Testicular Steroid Hormone Secretion in the Boar and Expression of Testicular and Epididymal Steroid Sulphatase and Estrogen Sulphotransferase Activity

Authors B. Hoffmann, A. Rostalski, H. M. Mutembei, S. Goericke-Pesch

Affiliation Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig-University Giessen, Germany

Key words
- boar
- estrogens
- sulphatase
- sulphotransferase

Abstract

Background: Spermatogenesis and epididymal function depend on testicular steroids with estrogens being important regulatory factors. However, testicular estrogen secretion shows distinct species specificities, with the boar being characterized by the production of high amounts of estrone [E1] and estronesulphate [E1S]. As the boar testis also expresses estrogen sulphatase [StS] and sulphotransferase [EST] the present paper is based on the hypothesis that local availability of biologically active estrogens results from an interplay between estrogen synthesis and local activities of StS and EST.

Methods: Blood was collected during castration of 37 boars, aged between 98 (peripubertal) to 2793 (old sexually mature) days, from the testicular vein and artery and peripheral circulation; E1, E1S, testosterone [T] and progesterone [P] were determined by established RIA-procedures. Similarly seminal plasma from 21 sexually mature boars was assessed. StS- and EST-activity were determined in testicular- and epididymal homogenates of 3 sexually mature boars (200 d) using ^{3}H-E1S resp. ^{3}H-E1 as substrate. Immunohistochemistry [IHC] was applied to locate EST-expressing cells in testes in relation to age and in the epididymis of 3 mature boars.

Results: Hormone data showed a high variability. A significant age dependent increase was only observed for E1 and E1S in the peripheral circulation with absolute values being highest for E1S (5–60 nmol/l), followed by T (2.6–14 nmol/l), P (0.5–1.48 nmol/l) and E1 (0.24–0.84 nmol/l). Testicular vein concentrations always exceeded those in the testicular artery with the differences being significant for E1 and P, group 1. Concentrations in the testicular artery and peripheral vein plasma were not different but higher ($p < 0.0001$) than those in seminal plasma with the exception of E1. StS activity was higher ($p < 0.001$) in the testis than the epididymis. EST activity was high in epididymal homogenates and at the level of detection in testis homogenates. IHC located EST in virtually all epididymal epithelial cells. In the testis the number of positive staining Leydig cells decreased ($p < 0.05$) from 72% in the premature to 57% in the mature boars.

Conclusion: The provision of biologically active estrogens to the testicular and epididymal compartment is controlled by a complex regulatory system, with the sulphatase pathway being an important component. P is a secretory product of the testis, E1 and E1S are not actively enriched in seminal plasma.

received 10.09.2008
first decision 10.06.2009
accepted 22.06.2009

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DOI http://dx.doi.org/10.1055/s-0029-1231082
Published online: September 30, 2009
Exp Clin Endocrinol Diabetes 2010; 118: 274–280

ISSN 0947-7349

Correspondence
B. Hoffmann
Klinik für Geburtshilfe Gynäkologie und Andrologie der Gross- und Kleintiere
Frankfurter Straße 106
35392 Giessen
Germany
Tel.: 0049 641 9938704
Fax: 0049 641 29328
bernd.hoffmann@vetmed.uni-giessen.de

Introduction

The importance of testicular testosterone production for spermatogenesis with Sertoli, Leydig and peritubular cells being the prime target cells as shown by the expression of the androgen receptor has been well established and has been addressed in a multitude of reviews (e.g. see Griswold and McLean 2006).

Ample evidence has also been obtained that estrogens are important factors permitting for normal testicular and male reproductive tract function (Hess and Carnes 2004).

Detection of aromatase in the Leydig cells of adult testes across species shows that these cells are the prime source of testicular estrogens. However, in some species aromatase is also expressed by Sertoli cells (Peters et al., 2003) and by both meiotic and post-meiotic germ cells (reviewed by Carreau et al., 2003). As identified by the expression of either or both the estrogen receptors ERα and ERβ, targets for estrogens within the testis

are somatic and germ cells, the efferent ductules and the epididymis (Hess and Carnes 2004, Mutembei et al., 2005). To this a clear role for estrogens in reabsorption of epididymal fluid, a process necessary to maintain normal fertility, has been demonstrated (Lubahn et al., 1993; Korach 1994; Hess et al., 2000,2001; Hess and Carnes 2004) but further information concerning their role in testicular function is still poor and in part controversial (see Mutembei et al., 2005), also because distinct species specificities are observed. Thus the boar and the stallion are unique for producing large quantities of free and conjugated estrogens with estrone sulphate (E1S) being the predominant estrogen (Raeside 1979; Claus, Hoffmann 1980).

In a recent study with boar testes (Mutembei et al., 2005), we could show a distinct expression of the ERα in spermatogonia and primary spermatocytes, distinctly less intense signals were observed in the secondary spermatocytes, round spermatids and Leydig cells. Expression of the ERβ was observed in virtually all Sertoli, Leydig and – contrary to the report of Lekhkota et al., 2006, – also germ cells, except for elongating/ed spermatids and a role of testicular estrogens as autocrine and/or intracrine regulatory factors was discussed. This hypothesis is supported by observations of Wagner et al., 2006, who reported on a partial restoration of spermatogenesis in boars actively immunized against GnRH following supplementation with estradiol-17β.

However, when addressing the role of estrogens as autocrine hormonally active regulatory factors, reference is made to their free, bioactive form whilst conjugated estrogens do not interact with the receptor (Hähnel et al., 1973, Kuiper et al., 1997). Thus in view of the fact that in the boar E1S is the predominant estrogen, the discussed role of testicular estrogens as paracrine and/or autocrine regulatory factors (Mutembei et al., 2005) may be questioned. However, consideration should be given to the fact that at the level of target cells or organs availability of biologically active estrogens may also be controlled by locally expressed estrogen sulphatase (StS) and sulphotransferase (EST).

StS is a product of the StS gene, located on the short arm of the X chromosome in humans and mice (Yen et al., 1987). Steroid sulphatase is primarily localized in the endoplasmic reticulum (Chang et al., 1986) and catalyses removal of the sulphate group from the 3-hydroxysteroid sulphates such as estrone-3-sulphate (E1S) leading to the formation of free, biologically active E1 (Purohit et al., 1994; Chandra 2003). Activity of StS has been demonstrated in the boar testis (Yamamoto et al., 1997) and in many other tissues such as the placenta (e.g. human: Dibbelt and Kuss 1984; sheep: Mason et al., 1889; cattle: Mattioli et al., 1984, Hoffmann et al., 2001).

EST is a member of the superfamily of cytosolic steroid sulphotransferases and has been shown to be expressed in many tissues (i.e. liver, gonads, adrenal, brain) (Falany et al., 1995). EST activity has also been reported for the porcine testes and liver (Cooke et al., 1983) and also for the bovine placenta (Hoffmann et al., 2001).

These findings suggest that availability and regulation of biologically active estrogens in the boar testes depends on an interplay between P450 aromatase, EST and StS. With this paper are presented data from a number of in part independent studies which were performed in order to gain further information on this regulatory system. Thus activity and expression of StS and EST was tested in porcine testes and epididymis in conjunction with the determination of E1S, E1, the predominant estrogens in the boar. To characterize testicular function further testosterone and progesterone concentrations were also determined. Blood collection was from the testicular artery and vein and from the peripheral circulation to allow conclusions on the secretory activity of the testis. For reasons of comparison also seminal plasma was examined.

Materials and Methods

Assessment of testicular steroid hormone secretion

Animals and collection of blood and semen samples

During castration of 37 boars attempts were made to collect blood from the testicular vein and artery, as well as from peripheral circulation (Vena auricularis or Vena cava cranialis or Vena jugularis externa, depending on accessibility). Animals were grouped according to their age into 4 groups: Gr. 1, 100 d (range: 98–116 d, peripubertal), Gr. 2, 150–200 d (range: 148–218 d, early sexually mature), Gr. 3, 250–365 d (range: 249–336 d, sexually mature), Gr. 4, >365 d (range: 365–2 793d, old sexually mature).

As is evident from **Table 1**, peripheral blood samples could be collected from all but one boars, successful sampling from the testicular vein and artery varied between 33–100 %.

For seminal plasma semen was collected from 21 boars aged 329 to 2 793 days, centrifuged (1 000 g, 4 °C) and 8 ml aliquots were stored at −20 °C until assay.

Hormone assay

Steroid hormone assay was by in-house competition radioimmunoassays as described earlier [Testosterone (T): Röcken et al., 1995; Hoffmann and Landeck 1999 – Estrone (E1), Estronsulphate (EIS): Hoffmann et al., 1997 – Progesterone (P): Hoffmann et al., 1973,1992]. Quality control included routine participation in the EQUAS programme of the society for Comparative Endocrinology and the monitoring of inter assay coefficients of variation which were around (low and high) 6–7.5 % for –T, 13.4–11.4 % for E1/E1S and 13.8–9.9 % for P.

Assessment of StS- and EST-activity

Animals and tissue preparation

Immediately following castration of three 200-day-old boars, testes with epididymides were placed in ice – cold phosphate buffer (PBS) and transferred to the laboratory within 15 min. Following a thorough rinsing with PBS testes and epididymides were separated.

Testes were cut in half and the mediastinum and the tunica albuginea were trimmed off. The remaining tissue was then cut in small cubes which were transferred into 20 ml centrifuge vials and further cut with a pair of scissors until a pulpy consistency had been reached.

Owing to the high content of fibrous tissue of the cauda epididymidis only the head and corpus were processed. Following trimming off of the lamina visceralis, the epididymal duct was rinsed with saline to remove as many spermatozoa as possible. Head and corpus were then cut in small pieces and further processed as indicated above. The tissue pulp was washed with Ringer solution until a clear supernatant was obtained.

Finally 5 g of the cut tissue (testis, epididymis), suspended in 15 ml Ringer-Hepes-buffer (ratio 39:1), were pre-homogenized using an Ultra-Turrax (IKA-Werk, Stauffen, Germany) by applying 3 consecutive 1 min bursts, followed by a final homogenization using a Potter-Elvejhem homogenisor. The obtained homogenate was then passed through a gauze filter consisting of

Table 1 Steroid concentrations determined in peripheral and testicular circulation in boars of different age groups (all values expressed as nmol/l, Xg (DF)).

Steroid	Age groups (days)	Peripheral Circulation		Testicular Circulation			
				testic. art.		testic. vein	
		Xg (DF)	n	Xg (DF)	n	Xg (DF)	n
estrone	100	0.24 (1.94)[a]	13	0.45 (1.47)[a]	8	1.10 (1.95)[b]	6
	150–200	0.32 (2.05)[a]	12	0.48 (2.42)	6	1.16 (2.77)[b]	13
	250–365	0.48 (1.23)	6	0.38 (1.83)	5	1.44 (2.01)	4
	>365	0.84 (1.62)	6	0,30 (1.48)	2	2.19 (2.16)	4
estronesulfate	100	5.06 (4.38)[a]	13	4.89 (3.06)	8	45.11 (4.14)[b]	6
	150–200	12.66 (4.50)[a]	12	21.34 (3.81)	6	48.24 (5.05)[b]	13
	250–365	15.23 (4.09)	6	11.18 (3.20)	5	38.46 (3.90)	4
	>365	60.23 (2.26)	6	39.13 (2.11)	2	221.20 (3.36)	4
testosterone	100	2.65 (2.61)[a]	13	4.14 (3.08)	8	113.72 (3.60)[b]	6
	150–200	6.47 (2.51)[a]	12	25.23 (2.75)	6	100.42 (15.04)[b]	13
	250–365	7.44(2.65)[a]	6	12.34 (3.41)	5	179.00 (3.96)[b]	4
	>365	14.08 (1.93)[a]	6	20.52 (1.19)	2	101.95 (1.63)[b]	4
progesterone	100	0.72 (1.68)[a]	13	0.46 (1.62)[a]	8	1.65 (1.82)[b]	6
	150–200	1.48 (1.93)	12	1.89 (2.17)	6	2.18 (2.21)	13
	250–365	0.55 (1.50)	6	0.65 (1.87)	5	1.50 (2.44)	4
	>365	1.27 (1.51)[a]	6	0.96 (1.06)	2	3.89 (1.71)[b]	4

[a,b]: figures with difference superscript differ with $p < 0.01–0.001$ in respect to site of sampling, n: number of blood samples collected

two layers prepared from a sterile gauze swab (Typ 17 EN 14079) to remove remaining fibrous tissue particles.
All preparatory steps were performed under sterile conditions and on ice in a laminar-flow-hood.

Assay of StS-Activity

As described previously (Hoffmann et al., 2001), 0.2 ml homogenate were added in duplicate to 1 ml Ringer-Hepes-Buffer containing 0.1 % BSA; 0.6 pmol ^{3}H-estrone sulphate (^{3}H-E1S) served as substrate. Incubation was at 37 °C in a water bath with constant shaking using sterile 15 ml glass tubes. To test for non-enzymatic transformation, control samples were set up with an equal volume of incubation buffer instead of homogenate (medium blank) and with heat- inactivated (30 min in boiling water) tissue homogenates (tissue blanks). The incubations were stopped at 0, 5, 15, 30 and 60 min by placing the incubation vials for 3 min in boiling water.
For analysis of the formed free ^{3}H-E1 the incubated samples were extracted with 4 x 3.5 ml toluene. The pooled extracts were dried and redissolved in scintillator (Zinsser Analytic GmbH, D-60489 Frankfurt) and the ^{3}H-activity counted. Similarly the ^{3}H-activity representing the non hydrolyzed ^{3}H-E1S in the remaining aqueous phase was counted; the two counts yielded the 100 % value for each tube, allowing calculation of the conversion rate.

Assay of EST-Activity

Except for the addition of 5 µmol 4-nitrophenylsulfat-potassium salt (competitive inhibition of StS) and 50 mmol 3'-phosphoadenosine-5'-phosphosulfate as a co-factor, (Gregory and Robbins 1960) and the use of 0.6 pmol ^{3}H-Estrone (^{3}H-E1) as substrate incubation and controls were the same as for StS.
As described earlier (Hoffmann et al., 2001) for analysis of the ^{3}H-E1S formed, samples were extracted with 4 × 3.5 ml toluene before and after enzymatic hydrolysis with 0.3 standard units arylsulphatase (β-glucuronidase/arylsulphatase from helix pomatia – Serva Feinbiochemika GmbH Co., Heidelberg, Germany). The first extraction provided the fraction of free non converted ^{3}H-E1 substrate, the second the fraction of the formed ^{3}H-E1S. The pooled extracts were dried, redissolved in scintillator and the ^{3}H-activity counted. As described above, EST-activity was expressed as percentage substrate conversion following calculation of the 100 % value.

Immunolocalization of EST and STS

Animals, tissue collection and sample preparation

Testes for immunohistochemistry (IHC) were collected at castration from healthy boars aged 50, 100, 150, and 250 days (five animals per age group). Sample collection and preservation for IHC were as previously described (Mutembei et al., 2005). Additionally the epididymides from three 250-day- old boars were preserved for IHC.

Immunohistochemical staining procedure

Indirect immunoperoxidase staining methods were employed using the streptavidin–biotin technique for signal enhancement as previously described (Mutembei et al., 2005). A polyclonal rabbit anti-mouse EST (Song et al., 1995) served as source for the primary antibody, it was used in a dilution of 1:2 500. Negative controls were set up using non-immune rabbit serum at an equal concentration to that of the primary antibody.
The positively staining cells were identified in three sections and the total number and the number of positive staining Leydig cells was counted at a 200-fold magnification.

Immunolocalization of STS

Results of the immunolocalization of StS in testes samples together with data on the enzyme kinetics have already been published (Mutembei et al., 2009), and hence will only be discussed in light of the observations presented here.

Statistics

Hormone data

All boars were considered as one group. To cope with the asymmetrical, nearly log normal distributions of hormone values in plasma, the geometric means (Xg) and dispersion factor (DF) were calculated. To test for differences an unpaired Student's t-test, Version Excel 97, Windows 98, Microsoft, was applied.

Enzymatic conversion

Data are presented as X ± SD. Student's t-test (paired and unpaired) was applied to test for differences.

Immunolocalization of EST in testes

For statistical evaluation, a three-factorial partial hierarchical analysis of variance to test for influence of age group [G], animal within age group [A (G)], section within animal and age group [S (AG)] using BMDP statistical software, program BMDP8V (Dixon 1993) was performed.

Results

A. Assessment of Steroid Hormone Concentrations

The results (Xg/DF) are summarized in **Table 1**. As is indicated by the deviation factors (DF) which range between 1.50–15.04, there was a high variability concerning the individual values obtained.

With $p < 0.01$ to $p < 0.001$, a significant age-dependent increase was observed for E1 and E1S in peripheral circulation.

At all three sampling sites E1S concentrations exceeded those of E1 with a mean factor of 49.7 ± 34.5 ($p < 0.02$). There were no differences between the concentrations determined in the testicular artery and in the peripheral circulation. In peripheral circulation absolute values were highest for E1S, followed by testosterone, progesterone and E1. A similar ranking was observed for the testicular vein.

For each steroid and age group positive arterio-venous differences showed up in testicular circulation, however, the difference was only significant for P4 and E1, 100-day age group (Gr. 1). When compared to peripheral circulation testicular vein concentrations were significantly higher for E1, Gr.1 and 2; E1S, Gr. 1 and 2; P4, Gr. 4 and T, Gr. 1-4.

With mean values [Xg(DF), nmol/l] of 3.14 (3.3) for E1S, 1.22 (2.20) for T and 0.39 (1.4) for P significantly ($p < 0.0001$) lower concentrations were determined for these steroids in seminal plasma than in peripheral circulation. E1 [0.64(4.9), nmol/l] was not different.

B. Assessment of EST- and STS-activity

While EST-activity was at the lower level of detection in testicular tissue and showed no changes over time, there was a highly significant ($p < 0.001$) conversion (44.1% after 60 min) of free ^{3}H-E1 into ^{3}H-E1S in epididymal homogenates (**Fig. 1**).

StS-activity showed highly significant ($p < 0.001$) conversion rates with both tissue homogenates. However, the substrate conversion rate was higher ($p < 0.01$) in the testis compared with the epididymis (**Fig. 2**).

C. Immunolocalization of EST

Testis

Positive staining was restricted to the cytoplasm of the Leydig cells. Intensity and the number of positive cells was higher for the premature boars (day-50 to -100) with 71.8 ± 1.6% of the Leydig cells being positive (**Fig. 3a, b**) than in mature boars, where the number of positive cells was significantly ($p \leq 0.05$) lower with 57.2 ± 0.5% (day-150 and -250) (**Fig. 3c, d**).

As shown in **Fig. 4**, immunohistochemistry gave a distinct cycloplasmic staining of virtually all epithelial cells of the epididymal duct.

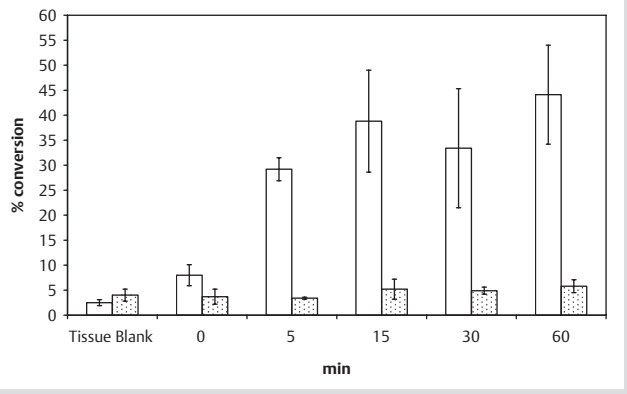

Fig. 1 Conversion (%) of 3H-Estrone into 3H-Estrone sulphate by porcine testis and epididymis (caput, corpus) -tissue homogenates (n = 3, age: 200 days, X ± SD).
epididymis, testis

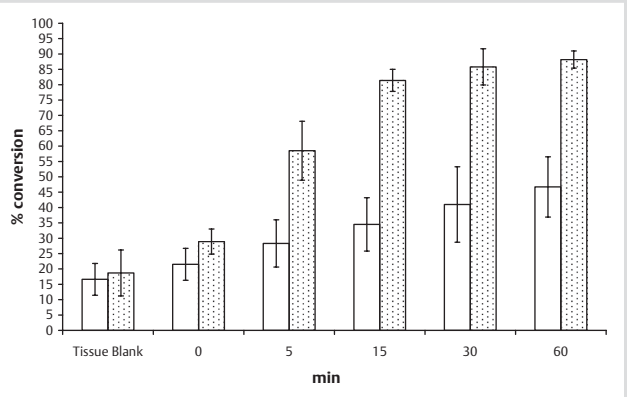

Fig. 2 Conversion (%) of 3H-Estrone sulphate into 3H-Estrone by porcine testis and epididymis (caput, corpus) -tissue homogenates (n = 3, age: 200 days; X ± SD).
epididymis, testis

Discussion

The porcine testis secretes large amounts of estrogens with E1 concentrations exceeding those of estradiol by a factor of 2–2.5 (Claus et al., 1985); the factor for the respective conjugated forms is around 12 (Rostalski 2005). In view of this situation and when taking into account the relative binding affinity of E1 of 20–48% as determined in in-vitro receptor preparations (Korenman et al., 1970, Kyrein 1976), E1 in the boar must be considered a potent estrogen, able to interact with both the ERα and ERß, as a potent competitor to estradiol.

As shown in the present study, E1S concentrations in testicular vein distinctly exceeded those of E1, though – due to the high individual variation – the difference was not always significant. This observation seems to indicate that the testis is the source of E1S. However, such a conclusion must be questioned as in the rete testis fluid concentrations of free total estrogens exceeded those of conjugated total estrogens (Claus et al., 1985), pointing towards a different site than the testis of sulphoconjugation. In this respect the observations on the expression StS and EST in testicular and epididymal tissue gain functional importance. Thus the present investigations have shown that in adult boars

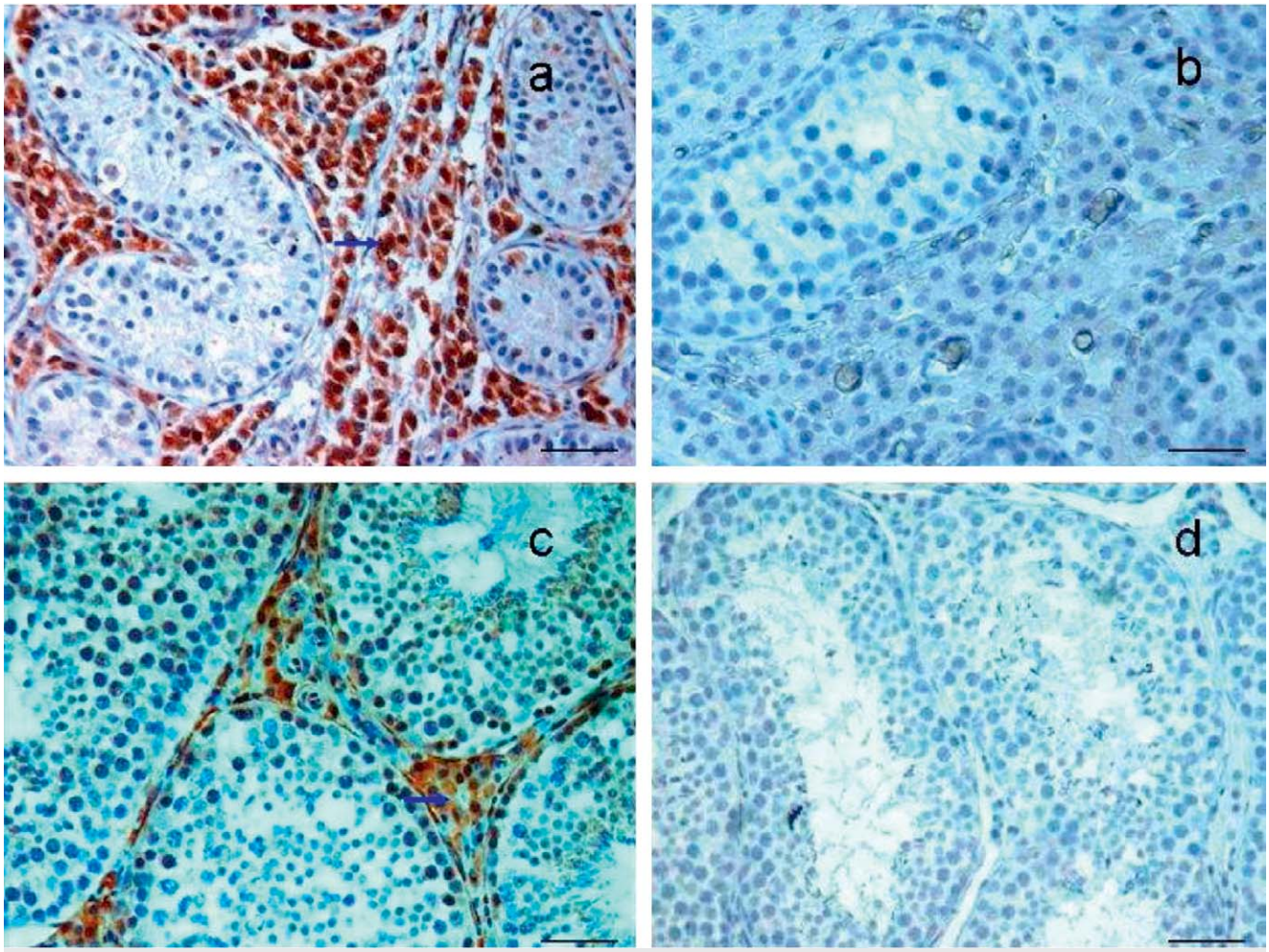

Fig. 3 Localisation of EST in porcine testicular tissue by immunohistochemistry. Signals are restricted to cytoplasm of Leydig cells (blue arrow). **a**: sexually immature boar (250 d), **b**: negative control, **c**: Sexually mature boar, **d:** negative control, Bars = 50 µm.

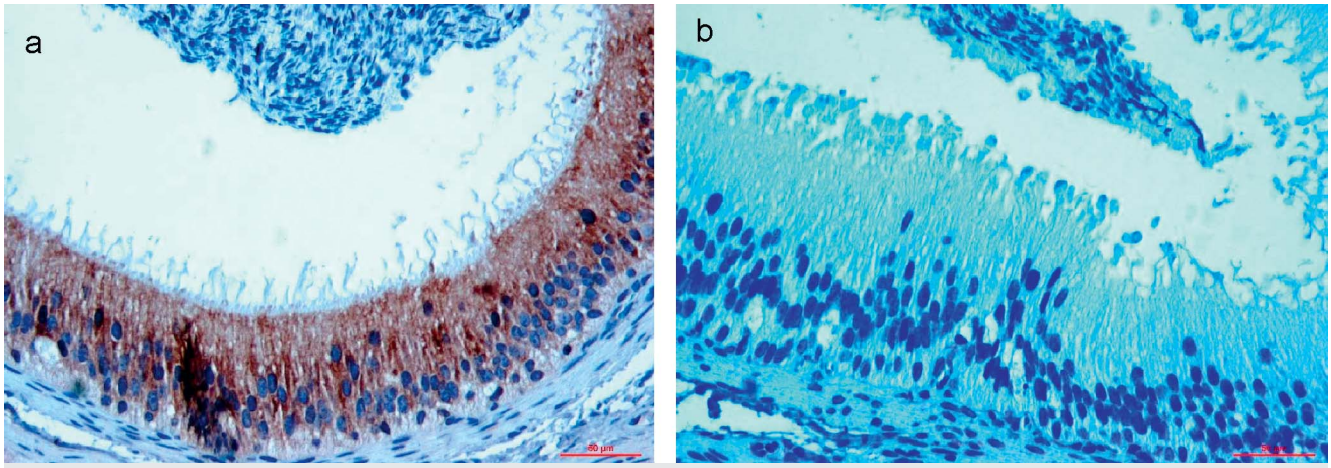

Fig. 4 Localisation of EST in the boar epididymis (head) of a sexually mature animal (250 d) by immunohistochemistry. **a:** Signals are restricted to cytoplasm of epithelial cells **b:** negative control, Bars = 50 µm.

EST-activity was high in epididymal homogenates while it was at the lower limit of detection in testis homogenates. In accordance with this observation, IHC confirmed a high expression of EST in the epididymal epithelium of adult boars. Other than the in-vitro data, IHC also localized EST in the testis. Expression was restricted to the Leydig cells and significantly decreased with age from peripubertal to adult boars. This decrease obviously resulted in a situation that no in-vitro substrate conversion rate over time could be demonstrated any more. Thus and in accordance with observations by Raeside et al., 1999, in adult boars the prime site of sulphoconjugation of E1 seems to be the epididymis (caput, body). The situation in pre/peripubertal boar, however, might be different, as IHC showed a stronger staining in these animals with more Leydig cells being positive.

A specific feature of the boar are the strong interconnections of the arterial and venous vasculature of testis and epididymis (Stoffel et al., 1990). This situation seems to allow transport of E1 from the testis into the epididymis, where it is transformed into E1S. As venous drainage of the epididymis is into the vena testicularis (Stoffel et al., 1990), the high concentrations of E1S occurring there seem to result from the testicular-epididymal vasculature.

In this study a high activity of StS was determined in testicular tissue and expression of the enzyme at the RNA and protein level as well as its kinetic specificities have recently been published (Mutembei et al., 2008). Thus any sulphoconjugated estrogen reaching the rete testis via backflow might be hydrolyzed, a mechanism which would provide biologically active estrogens for targeting estrogen receptor-expressing cells, e.g. Leydig- and germ cells. StS activity was also found in epididymal homogenates and the expression of both enzymes, StS and EST, at the same location indicates that availability of E1 is controlled by a complex and locally expressed sulphatase pathway.

Measurements of steroids in testicular and peripheral circulation revealed that P is a testicular secretory product. Interestingly, in prepubertal boars, prespermatogonia express the progesterone receptor and a functional role for P in spermatogenesis has been discussed (Kohler et al., 2007).

The present data further reveal that there is no difference in the levels of steroid hormones determined in the testicular artery and peripheral circulation; with the exception of E1 even lower concentrations were determined in seminal plasma. Thus the occurrence of estrogens in seminal plasma seems not to result from an active secretory process (Claus et al., 1985) but rather from a distribution following general pharmacokinetic principles.

A significant effect of age was only observed for E1S and E1 in the peripheral circulation; concentrations of both steroids increased from day 100 to day >365. Owing to the large individual variations the same observation was not significant for the other two sampling sites, the testicular vein and artery. The data also seem to indicate that, e.g. when compared to T, the increase of E1S is delayed and that highest concentrations are only reached after one year of age.

In conclusion our data indicate that provision of biologically active estrogens to the testicular and epididymal compartment is controlled by a complex regulatory system, with the sulphatase pathway and the boar specific vascular interconnections being important components. P is a secretory product of the testis and there is no active enrichment of E1 and E1S in seminal plasma.

Acknowledgement

The authors greatly appreciate the support of Dr. J. Pürstl, Zucht- und Besamungsunion Hessen e.G. for providing boar seminal plasma and of Dr. W. C. Song, University of Pennsylvania, Philadelphia, USA, for the generous gift of EST-antibody.

Conflict of Interest: None.

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