

IMMUNOHISTOCHEMICAL LOCALIZATION OF AROMATASE ENZYME IN THE TESTES OF 12-WEEK OLD COCKERELS FED A DIET EITHER DEPLETED OF OR SUPPLEMENTED WITH VİTAMİN-D

İbrahim KÜRTÜL*

Geliş Tarihi : 30.11.2000

Summary: This study attempts to localize the aromatase enzyme in chicken testes and to determine the effects on it of vitamin D-depletion. White leghorn male chicks were fed adiet from 1 day to 12 weeks of age. The testes from both groups were removed, fixed and cut for immunohistochemistry. Immunohistochemical observations showed no specific staining in the vitamin D-depleted chicken testes since there was no developed germ cell possessing aromatase activity. In vitamin D-repleted chicken testes, the specific immunomarker for aromatase activity seemed to be present in close proximity to the lumen of tubules seminifer convolutes. In the brain of the mature male turkey used as a positive control, there was specific staining, indicating the specificity of the primary antibody used. To date this study is the first to reveal the effect of dietary vitamin D-depletion on aromatase activity in chicken testes. More detailed studies should be conducted on more mature chickens.

Key Words: Aromatase, chicken, testis, vitamin D.

Aromataz Enziminin 12 Haftalık D Vitamini Eklenmiş ve Eklenmemiş Rasyonlarla Beslenen Horoz Testislerindeki İmmunohistokimyasal Lokalizasyonu

Özet: Bu çalışmanın amacı 12 haftalık horoz testislerinde aromataz enziminin lokalizasyonunu ve D vitamini yokluğunun etkisini ortaya çıkarmaktır. Bu amaçla beyaz Leghorn erkek civcivler D vitamininden yoksun ve D vitamini katkılı iki rasyonla iki grup halinde 1. günden 12. haftaya kadar beslendi. İki gruptan da testisler alınarak tartıldı ve boyama için tespit edilerek kesildi. İmmunohistokimyasal incelemede D vitamininden yoksun rasyonla beslenen hayvanların testislerinde spesifik bir boyamaya rastlanmadı. D vitamini katkılı rasyonlarla beslenen hayvanların testislerinde ise lumen'e yakın bölgelerde spesifik reaksiyonlar başlamış gibiydi. Kontrol olarak kullanılan ergin hindi beyinlerinde ise spesifik reaksiyonlar gözlemlendi. Bu çalışmanın sonuçları horoz testislerinde aromataz aktivitesinin 12. hafta civarında ortaya çıkmaya başladığını düşündürmektedir. D vitaminin aromataz üzerindeki etkisini ortaya koymak için daha gelişmiş horoz testisleri üzerinde daha geniş çalışmalar yapılmalıdır.

Anahtar Sözcükler: Aromataz, horoz, testis, D vitamini.

INTRODUCTION

Aromatase is an enzyme which is responsible for the conversion of testosterone to estrogen. Estrogen production has been shown in various tissues of many species including human¹⁻³, rats⁴⁻⁹, and ferrets^{10,11}. It has been found that human adipose tissue is capable of producing estrogen by aromatization of androstenedione². Immunohistochemical localization, using a monoclonal antibody, has shown aromatase enzyme to be within the Leydig cell cytoplasm of normal adult human testes³.

A study carried out in mature rats has re-

vealed that aromatase activity in adult rats is regulated by luteinising hormon, LH⁴. An in vitro study has indicated in immature rats that, estradiol level can be stimulated by follicle stimulating hormone (FSH)-dependent aromatase found in the Sertoli cells; however, LH in mature rats is more potent than FSH⁵. Likewise, results of another study have showed that there was a significant increase in the level of aromatase activity in the Leydig cell but a decrease in the Sertoli cell with the age in the rat testes⁷. In a later study it has been suggested that Sertoli cells function in the stimulan of aromatase

* Department of Anatomy Faculty of the Veterinary Medicine, Ankara University, Ankara-TÜRKİYE

activity in Leydig cells of adult rats⁹.

High levels of aromatase activity have been found in hypothalamic and limbic nuclei of the rat brain, where the enzyme has been suggested to play an important role in both behavior and neuroendocrine function of androgens⁶. Results of another study have suggested that aromatase activity in the hypothalamus-preoptic area is regulated by testosterone or one of its metabolites⁸. It has been shown that the brains of both sexes of ferrets have the capability of synthesizing estrogen during the perinatal period of sexual differentiation¹⁰. Later⁹, it was shown that the level of estrogen in the brain is augmented for the feedback control of LH secretion and regulation of sexual behavior in adult ferrets¹¹.

Reproductive behaviors and masculine aggressive behaviors in vertebrates are regulated by testosterone. Although testosterone is dominant in male behaviors, it is now well known that for the full expression of testosterone action in the brain, aromatization of the intact molecule to the estrogen is needed¹². This study¹² demonstrated that aromatization compromises assertive behavior in Japanese quail, and is necessary for the activation of aggressiveness in this species.

Most recent studies have shown that germ cells of the mouse testis¹³, and rooster epididymal sperm and testicular germ cells¹⁴ contain P450_{arom} by immunocytochemistry using a polyclonal antihuman placental cytochrome P450_{arom} antibody. In a study done using male and female siblings, the effects of aromatase deficiency caused by a gene mutation have shown that estrogen is essential for normal skeletal maturation in women as well as in men¹⁵.

Thus, the study presented here is an attempt to reveal immunohistochemical localization of aromatase enzyme in the testes of vitamin D-depleted and repleted chicken at 12-week of age.

MATERIAL and METHODS

White leghorn male chicks were raised from 1 day to 12 weeks of age in two different groups. Each group was fed one of the following diets: vitamin D-depleted diet + 3% calcium and

vitamin D-repleted diet + 3% calcium. The diets had already been analyzed for the levels of vitamin-D and Ca. There were 9 chickens in each group. Chickens were weighed before they were euthanized by Sodium Pentobarbital.

For fixation and immunohistochemical localization of aromatase enzyme, testes were removed from chickens and were fixed for 2 weeks by the use of the freeze substitution technique at -70 °C, using absolute ethanol containing 0.5 % glutaraldehyde. Fixed tissues then were processed and embedded in paraffin by the use of described techniques^{16,17}. Five-micrometers in thickness of tissue sections from each group were serially cut for the immunohistochemical localization of aromatase.

Tissue sections were first deparaffinized and incubated with 5 % urea. Secondly, the slides were incubated with 5 % normal goat serum, NGS, and a polyclonal rabbit antihuman placental aromatase antibody (P450_{arom}, a kind gift from Dr. Yoshio Osawa, Hauptman-Woodward Medical Research Institute, Buffalo, New York 14203). Control slides made from the tissue of mature male turkeys were also incubated in normal rabbit serum, NRS. Finally, they were incubated with biotinylated goat anti-rabbit IgG, with streptavidin, and with 0.05 % 3,3'-diaminobenzidine hydrochloride (DAB) & 0.01 % hydrogen peroxide. Tissue sections of mature male turkey brain were used as positive controls.

RESULTS

Specific immunomarker for aromatase activity in the vitamin D-repleted chicken testis in the sufficient morphological development seems to be present nearer the lumen of seminiferous convoluted tubuli (Fig. 1). Immunocontrol of section adjacent to that in Figure 1, stained with non-immune serum is displayed in Figure 2. There was no specific staining for aromatase activity in the vitamin D-depleted chicken testes (Fig. 3). Immunocontrol of section adjacent to that in Figure 3, stained with non-immune serum has also shown no specific staining for aromatase activity. In the hypothalamus-preoptic area of mature male turkey brain used as a positive control, there was a specific staining that con-

firmed the effectiveness the primary antibody used (Fig. 4). Immunocontrol of section adjacent to that in Figure 4., stained with non-immune serum is demonstrated in Figure 5. Adjacent section slides incubated with NRS did not show any specific staining, again indicating effectiveness of the primary antibody used in the experiment.

DISCUSSION

Aromatase activity in the testes of vitamin D-depleted and -repleted 12-week old chickens was studied. Immunohistochemical observations showed that there was no specific staining in the vitamin D-depleted chicken testes. In vitamin D-repleted chicken testes, specific immunomarker for aromatase activity seems to be present nearer the lumina of seminiferous convoluted tubuli. Pachytene spermatocytes and spermatids are found deeper in the seminiferous convoluted tubuli. For this, immunomarker for aromatase activity may be non-specific. This may still indicate that aromatase begins to appear in more mature germ cells (at least at the level of pachytene spermatocytes and spermatids), which is approximately when they begin to appear (12-weeks age in domestic chicken, as in our study). In the brain of mature male turkey used as a positive control, there was specific staining, indicating that the primary antibody used was specific to aromatase.

Aromatase is responsible for the conversion of testosterone to estrogen. The presence of estrogen in the testes of vertebrates is believed to depend on the conversion of testosterone to estrogen by aromatase enzyme. Tissues of various species have been documented to contain this enzyme such as human^{2,3} rats^{5,6} and rooster¹⁴.

A recent study has suggested that vitamin D, along with glucocorticoids, enhances the enzymatic activity and expression of mRNA for P450_{arom} gene expression¹⁸. Estrogen has been suggested to have an important role in the control of spermatogenesis¹². In our study, if there is an inhibition of aromatase activity by vitamin D-depletion, decreased estrogen production also may be the cause of deterioration of the male germ cells seen in the seminiferous convoluted tubuli in the testes of vitamin D-depleted chickens. In other words, germ cells in the vitamin

D-depleted chicken testes have not yet reached at the level of the aromatase production activity.

Aromatase enzyme activity is regulated by LH in the testes. An in vitro study⁵ has indicated that in the immature rat testis FSH plays an important role on the aromatase activity in the Sertoli cells. However, in the mature rat testis, LH is more potent than FSH. Aromatase is present in hypothalamic and limbic nuclei of the brain, where the enzyme is thought to affect both behavior and neuroendocrine function of androgens.

Aromatase activity in the germ cells and sperm cells of the mature mouse¹³ and the rooster¹⁴ was shown. In both studies, the enzyme was present in pachytene spermatocytes and further developed germ cells. These germ cells usually begins to appear in around 12-week-old chicken testes. In our study, since there was no immunostaining for aromatase activity in the seminiferous tubules of the testes from vitamin D-depleted chicken, it can be concluded that these spermatocytes were premature for aromatase activity. The chickens that were used in the present study were 12-weeks old. Apparently, to reveal the effects of vitamin D-depletion on aromatase activity in chicken testis, further studies should be conducted in more mature chickens at molecular level.

REFERENCES

1. Thompsom EA and Siitteri PK: Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microzomes during aromatization of androstenedione. *The Journal of Biological Chemistry*, 249: 5364-5372, 1974.
2. Ackerman GE, Smith ME, Mendelson CR, McDonald PC and Simpson ER: Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. *Journal of Clinical Endocrinology and Metabolism*, 53: 412-417, 1981.
3. Inkster S, Yue W and Brodie A: Human testicular aromatase: immunocytochemical and biochemical studies. *Journal of Clinical Endocrinology and Metabolism*, 80: 1941-1947, 1995.
4. Valladares LE and Payne AH: Induction of testicular aromatization by luteinizing hormone in mature rats. *Endocrinology*, 105: 431-436, 1979.
5. Rommerts FFG, Jong FH, Brinkman AO and Mojen HS: Development and cellular localization of rat testicular aromatase activity. *Journal of Reproduc-*

- tion and Fertility, 65: 281-288, 1982.
6. Roselli CE, Ellinwood WE and Resko JA: Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism. *Endocrinology*, 114: 2183-2189, 1984.
 7. Chon-Hwa Morris T, Aquilano DR and Dufau ML: Cellular localization of rat testicular aromatase activity during development. *Endocrinology*, 116: 38-46, 1985.
 8. Roselli CE, Horton LE and Resko JA: Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology*, 117: 2471-2477, 1985.
 9. Carreau S, Papadopoulos V and Drosowsky MA: Stimulation of adult rat leydig cell aromatase activity by a sertoli cell factor. *Endocrinology*, 122: 1103-1109, 1988.
 10. Tobet SA, Shim JH, Osiecki ST, Baum MJ, and Canick JA: Androgen aromatization and 5 α -reduction in ferret brain during perinatal development: Effects of sex and testosterone manipulation. *Endocrinology*, 116: 1869-1877, 1985.
 11. Weaver CE and Baum MJ: Differential regulation of brain aromatase by androgen in adult and fetal ferrets. *Endocrinology*, 128: 1247-1254, 1991.
 12. Schlinger BA and Callard GV: Aromatization mediates aggressive behavior in quail. *general and comparative endocrinology*, 79: 39-53, 1990.
 13. Nitta H, Bunick B, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K and Bahr JM: Germ cells of the mouse testis express p450 aromatase. *Endocrinology*, 132: 1396-1401, 1993.
 14. Kwon S, Hess RA, Bunick D, Nitta H, Janulis L, Osawa Y, and Bahr JM: Rooster testicular germ cells and epididymal sperm Contain P450 aromatase. *Biology of Reproduction*, 53: 1259-1264, 1995.
 15. Morishima A, Grumbach MM, Simpson ER, Fisher C, and Qin K: Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *Journal of Clinical Endocrinology and Metabolism*, 80: 3689-3698, 1995.
 16. Inpanbutr N, Reiswig JD, Bacon WL, Slemmons RD, and Lacopino AM: Effect of vitamin D on testicular cabp28k expression and serum testosterone in chickens. *Biology of Reproduction*, 54: 242-248, 1996.
 17. Inpanbutr N and Taylor AN: Expression of calbindin-d28k in developing and growing chick testes. *Histochemistry*, 97: 335-339, 1992.
 18. Tanaka S, Haji M, Takayanagi R, Tanaka S, Sugioka Y, and Nawata H: 1,25-Dihydroxyvitamin D₃ enhances the enzymatic activity and expression of the messenger ribonucleic acid for aromatase cytochrome P450 synergistically with dexamethasone depending on the vitamin D receptor level in cultured human osteoblasts. *Endocrinology*, 137: 1860-1869, 1996.

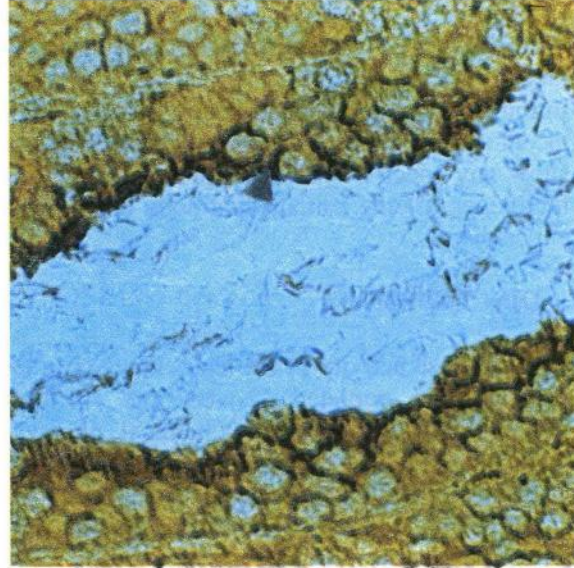


Figure 1. Specific staining for aromatase activity seems to be present nearer the lumen of seminiferous convoluted tubuli in the vitamin D-repleted chicken (►)X 400.

Resim 1. D vitamini katkılı yemle beslenen horozlarda aromataz aktivitesi için spesifik boyama tubules seminifer convolutes'in özellikle lümenine yakın kısımlarda görülüyor gibi X 400.

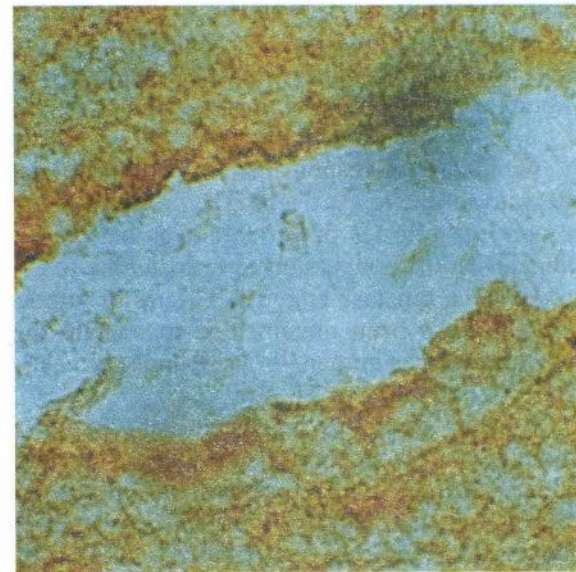


Figure 2. Immunocontrol of sequential of figure 1, displaying no specific staining. X400.

Resim 2. Şekil 1'deki görüntünün immun kontrol boyamasında herhangi bir spesifik boyama gözükmemekte. X 400.

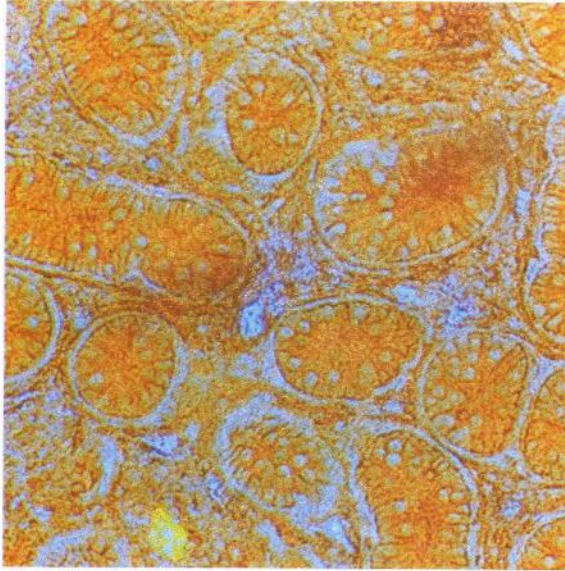


Figure 3. No specific staining for aromatase activity in the vitamin D-depleted chicken. X400.

Resim 3. D vitamini ilave edilmeyen rasyonla beslenmiş horozlarda aromataz aktivitesi için herhangi bir spesifik boyama gözükmemekte. X400.

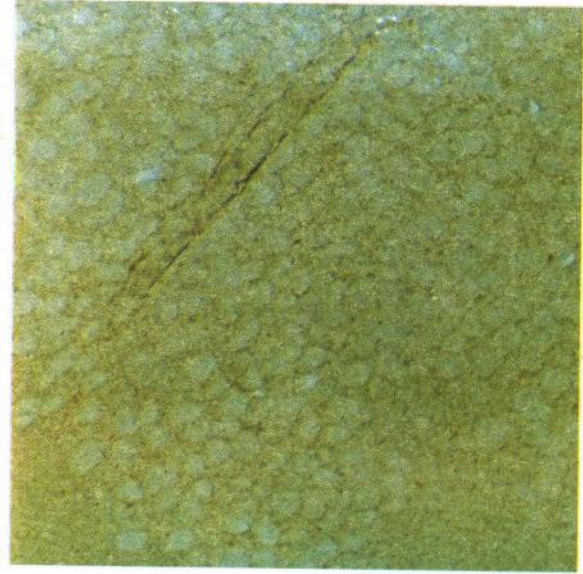


Figure 5. Immunocontrol of sequential of figure 4, indicating effectiveness of primary antibody used in the study. X400.

Resim 5. Şekil 4'deki görüntünün immün kontrol boyamasında herhangi bir spesifik boyama gözükmemekte. X400.

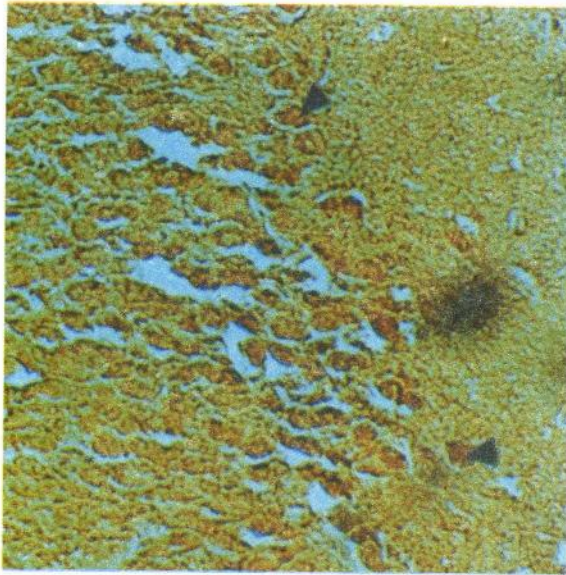


Figure 4. Specific staining for aromatase activity in the brain of turkey, (▶) (positive control). X400.

Resim 4. Hindi beyninde aromataz aktivitesini gösteren spesifik boyama, (pozitif kontrol). x400.