe deer and californian sea lion [21,33,37,47,54,56,58,62,63,72]. In non-mammalian vertebrates, ERs have been found in the reproductive systems of fish [12,45,67], amphibians [8], lizards [18], turtles [31,55], and birds [41,49].

Although roosters represent one of the avian species most extensively used for commercial and experimental purposes, the occurrence and functional role of the estrogen responsive system in their genital organs are still poorly determined. A possible role for estrogen receptor signaling in this species was first proposed when high levels of the enzyme P450 aromatase were found in the germinal cells in the testis, as well as in the sperm traversing the male tract [42]. More recently, it was shown that testicular ER\(\alpha\) levels change in immature, mature and aged roosters, being more highly expressed in sexually active animals, thus confirming that ER may be important for modulating rooster testicular functions [32]. There is only one report on ER expression in avian male reproductive tracts; however, the authors did not differentiate ER\(\alpha\) and ER\(\beta\) [41]. To address this issue is highly important, since, although ER\(\alpha\) and ER\(\beta\) shares structural similarity, they also present important functional differences [22,34,40].

A remarkable characteristic of the rooster male tract is the prominence of the efferent ductules, which compose up to 50% of the epididymal region, contrasting with a short and non-differentiated epididymal duct [3,50]. The avian efferent ductules are responsible for reabsorption of more than 90% of the fluid coming from the testis, with a rate of reabsorption that is greater than in mammals [20]. Regulation of the fluid reabsorption is a known function attributed to estrogens in the mammalian efferent ductules [35,36]. However, the molecular mechanism regulating this important function is still not determined for birds. It is consensual in the literature that, among the male genital tract of mammals, the efferent ductules are the most sensitive to estrogens, presenting higher labeling for ER\(\alpha\) [29,37,47,48,62]. Higher ER\(\alpha\) expression is also observed in turtle efferent ductules [31,55], but it is still uncertain for roosters. Further investigation detailing the distribution of both estrogen receptors in the rooster epididymal region would be helpful to clarify whether or not there is different sensitivity to estrogens that would predict functional differences within the reproductive tracts. Therefore, to contribute with information about occurrence and precise cellular distribution of ER\(\alpha\) and ER\(\beta\) in the testis and each segment composing the epididymal region of roosters is the aim of the present study.
2. Materials and methods

2.1. Animals

The study was performed on the epididymal region of adult roosters (\textit{Gallus gallus}) (\(n = 10\)) in reproductive activity obtained from commercial sources and housed at the Federal University of Minas Gerais facilities. Since it is difficult to determine the precise age of the animals, parameters as the body weight as well as absolute and relative testis weights were taken to emphasize the homogeneity of the group (Table 1). The animals received water and commercial chow (Sociê Guyomarç’h, Brazil) ad \textit{libitum}. Principles of research involving animals followed those published by the local ethical committee (http://www.ufmg.br/bioetica/coep).

2.2. Tissue preparation

Roosters were weighed, anesthetized (i.p. sodium pentobarbital 50 mg/kg of body weight) and perfused intracardially with saline solution followed by 10% neutral buffered formalin (NBF) for immunohistochemistry studies. For Western Blotting analysis, animals were perfused with saline solution only, and after the dissection of the testis and epididymal region, fragments of tissues were frozen in liquid nitrogen.

2.3. Western blotting

Western blotting analyses were performed in order to investigate the expression of ER\(x\) and ER\(\beta\) in the testis and epididymal region of roosters, as well as to test the specificity of the antibodies used. Fragments of tissue frozen in liquid nitrogen (\(n = 6\)) were macerated in dry ice and submitted to total protein extraction. After this step, the samples were subjected to electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes. The membranes were blocked with 10% normal goat serum (NGS) and incubated with rabbit anti-ER\(\alpha\) (clone 60C, Millipore, USA) or anti-ER\(\beta\) (NCL-ERbeta, Novocastra, USA) antibodies diluted at 1:300 or 1:150, respectively. After several washes in phosphate buffer saline (PBS) – tween 0.05%, the membranes were incubated with the avidin–biotin complex (Vectastain ABC kit – Vector Laboratories, USA) diluted at 1:300 or 1:150, respectively. Non-specific binding was blocked with PBS instead of the primary antibody. Following the incubation of sections with the biotinilated goat anti-rabbit (for ER\(x\)) or goat anti-mouse (for ER\(\beta\)) secondary antibodies (Dako, USA) diluted at 1:1000, the membranes were then incubated with the avidin–biotin complex (Vectastain Standard ABC kit – Vector Laboratories, USA) for 30 min and the immunolabeling was visualized with a solution of 0.1% 3,3’-diaminobenzidine in PBS containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H\(_2\)O\(_2\). Both antibodies used in this technique were raised against a synthetic peptide of human ER\(x\) or ER\(\beta\), which share, respectively, 98% and 96% of similarity with chicken sequences according to BLASTp analysis [5,6].

2.4. Immunohistochemistry

Aiming to determine the cellular and regional distribution of ER\(x\) and ER\(\beta\) proteins, fragments of the testis and epididymal regions fixed in NBF (\(n = 4\)) were embedded in paraffin, sectioned (5 \(\mu\)m) and used for immunohistochemistry. Sections were blocked for endogenous peroxidase activity with 0.6% H\(_2\)O\(_2\) in methanol and subjected to a standard protocol for antigen retrieval in citrate buffer and microwave. Then, non-specific activity was blocked by the incubation of slides in 10% NGS prior to the incubation with the anti-ER\(x\) antibody (clone 60C, Millipore, USA) diluted at 1:50 for 12 h or with the anti-ER\(\beta\) antibody (NCL-ERbeta, Novocastra, USA) diluted at 1:25 for 48 h. For negative control, sections were incubated with PBS instead of the primary antibody. Following the incubation of sections with the biotinilated goat anti-rabbit (for ER\(x\)) or goat anti-mouse (for ER\(\beta\)) secondary antibodies (Dako, USA) diluted at 1:50, sections were exposed to the avidin–biotin complex (Vectastain ABC kit – Vector Laboratories, USA). After this step, the immunoreactions were visualized using diaminobenidine containing 0.01% H\(_2\)O\(_2\) in 0.05 M Tris–HCl buffer, pH 7.6. Sections were slightly counterstained with Meyer’s hematoxilin.

2.5. Morphometry

The differences in ER\(x\) and ER\(\beta\) immunostaining intensity among the segments composing the epididymal region were quantified by using computer-assisted image analysis [26,66]. Digital pictures of 5 random sections of each segment analyzed were taken, converted to grayscale mode and then inverted. The images were exported to Image-Tool software (Version 3.00; University of Texas Health Sciences Center, USA), for quantitative analysis. The stained nucleus were traced and measured and the pixel intensity was determined for the traced areas. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells and subtracted from values detected in the labeled regions. ER\(x\) and ER\(\beta\) staining were quantified in 50 cells of each segment studied per animal (\(n = 4\)).

2.6. Statistical analysis

Differences in the immunostaining intensity of ER\(x\) and ER\(\beta\) in the rooster epididymal region were assessed by Student’s t-test (for ER\(x\)) or analysis of multiple variance (ANOVA) followed by the Newman-Keuls post hoc test (for ER\(\beta\)). Differences were considered statistically significant when \(P < 0.05\).

3. Results

3.1. Western blotting

The ER\(x\) and ER\(\beta\) antibodies recognized a single protein band of about 67 kDa and 54 kDa, respectively, in the total protein extracts of both testis and epididymal region (Fig. 1). These molecular weights are in agreement with those previously described for both proteins in different species [31,32,47,54,72]. However, since

<table>
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<tr>
<th>Table 1</th>
<th>Body and testis/epididymal region weights of roosters.</th>
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<tr>
<td>Body weight (kg)</td>
<td>Testis/epididymal region absolute weight (g)</td>
</tr>
<tr>
<td>Fixed(^a)</td>
<td>Frozen(^b)</td>
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<tr>
<td>2.8 ± 0.2</td>
<td>25 ± 4</td>
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Data are presented as mean ± SEM.

\(^a\) Tissues fixed in NBF for immunohistochemistry (\(n = 4\)).

\(^b\) Tissues frozen in liquid nitrogen for Western blotting (\(n = 6\)). BW = Body weight.

**Fig. 1.** Western blotting analysis of ER\(x\) and ER\(\beta\) expression in (A) testis and (B) epididymal region of roosters; \(n = 6\).
rooster ERα and ERβ proteins are not commercially available, it is difficult to fully determine the antibodies specificity by direct means.

3.2. Immunoreactivity for ERα

ERα protein was found on the nuclei of epithelial cells in the proximal efferent ductules, distal efferent ductules and epididymal duct; however, with remarkable differences in the intensity and pattern of the immunoreaction (Table 2 and Fig. 2A–D). In the efferent ductules, only the non-ciliated cells lining the epithelia were positive for ERα, whereas the ciliated cells were not reactive for this protein. Regarding the proximal efferent ductules, a weak and intermittent staining for the receptor was observed, as only a few non-ciliated cells, scattered in the epithelia, were positive. On the other hand, the frequency of positive non-ciliated cells in the distal efferent ductules was higher and the cells presented the strongest staining for ERα among all segments analyzed (Fig. 3A). Finally, the principal and basal cells of the epididymal duct showed a moderate immunostaining for ERα protein. The testis, rete testis epithelial cells as well as connective tissue cells in the epididymal region were not immunoreactive for ERα (Table 2 and Fig. 2). Since ERα reaction was negative in the rete testis and intermittent in the proximal efferent ductules, these segments were not included in the morphometrical analysis.

3.3. Immunoreactivity for ERβ

ERβ immunostaining was found in the nuclei of Leydig cells, as well as endothelial and smooth muscle cells of blood vessels, in the testis interstitium (Table 2 and Fig. 4A and B). Within the tubular compartment, ERβ was found in Sertoli cells, peritubular myoid cells and some germ cells, including spermatogonia and early spermatocytes. The staining intensity in the spermatogonia did not differ among different sections of the seminiferous tubules; however, the Sertoli cells and spermatocytes immunoreactivity varied considerably. Round and elongated spermatids were negative for ERβ.

Labeling for ERβ protein was also observed in the nuclei of cells lining the rete testis, proximal and distal efferent ductules as well as in the epididymal duct with no detectable regional differences between segments (Table 2, Fig. 3B, Fig. 4C–F). In the rete testis, ERβ staining was observed in the nuclei of cuboidal epithelial cells and in luminal cells morphologically similar to macrophages. In the efferent ductules, positivity was observed in the non-ciliated and...
ciliated cells in both proximal and distal segments. When comparing the cell types lining the epithelia, it was evidenced that ERβ expression was higher in the non-ciliated cells than in the ciliated cells in both segments (Fig. 3C). Regarding the epididymal duct, ERβ immunostaining was found on the nuclei of basal and principal cells with no evident differences in the staining intensity between both cell types. Some cells of the connective tissue were also positive for this receptor.

4. Discussion

The present study showed for the first time that estrogen receptor ERα and ERβ present differential cellular and regional distribution in the testis and epididymal region of roosters. ERβ was the predominant receptor detected in the testis, being expressed in the somatic and some germ cells. In the epididymal region, the most intense reaction for ERα was found in the distal efferent ductules, whereas ERβ was strongly expressed in all segments. Together, these results indicate that differential estrogen signaling via ERs may occur in testis and male tract of roosters.

The occurrence of ERα and ERβ in the rooster extratesticular excurrent ducts with highest ERα immunostaining observed in the efferent ductules is in agreement with other findings in mammalian and non-mammalian species [31,37,47,48,55,63]. It has been proven that estrogens rather than androgens control the bulk of testicular fluid reabsorption which occurs in the mammalian efferent ductules, as estrogens mediate the regulation of several key proteins involved in this process [36,43,51,53,57,71]. Similar to mammals, the avian efferent ductules are responsible for the reabsorption of more than 90% of the testicular fluid [2,20,50], in a process involving Na+/K+ ATPase, carbonic anhydrase, Na+/H+ exchanger (NHE3) and aquaporins [9,70]. Most of these proteins have been shown to be modulated by estrogen in mammals [43,51,53,57,61,71]. Therefore, it is plausible to infer that the estrogen responsive system may also have a role in regulating avian efferent ductules reabsorption. Corroborating this point of view, there is evidence that estrogenic/antiestrogenic compounds affects the structure of the avian epididymal region, as well as testis, including formation of blisters filled with fluid or seminiferous tubules dilation and reduced semen formation [11,13,30,50,69]. These testicular alterations are suggestive of disruption of efferent ductule reabsorative function in roosters with consequent back up of fluid in the testis, similar to that found in mammals with disruption of estrogen action [36,52,53].

Higher ERα labeling was found in the distal efferent ductules whereas the proximal segment presented a slight and intermittent immunopositivity. Differential expression of ERs does not appear to be restricted to roosters, as higher immunoreactivity for this receptor in the distal efferent ductules has also been observed in turtles [55]. Differences in distribution of other important receptors, such as androgen and vitamin D3 receptors, has already been described between proximal and distal efferent ductules of roosters [25,26]. One possible explanation for these differences can be the distinct embryological origin of both segments, as the proximal efferent ductules develop from the capsule of mesonephric corpuscles, whereas the distal efferent ductules originate from the mesonephric tubules [14,15]. Therefore, despite the common name, these ductules present considerable morphological differences and thus may play different functional and physiological roles in avian reproduction as already suggested [1,2].

Principal and basal cells of the epididymal duct were positive for ERα and ERβ, even though the ERα labeling was just slight in both cell types. Indeed, the occurrence of ERα protein within the epididymal duct has been controversial, as it was reported in some species [4,31,35,47,55,72] but appears absent in others [33,35,47,48,56,63]. The functions of estrogen receptors in the epididymal duct are still a matter of debate, but there is evidence that ERα may play a role in the maintenance of normal luminal milieu, by regulating the fluid osmolality and acidification, which is essential for sperm maturation and function [38,39]. The avian epididymal region is also thought to play an important role in the composition of luminal fluid to provide sperm maturation, since sperm undergoes changes in the pattern of surface protein expression that are correlated with changes in fluid composition [28]. The changes in avian epididymal fluid include creation and maintenance of a hyper-osmotic environment [19], a function compatible with the expression of specific water and ion transporters [9,70], known to be regulated by estrogen. Therefore, a speculative role for estrogen in the avian epididymal duct may be related to regulation of...
fluid/ion transporters involved in the maintenance of the proper luminal environment.

Within the rooster testis, ERβ was detected in most somatic cells and some germ cells, which is consistent with the findings for other species [8,10,16,18,47,54,62,65,72]. Contrasting with ERα, no labeling above background was observed for ERα in the rooster testis (even after using several different antibodies, data not shown), even thought the protein was detected by Western blotting assays. This result may be explained by differences in assay sensitivity, as Western blotting is known to be more sensitive for detection of small amounts of proteins than immunohistochemistry. In addition, ERα mRNA has been reported in the testis of roosters [68], being consistent with the protein expression found in this study by Western blotting. In agreement with our results, weak signal in ERα immunohistochemistry or negativity at all has been described for other species, including goat, roe deer, bat, human and non-human primates [10,33,54,62,63]. Estrogen action has been associated with spermatogenic cell proliferation, development and survival in the testis of different vertebrate species [17,23,44,45,64]. These functions appear to be mediated by factors produced by Sertoli cells [46], by a direct action of estrogen on germ cells or both [24]. Considering the ubiquitous cell distribution in the testis, it is believed that ERβ is the estrogen receptor subtype directly involved in this testicular estrogen signaling.

In conclusion, ERα and ERβ have distinct pattern of expression in the testis and epididymal region of roosters, as ERα expression was more pronounced in the distal efferent ductules whereas ERβ was more widely distributed along the rooster male genital organs. These data confirm the assumption that all components of the epididymal region may be a target for estrogen action, but cellular- and regional-specific functions for estrogens acting through ERα and ERβ are predicted.

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References

A. Blomqvist, C. Berg, L. Holm, I. Brandt, Y. Ridderstale, B. Brunstrom


J. Bouma, J.J. Nagler


J. Bouma, J.J. Nagler


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