Involvement of glucocorticoids in testicular involution after active immunization of boars against GnRH

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Abstract

Active GnRH immunization of boars inhibits LH and testicular steroids but the consequences for spermatogenesis are unknown. Six boars were immunized three times against GnRH at 20, 24 and 28 weeks. Another six boars served as controls. Plasma LH and FSH were determined at 28 and 31 weeks. Testosterone and cortisol were determined before killing the pigs at 32 weeks. Tissue samples were taken for histology and fluid from the seminiferous tubuli for steroid determination. Individual germ cells were counted in histological sections. The glucocorticoid receptor (GCR), mitosis of spermatogonia and apoptosis were characterized by immunocytochemistry. Immunization reduced LH and testosterone to base levels whereas FSH was not changed. Testis weight was reduced by 64% due to a loss of Leydig cell cytoplasm (90.3%) and a decrease of tubule diameters (60.6%). Except for A-spermatogonia, all other spermatogenic cells were reduced by about 60%. Mitosis was reduced in immunized boars. Expression of GCRs was limited to spermatogonia and differed between immunized boars (8% of spermatogonia) and controls (2%). In the controls, androgen concentrations in tubular fluid were tenfold higher compared with immunized boars. Cortisol concentrations were of the order of 40 nmol/l both in the tubular fluid and blood plasma. These concentrations did not differ between groups. Apoptosis occurred only in spermatogonia and pachytene spermatocytes and was twofold higher in immunized boars compared with controls. Thus the availability of glucocorticoids in the tubuli and the expression of GCRs initiate apoptosis, which in turn reduces sperm yield. Testosterone is known to be an inhibitor of GCR expression, thus increasing the efficiency of spermatogenesis.

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Introduction

Earlier studies with hypophysectomized animals clearly demonstrated that the regulation of spermatogenesis depends primarily on an interaction between follicle-stimulating hormone (FSH) and testosterone (Steinberger 1971, McLachlan *et al.* 2002). FSH plays a key role in the development of the immature testis by stimulating Sertoli cell proliferation and later progression of A- to B-spermatogonia (Billig *et al.* 1995, Franca *et al.* 2000). Testosterone alone can maintain complete spermatogenesis, but the synergistic action of FSH is necessary to normalize quantitative aspects of spermatogenesis (Steinberger 1971, Bartlett *et al.* 1989, McLachlan *et al.* 1996). This effect is explained by an FSH-dependent formation of the androgen-binding protein (ABP) by the Sertoli cells, which accumulate androgens in the tubuli (Hansson *et al.* 1975, Weddington *et al.* 1975).

Species-specific differences exist in testicular morphology in the pig. In the boar testes the total volume of

the Leydig cell compartment is unusually high and represents about one-third of the total testis volume (Groth & Claus 1977). In addition, an ABP could not be demonstrated (Jegou & Le Gac-Jegou 1978), but testosterone concentrations in tubular fluid are high and exceed blood plasma concentrations by a factor of 10–20. In addition, 147 nmol/l of 17 β -oestradiol were measured in tubular fluid (Claus *et al.* 1985). The high tubular concentrations of oestradiol do not necessarily suggest a role for spermatogenesis because they are transferred via the ejaculate into the female genital tract thus optimizing oestrogen-dependent mechanisms leading to fertilization (Claus, 1990). More recently we determined cortisol in tubular fluid and found mean concentrations of 33.33 nmol/l fluid in four boars (R Claus & B Möck, unpublished observations). In rats, testicular effects of glucocorticoids were attributed to a stress-related reduction of androgen formation by the Leydig cells (Evain *et al.* 1976, Ge *et al.* 1997), which also contain glucocorticoid receptors (GCRs) (Weber *et al.* 2000). GCRs were also found in several types

of germ cells and occasionally in Sertoli cells (Schultz *et al.* 1993, Konrad *et al.* 1998). The frequency of GCR-positive cells was much higher in the tubuli (80%) compared with the interstitium (20%) as again determined in rats (Biagini *et al.* 1995). The glucocorticoid–GCR system is related to germ cell apoptosis in the tubuli because application of the synthetic glucocorticoid dexamethasone increased the number of apoptotic germ cells, whereas the application of mifepristone, a GCR antagonist, dramatically decreased germ cell apoptosis even if dexamethasone was additionally applied (Yazawa *et al.* 2000).

Apoptosis of testicular cells, including germ cells, is the central phenomenon for seasonal involution of testis as shown for several species (for a review, see Young & Nelson 2001). It is also a surveillance mechanism within the tubuli to detect and eliminate defective cells in various stages of development via the Fas receptor (Francavilla *et al.* 2002). In addition, glucocorticoid-dependent apoptosis of spermatogonia and primary spermatocytes (Billig *et al.* 1995, Yazawa *et al.* 2000, Heninger *et al.* 2002) might be a fine-tuning mechanism to adapt sperm yield to the actual requirements for mating. Apoptosis therefore seems to be a key regulator with regard to the fact that under normal physiological conditions final sperm yield represents only about 50% of original spermatogonia in many species (Clermont 1962, Johnson *et al.* 2000). The assumption of a regulatory role of the glucocorticoid–apoptosis chain also provides a better understanding of androgenic effects on spermatogenesis because it is known that androgens can modify cortisol activity by down-regulation of GCR expression in various tissues (Kerr *et al.* 1996, Chen *et al.* 1997). Such an assumption, however, requires additional confirmation. Therefore it was the aim of the study to inhibit steroid synthesis in the Leydig cells of boars by immunization against gonadotrophin-releasing hormone (GnRH), which suppresses luteinizing hormone (LH). The determination of mitosis, GCR expression and apoptosis compared with untreated boars should then provide information on the likelihood of such an assumption.

Materials and Methods

Experimental design

Characteristics of spermatogenesis were determined by comparing hormone concentrations in peripheral blood and fluid of the tubuli, as well as histological criteria in the testes of GnRH-immunized boars ($n = 5$) with untreated boars (controls, $n = 5$). The time schedule for immunization was based on the following considerations.

In the domestic pig, the main pubertal spurt occurs at around 17 weeks of age. Therefore the first immunization was performed at 20 weeks so that initiation of spermatogenesis by gonadotrophins remained undisturbed. Based on earlier experiences (Metz *et al.* 2002), a booster injection (24 weeks of age) 4 weeks after the initial

immunization ensures that gonadal steroid concentrations are minimal 1 week later (25 weeks of age). Due to the duration of spermatogenesis and epididymal maturation, a definite effect of treatment on sperm production should be obvious 7 weeks later. In the midst of this 7-week period (28 weeks of age) a second booster injection was given to ensure a continued inhibition of GnRH. Testes were then obtained after killing the boars at an age of 32 weeks.

Ethics of experimentation

All steps of the animal experiments, including cannulation, immunization, sampling and killing were approved by the local animal welfare commission.

Animals, housing and sampling

The German landrace boars were obtained from the university herd and were kept individually in stalls (1.9×2.8 m). The experiment was performed in two consecutive replicates. The replicates were performed to limit the group size and thus to ensure extensive care of the animals. They had acoustic and visual contact with each other. The animals were fed a ration with 10 MJ metabolizable energy/kg of feed and 13.9% crude protein at an amount of 3 kg per day, which was offered in two portions in the morning and late afternoon. The average weight of boars increased from 82 ± 3.4 kg (20 weeks of age) up to 140 ± 5.3 kg (32 weeks).

For immunization, portions of 2 ml Improvac (CSL Limited ACN, Victoria 3052, Australia) were injected subcutaneously behind the earbase as recommended by the supplier and described previously (Metz *et al.* 2002). Two days after the first booster injection all animals were fitted with indwelling cephalic vein catheters, at an age of 24 weeks as described previously (Claus *et al.* 1990).

At an age of 32 weeks the pigs were killed by intravenous infusion of 0.2 ml Eutha77 per kilogram body weight (Essex Pharma, Munich, Germany).

Blood samples were collected over the 7-week period in heparinized vials at 0800 h prior to feeding but only samples collected the last 2 days prior to killing were used for steroid determination. After each sample collection the catheters were rinsed with heparinized saline, and the blood was centrifuged and stored at -22°C until assayed for testosterone and cortisol. To determine the effect of immunization on circulating concentrations of LH and FSH, blood sampling every 20 min over a 12-h period was carried out at 3 weeks and again 2 days before killing the animals. Mean plasma concentrations were calculated and used to characterize immunization effects on gonadotrophins.

Immediately after killing, the left testis was removed and the weight was determined. The testes were cut in two halves across the longitudinal axis. Two 1 g specimens of tissue were taken from the middle area of the parenchyma for later histological evaluation. They were fixed

either in Bouin's solution or in 37% formaldehyde. Bouin-fixed samples were used for gross morphology and formaldehyde-fixed samples were used for the immunocytochemical determinations. The other half of the testis was gently squeezed and the resulting fluid dripped into collection tubes as described earlier (Claus *et al.* 1985). The fluid was inspected microscopically and the sperm content confirmed that the tubuli predominantly were the source of the fluid. It was centrifuged and the clear supernatant stored deep frozen at -22°C for later steroid determination. All samples for immunohistochemistry could be obtained within 5 min after killing.

Analytical procedures

Determinations of LH and FSH were performed by RIA as published earlier (Claus *et al.* 1990) with the exception that in the case of LH the first antibody was pre-incubated with the sample before adding the tracer. LH (AFP: 11043B) and FSH (AFP: 10640B) for iodination were obtained from Dr Parlow (NIDDK, Torrance, CA, USA).

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 1:150 000.

The reliability criteria were as follows: for LH the sensitivity was 0.1 fmol per tube corresponding to 1.0 pmol/l blood plasma. The intraassay coefficient of variation was 7.9% and the interassay coefficient 8%. For FSH the sensitivity was 0.12 fmol per tube, corresponding to 1.2 pmol/l blood plasma. The intraassay coefficient of variation was 11% and the interassay coefficient 12%.

Testosterone was determined by RIA in blood plasma samples (Bubenik *et al.* 1982). Only samples from the last 2 days prior to killing were evaluated for comparison with testis status. Cortisol was also measured by a standard RIA after solvent extraction from the binding protein (Claus & Weiler 1996). In addition, determinations of these steroids were performed in fluid from the tubuli after solvent extraction as described earlier (Claus *et al.* 1985).

The antiserum against testosterone had been raised against testosterone-3-CMO-BSA and revealed cross-reactivity only with 5α -dihydrotestosterone (28.5%). The sensitivity was 0.14 nmol/l plasma. The intraassay coefficient of variation varied between 3.9% and 6.7% depending on concentration and between 7.2% and 18% for the interassay variation coefficient. The latter figure refers to the immunized group where concentrations were close to the detection limit.

The antiserum for cortisol determination had been raised against cortisol-11 β -HS-BSA, and revealed a cross-reactivity with cortisone (18.26%) and deoxycorticosterone (9%). The coefficients of variations were 5% for intraassay and 12% for interassay variation. It had been clarified earlier that the sampling time reflects the diurnal mean for cortisol (Claus & Weiler 1996).

Histological procedures

The Bouin-fixed samples were conventionally dehydrated and embedded in paraffin (Romeis 1989). Sections of 4 μm were cut with a Leica sliding microtome (SM 2000R, Nussbach, Germany), and the slides were stained with haematoxylin–eosin. The average diameter of the tubuli was determined by planimetry.

For characterization of the change of the Leydig cell area, a total of five interstitial areas that were surrounded by three neighbouring tubuli were chosen at random and the number of Leydig cell nuclei was counted. The number of Leydig cells was not remarkably different between controls and immunized boars.

The diameter of the nuclei and the diameter of the complete Leydig cells were determined and the volumes calculated under the simplified assumption of a spherical shape of both cells and nuclei.

To characterize spermatogenic activity, three sections of each testis were evaluated for the following spermatogenic cells: A-spermatogonia, B-spermatogonia, pachytene spermatocytes, round spermatids, and elongated spermatids. For the quantitative distribution of spermatogenic cell types all sections were evaluated by the same person.

Because complete spermatogenesis in the boar can be separated into eight stages (Swierstra 1968), five representative round tubuli for each of these eight stages were localized in the three sections and the cells were counted, so that the quantitative data for each cell type are based on 40 tubuli in each boar.

Immunocytochemical evaluation

The formaldehyde-fixed samples were treated in the same way as the Bouin-fixed samples.

To characterize mitosis, the histoprime monoclonal antibody against Ki67 MIB-1 (Canon, Wiesbaden, Germany) was used as described in detail previously (Mentschel *et al.* 2001). Counterstaining was performed by haematoxylin (Merck, Darmstadt, Germany). Because the antibody also stains meiotic stages of spermatogenesis, only stained spermatogonia were counted, whereas stained spermatocytes were ignored. Staining for mitosis, however, does not allow differentiation of A- and B-spermatogonia.

Apoptotic cells were identified by a modified terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling (TUNEL) assay (Gavrieli *et al.* 1992) that leads to staining of apoptosis-specific DNA fragments. The staining reaction was based on the *in situ* cell death detection kit POD (Boehringer, Mannheim, Germany). Details of the procedure were described earlier (Mentschel *et al.* 2001). Again, haematoxylin was chosen for counterstaining. The stained cells were attributed to the relevant types of germ cells.

For the immunocytochemical detection of the porcine GCR (pGCR), a polyclonal antibody against pGCR had been raised in rabbits (Gutscher *et al.* 2001). It was

directed against 135 amino acids from the N-terminal ending of the modulatory region which had been expressed in *E. coli*. As described previously (Gutscher *et al.* 2001), this specific antiserum detects GCR both in the cytoplasm and the nucleus. The immunocytochemical procedure has been described in detail previously, including the confirmation of the specificity of the antiserum by Western blot (Gutscher *et al.* 2001). The sections were again counterstained with haematoxylin.

For quantitative analysis of mitosis, GCR and apoptosis, another three sections were evaluated and only round tubuli were counted. Counting of positively stained cells again was referred to the individual types of spermatogenic cells. An average of 100 tubuli that had not been pre-selected for the stages of spermatogenesis was evaluated for each testis and criterion.

Statistical evaluation

The gonadotrophin concentrations represent the arithmetic means±S.E.M. from two windows with 37 samples from five boars each. The steroid concentrations are given as arithmetic means±S.E.M. from five boars each represented by two blood samples. The histological data are represented as arithmetic means±S.E.M. of at least 40 tubuli. The parameters from the immunocytochemical evaluation represented the arithmetic means±S.E.M. from 100 counted tubuli. All data were tested for normal distribution using the Kolmogorov–Smirnov test. They were analysed using the mixed model analysis of the Statistical Package for the Social Sciences (version 11, SPSS, Chicago, IL, USA). The following model was used

Y_{ijk} = μ + α_i + β_j + (αβ)_{ij} + e_{ijk}

where Y_{ijk} = mean count of kth animal of ith group within jth replicate, μ = general effect, α_i = main effect of ith group, β_j = main effect of jth replicate, (αβ)_{ij} = group × replicate interaction, and e_{ijk} = residual error.

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

Results

Gross morphology

The immunization against GnRH had significant effects on the testis size. The mean weight was significantly reduced by 67% compared with controls but individual boars reacted differently so that testis weights varied between 315 g and 400 g (mean 343 ± 13.8 g) in the controls and between 95 g and 150 g (mean 114 ± 12.1 g) in the immunized boars. This reduction was explained by a decrease of the mean area of tubuli (controls: 0.068 ± 0.008 mm²; immunized boars: 0.025 ± 0.002 mm²; P < 0.01) by 60.6%. Additionally, the average size of the individual Leydig cells was remarkably reduced due to a severe reduction in the amount of cytoplasm (controls: 2098.36 ± 164.27 μm³; immunized boars: 203.39 ± 24.88 μm³; P < 0.001) and nuclei (controls: 155.74 ± 5.24 μm³; immunized boars: 56.41 ± 6.31 μm³; P < 0.001). The number of Leydig cell nuclei that were encircled by the neighbouring tubuli was not obviously altered (controls: 35.46 ± 3.48 cells; immunized boars: 38.56 ± 3.16 cells; not significant).

Hormone concentrations

The reduction in the functional state of Leydig cells and thus the immunization success was confirmed by the testosterone concentrations (see Table 1). In the controls mean testosterone concentrations were significantly higher than in the immunized boars, so that immunization led to a decrease by 97.4%. Testosterone in the tubular fluid (Table 1) was decreased by 91% in the immunized boars. In this fluid the concentrations were about 40-fold higher compared with blood plasma concentrations 2 days earlier.

The reduction of testosterone was explained by a reduction of LH, as also shown in Table 1. In contrast to this 77% decrease of LH, FSH concentrations were not influenced by immunization, and the mean concentrations did not differ significantly between controls and immunized boars (Table 1).

The mean cortisol concentrations (Table 1) in blood plasma were similar in controls compared with immunized

Table 1 Concentrations of hormones in peripheral blood plasma and tubular fluid of control and GnRH-immunized boars.

	Control boars (n = 5)	Immunized boars (n = 5)	Significance P <
Testosterone			
(nmol l ⁻¹) blood plasma*	13.6 ± 1.18	0.35 ± 0.035	0.001
(nmol l ⁻¹) tubular fluid†	586.0 ± 31.2	54.3 ± 2.6	0.011
Cortisol			
(nmol l ⁻¹) blood plasma*	45.3 ± 4.7	32.5 ± 8.6	NS
(nmol l ⁻¹) tubular fluid†	41.0 ± 4.3	30.3 ± 4.8	NS
LH (pmol l ⁻¹)‡	4.3 ± 0.26	1.0 ± 0.3	0.001
FSH (pmol l ⁻¹)‡	9.75 ± 1.22	11.70 ± 2.20	NS

* Arithmetic means±S.E.M. from five boars each represented by two blood samples.
† Arithmetic means±S.E.M. from five boars each represented by one sample of tubular fluid.
‡ Arithmetic means±S.E.M. from two windows with 37 samples for five boars each, 3 weeks apart.
NS, Not significant.

boars. In addition, cortisol concentrations in the tubular fluid collected 2 days later after killing were of the same order as determined in blood plasma and did not differ significantly between groups.

Comparison of spermatogenic cell types

Data on the absolute number of spermatogenic cell types in the tubuli of controls and immunized boars are given in Table 2. In addition, Figs 1 and 2 give representative examples of spermatogenesis of the two groups. Immunization did not significantly change the frequency of A-spermatogonia, whereas all other cells were significantly reduced due to immunization. When taking all cell types together, the average reduction was 59.7% in treated boars. Individual cell types did not differ in their sensitivity to testosterone withdrawal. The reduction in the immunized boars compared with controls was 63.0% for round spermatids, followed by B-spermatogonia (62%), pachytene spermatocytes (61.5%) and elongated spermatids (57.0%).

Results from immunocytochemistry

The absolute number of positively stained cells per tubule is given in Table 3 for mitosis, GCR and apoptosis in the two groups of boars. Representative examples for GCR and apoptosis from the immunized group are additionally shown in Figs 3 and 4. The data in Table 3 reveal significant differences for mitosis, GCR and apoptosis. Whereas mitotic rate was about twofold higher, the frequency of GCR-positive cells was nearly fourfold lower in controls compared with immunized boars. Immunization also led to a twofold increase in the frequency of apoptotic cells in the immunized boars. The distribution of different cell types is shown in Table 4. GCR could be demonstrated for A- and B-spermatogonia, but was not observed in any of the other cell types within the tubuli. Apoptosis could be counted in A- and B-spermatogonia and additionally in pachytene spermatocytes whereas its occasional staining in only a few round and elongated spermatids did not allow a frequency distribution to be calculated.

Discussion

The immunization protocol effectively inhibited GnRH over the whole experimental period as indicated by the

Figure 1 Spermatogenesis in a non-immunized control boar representing stage VIII of the seminiferous cycle. A, A-spermatogonia; B, B-spermatogonia; P, pachytene spermatocyte; RS, round spermatid; ES, elongated spermatid.

Figure 2 Spermatogenesis in a GnRH-immunized boar representing stage VIII of the seminiferous cycle. A, A-spermatogonia; B, B-spermatogonia; P, pachytene spermatocytes; RS, round spermatids; ES, elongated spermatids.

decreased blood plasma concentrations of both LH and testosterone. FSH concentrations, however, were not decreased. Apparently, considerable species differences exist. In rodents, GnRH immunization led to a decrease of FSH in blood plasma down to non-detectable levels (McLachlan *et al.* 1994a). In other species including sheep, goat, bull and horse, FSH concentrations were decreased to a varying degree but levels that suggest a

Table 2 Frequency of individual spermatogenic cell types in control boars and immunized boars.

Cell type (cells per tubule)*	Control boars (n = 5)	Immunized boars (n = 5)	Significance P <
A-spermatogonia	8.18 ± 0.4	7.8 ± 0.4	NS
B-spermatogonia	42.1 ± 3.8	16.12 ± 1.7	0.002
Pachytene spermatocytes	54.9 ± 3.9	21.17 ± 1.6	0.001
Round spermatids	130.65 ± 8.8	48.92 ± 6.1	0.002
Elongated spermatids	77.8 ± 2.9	33.18 ± 1.05	0.001

*Arithmetic means ± S.E.M. from 40 tubuli of each boar.
NS, Not significant.

Table 3 Cells stained positive for either mitosis, GCR or apoptosis in control and immunized boars.

	Stained cells/tubulus		Significance <i>P</i> <
	Control boars (<i>n</i> = 5)	Immunized boars (<i>n</i> = 5)	
Mitosis*†	14.31 ± 1.07	7.70 ± 0.69	0.017
GCR†	0.67 ± 0.2	2.48 ± 0.54	0.033
Apoptosis†	0.29 ± 0.02	0.59 ± 0.03	0.001

* To exclude stained cells due to meiosis, only cells before preleptotene spermatocytes were regarded.

† Arithmetic means ± S.E.M. from 100 counted tubuli of each boar.

Figure 3 Examples of GCR in the tubuli of a GnRH-immunized boar. White arrows point to positively stained cells. **Figure 4** Examples of apoptosis in the tubuli of a GnRH-immunized boar. White arrows point to positively stained cells.

physiological function were still maintained (Rabb *et al.* 1990, Brown *et al.* 1994, Godfrey *et al.* 1996, Finnerty *et al.* 1998). For the pig it was reported that immunization inhibited LH, whereas plasma and pituitary FSH contents were not affected at all (Awoniyi *et al.* 1988). In another study a remarkable reduction of FSH in response to immunization was found (Caraty & Bonneau 1986). Except the

latter study, the data suggest that GnRH is not the main determinant for FSH in the pig and other mechanisms seem to be involved, such as a separate releasing hormone for FSH (Awoniyi *et al.* 1988, Padmanabhan & McNeilly 2001). Its existence, however, has not been proven so far. A role for activin expression in the pituitary is less likely because its stimulating effect on FSH is again mediated by GnRH (Peng & Mukai 2000). An alternative explanation might be a different sensitivity of LH and FSH to negative feedback effects of steroids at the pituitary level.

The role of FSH is not limited to the pubertal initiation of spermatogenesis. It was shown in hypophysectomized animals that FSH supports mitotic divisions of spermatogonia either by optimizing Sertoli cell function in general (Kerr *et al.* 1992, McLachlan *et al.* 1995, Yazawa *et al.* 2002), or by improving the availability of mitosis-regulating growth factors (Spiteri-Grech & Nieschlag 1992, de Rooij 2001). It was also shown, however, that in the absence of androgens, cell development does not proceed beyond round spermatids, which are either sloughed into the lumen or phagocytosed by the Sertoli cells after entering apoptosis (Russell & Clermont 1977, Muffly *et al.* 1994). Testosterone, in contrast, can maintain spermatogenesis up to spermiation of mature spermatozoa without the presence of FSH (Sharpe 1989, McLachlan *et al.* 1994b) even if the total number of sperm is decreased compared with intact animals. In our study FSH was maintained in the immunized boars so that the differences between groups could be directly attributed to testosterone formation.

The data from both groups of boars showed that mitosis was reduced in the immunized boars, but was still maintained at a remarkable level. The differences between groups might be attributed to the absence of testosterone in the immunized boars. Testosterone has a trophic effect on the Sertoli cells and thus is likely also to support mitotic divisions (McLachlan *et al.* 1994b).

It appears that testosterone exerts its regulating function on spermatogenesis primarily by modifying the cortisol-dependent apoptosis in the germinal epithelium. Apoptotic processes throughout spermatogenesis have been described for virtually all types of germ cells (Russell & Clermont 1977, Henriksen *et al.* 1996, Shetty *et al.* 1996, Print & Lakoski-Loveland 2000, Yazawa *et al.* 2000, Young & Nelson 2001, Heninger *et al.* 2002) but its role during ongoing spermatogenesis is not definitely known.

The main steps in apoptosis can be separated into initiation of apoptosis, expression of regulatory proteins with either anti- or pro-apoptotic functions, and finally execution of apoptosis by a variety of caspases and endonucleases (Distelhorst 2002). Differences mainly exist in the initiation of apoptosis. It could be mediated by expression of the Fas receptor which, for example, signals abnormal cell development, thus leading to the elimination of defective cells (Francavilla *et al.* 2002). Mitochondrial dysfunctions may initiate apoptosis in response to a shortage in energy supply (Mentschel & Claus 2003)

Table 4 Association of the frequency of GCR staining or apoptosis staining with individual spermatogenic cell types.

Phenomenon	Cell type	Control boars (n = 5) (positive cells/tubulus)	Immunized boars (n = 5) (positive cells/tubulus)	Significance P <
GCR*	A-spermatogonia	0.31 ± 0.13	0.65 ± 0.18	NS
	B-spermatogonia	0.69 ± 0.17	1.86 ± 0.39	0.042
Apoptosis*	A-spermatogonia	0.04 ± 0.01	0.03 ± 0.01	NS
	B-spermatogonia	0.14 ± 0.03	0.21 ± 0.04	0.049
	Pachytene spermatocytes	0.17 ± 0.02	0.31 ± 0.03	0.031

*Arithmetic means ± S.E.M. of 100 counted tubuli of each boar.
NS, Not significant.

whereas apoptosis, which is released by the activated GCR, is part of a normal regulatory process and adapts cell production by mitosis to the actual physiological demands (Distelhorst 2002). Within the spermatogenic process Fas-regulated apoptosis typically occurs in more advanced cells in the adluminal compartment of the tubuli (Francavilla *et al.* 2002). In our study only a very few apoptotic cells were found in this compartment whereas the highest frequency of apoptotic cells in both the controls and the immunized boars could be attributed to spermatogonia and, even more markedly, to pachytene spermatocytes. The highest frequency of GCR-positive cells was detectable in the preceding B-spermatogonia. Such a sequence between GCR expression and apoptosis suggests that the regulatory decision of later sperm yield is made early in spermatogenesis so that B-spermatogonia and pachytene spermatocytes may be subject to apoptosis. The assumption of a regulatory role of cortisol on spermatogenesis is further supported by experiments where apoptosis within the germinal epithelium was induced by exogenous application of dexamethasone (Yazawa *et al.* 2000). In our study the presence of cortisol in the lumen of the tubuli ensured the availability of the ligand for the receptor as also confirmed by staining of the nuclei. A cortisol-induced loss of early spermatogenic cells is likely to be an important fine-tuning mechanism to avoid both overload of Sertoli cells (Huckins 1978, Johnson *et al.* 2000) and the presence of excessive sperm number in the ejaculate and thus an increased risk of polyspermy.

The ultimate regulator of this mechanism is the presence of androgens. As shown for the rat, several stages of spermatogenic and Sertoli cells express the androgen receptor (Kimura *et al.* 1993, Zhou *et al.* 1996, Arenas *et al.* 2001) but the mode of action is not definitely known. In other tissues, androgens may improve protein synthesis but additionally they are well known to modulate GCR expression via androgen-responsive elements on the DNA (Haelens *et al.* 1999). In addition, androgen receptors and glucocorticoid receptors can form heterodimers that modify effects on the DNA (Chen *et al.* 1997). The resulting down-regulation of catabolic effects (Mayer & Rosen 1975, Danhaive & Rousseau 1988) is part of the overall anabolic function of androgens in meat-producing animals including pigs (Metz *et al.* 2002). Similar effects of the glucocorticoid-androgen system within the testes

have not yet been investigated. They might provide new aspects for the regulation of spermatogenesis but require additional investigation, including the application of testosterone to GnRH-immunized boars.

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