











**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* proteininin erkek domuz testisinde lokalizasyonu, (4a) *CYP19*'un Leydig hücrelerinde immunoflorasan tesbiti, (4b) Hücre çekirdeklerinin 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<sup>13</sup> and stallion<sup>1</sup>. The porcine *CYP19* gene is expressed in a tissue-specific fashion in three principal sites, the gonads, the placenta, and the preimplantation blastocyst<sup>14</sup>. Tissue-specific expression of the *CYP19* promoted survival of the *CYP19* genes<sup>15</sup>. 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<sup>16</sup>. Testis is major source for *CYP19* enzyme and corresponds to daily sperm production<sup>11</sup> is supporting our findings. *CYP19* enzyme catalyses the synthesis of estrogens from androgens and play roles in the sexual development, reproduction and in behaviour<sup>14</sup>. In the mammalian testis, gonadotropins and testosterone together with numerous locally-produced factors are responsible for the induction and/or the maintenance of spermatogenesis<sup>7</sup>. Levallet et al.<sup>17</sup> and Janulis et al.<sup>18</sup> showed that the highest amount of *CYP19* mRNA in testis is related to the estrogen production. Gist et al.<sup>19</sup> 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)<sup>20</sup>. *CYP19* deficient mice indicated that spermatogenesis required the presence estradiol-17 beta (E<sub>2</sub>). E<sub>2</sub> 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<sup>8,20,21</sup>. 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<sup>22</sup>. 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<sup>23</sup>, horses<sup>6,24</sup>, ram<sup>25</sup> and human<sup>13,26</sup>. However, some studies detected immunoreactive *CYP19* in both the Leydig cells and seminiferous tubules in rat<sup>17,27</sup>, mouse<sup>28</sup> and rooster<sup>29</sup>. Importantly, this study confirmed that immunoreactive *CYP19* is restricted only to the Leydig cells in the testis in mammalian species like horses<sup>5,30</sup>, pig<sup>16,23</sup> and rams<sup>25</sup>. It has been reported that the major function of Leydig cells is to produce estrogen and being the source of estrogen biosynthesis in rat<sup>30</sup> and human<sup>31</sup>. Moreover, Hess et al.<sup>30</sup> 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<sup>1</sup> but in immature rat before puberty it is found more in Sertoli cell<sup>30</sup>. 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.

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## REFERENCES

1. Lemazurier E, Sourdain 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.