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Effects of Estradiol Infusion in GnRH Immunized Boars on Spermatogenesis

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Abstract

Active immunization of boars against gonadotropin-releasing hormone (GnRH) inhibits luteinizing hormone (LH) and testicular steroids, so that mitosis of spermatogonia is reduced and apoptosis increased. To clarify whether high amounts of estrogens which are synthesized in the boar testis support spermatogenesis, a group of 6 boars was immunized against GnRH and then infused for 7 weeks with estradiol (E_2-17ß). For comparison, intact boars and immunized boars were infused with saline only. Testicular tissue was then analyzed by immunocytochemistry for apoptosis (TUNEL, EM), mitosis (Ki67), and estrogen receptor α (ERα). The specificity of ERα staining was confirmed by RT-PCR and Western blot. Immunization decreased LH and testosterone to minimal concentrations in immunized and $E_2$17ß-infused immunized boars, whereas follicle-stimulating hormone (FSH) was not significantly altered. Estradiol decreased to base levels after immunization. Infusion increased E_2-17ß in peripheral blood plasma of the immunized boars to physiological levels. Except for A-spermatogonia, all spermatogenic cells decreased after immunization by about 60%. After estradiol infusion, cell counts increased again and were intermediate between control and immunized boars. Mitosis of spermatogonia was reduced by nearly 50% due to immunization but was partly restored by E_2-17ß infusion. Expression of ERα was localized in

spermatogonia, suggesting stimulation of mitosis, which was further confirmed due to its predominant occurrence in stage I of the seminiferous epithelial cycle (main stage of cell division). Apoptosis was minimal in boars but elevated in the other 2 groups. Data showed that estrogens in physiological concentrations supported mitosis but were not sufficient to normalize sperm production because apoptosis was still high.

Key words: Estrogen receptor, spermatogenic stage, apoptosis, mitosis

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Testicular androgens are responsible for the maintenance of male reproductive functions, including spermatogenesis. In addition, small amounts of estrogens are measurable in peripheral plasma of males. Such trace amounts may result from steroidogenesis in the testes but also in cells of the adrenal cortex (Conley et al, 1996), as shown for many species, leading to peripheral concentrations on the order of 5–20 pg/mL (Eiler and Graves, 1977; Overpeck et al, 1978; Melnyk et al, 1992; Bujan et al, 1993). Several cell types in the testes were shown to express aromatase activity, such as Sertoli cells, Leydig cells, germ cells, and peritubular cells, depending on the species (Brodie and Inkster, 1993; Tsubota et al, 1993; Almadhidi et al, 1995; Levallet et al, 1998; Carreau et al, 2001; Fra˛cek et al, 2001; for review: O'Donnell et al, 2001). Testicular synthesis also explains the occurrence of small amounts of estrogens in semen as reported, eg, for the rat (Free and Jaffe, 1979), the bull (Ganjam and Amann, 1976; Eiler and Graves, 1977), and man (Bujan et al, 1993; Luboshitzky et al, 2002). Concentrations are on the order of 150 pg/mL (Reiffsteck et al, 1982). Studies in rats demonstrated the occurrence of estrogen receptors α and ß (ERα, ERß) both in Sertoli cells and in several types of spermatogenic cells, so that a role of estrogens for spermatid adhesion to Sertoli cells was suggested (Miura et al, 1999; Ebling et al, 2000; Pelletier et al, 2000; Hess, 2003).

In a few species, high amounts of estrogens are synthesized in the Leydig cells and are measurable in high concentrations in peripheral blood plasma, as shown for the stallion (Raeside, 1969; Setchell and Cox, 1986; Claus et al, 1992; Lemazurier et al, 2001) and the boar (Velle, 1958). In the latter species, peripheral concentrations may reach nearly 280 pg/mL blood plasma (Claus and Hoffmann, 1980). In the mature boar, aromatase activity occurs exclusively in the Leydig cells (Fra˛cek et al, 2001; Mutembei et al, 2005). Sertoli cell expression of aromatase is limited to a narrow period of fetal development (Claus et al, unpublished data). Substitution experiments with castrated boars demonstrated that the synergistic action of androgens and estrogens is necessary to ensure normal function of accessory sex glands, male libido, and sexual behavior (Joshi and Raeside, 1973; Parrot and Booth, 1984; Booth, 1988), and also the establishment of the anabolic potential (van Weerden and Grandadam, 1976).

In the boar, high amounts of total estrogens also occur in the fluid of spermatogenic tubules. They are primarily represented by 17ß-estradiol (E_2-17ß), which may reach concentrations of 41 ng/mL in this fluid (Claus et al, 1985). A high proportion of E_2-17ß is transferred into the semen, so that the total amounts per ejaculate may reach 110 ng. These amounts influence female reproductive functions such as sperm transport and ovulation (Claus, 1989, 1990). The high tubular concentrations of estrogens additionally suggest an effect on spermatogenesis. An individual function of estrogens and a possible synergism with androgens remain to be clarified. Two types of estrogen receptors are well described, estrogen receptors α and ß (ERα, ERß). In the boar the presence of the receptor types in different cells within the testes was investigated by immunocytochemistry and the ERß demonstrated in Sertoli cells (Mutembei et al, 2005), germ cells (Rago et al, 2004; Mutembei et al, 2005), and Leydig cells (Mutembei et al, 2005). ERα immunoreactivity was shown in germ cells (Rago et al, 2004; Mutembei et al, 2005) and Leydig cells (Rago et al, 2004; Mutembei et al, 2005). A more

detailed study, however, using in situ hybridization combined with laser-assisted cell picking revealed a clear separation between expression of ERα and ERß between cell types, so that ERα expression was found to be limited to spermatogonia and spermatocytes, whereas ERß expression occurred only in Sertoli cells (Lekhkota et al, 2005). It appears, therefore, that E_2-17ß may be directly involved in the regulation of mitotic activity of early stages of spermatogenesis, but a possible effect cannot be separated from an effect of androgens so far. Active immunization of boars with potent antigens against gonadotropin-releasing hormone (GnRH) leads to an inhibition of luteinizing hormone (LH), so that androgen and estrogen formation in the testes drops (Metz et al, 2002). Follicle-stimulating hormone (FSH) concentrations are not influenced by active immunization, so that blood plasma concentrations do not differ significantly between normal and immunized boars (Awoniyi et al, 1988; Wagner and Claus, 2004). In consequence, immunization allows us to study spermatogenesis in the absence of steroids and additionally to perform substitution experiments to clarify individual or combined effects of androgens and estrogens.

So far the effects of immunization on spermatogenesis have been compared with normal control boars (Wagner and Claus, 2004). It was found that steroid withdrawal leads to a reduction of the mitotic rate of spermatogonia. In addition, the expression of the glucocorticoid receptor in spermatogonia was increased so that apoptosis was elevated in spermatogonia and spermatocytes. In consequence, reduced mitosis and increased apoptosis led to a decrease of spermatogenic activity in the absence of testicular steroids (Wagner and Claus, 2004).

Based on these effects, the present paper reports the consequences of a continuous infusion with E_2-17ß on ERα-mediated effects on spermatogenesis in immunized boars.

Material and Methods

Experimental Design

The study was performed with 6 postpubertal German landrace boars which were GnRH-immunized and then infused via peristaltic pumps for 7 weeks with physiological saline with the addition of E_2-17ß (E_2-17ß-infused immunized boars). The data obtained were compared with those from 2 other groups which were either normal boars (control boars; n = 5) or immunized boars (n = 5). The time schedule for immunization of the E_2-17ß-infused boars was the same as for the immunized controls: the initial immunization was performed at 20 weeks of age so that initiation of spermatogenesis by gonadotropins remained undisturbed. Booster injections were given 4 and 8 weeks after the initial immunization. At the age of 24 weeks, the boars were fitted with indwelling cephalic vein catheters on both sides of the neck with an established method (Claus et al, 1990). One catheter was used for infusion, the other for uncontaminated blood sampling. The infusion of saline started at 25 weeks of age, when the endogenous formation of testicular steroids was already suppressed by immunization. Saline infusion was continued for 1 week in all 3 groups of boars. Thereafter, in the estradiol-infused immunized boars the saline was substituted first with a high dose of E_2-17ß (0.81 mg/mL saline) for 1 week to simulate the peripubertal rise of steroids normally occurring in the boar (Schwarzenberger et al, 1993) and then continued with a lower dose (0.20 µg/mL saline) for another 5 weeks. Infusion of a volume of 100 mL/h was performed with peristaltic pumps. The control groups only received saline through their catheter.

All steps of the animal experiments, including cannulation, immunization, infusion, sampling of

blood, and killing, were approved by the local animal welfare commission.

Animals, Housing, and Sampling

German landrace boars from the university herd were kept individually in stalls (1.9 x 2.8 m). The animals were fed a diet with 10 MJ metabolizable energy/kg feed and 13.9% crude protein at an amount of 3 kg per day. The average weight of the boars increased from 82 ± 3.4 kg (mean ± SEM: 20 weeks of age) to 140 ± 5.3 kg (32 weeks) during the experiment. As a vaccine, portions of 2 mL Improvac (CSL Limited ACN, Victoria, Australia) were injected as recommended by the supplier.

Blood samples were collected over the 7-week period daily at 0800 hours prior to feeding and used for radioimmunological determination of E_2-17ß in plasma extracts with a highly specific antiserum which had been raised in rabbits against E_2-17ß-6-CMO-BSA (Claus et al, 1983). This antiserum revealed cross reactivities only with E_2-17α (0.4%) and with estrone (0.43%). The sensitivity was 11.5 pg/mL. The intra-assay coefficient of variation was 18%, for a concentration of 86 pg/mL. The interassay variation varied depending on the concentration between 10% and 16%.

Testosterone was also determined radioimmunologically (Bubenik et al, 1982). The antiserum against testosterone had been raised against testosterone-3-CMO-BSA and revealed cross-reactivity only with 5a-dihydrotestosterone (28.5%). The sensitivity was 0.04 ng/mL plasma. The intra-assay coefficient of variation varied between 3.9% and 6.7% depending on the concentration and between 7% and 18% for the interassay variation coefficient. The latter figure refers to the immunized group, where concentrations were close to the detection limit.

For LH and FSH determination, window sampling was performed 5 times over the experimental period. For each window, blood was drawn every 20 minutes for 12 hours (0800 till 2000 hours). Determinations of LH and FSH were performed by RIA as published earlier (Claus et al, 1990), with the exception that in case of LH the first antibody was preincubated with the sample before adding the tracer. LH (AFP: 11043B) and FSH (AFP: 10640B) for iodination were obtained from Dr. Parlow (NIDDK, Torrance, Calif). From the same source the species-specific antisera (LH: AFP 15103194; FSH: AFP 2062096Rb) had been obtained. The LH antiserum could be used at a final dilution of 1:666 000. The FSH antiserum was used at a dilution of 1:200 000.

The reliability criteria were as follows: For LH the sensitivity was 25 pg/mL blood plasma. The intra-assay coefficient of variation was 7.9% and the interassay coefficient 8%. For FSH the sensitivity was 36 pg/mL blood plasma. The intra-assay coefficient of variation was 11% and the interassay coefficient 12%.

The pigs were killed by intravenous infusion of 0.2 mL Eutha 77/kg body weight (Essex Pharma, Munich, Germany). Testes were collected, the epididymides removed, and the weight determined. Samples for histology and immunocytochemistry were taken from the testes within = minutes after killing and fixed either in Bouin solution or 4% formaldehyde. For confirmation of the specificity of apoptosis staining, additional samples were fixed in 2.5% glutaraldehyde for later analysis by electron microscopy.

Histological Evaluations

All evaluations are based on = animals for control and immunized boars and on 6 animals for the $E_2$17ß-infused immunized boars.

The Bouin-fixed samples were used to characterize the spermatogenic activity by counting the

following spermatogenic cells: A-spermatogonia, B-spermatogonia, primary spermatocytes, round spermatids, and elongated spermatids. Because complete spermatogenesis in the boar can be separated into 8 stages (Swierstra, 1968), 5 representative round tubules for each of stages I–V and VIII were localized in the sections. Because stages VI and VII cannot be differentiated with certainty by light microscopy, the resulting data were combined (stage VI/VII). The cells were counted so that the quantitative data for each cell type are based on 40 tubules in each boar. Details of the histological procedure were given earlier (Wagner and Claus, 2004).

The immunocytochemical staining of Ki67 (mitosis) was performed in formaldehyde-fixed tissue as described (Mentschel et al, 2001). Apoptosis was determined also in formaldehyde-fixed samples using the TUNEL reaction as modified by Gavrieli et al (1992). The determination of the estrogen receptor α (ERα) was based on a polyclonal antibody raised in rabbits against a synthetic 67 kda N-terminal epitope of the human estrogen receptor protein (Ab 17, Lab Vision Corporation, Fremont, Calif). It was shown by the supplier that it also detects the porcine ERα. It was used at a dilution of 1:150. Counterstaining was performed by hematoxylin. Staining of the ERα was also extended to slices from paraffinembedded testis samples from control boars and immunized boars. For immunocytochemical evaluation of testis, generally 2 slides with 3 tissue cross sections were scored for each animal. Each immunocytochemical parameter was counted in 100 tubules per slide and is given as "positive cells per tubule." The tubules were selected randomly, and counting was performed by 2 independent observers. Mitosis (Ki67-stained cells) and the ERα expression were additionally referred to the stages of the seminiferous epithelial cycle.

Western Blot Analysis of the ERα

To confirm the specificity of the antiserum for the ERα, Western blot analysis was carried out in porcine testicular tissue that had been stored at −80 °C. For protein extraction the tissue was transferred into ice-cold lysis buffer (2 mL/g tissue; 10 mmol Tris-HCl, pH 7.5; 20 mmol sodium molybdate; 10 mmol dithiothreitol; 10% glycerol; 0.05% Triton X-100; 1 mmol EDTA; 2 mmol PMSF) containing protease inhibitors (Aprotinin, 10 µg/mL; Leupetin, 10 µg/mL; Pepstatin A, 10 µg/mL; AppliChem, Darmstadt, Germany). Tissue was homogenized with a bead mill (Mikrodismembrator U; B. Braun, Melsungen, Germany) for 20 seconds at 2000 rpm, centrifuged (10 min, 9500 g, 4 °C) and the protein content in the supernatant determined (Bradford, 1976).

Equal amounts of protein were boiled in loading buffer (60 mmol Tris, pH 6.8; 15% SDS, 35% glycerol, 25% ß-mercaptoethanol; 0.1% bromphenol blue) for 5 minutes. Electrophoresis (50 µg protein/lane) was performed with a 12% SDS-polyacrylamide separating gel and a 5% stacking gel. For calibration a prestained protein ladder (10–180 kDa; MBI Fermentas, St Leon-Rot, Germany) was additionally loaded onto the gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (0.45 µm, Hybond-P; Amersham, Freiburg, Germany) using the Semi-Phor semidry blotting chamber (Hoefer Scientific Instruments, San Francisco, Calif). For blocking, the membranes were incubated in PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk powder at 4 °C overnight.

The ERα antibody was then added and incubated for 2.5 hours at room temperature at a dilution of 1:2000. After washing, the second biotinylated antibody (sheep against rabbit-IgG) was added and incubated for 2 hours at a dilution of 1:2000. After washing, the streptavidine-biotin-horseradishperoxidase-complex (ABC-complex; DAKO, Hamburg, Germany) was added at a dilution of 1:5000 and incubated for 1 hour at room temperature. The visualization was carried out with a luminol-based detection reagent (250 mmol luminol; 80.4 mmol p-coumaric acid 1M Tris/HCl). The chemiluminescent signal was documented on an X-ray film. The Western blot analysis was repeated twice.

Confirmation of ERα by RT-PCR

The occurrence of the ERα mRNA was additionally confirmed by RT-PCR. Total cellular RNA was isolated from testicular tissue using TRIzol Reagent (Life Technologies, Karlsruhe, Germany). RNA samples were evaluated for purity and integrity using absorbance (A260/A280) and r-RNA (28s/18s) ratios, respectively. Primers for ERα were based on a porcine sequence (GenBank accession number AF035775). The following primers were used: forward primer 5'-GCT CCT GTT TGC TCC TAA C—'9, reverse primer 5'-GAC ACG GTG GAT ATG GTC—'9. PCR protocols were optimized for amplification within the exponential portion of the curves, as described for other genes in our laboratory. PCR was performed in 50-µL reactions containing 1 µL of reverse transcription mixture, Taq DNA Polymerase, 2 mol $MgCl_2$,10 x PCR-buffer, 10 mmol dNTPs, and 10 pmol/µL primers. Reactions were incubated for 3 minutes at 95 °C, followed by cycles of 1 minute at 94 °C, 2 minutes at 56 °C, 1 minute 30 seconds at 72 °C. The number of cycles was 35. After cycles were completed, reactions were incubated at 72 °C for 10 minutes, which was followed by cooling to 4 °C. A set of negative controls was processed in a similar manner, except that reverse transcriptase was replaced by water in reverse transcription reactions. Products were separated by electrophoresis in 2% agarose gel, visualized by ethidium bromide staining, and documented on Polaroid T 57 film. The RT-PCR procedure was repeated 10 times. For Western blot and RT-PCR different sample preparations were used.

Statistical Evaluation

All data represent the arithmetic means ± SEM from 5 boars in the control boars and the immunized boars and from 6 boars in the E_2-17ß infused immunized group.

For the testosterone concentrations, each boar is represented by 22 blood samples; estradiol concentrations represent 50 blood samples from each boar. The concentrations of LH and FSH represent the arithmetic means ± SEM from 2 windows, with 37 samples from 5 boars each. The histological data are given as arithmetic means ± SEM of at least 40 tubules from each boar. The immunocytochemical evaluation refers to the arithmetic means ± SEM of 100 counted tubules per animal.

All data were tested for normal distribution by the Kolmogorov-Smirnov test.

They were further analyzed using the mixed model analysis of the Statistical Package for the Social Sciences (version 11; SPSS, Chicago, Ill).

The following model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\ \beta)_{ij} + e_{ijk}$$

Y_{ijk} = mean count of kth animal of ith group within jth replicate

µ = general effect

α_i = main effect of ith group

$ß_j$ = main effect of jth replicate

$(\alpha\ ß)_{ij}$ = group x replicate interaction

e_{ijk} = residual error

In this design, the animal was assumed to be chosen at random.

Results

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Immunization led to a considerable reduction in testicular size, due to both the loss of cytoplasm in the Leydig cells and a reduction of spermatogenic cells and of the tubular diameter in consequence. The mean weight (±SEM) of 1 testis was 376.4 ± 25.8 g in control boars, 94.8 ± 6.9 g in immunized boars, and 145.0 ± 17.0 g in E_2-17ß-infused immunized boars.

Hormone Concentrations

Concentrations of LH, FSH, testosterone, and estradiol are summarized in Table 1. LH concentrations decreased considerably, from 130 to 27 pg/mL respectively. Infusion of estradiol had no effect on LH concentrations in immunized boars.

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Table 1. *Concentrations of hormones in peripheral blood plasma of control boars, GnRH-immunized boars, and estradiol-infused immunized boars (arithmetic means ± SEM)**

In contrast, FSH concentrations were not influenced neither by GnRH-immunization nor the additional supply of estradiol. Testosterone in the control boars revealed mean concentrations of 4 ng/mL. The variation is mainly explained by differences in the concentrations between individuals (range of mean levels: 1.3–5.4 ng/mL). Immunization effectively decreased testosterone concentrations, but as expected, they were not influenced by E_2-17ß infusion.

The mean E_2-17ß concentrations were 106 pg/mL in the control boars, with an age-dependent increasing tendency along the 50-day sampling period. E_2-17ß values differed considerably between individual boars, so that the range of mean estradiol concentration was 54–284 pg/mL.

Immunization decreased E_2-17ß concentrations from 106 to 12 pg/mL (range: <12 pg/mL to 36 pg/mL). The infusion of estradiol led to an immediate rise in plasma. The infusion of high amounts of E_2-17ß led to a mean level of 512.4 ± 49.1 pg/mL. After lowering the amount of E_2-17ß, the concentration decreased to a mean concentration of 234 pg/mL, which was 1.6-fold higher than the control boars.

Germ Cells

A comparison of the frequency of the individual spermatogenic cell types is given in Table 2. For B-spermatogonia, primary spermatocytes, and round and elongated spermatids, the absence of testicular steroids in the immunized boars led to a significant decrease of cell counts compared to control boars. In the estrogen-infused boars the frequency of these cells increased again, but the counts were intermediate between control boars and immunized boars. In consequence, all counts in the E_2-17ß-infused pigs differed significantly when compared to both the control and the immunized boars.

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Table 2. *Frequency of individual spermatogenic cell types (cells per tubule) in control boars, immunized boars, and estradiol-infused immunized boars; values represent arithmetic means ± SEM from all stages of the seminiferous epithelial cycle**

A-spermatogonia, however, did not differ significantly between controls and immunized boars. E_2-17ß-infused immunized boars had the lowest number of A-spermatogonia, which differed significantly compared to both control and immunized boars. Thus it appears that cell counts had shifted from A- to B-spermatogonia due to estradiol infusion.

Results From Immunocytochemistry, RT-PCR, and Western Blot

An example for the ERα staining is shown for an immunized boar in Figure 1. As can be seen, ERα-staining is clearly separated from the neighboring cells and specifically occurs in spermatogonia. The specificity was additionally confirmed by Western blot and RT-PCR. Western blot analysis confirming the specificity of the antiserum had been performed by the supplier with human breast carcinoma cells and is additionally verified for testicular tissue in Figure 2. The bands of the expected size confirmed the applicability of the antiserum for boar testicular tissue.

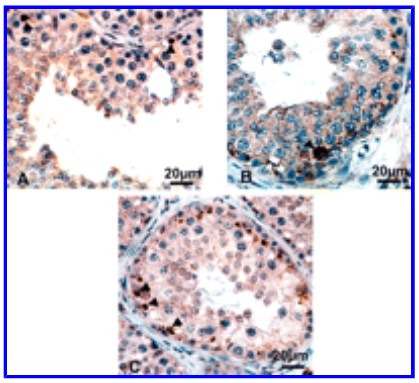

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Figure 1. Examples of ERα localization in testicular tissue of a control boar **(A)**, an immunized boar **(B)**, and an $E_2$17ß-infused immunized boar **(C)**. Arrows point to positively stained B-spermatogonia.

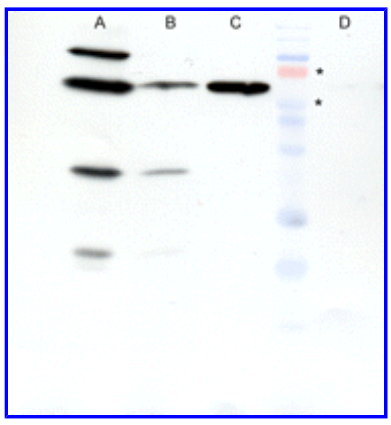

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Figure 2. Western blot analysis of the ERα in testicular tissue of a control boar **(A)**, an immunized boar **(B)**, and an E_2-17ß-infused immunized boar **(C)**. Porcine muscle tissue **(D)** served as negative control. Asterisks refer to the 73-kDa and 54-kDa bands of the prestained protein ladder (10–180 kDa). The exposed film was overlaid with the blot so that the prestained protein ladder is also visible on the scanned image.

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RT-PCR gave bands of the expected size of 222 bp and thus confirmed the expression of the ERα mRNA (Figure 3).

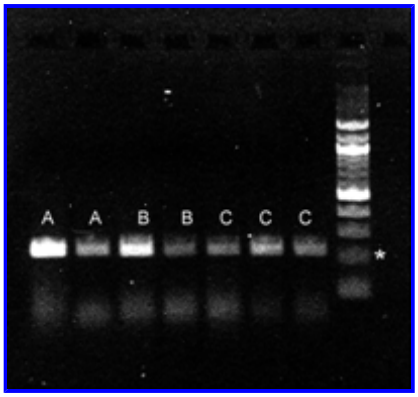

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Figure 3. Gel showing examples from RT-PCR of the ERα in testicular tissue homogenate of control boars (lanes A), immunized boars (lanes B), and $E_2$17ß-infused immunized boars (lanes C). The bands with the same electrophoretic motility reveal the expected size of 222 bp. An asterisk marks the 200-bp rung of the 100-bp ladder.

ERα-staining in the 3 groups of boars revealed that positive stained cells only occur within the tubules, but not in interstitial cells and Sertoli cells. Within the tubules, positive staining was primarily observed for A- and B-spermatogonia and only occasionally in more advanced spermatogenic cells. The occurrence of ERα in spermatogonia suggests a role for mitosis, so the relationships between ERα-expression and mitosis were analyzed for boars and referred to the stages of the seminiferous epithelial cycle, as shown in Figure 4. ERα was mainly detected in stage I. The number of Ki-67-positive cells which indicate the initiation of mitotic activity is included in Figure 4 and reveals highest cell counts in stage VIII. Because the stage-dependent pattern of ERα–staining did not differ between the other 2 groups, further quantification of ERα-positive cells was limited to stage I of the seminiferous epithelial cycle. These data and the number of cells undergoing mitosis and apoptosis are summarized for the 3 groups in Table 3. In Figure 5 an example of apoptosis is revealed by electron microscopy and confirms the occurrence of apoptosis in early stages of spermatogenesis.

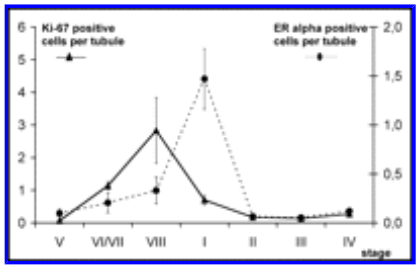

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Figure 4. Mitotic (Ki67-positive) cells and ERα positive cells per tubule in intact control boars, referred to the stages of the seminiferous epithelial cycle. The stage with the highest mitotic rate (stage VIII) and the maximal expression of the estrogen receptor (stage I) is centralized in the curve. Data represent the arithmetic mean of the 5 control boars.

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Table 3. *Mitosis, apoptosis, and estrogen receptor alpha (Stage I) (positive cells per tubule) in control, immunized, and estradiol-infused immunized boars (arithmetic means ± SEM)**

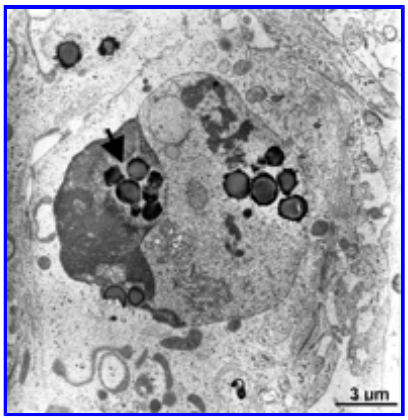

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Figure 5. Example of germ cell apoptosis in an immunized boar by electron microscopy. Note the apoptotic bodies of the germ cell. (magnification 7000x).

In the estradiol-infused immunized boars, the numbers of mitotic cells was intermediate compared to control boars and immunized boars (Table 3). In consequence, mitosis in E_2-17ß-infused immunized boars again differs significantly from the other 2 groups. No significant difference was detectable when comparing apoptosis in immunized and estradiol-infused boars, whereas apoptosis in the control boars was significantly lower. No significant differences exist in the expression of the ERα between the 3 groups, although mean positive cell counts are more than 2-fold in the E_2-17ß-infused boars compared to the immunized boars.

Discussion

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As reported earlier, immunization against GnRH led to a nearly complete inhibition of LH but not FSH, so that changes in spermatogenesis can be specifically attributed to the lack of testicular steroids (Metz et al, 2002; Wagner and Claus, 2004). Remaining concentrations of E_2-17ß were on the order of 12 pg/mL and thus represent the limit of sensitivity of the assay (11.5 pg/mL). Infusion of E_2-17ß did not alter the minimal testosterone concentrations but increased circulating E_2-17ß concentrations up to physiological levels. The concentrations obtained were higher compared to control boars, but were of an order which regularly can be determined in older boars (Claus et al, 1983). Thus the experimental design allowed the study of a selective effect of estradiol on spermatogenesis in the boar.

An alternative approach had been chosen by knockout experiments in mice inhibiting either the aromatase activity, and thus aromatization of androgens to estrogens (Fisher et al, 1998; Robertson

et al, 1999), or alternatively the estrogen receptor (Lubahn et al, 1993; Krege et al, 1998; for review see O'Donnell et al, 2001). In both cases possible effects of estrogens on different traits of male reproductive physiology had to be studied in the presence of androgens. More recently E_2-17ß formation was inhibited with an aromatase inhibitor, but effects on spermatogenesis were not investigated (At-Taras et al, 2005).

In our study the expression of ERα in boar testis was additionally confirmed by RT-PCR and by Western blot analysis in all 3 groups. In Western blot the preparations from the nonimmunized control boars obviously led to additional bands which may be attributed to a different endocrine background (eg, due to the presence of androgens). The underlying substances were not further identified. In addition, we could demonstrate previously that ERα expression is limited to early stages of germ cell development (Lekhota et al, 2005). ERα expression in the noninfused immunocastrates shows that it is partly independent from the presence of the ligand. A tendency for elevated receptor density in E_2-17ß-infused boars may be due to an additional ligand-dependent expression. The ERα could be exclusively localized in A- and B-spermatogonia. Staining, however, was focused on spermatogonia in stage I of the seminiferous epithelial cycle, which represents the main stage of spermatogonial division (Frankenhuis et al, 1982; Garcia-Gil et al, 2002). Stages VI/VII and VIII also belong to the mitotic stages, but mainly represent the G1 and S-phase, whereas actual division (M-phase) occurs in stage I (Guraya, 1987) where also ERα was detected in the present study. A mitosis-supporting effect of estrogens for spermatogenesis is not surprising, because their promotion of cell division is well known for a variety of other tissues, including estrogen-dependent tumors (Altucci et al, 1997; Zhang et al, 1998). In such tissues it was also found that estrogens promote cells to finish the G1-phase and to enter the M-phase (Leung and Potter, 1987; Zhang et al, 1998). A role of estrogens for mitosis of spermatogonia was also assumed for the stallion (Sipahutar et al, 2003) and for rodents and the eel (O'Donnell et al, 2001; Miura et al, 1999).

By the use of subcutaneous E_2-17ß Silastic implants it was shown in GnRH-deficient mice that the treatment led to an increase of the tubular volume (Ebling et al, 2000), as also shown in the hamster (Pak et al, 2002). In the latter study the effect was attributed to a spermatogenesis-initiating effect of estrogens.

Quantitative analysis revealed that counts of the individual spermatogenic cells other than A-spermatogonia were intermediate in the E_2-17ß-infused immunized boars, compared to both boars with significantly higher and immunized boars with significantly lower cell counts.

These data thus additionally support a role of E_2-17ß in spermatogenesis in the boar. This does not mean necessarily that overall production of sperm is improved in E_2-17ß-infused immunized boars compared to immunized boars, because counteracting apoptosis in the following spermatogenic cells was not significantly different between these 2 groups. In control boars, however, apoptosis was markedly reduced, suggesting an additional role of androgens. It was not possible to compare sperm counts in ejaculates, because semen collection attempts were unsuccessful both in immunized and E_2-17ß-infused immunized boars. Taken together, the data show that in the boar ERα is localized only in spermatogonia. Infusion of E_2-17ß led to an increase of mitosis in the tubules of GnRH-immunized boars, indicating that estrogens are necessary for germ cell renewal in the testis of boars.

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