Kafkas Univ Vet Fak Derg
18 (2): 243-248, 2012
DOI:10.9775/kvfd.2011.5370

RESEARCH ARTICLE

Investigation on Porcine Aromatase *(CYP19)* as a Specific Target Gene for Boar Testis

Mehmet Ulaş ÇINAR * Asep GUNAWAN *

* Institute of Animal Sciences, Animal Breeding and Husbandry Group, University of Bonn, 53115 Bonn, GERMANY

Makale Kodu (Article Code): KVFD-2011-5370

Summary

Cytochrome P450 aromatase is the key enzyme in estrogen biosynthesis, encoded by *CYP19* gene. Impairment of spermatogenesis associated with a decrease in sperm motility and inability to fertilize oocytes in mice due to the lacking of *CYP19* was observed. However, it is little known about the *CYP19* roles in boar spermatogenesis and fertility. Therefore, the aim of this research was to investigate the mRNA and protein expression of CYP19 in boar reproductive tissues from boars with different sperm quality. For mRNA and protein expression study, a total of six boars were divided into two groups with Group 1 (G-I) and Group 2 (G-II) where G-I was characterized for relatively a better sperm quality. The result showed that the *CYP19* transcript was not expressed throughout the male reproductive system. mRNA expression of CYP19 was higher only in testis. *CYP19* expression was similar in testis collected from G-I and G-II boars. The *CYP19* protein expression results from western blot were different with the results of qRT-PCR. The CYP19 protein was higher in testis collected from G-II than G-I boars. The CYP19 protein localization in testis showed a strong staining only in the cytoplasm Leydig cell. These results shed new light on the roles of porcine CYP19 in spermatogenesis as a specific target gene for testis.

Keywords: *mRNA, Protein, Immunofluorescence, Testis, Boar spermatozoa*

Erkek Domuzlarda Testisler İçin Hedef Bir Gen Olan Aromataz *(CYP19)* Üzerine Araştırma

Özet

Sitokrom P450 aromataz östrojen üretiminde önemli bir enzim olup, *CYP19* geni tarafından ifade edilir. *CYP19* geninin yokluğunda farelerde sperm üretiminin zarar gördüğü ve ilişkili olarak spermlerin yüzme ve yumurtaları dölleme kabiliyetinin azaldığı gözlenmiştir. Buna karşılık CYP19'un domuz sperm üretiminde ve yumurtaların döllenmesinde nasıl bir rol oynadığı bilinmemektedir. Bu nedenle, bu calışmanın amacı CYP19 mRNA ve protein ifadesini farklı sperm kalite özelliklerine sahip olan erkek domuzların üreme organı dokularında araştırmaktır. mRNA ve protein ifadesi çalısması için altı erkek domuz, grup 1 (G-I) ve grup 2 (G-II) olarak iki gruba ayrılmıştır. G-I hayvanlar göreceli olarak G-II hayvanlara göre daha iyi sperm özelliklerine sahiptir. Sonuçlar *CYP19* mRNA ifadesinin tüm erkek üreme organlarında ifade edilmediğini göstermiştir. *CYP19'un* mRNA ifadesi en çok testis dokularında gözlenmiştir ve ayrıca G-I ve G-II domuzlardan alınan testis örneklerinde aynı düzeyde tespit edilmiştir. CYP19'un Western-Blot protein ifadesi sonuçları qRT-PCR sonuçlarından farklı olarak gözlenmiştir. CYP19 protein ifadesi G-II hayvanlarda G-I hayvanlara göre daha yüksek düzeyde gözlenmiştir. Testiste CYP19 protein lokalizasyonu sitoplazma Leydig hücrelerinde güçlü bir sinyal göstermiştir. Sonuçlar, *CYP19* geninin domuzlarda sperm üretimi için testislerde spesifik bir hedef gen olduğuna dair yeni bulgular ortaya koymuştur.

Anahtar sözcükler: *mRNA, Protein, İmmunfloresans, Testis, Domuz spermatozoa*

INTRODUCTION

Aromatase is the only enzyme responsible for the irreversible bioconversion of androgens into estrogens. This enzyme is a complex composed of an ubiquitous NADPH cytochrome P450 reductase and a specific cytochrome P450 aromatase encoded by the CYP19 gene [1]. Estrogens have been for a long time considered as a specific female hormone; however, the presence of estrogens in the male gonad is now well documented [2]. Indeed the androgen/estrogen balance is essential for normal sexual development and reproduction in mammals. In

İletişim (Correspondence)
+49 228 733583
ucin@itw.uni-bonn.de

the mammalian testis, maintenance of this balance is under a fine tuning via endocrine and paracrine factors, but is also related to the aromatase activity [2]. Although in humans and some higher primates, there is a more extensive distribution of estrogen biosynthesis including placenta, adipose tissue, liver and skin [3], according to the steroid levels assayed in the testicular artery and vein, testes are a major source of estrogens [4] and aromatase has been immunolocalized in Leydig cells [5,6]. In the testis of mammals, gonadotropins and testosterone together with numerous locally-produced factors are responsible for the induction and/or the maintenance of spermatogenesis [7].

Deficieny of CYP19 and effects on spermatogenesis were shown in different mammalian species. Impairment of spermatogenesis associated with a decrease sperm motility and inability to fertilize oocytes was reported due to lacking of *CYP19* gene in mice [8]. In buffalo, higher expression of *CYP19* was found in spermatozoa obtained from good quality of semen as compared to spermatozoa from poor quality of semen [9]. Similarly, the higher expression *CYP19* was found in motile spermatozoa as compared to non-motile [10]. CYP19 mRNA and protein expressed higher in adult stallions compared to colts [1]. In the adult stallions, the testis, among the tissues analyzed, found to be the major source of aromatase that shows gene expression is specifically enhanced at this level, and is responsible for the high estrogen synthesis [1].

Testis is responsible for the induction and/or the maintenance of spermatogenesis and is the major source for CYP19 enzyme [11]. However, there is no information regarding the expression of *CYP19* in reproductive tissue from different quality of boar sperm and very few known about the role of *CYP19* in boar spermatogenesis. Therefore, this research was aimed to investigate the mRNA and protein expression of CYP19 in boar reproductive tissues from boars with different sperm quality.

MATERIALS and METHODS

Samples for mRNA and Protein Expression Analysis

Boars from the artificial insemination station SuisAG (Sempach, Switzerland) were selected based on extreme phenotypes [high/low sperm concentration (SCON), sperm motility (SMOT), and sperm volume (SVOL)]. The SCON (average sperm concentration) was highly negative (r = -0.8) correlated with SVOL (average semen volume), whereas SCON was highly positive (r = 0.7) correlated with SMOT (average sperm motility). Moreover, SVOL was highly negative (r = -0.8) correlated with SMOT. Therefore, grouping was done on the basis of SCON, SVOL and SMOT *(Table 1)*. A total of six animals were selected and equally divided into group I (G-I) with high SCON (>262.32 $\times 10^6$ ml), high SMOT (>76.59%) and low SVOL (<215.24 ml/ejaculation) and group II (G-II) with low sperm concentration and motility, and high sperm volume *(Table 1)*. The difference between the two groups was calculated using proc t-test in SAS. There were differences for SCON (P<0.05) and for SVOL (P<0.01) between G-I and G-II, whereas for the SMOT the difference was not significant (P = 0.12). Reproductive tissues (testis, head of epididymis, body of epididymis, tail of epididymis, vas deferens, bulbourethral gland, vesicular glands and prostate gland), non reproductive tissues (brain, liver and skeletal muscle tissue) and semen samples (spermatozoa) of six boars (Duroc, Large White and Landrace) were collected for the mRNA and protein study [12].

Semi-Quantitative PCR

Total RNA was isolated using TRI Reagent (Sigma-Aldrich) from different reproductive and non reproductive tissues of breeding boars mentioned in previous chapter. RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated using on-column RNase-Free DNase set (Promega) and quantified sphectrophotometrically (Nano Drop, ND8000 Thermo Scientific). Furthermore, RNA integrity was checked by 2% agarose gel electrophoresis. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen). cDNA amplification was performed by using specific forward and reverse primers (forward: 5´-ttag caagtcctcaagtgtg -3´ and reverse: 5´- ccaggaagaggttgtt agag-3´) derived from porcine *CYP19* sequence (GenBank accession: U37311). Amplification was performed with an initial heating at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, annealing temperature at 52°C for 30 sec and 72°C for 30 sec, on the PCR Thermal Cycler (Bio-Rad). PCR product were electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide. Amplification of *GAPDH* (forward: 5´-acccagaagactgtgga tgg-3´ and reverse: 5´-acgcctgcttcaccaccttc-3´) (GenBank accession No. AF017079) served as housekeeping gene.

Table 1. *Means, standard errors (S.E.), number of boars and ranges of traits selected for mRNA and protein expression study*
Tablo1. *mRNA ve protein ifadesi için seçilen erkek domuzlara ait özelliklerin ortalamaları, standart hataları, erkek domuz sayıları ve örneklem genişliği*

Traits	Selected Animals (n = 6)		G-I (n = 3)		G-II (n = 3)	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
SCON (10^6/ml)	262.32	87.97	335.94	50.78	188.70	22.54
SVOL (ml)	215.24	34.93	185.07	16.33	245.40	7.42
SMOT (%)	76.59	3.71	79.03	1.89	74.14	3.60

Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR, total RNA and cDNA synthesis from different reproductive tissues of two divergent groups of animals (G-I and G-II) were done as described above. The same primers pair used in semi-quantitative PCR were also used in qRT-PCR. Nine-fold serial dilution of plasmids DNA were prepared and used as template for the generation of the standard curve. In each run, the 96-well microtiter plate contained each cDNA sample, plasmid standards for the standard curves and no-template control. To ensure repeatability of the experiments, each plate was run in three replications. Quantitative real-time RT-PCR (qRT-PCR) was set up using 2 µl first-strand cDNA template, 7.6 µl deionized H_2O, 0.2 µM of upstream and downstream primers and 10 µl 1× Power SYBR Green I master mix with ROX as reference dye (Bio-Rad). The thermal cycling conditions were 3 min at 94°C followed by 40 cycles of 20 sec at 94°C and 1 min at 60°C. Experiments were performed using the ABI prism®7000 (Applied Biosystems) qRT-PCR system. An amplification-based threshold and adaptive baseline were selected as algorithms. The housekeeping gene *GAPDH* (forward: 5´-acccagaagactgtggatgg-3´ and reverse: 5´-acgcctgcttcaccaccttc-3´) derived from porcine sequence (GenBank accession No. AF017079) was used for the data normalization. Final results were reported as the relative abundance level after normalizing with mRNA expression level of the housekeeping gene. Differences in CYP19 mRNA expression were analyzed with the simple t-test in SAS software (SAS Institute Inc., ver. 9.2). Values of $P<0.05$ were considered to indicate statistically significant differences.

Western Blotting

Total protein was isolated from the tissues of the six boars which were also used for mRNA isolation. Total protein was isolated by using TRI Reagent (Sigma-Aldrich), before protein separation in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (gradient 4-18%) and transferring onto a nitrocellulose membrane (Amersham Biosciences). The membranes were further kept in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% Polyvinylpyrolidone) for 1 h at room temperature; the membrane was incubated overnight at 4°C with the anti-CYP19 antibody purified from goat polyclonal antibody (Cat.nr.14245; Santa Cruz) in the blocking medium (diluted 1:500). Non-specific binding of antibody was washed off with six changes of 0.1% PBST. The horseradish peroxidase conjugated donkey anti-goat IgG secondary antibody (Cat.No. Sc2020; Santa Cruz) was used as the secondary antibody (diluted 1:5000). The membrane was incubated for 1 hour at room temperature with secondary antibody, followed by washing with six changes of 0.1% PBST. The chemiluminesce was detected by using the ECL plus western blotting detection system (Amersham Biosciences) and visualized by using Kodak BioMax XAR film (Kodak). GAPDH was used as a loading control and for normalization. The membrane was stripped by incubation in 2% SDS, 100 mM Tris-HCl, 0.1% beta-mercaptoethanol for 30 min at 60°C and re-probed with GAPDH antibody (Cat.No. Sc20357; Santa Cruz).

Protein Localization by Immunofluorescence

Due to the limitations of fresh samples from G-I and G-II boars, we collected fresh testis sample from a healthy breeding boar after slaughtering for protein localization. Immunofluorescence staining was performed on 8 µm cryostat sections of snap frozen tissues. All sections were kept in -80°C for further analysis. To block unspecific staining, sections were incubated for 30 minutes at room temperature with 5% bovine serum albumin in PBS (50 nM sodium phosphate, pH 7.4; 0.9% NaCl). Sections were incubated overnight at 4°C with the CYP19 goat polyclonal primary antibody (Cat.nr.14245; Santa Cruz) diluted at 1:50 in PBST followed by six times (10 min to time) washing with PBS. Then, the sections were incubated 1 hour at room temperature with the biotinylated donkey anti-rabbit IgG-B conjugated with fluorescein isothiocynate (FITC) reactive water-soluble fluorescent dye (Cat nr. Sc2090; Santa Cruz) (dilution 1:200) which was used as a secondary antibody for CYP19. Then sections were washed six times (10 min to time) with PBS. Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss). In case of negative controls, PBS was used instead of the primary antibody.

RESULTS

mRNA Expression by Semi-Quantitative PCR

Among reproductive and non-reproductive tissues *CYP19* gene expression was detected only in testis *(Fig. 1)*. When semi-quantitative PCR was applied in reproductive tissues from G-I and G-II boars, CYP19 mRNA expression was observed in testis among all different reproductive tissues *(Fig. 2a)*. The semi-quantitative reverse transcription PCR result of *GAPDH* showed no remarkable differences among tissues *(Fig. 1 & Fig. 2a)*.

mRNA and Protein Expression Study in Testis from G-I and G-II Boars

Semi-quantitative PCR results showed that the *CYP19* mRNA was highly expressed in testis samples from G-I and G-II boars *(Fig. 2a)*. Results of semi-quantitative PCR overlapped with the results of the qRT-PCR *(Fig. 2b)*. qRT-PCR showed that no significant mRNA expression difference in testis between G-I and G-II boars *(Fig. 2b)*. CYP19 protein with 58 kDa molecular weight was detected in testis of G-I and G-II boars *(Fig. 3a)*. Protein expression result of western blot appeared to be inconsistent with

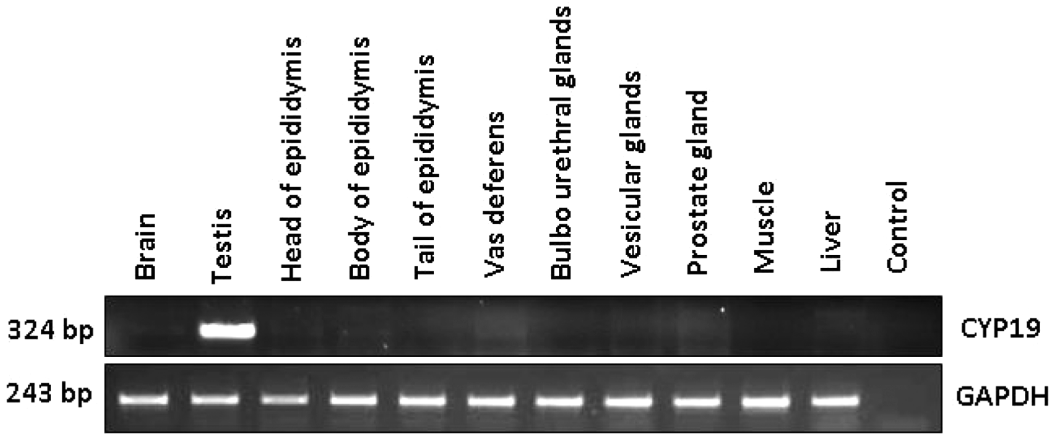

Fig 1. mRNA expression of *CYP19* in reproductive and non-reproductive tissues by semi-quantitative PCR

Şekil 1. *CYP19* geninin üreme ve üreme dışı dokularda yarı-kantitatif PCR mRNA ifadesi

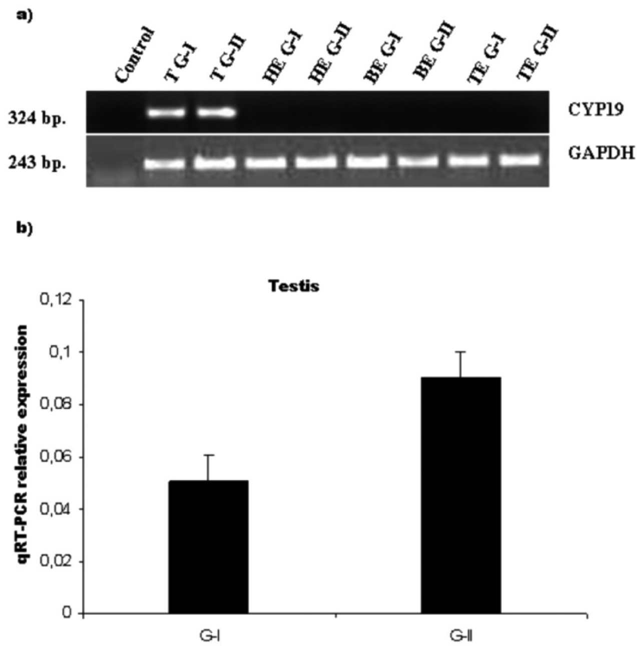

Fig 2. mRNA expression of *CYP19* in reproductive tissues (testis, head, body and tail of the epididymis), (2a) CYP19 mRNA expression in different reproductive tissues from G-I and G-II boars by semi-quantitative PCR, (2b) *CYP19* mRNA expression in different reproductive tissues from G-I and G-II boars by qRT-PCR

Şekil 2. *CYP19* geninin üreme dokularında (testis, epididimisin baş, vücut, ve kuyruk kısmı) mRNA ifadesi, (2a) *CYP19* mRNA ifadesinin üreme dokularında yarı-kantitatif PCR ile G-I ve G-II erkek domuzlarda gösterimi, (2b) *CYP19* mRNA ifadesinin üreme dokularında qRT-PCR ile G-I ve G-II erkek domuzlarda gösterimi

the results of the qRT-PCR *(Fig 3b & Fig. 2b)*. The western blot result showed that the CYP19 protein expression was higher in the testis of G-II boars compared to G-I boars *(Fig. 3b)*.

Localization of CYP19 Protein in Boar Reproductive Tissues by Immunofluorescence

Sections of testis were stained through the same

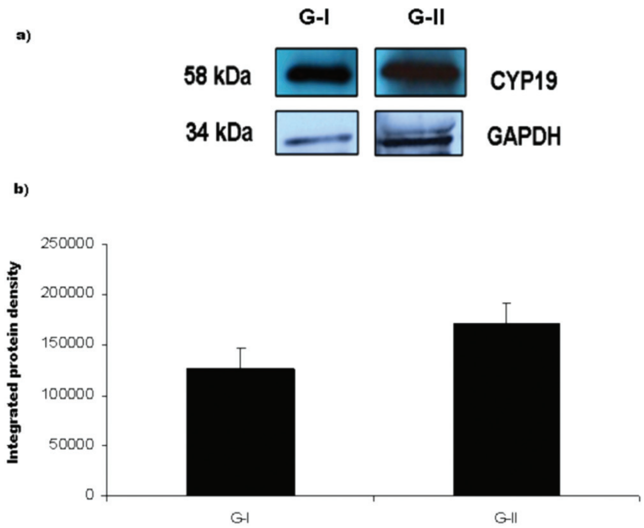

Fig 3. CYP19 protein expression in testis from G-I and G-II boars by Western Blotting, (3a) Western-Blot results of CYP19 and GAPDH proteins in testis from G-I and G-II boars, (3b) Quantification of Western-Blot results between two group boars

Şekil 3. G-I ve G-II erkek domuzlarda CYP19 protein ifadesinin Western-Blot ile gösterilmesi, (3a) G-I ve G-II erkek domuzlarda CYP19 ve GAPDH proteinlerinin Western-Blot ile gösterilmesi, (3b) Western-Blot sonuçlarının iki grup erkek domuz arasında sayısallaştırılmış görüntüsü

optical panel for the cell surface CYP19 protein expression *(Fig. 4)*. Immunoreactive CYP19 protein was observed as strong staining only in the cytoplasm of the Leydig cells in testis. No immunostaining was detected in Sertoli cells *(Fig. 4a)*.

DISCUSSION

CYP19 mRNA and Protein Expression in Boar Reproductive Tissues

In this study, we measured the *CYP19* mRNA gene expression in various boar tissues for the first time. Moreover, CYP19 gene was measured in various reproductive organs from boars with divergent sperm quality traits.

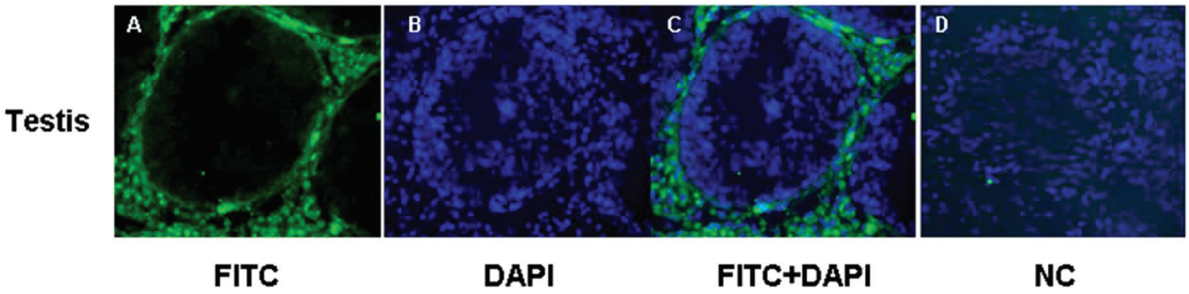

Fig 4. Localization of *CYP19* protein in testis of boar, (4a) Immunofluorescence detection of CYP19 in Leydig cells. Leydig cells were stained with *CYP19* (arrows), (4b) The cell nuclei were counterstained with DAPI, (4c) Merged images, (4d) Negative control

Şekil 4. *CYP19* proteinin erkek domuz testisinde lokalizasyonu, (4a) *CYP19*'un Leydig hücrelerinde immunoflorasan tesbiti, (4b) Hücre cekirdeklerinin DAPI ile işaretlenmiş görüntüsü. (4c) Birleştirilmiş görüntü, (4d) Negatif kontrol

mRNA and protein expression of CYP19 was measured quantitatively by using qRT-PCR and western-blot respectively. CYP19 protein was also localized in testis section to proof the presence of this protein in Leydig cells. Results demonstrated that the porcine *CYP19* mRNA expression was observed only in testis *(Fig. 2 & Fig. 3)* and *CYP19* transcript was not widespread throughout the male reproductive tract. Importantly, *CYP19* expressed highly in testis of boars collected from G-I and G-II.

In our study *CYP19* transcript was not detected throughout the male reproductive system except in testis which is in accordance with the previous reports describing that *CYP19* expression is found only in testis in human [13] and stallion [1]. The porcine *CYP19* gene is expressed in a tissue-specific fashion in three principal sites, the gonads, the placenta, and the preimplantation blastocyst [14]. Tissue-specific expression of the *CYP19* promoted survival of the *CYP19* genes [15]. Although promoter that drives ovarian *CYP19* expression is well conserved in mammalian species, expression in the pig testis is driven by a different promoter than that utilized in the ovary [16]. Testis is major source for CYP19 enzyme and corresponds to daily sperm production [11] is supporting our findings. CYP19 enzyme catalyses the synthesis of estrogens from androgens and play roles in the sexual development, reproduction and in behaviour [14]. In the mammalian testis, gonadotropins and testosterone together with numerous locally-produced factors are responsible for the induction and/or the maintenance of spermatogenesis [7]. Levallet et al.[17] and Janulis et al.[18] showed that the highest amount of *CYP19* mRNA in testis is related to the estrogen production. Gist et al.[19] detected *CYP19* in the testis and suggested that testicular estrogens might have a regulatory influence on the spermatogenesis in the testis. Investigation on spermatogenesis in knockout mice (ArKO) revealed that lack in functional aromatase (CYP19) enzyme is unable to convert C_{19} steroids (androgens) to C_{18} steroids (estrogens) [20]. CYP19 deficient mice indicated that spermatogenesis required the presence estrodiol-17 beta (E2). E2 is necessary to stimulate glucose uptake, oxidative metabolism and motility. CYP19 deficient mice (ArKO) are reported to have disrupted spermatogenesis associated with a decrease in sperm motility and inability to fertilize oocytes [8,20,21]. The presence of *CYP19* transcripts could be a marker of male gamete quality since existence of it reported to be influence the motility and the acrosome reaction [22]. However, our results showed that CYP19 mRNA and protein expression are tended to be higher in G-II boars but the mRNA and protein expression differences between G-I and G-II were not statistically significant. Moreover, it is important to note that all boars used in this study were used for breeding purpose by the breeding company which means all boars were good enough. The differences for SCON, SVOL and SMOT between two groups of boars were not extreme. The G-II boars had comparatively poor quality semen when compared to G-I.

Protein Localization of CYP19

Immunoreactive CYP19 protein was observed strong staining only in cytoplasm of Leydig cells in testis. These results are in good agreement with the previous study in boar [23], horses [6,24], ram [25] and human [13,26]. However, some studies detected immunoreactive CYP19 in both the Leydig cells and seminiferous tubules in rat [17,27], mouse [28] and rooster [29]. Importantly, this study confirmed that immunoreactive CYP19 is restricted only to the Leydig cells in the testis in mammalian species like horses [5,30], pig [16,23] and rams [25]. It has been reported that the major function of Leydig cells is to produce estrogen and being the source of estrogen biosynthesis in rat [30] and human [31]. Moreover, Hess et al.[30] showed in male horse that there is an age-dependent shift in the localization of immunoreactive CYP19 from the Leydig cell to Sertoli cells. In adult animals, highest CYP19 activity are found in the Leydig cells [1] but in immature rat before puberty it is found more in Sertoli cell [30]. The boar used for localization in this study was an adult breeding boar which supports our finding for localization of CYP19 only in the Leydig cells. The mRNA and protein expression study of the CYP19 imply that it may have a role in spermatogenesis and specific target gene in testis in pigs. Therefore, the results of this study could be valuable to shed light on the roles of CYP19 in spermatogenesis in boars.

ACKNOWLEDGMENTS

This study was financially supported by FBF (Förderverein Biotechnologieforschung e. V.), Bonn, Germany.

REFERENCES

1. Lemazurier E, Sourdaine P, Nativelle C, Plainfosse B, Seralini GE: Aromatase gene expression in the stallion. *Mol Cell Endocrinol*, 178 (1-2): 133-139, 2001.

2. Carreau S, Silandre D, Bourguiba S, Hamden K, Said L, Lambard S, Galeraud-Denis I, Delalande C: Estrogens and male reproduction: A new concept. *Braz J Med Biol Res*, 40 (6): 761-768, 2007.

3. Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE, Hinshelwood MM, Graham-Lorence S, Sun TJ, Fisher CR, Qin KN: Aromatase expression in health and disease. *Recent Progress in Hormone Research, Proceedings of the 1996 Conference*, Vol. 52, 52, 185-214, 1997.

4. Setchell BP, Cox JE: Secretion of free and conjugated steroids by the horse testis into lymph and venous blood. *J Reprod Fertil*, 32, 123-127, 1982.

5. Almadhidi J, Seralini GE, Fresnel J, Silberzahn P, Gaillard JL: Immunohistochemical localization of cytochrome-P450 aromatase in equine gonads. *J Histochem Cytochem*, 43 (6): 571-577, 1995.

6. Eisenhauer KM, Mccue PM, Nayden DK, Osawa Y, Roser JF: Localization of aromatase in equine Leydig-cells. *Domest Anim Endocrin*, 11 (3): 291-298, 1994.

7. Saez JM: Leydig-Cells - Endocrine, Paracrine, and Autocrine Regulation. *Endocr Rev*, 15 (5): 574-626, 1994.

8. Robertson KM, O'Donnell L, Jones MEE, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER: Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proceedings of the National Academy of Sciences of the United States of America*, 96 (14): 7986-7991, 1999.

9. Singh D, Tiwari A, Kumar OS, Sharma MK: Expression of cytochrome P450 aromatase transcripts in buffalo *(Bubalus bubalis)*-ejaculated spermatozoa and its relationship with sperm motility. *Domest Anim Endocrin*, 34 (3): 238-249, 2008.

10. Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpino AVR, Ando S: Estrogen receptor (ER)alpha and ER beta are both expressed in human ejaculated spermatozoa: Evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J Clin Endocr Metab*, 89 (3): 1443-1451, 2004.

11. Hoffmann B, Landeck A: Testicular endocrine function, seasonality and semen quality of the stallion. *Anim Reprod Sci*, 57 (1-2): 89-98, 1999.

12. Kaewmala K, Uddin MJ, Cinar MU, Grosse-Brinkhaus C, Jonas E, Tesfaye D, Phatsara C, Tholen E, Looft C, Schellander K: Association study and expression analysis of CD9 as candidate gene for boar sperm quality and fertility traits. *Anim Reprod Sci*, 125 (1-4): 170-179, 2011.

13. Inkster S, Yue W, Brodie A: Human Testicular Aromatase - Immunocytochemical and Biochemical-Studies. *J Clin Endocr Metab*, 80 (6): 1941-1947, 1995.

14. Lauber ME, Sarasin A, Lichtensteiger W: Sex differences and androgen-dependent regulation of aromatase (CYP19) mRNA expression in the developing and adult rat brain. *J Steroid Biochem*, 61 (3-6): 359-364, 1997.

15. Corbin CJ, Hughes AL, Heffelfinger JR, Berger T, Waltzek TB, Roser JF, Santos TC, Miglino MA, Oliveira MF, Braga FC: Evolution of suiform aromatases: Ancestral duplication with conservation of tissue-specific expression in the collared peccary (Pecari tayassu). *J Mol Evol*, 65 (4): 403-412, 2007.

16. Conley AJ, Corbin CJ, Hinshelwood MM, Liu Z, Simpson ER, Ford JJ, Harada N: Functional aromatase expression in porcine adrenal gland and testis. *Biol Reprod*, 54 (2): 497-505, 1996.

17. Levallet J, Bilinska B, Mittre H, Genissel C, Fresnel J, Carreau S: Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. *Biol Reprod*, 58 (4): 919-926, 1998.

18. Janulis L, Bahr JM, Hess RA, Janssen S, Osawa Y, Bunick D: Rat testicular germ cells and epididymal sperm contain active P450 aromatase. *J Androl*, 19 (1): 65-71, 1998.

19. Gist DH, Bradshaw S, Morrow CMK, Congdon JD, Hess RA: Estrogen response system in the reproductive tract of the male turtle: An immunocytochemical study. *Gen Comp Endocr*, 151 (1): 27-33, 2007.

20. Fisher CR, Graves KH, Parlow AF, Simpson ER: Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proceedings of the National Academy of Sciences of the United States of America*, 95 (12): 6965-6970, 1998.

21. Robertson KM, Simpson ER, Lacham-Kaplan O, Jones MEE: Characterization of the fertility of male aromatase knockout mice. *J Androl*, 22 (5): 825-830, 2001.

22. Carreau S, Delalande C, Galeraud-Denis I: Mammalian sperm quality and aromatase expression. *Microsc Res Techniq*, 72 (8): 552-557, 2009.

23. Mutembei HM, Pesch S, Schuler G, Hoffmann B: Expression of oestrogen receptors alpha and beta and of aromatase in the testis of immature and mature boars. *Reprod Domest Anim*, 40 (3): 228-236, 2005.

24. Hess RA, Carnes K: The role of estrogen in testis and the male reproductive tract: A review and species comparison. *Anim Reprod*, 1 (1): 5-30, 2004.

25. Bilinska B, Lesniak M, Schmalz B: Are ovine Leydig cells able to aromatize androgens? *Reprod Fert Develop*, 9 (2): 193-199, 1997.

26. Brodie A, Inkster S: Aromatase in the Human Testis. *J Steroid Biochem*, 44 (4-6): 549-555, 1993.

27. Carpino A, Pezzi V, Rago V, Bilinska B, Ando S: Immunolocalization of cytochrome P450 aromatase in rat testis during postnatal development. *Tissue Cell* 2001, 33 (4): 349-353.

28. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, Bahr JM: Germ-cells of the mouse testis express P450 aromatase. *Endocrinology*, 132 (3): 1396-1401, 1993.

29. Kwon S, Hess RA, Bunick D, Nitta H, Janulis L, Osawa Y, Bahr JM: Rooster testicular germ-cells and epididymal sperm contain P450 aromatase. *Biol Reprod*, 53 (6): 1259-1264, 1995.

30. Hess RA, Ruz R, Gregory M, Smith CE, Cyr DG, Lubahn DB, Hermo L: Role of the estrogen receptor alpha in sperm production and motility in mice. *Biol Reprod (Special Issue)*: 131, 2004.

31. Payne A, Kelch R, Musich S, Halpern M: Intratesticular site of aromatization in the human. *J Clin Endocrinol Metab*, 42, 1081-1087, 1976.