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Effects of an anti-gonadotrophin releasing hormone vaccine on the morphology, structure and function of bull testes

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ABSTRACT

Castration reduces aggressive and sexual behaviour and provides better carcass quality in bull calves. Vaccination against gonadotrophin-releasing hormone (GnRH) is used as an alternative to surgical castration for the purposes of reducing pain and distress in the animals. Currently, no anti-GnRH vaccine has been authorized for use in cattle in the European Union. The aim of the present study was to assess the effect of an anti-GnRH swine-specific vaccine (Improvac®, Zoetis, USA) on the morphology, structure and function of bull testes. Animals were vaccinated at days 1, 21 and 104 of the experimental period and were classified based on their live weight into the following two groups: LIGHT (172.9 ± 30.00 kg) and HEAVY (323.8 ± 37.79 kg). The scrotal circumference was measured on day 1 and prior to slaughter (day 164). At slaughter, the sperm motility and concentration in the caudae epididymis were assessed. Testes were weighed, measured and examined using ultrasound, and then tissue samples were collected and fixed in formalin. Histological and immunohistochemical studies were performed on the testes to measure the diameter of the seminiferous tubules and assess the testicular cell populations. The results revealed that suppression of testicular development was associated with the use of the Improvac® vaccine, which resulted in a smaller size of the testes and impaired spermatid production. However, the effect of Improvac® was more pronounced and consistent in calves vaccinated at a low live weight than at a heavy live weight, which suggested that vaccination is more effective when calves are vaccinated before or early during puberty. However, testes from calves vaccinated at a low live weight were more prone to the development of intraluminal concretions in the seminiferous tubules.

1. Introduction

Castration is a common practice that is used in pigs to avoid the presence of boar taint as well as aggressive behaviour in intact males. To a lesser extent, castration is also used in bull calves, especially when they are bred extensively, to reduce aggressive and sexual behaviour and to ensure better carcass quality [1]. During the last few years, vaccination against gonadotrophin-releasing hormone (GnRH) has been used as an alternative to surgical castration to reduce pain and distress in the animals [2–4]. Gonadotrophin-releasing hormone is secreted by hypothalamic neuroendocrine cells and regulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), which control the secretion of testosterone and spermatogenesis. Immunocastration stimulates the production of antibodies that neutralize GnRH; as a consequence, the secretion of FSH and LH is inhibited and, thus, growth, maturation and testicular functioning are disrupted [4–6].

Improvac® (Zoetis, USA) is the first anti-GnRH vaccine approved for widespread use in pigs. While Improvac® is authorized for use in the European Union and is currently available in more than 60 countries, an anti-GnRH vaccine that has been designed for use in cattle (Bopriva®, Pfizer Animal Health, Australia) has not yet been authorized for use. A decrease in testicular development has been described in pigs immunized with Improvac® and Bopriva® [7–9]

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and in calves immunized with Bopriva® [10]. In those studies and in many others [2,3,6,11,12], vaccination against GnRH was shown to be effective in reducing the levels of testosterone in peripheral blood in pigs and bulls. The aim of the present study is to assess the effects of the swine-specific vaccine Improvac® on the morphology, structure and function of the testes in bulls vaccinated at two different live weights (LW).

2. Material and methods

2.1. Animals, vaccination and sampling

This study was conducted at the Centro de Investigación y Tecnología Agroalimentaria (CITA) of Aragón (Zaragoza, Spain). All procedures were approved by the Animal Ethics Committee at CITA. The care and use of animals were performed in accordance with the current normative guidelines (RD 53/2013), which meet the requirements given in Union Directive 2010/63 for the protection of animals used for experimental and other scientific purposes.

A total of 16 male calves of the "Serrana de Teruel" breed were used in the present study. The "Serrana de Teruel" is a local endangered breed raised in mountainous areas in the centre of Spain, that is seldom handled [1]. The animals used in the present study formed part of a previous study to assess the effect of the Improvac® on the carcass traits and meat quality of bulls [12]. A 2x2 experimental design was followed. The calves were randomly assigned to two groups of equal size depending on their LW at the beginning of study: the light group (LIGHT; n = 8, 172.9 ± 30.00 kg LW and 7.7 ± 1.12 months of age) and the heavy group (HEAVY; n = 8, 323.8 ± 37.79 kg LW and 10.6 ± 2.11 months of age). Half of the calves in each group were randomly selected to serve as a control (C, n = 8 (4 LIGHT-C + 4 HEAVY-C)) group, while the other calves were placed into the vaccinated group (VA, n = 8 (4 LIGHT-VA + HEAVY-VA)). Based on the commercial Bopriva® planning, the calves were vaccinated subcutaneously with 2 mL of Improvac® on days (d) 1, 21 and 104 of the experimental period. The animals were fed a commercial concentrate and barley straw *ad libitum* for 164 days ±2.9 days of the experimental period. The scrotal circumference was determined using measuring tape on the day that the first dose of Improvac® (d 1) was administered and immediately prior to slaughter. The animals were slaughtered at the end of experimental period by a commercial European Union licensed abattoir. Testes with epididymides were removed at the slaughterhouse within 30 min after slaughter and stored at 15 °C prior to processing at the laboratory.

2.2. Macroscopic analysis

The testes were dissected away from the epididymides and extraneous tissue and weighed. Both the testis length and width were measured using a Vernier calliper, while the longitudinal and cross-section circumferences were measured using measuring tape. The volume of each testes was determined via submersion in a graduated cylinder filled with normal saline.

2.3. Ultrasound examination

Ultrasound examination (Aloka SSD-500, Aloka, Spain) of each testis was conducted using a 7.5 MHz linear probe to determine the level of the echogenicity of the testicular parenchyma, the testicular width, and the thickness of the mediastinum and anterior and posterior parenchyma. The ultrasound settings (focus, gain, brightness and contrast) were standardized and used for all the examinations. After placing the testis in an anatomic position, the probe was placed on the back, aligned with the sagittal plane (over the posterior parenchyma, parallel to the longitudinal axis of testis) to measure the mediastinum and the parenchyma thicknesses [13], and it was aligned with the horizontal plane (parallel to the transversal axis) to measure the testicular width. To quantify the echotexture based on the ultrasound images, the largest representative area in the posterior testicular parenchyma was selected, avoiding shadows or any artefacts. The resultant images were processed using ImageJ v1.48 (National Institutes of Health, USA). The pixel intensity was measured using a 255 point scale [0 (anechoic) to 255 (hyperechoic) [14];, and the result was expressed as a percentage.

2.4. Epididymal sperm motility and concentration

Within the dissected caudae epididymis, multiple incisions with a scalpel blade were made [15]. After epididymal milking, the spermatozoa were aspirated using a micropipette and added to 1:5 diluted BioXcell medium (IMV Technologies, France). Serial dilutions were conducted to obtain the 1:400 and 1:4000 dilutions that were used for the assessments of motility and concentration, respectively. A Makler chamber and a Neubauer chamber were utilized to determine the progressive motility and spermatozoa concentration, respectively, using a phase contrast microscope. All materials and equipment were heated to 37 °C in an incubator prior to use.

2.5. Histological analysis

A histological analysis was used to examine the changes in testicular cell populations and the diameter of the seminiferous tubules in C and VA animals. A cross-section from the central part of each testis was fixed in 10% formalin, trimmed and processed according to standard procedures. For the histological examinations, 4 μm tissue sections were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stain.

The diameters of the seminiferous tubules in the H&E tissue sections were measured at 50× magnification using AxionVision 4 image processing software (Zeiss, Germany). Each testis section was divided into 4 areas and 3 randomly chosen seminiferous tubules in each area were assessed (24 tubules per animal). Only round tubule cross-sections were measured and two perpendicular measures were made for each tubule to obtain the average value.

2.6. Immunohistochemistry

The following primary monoclonal mouse antibodies were used: an anti-vimentin (clone V9), which was used as a marker for Sertoli cells, and an anti-human Ki-67 (dilution 1:50), which was used as a marker for proliferating cells in testes [16]. Vimentin is an intermediate filament protein present in the cytoskeleton of the Sertoli cells, and the Ki-67 antigen is a nuclear protein expressed during all active phases of the cell cycle.

Tissue sections (4 μm) were deparaffinized and hydrated using routine methods and then subjected to an antigen retrieval procedure that involved heating tissue sections at 96 °C for 20 min in EnVision™ FLEX Target Retrieval solution. After antigen retrieval, the tissue sections were immunolabelled using an automated immunostainer that performs the following steps interceded by washes with Tris-buffered saline (TBS): 1) addition of blocking solution for 5 min to block endogenous peroxidases; 2) incubation for 30 min with primary antibodies; 3) incubation for 30 min with EnVision™ FLEX solution for the purposes of visualization; 4) staining for 10 min with diaminobenzidine, which serves as the chromogen. Finally, the sections were washed in distilled water and counterstained with haematoxylin for 20 s. All incubations were

performed at room temperature and all immunohistochemical products and antibodies were obtained from Dako Denmark A/S (Denmark).

Based on the antibody manufacturer's instructions, tonsil tissue sections were used for the positive control. As a negative control, testes tissue sections (VA and C) were subjected to the same procedure in which the primary antibody was replaced with a negative control antibody (Universal Negative Control Mouse, Dako Denmark A/S, Denmark).

2.7. Statistical analysis

All statistical analyses were conducted using the SAS statistical package v 9.3 (SAS Institute Inc., NC, Cary, USA). The normality of the data residuals was confirmed using the UNIVARIATE procedure ($P > 0.05$) for all variables, except for the spermatozoa concentration, which was expressed as a logarithm.

The scrotal circumference, length, width, length circumference and width circumference, weight and volume of the testis, seminiferous tubule diameter, anterior and posterior parenchyma thickness, mediastinum thickness, progressive motility and spermatozoa concentration were analysed using the MIXED procedure for repeated measures using values from the left and right testes of each animal, with the exception of the scrotal circumference. The fixed factors were the vaccine treatment (VA vs. C) and the LW at the start of the experimental period (LIGHT vs. HEAVY). The sampling day was considered to be a within-subject effect (only for the scrotal circumference) and the animal as a random effect (experimental unit). The least square means were estimated for each fixed effect when no interaction was found between them. In case of scrotal circumference and progressive motility, the least square means are presented in four categories due to the interaction between LW and vaccine treatment. Pair-wise comparisons of the means were performed using the probability of difference (PDIFF) option of the LSMEANS procedure. The level of significance for all tests was denoted by $P < 0.05$.

3. Results

3.1. Effect of the anti-GnRH vaccine on the morphology of the testes

A three-way interaction was observed for the LW, vaccine treatment and time for the scrotal circumference measures. The scrotal circumferences of the C animals increased between the administration of the first anti-GnRH vaccine dose (day 1) and slaughter (day 164); for VA animals, no statistically significant differences were observed (Table 1). However, the scrotal circumference growth for the LIGHT-C group was almost four-fold greater than that of the HEAVY-C group (54.9% vs. 14.8%, respectively); on day 164, the LIGHT-C calves had similar values to those of the HEAVY-C calves ($P > 0.05$).

Vaccination with Improvac® affected the size of the testes, since the weight, length, width, perimeter measurements, and volume of the testes were lower in VA calves than in C calves ($P < 0.0001$) at slaughter. Moreover, the LW at the start of experimental period affected the morphology of the testes, as demonstrated by the fact that the HEAVY calves presented higher morphology measurement values than the LIGHT calves at slaughter ($P < 0.01$; Table 2).

3.2. Effect of the anti-GnRH vaccine on the ultrasound structure of the testes

The C calves exhibited higher values than the VA calves for all measurements of the testes obtained using ultrasound. Based on the LW category, the HEAVY animals exhibited higher testicular width and anterior parenchyma values than the LIGHT animals ($P < 0.01$), while no differences were found for the mediastinum and posterior parenchyma measurements ($P > 0.05$; Table 2).

Regarding the pixel intensity of the testicular parenchyma, neither the vaccine treatment ($55.2\% \pm 6.10$ vs. $64.7\% \pm 7.88$ for C and VA, respectively; $P > 0.05$) nor the LW category ($60.6\% \pm 7.88$ vs. $59.3\% \pm 6.10$ for LIGHT and HEAVY calves, respectively; $P > 0.05$) influenced the echogenicity of the posterior parenchyma in the testes at slaughter.

3.3. Effect of the anti-GnRH vaccine on epididymal spermatozoa progressive motility and concentration

Spermatozoa progressive motility was shown to be influenced by vaccine treatment and the LW. Calves from the HEAVY-C group demonstrated higher motility percentages than calves from the LIGHT-C group, while the VA groups (both HEAVY and LIGHT) exhibited the lowest values ($P < 0.05$; Table 1). The epididymal spermatozoa concentration was affected by vaccine treatment, as demonstrated by the fact that the C group exhibited a 9.9 ± 0.81 logarithm unit concentration (approximately 8.7×10^9 counts/mL), while the VA group exhibited a 1.1 ± 0.81 logarithm unit concentration (approximately 0.14×10^2 counts/mL; $P < 0.0001$). Although the HEAVY animals exhibited higher concentration values than the LIGHT animals, no significant differences were found that were related to the LW category [6.2 ± 0.81 logarithm unit concentration (approximately 1.4×10^6 counts/mL) vs. 4.9 ± 0.81 logarithm unit concentration (approximately 8.6×10^4 counts/mL), respectively; $P > 0.05$).

3.4. Effect of the anti-GnRH vaccine on the histology of the testes

The vaccine treatment and the LW category significantly affected the diameters of the seminiferous tubules (Table 2). The C and HEAVY calves exhibited higher values than the VA and LIGHT

Table 1
Mean scrotal circumference measurements at the beginning (day 1) and at the end of the experimental period (day 164) and mean epididymal spermatozoa progressive motility after slaughter according to the interaction between live weight and vaccine treatment fixed effects.

	Live weight x Vaccine treatment				
	LIGHT-C	LIGHT-VA	HEAVY-C	HEAVY-VA	sed
n =	4	4	4	4	
Scrotal circumference at day 1 (cm)	$20.4^{b,2}$	20.9^{b}	$32.4^{a,2}$	28.4^{a}	1.58
Scrotal circumference at day 164 (cm)	$31.6^{ab,1}$	21.9^{c}	$37.2^{a,1}$	30.2^{b}	1.58
Progressive motility (%)	18^{b}	0^{c}	36^{a}	1^{c}	4.9

LIGHT: light live weight at the start of experimental period. HEAVY: heavy live weight at the start of experimental period. C: control group. VA: vaccinated group.
Sed: standard error of the difference. n: number of animals.
Differences between groups in the same row: a, b, c: $P < 0.01$.
Differences between groups in the same column: 1, 2: $P < 0.05$.

Table 2
Mean of the morphological and ultrasound measurements of testis at slaughter, according to the live weight and vaccination category as independent fixed effects.

	Live Weight			Treatment		
	HEAVY	LIGHT	sed	CONTROL	VACCINATED	sed
n =	8	8		8	8	
Morphological measurements						
Length (cm)	10.2[a]	8.7[b]	0.33	11.2[a]	7.7[b]	0.33
Width (cm)	5.8[a]	4.7[b]	0.18	6.3[a]	4.2[b]	0.18
Length circumference (cm)	26.7[a]	22.3[b]	0.99	29.2[a]	19.8[b]	0.99
Width circumference (cm)	16.6[a]	13.9[b]	0.57	18.4[a]	12.4[b]	0.57
Testicular weight (g)	208.0[a]	130.7[b]	16.16	253.9[a]	84.9[b]	16.16
Testicular volume (ml)	186.9[a]	121.5[b]	20.21	227.8[a]	80.6[b]	20.21
Seminiferous tubule diameter (μm)	216.3[a]	181.9[b]	9.71	245.4[a]	158.2[b]	9.71
Ultrasound measurements						
Anterior parenchyma (cm)	2.1[a]	1.6[b]	0.13	2.3[a]	1.4[b]	0.13
Mediastinum (cm)	0.4	0.4	0.06	0.5[a]	0.3[b]	0.06
Posterior parenchyma (cm)	1.9	1.6	0.16	2.3[a]	1.3[b]	0.16
Testicular width (cm)	4.4[a]	3.6[b]	0.24	5.1[a]	3.0[b]	0.24

LIGHT: light live weight at the start of experimental period. HEAVY: heavy live weight at the start of experimental period.
Sed: standard error of the difference. n: number of animals.
Differences between groups: a, b: $P < 0.01$.

calves ($P < 0.001$ and $P < 0.05$, respectively). Regarding the testicular cell populations, all C calves exhibited fully developed spermatogenesis (Fig. 1A), which is characterized by the presence of spermatogonia, spermatocytes, round and elongated spermatids and Sertoli cells in the seminiferous epithelium. Small groups of normal Leydig cells were observed within the interstitial tissue. In contrast, the testes of all the VA calves except two exhibited microscopic features consistent with severe testicular hypoplasia [17], and all of the seminiferous tubules that were examined were abnormal. The testicular histology reflected a complete absence of spermatogenesis with a predominance of Sertoli cells in the seminiferous epithelium (Fig. 1B). Only a few spermatogonia were observed in the basal compartment of the seminiferous epithelium, and spermatocytes and other immature cells were frequently present in the lumen. Leydig cells had lost their cytoplasmic eosinophilia and were difficult to distinguish from interstitial fibroblasts. Two of the VA calves from the HEAVY group exhibited normal microscopic features consistent with active spermatogenesis (Fig. 1C).

Three VA calves in the LIGHT group and 1 in the HEAVY group exhibited the presence of intraluminal round concretions that were positive for periodic acid-Schiff (PAS) staining (Fig. 1D), which indicated a high polysaccharide content [18]. The concretions were of variable size, and the larger concretions exhibited a laminated concentric structure. In all cases, the concretions were surrounded by cells that had detached from the epithelium. No concretions were observed in the C calves.

3.5. Effect of the anti-GnRH vaccine on the immunohistochemistry of the testes

In C calves, vimentin-positive Sertoli cells were found from the basal to the adluminal compartment of the seminiferous epithelium and were evenly distributed between the spermatogenic cells, which comprised the majority of the cells. Vimentin immunolabelling in Sertoli cells was observed to exhibit perinuclear localization with characteristic apical filament projections (Fig. 2A). In this group of animals, Ki-67 antigen staining revealed the presence of a large number of active spermatogonia and spermatocytes in the basal half of the seminiferous epithelium (Fig. 2B). The same immunohistochemical results were observed in the 2 VA calves in the HEAVY group that exhibited active spermatogenesis (as previously described in the histology section). In the rest of the VA group calves, the seminiferous epithelium was immunolabelled with vimentin almost in its entirely, which indicated that it was largely composed of Sertoli cells (Fig. 2C). In these cells, vimentin immunolabelling was also observed the perinuclear region, but many more projections were found than in C calves. Only a few vimentin-negative cells, which corresponded to spermatogonia, were observed in the basal seminiferous epithelium); moreover, only a few of these spermatogonia were mitotically active, as indicated by Ki-67 immunolabelling (Fig. 2D).

In addition, the cells that surrounded the PAS-positive concretions detected in the lumen of the seminiferous tubules in four VA group calves were also positive for immunolabelling for vimentin (Fig. 2E and F).

4. Discussion

In accordance with previous studies that have evaluated Improvac® in swine and the cattle-specific vaccine Bopriva® in cattle and swine [7–10], our results demonstrate that the immunization of calves with Improvac® suppresses testicular development. These results are in line with those obtained in a previous study, in which we describe that plasma testosterone concentration was suppressed by Improvac® to below the limit of detection [12].

In the present study, the effect of Improvac® on the parameters assessed was more pronounced and consistent among calves vaccinated at a low live weight (LIGHT group) than at a heavy live weight (HEAVY group) at the start of treatment, which suggests that vaccination is more effective when calves are vaccinated before puberty. These results are in agreement with previous studies in pigs showing that early immunocastration with Improvac® at age 10 weeks caused a more severe disruption of testicular structure and function than standard vaccination at age 16 [8,19].

Puberty occurs in calves between 37 and 50 weeks of age; a scrotal circumference of 28 cm is often used to indicate the onset of puberty. Between 25 weeks of age and until puberty, a rapid phase of testes growth occurs that is characterized by proliferation and differentiation of germ cells and the differentiation of mature Sertoli cells [20]. Calves from the LIGHT group were vaccinated during this phase (scrotal circumference 20.9 ± 1.12 cm) and in all of them testicular growth seemed to be suppressed. Their scrotal circumferences were almost the same at the beginning of the experimental period and at slaughter, and remained in both measurements below the scrotal circumference that indicates the

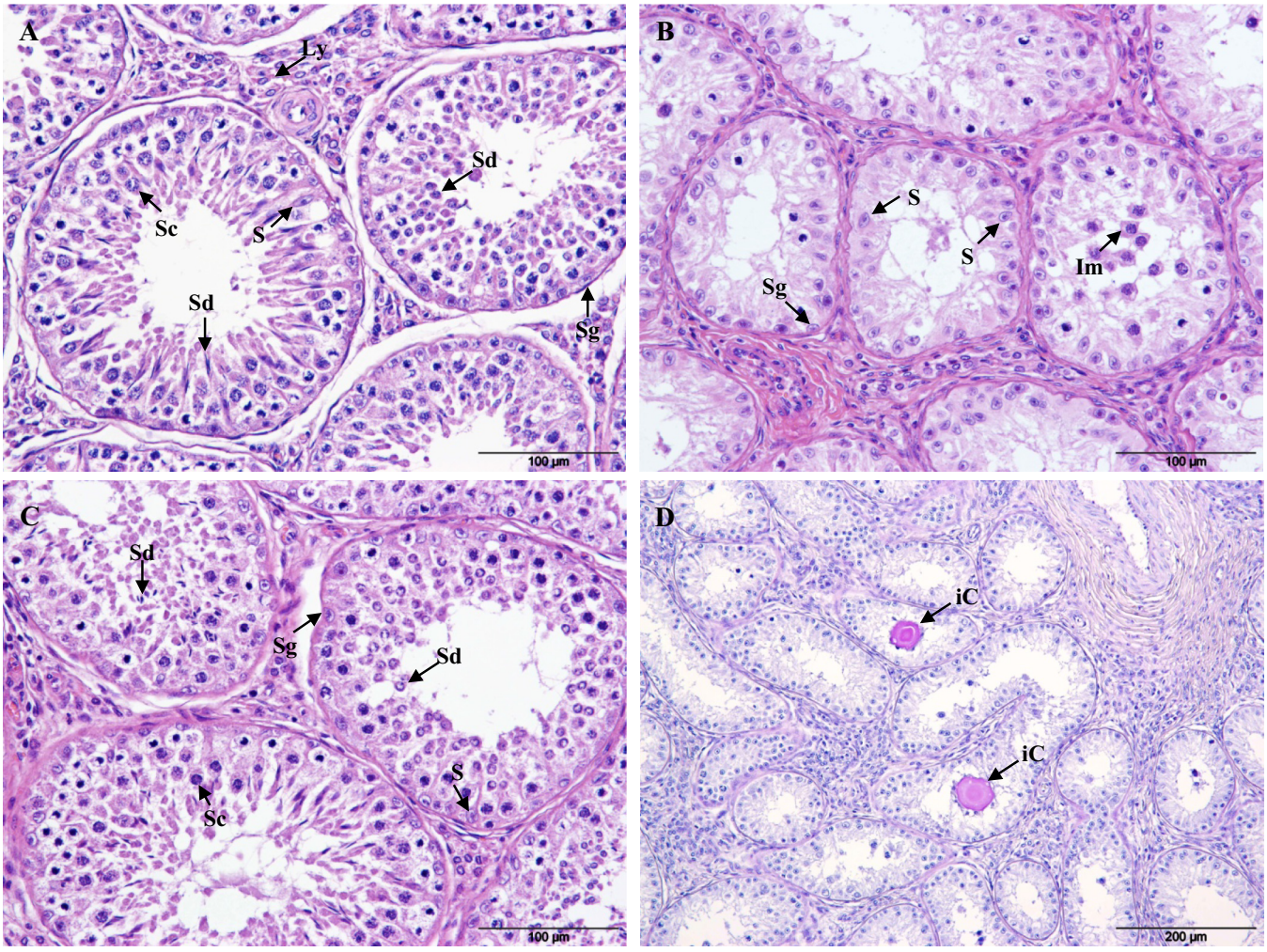

Fig. 1. Optical microscopy images. Histological appearance of testicular tissue from control (A) and vaccinated calves (B–D). (A) H&E stain. The control calf shows normal seminiferous tubules and Leydig cells in the peritubular tissue. In the seminiferous epithelium, fully developed spermatogenesis with easily discernible Sertoli cells, spermatogonia, spermatocytes and spermatids can be observed. (B) H&E stain. The vaccinated calf presents reduced-diameter of the seminiferous tubules. The seminiferous tubules show a complete absence of spermatogenesis with a predominance of Sertoli cells in the seminiferous epithelium, few spermatogonia in the basal compartment and immature cells in the lumen. (C) H&E stain. A vaccinated calf from the HEAVY group presents normal seminiferous tubules with fully developed spermatogenesis, similar to control calves. (D) PAS stain. In the seminiferous tubules from a vaccinated calf, intraluminal round PAS-positive concretions can be observed. S: Sertoli cell; Sg: spermatogonia; Sc: spermatocytes; Sd: spermatids (round and elongated); Im: immature cells; iC: intraluminal concretions.

onset of puberty. In addition, testes weight, volume and the diameter of the seminiferous tubules at slaughter were reduced by a mean value of 73.3%, 74.6% and 45.6%, respectively, in LIGHT-VA animals when compared to LIGHT-C animals. According to previous studies, these results suggest that a marked hypoplasia of the testes is associated with the use of the Improvac® vaccine in calves [6,9,10,21,22]. Moreover, the VA calves ceased to have spermatogenic capacity, as only insignificant spermatozoa concentration was observed within the caudate epididymis. The testes histology for all of the LIGHT-VA calves was dramatically affected and reflected a major disruption of spermatogenesis. However, the presence of some mitotic spermatogonia in the seminiferous epithelium suggests that the testes could return to a normal state. The response to the anti-GnRH vaccine is believed to be temporary [8,10,11,23,24]. In the present study, the effects of Improvac® on calves vaccinated before puberty lasted for at least 23 weeks after the first immunization, but further studies are required to determine whether these animals have the ability to totally recover or if their testicular function has been irreversibly affected. Janett et al. [24] observed that calves vaccinated with Bopriva® at the age of 3 weeks were able to attain spermatogenic capacity at slaughter (when the animals were 68 weeks old), although a marked delay in testicular development was observed. In contrast, studies in boars suggest that early vaccination may irreversibly affect testicular function [8,19].

In the case of the HEAVY-VA group, the scrotal circumference measurements (28.4 ± 1.12 cm) indicate that the calves were probably near or had reached puberty before the first vaccine dose. At slaughter, these scrotal circumference values were still above the threshold for puberty but did not reflect significant growth in the scrotal circumference during the experimental period. In this group of calves, testicular growth seemed also to be reduced, but a high degree of individual variability was observed in the parameters that were studied. Two animals in this group had values that were nearly identical to those of calves from the LIGHT-VA group (70.6%, 69.0% and 41.0% reduction in testes weight, volume and the diameter of the seminiferous tubules, respectively), as well as the presence of arrested spermatogenesis. In the remaining 2 calves, the reduction in the testes weight, volume and diameter of the seminiferous tubules was lower (48.6%, 45.6% and 20.0%,

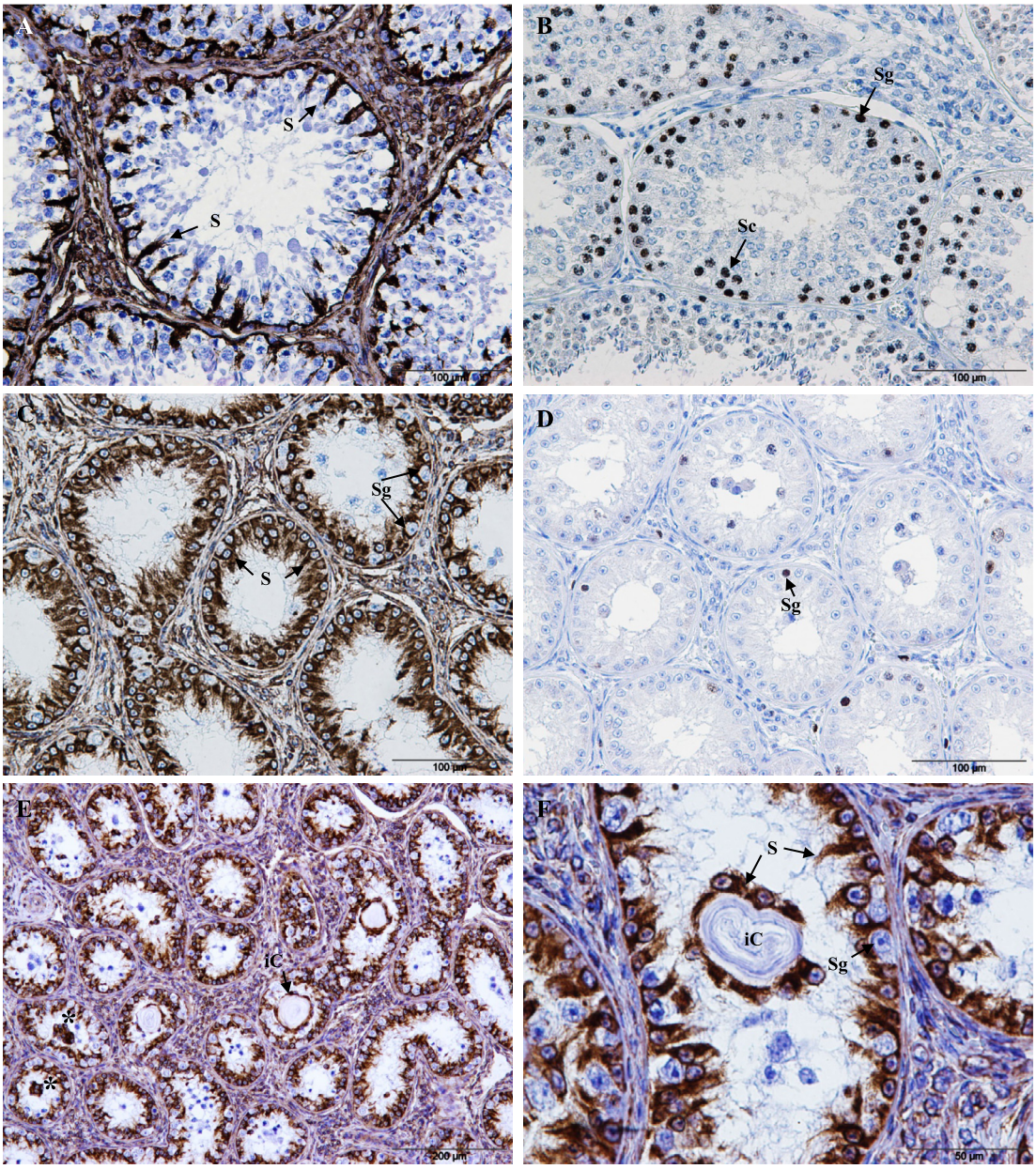

Fig. 2. Optical microscopy images. Histological appearance of testicular tissue from control (A–B) and vaccinated calves (C–F). Immunohistochemistry for vimentin (A, C, E, F) and Ki-67 (B, D). In control and vaccinated calves, vimentin is expressed in the interstitium and in Sertoli cells. (A) In the seminiferous epithelium of the control calf, the vimentin-positive Sertoli cells are evenly distributed between the spermatogenic cells. Vimentin immunolabelling exhibits perinuclear localization with characteristic apical filament projections. (B) In the seminiferous epithelium of a control calf, a large number of Ki-67 positive spermatogonia and spermatocytes are present. (C) The seminiferous epithelium of the vaccinated calf is composed mostly of Sertoli cells with just few spermatogonia in the basal compartment. (D) In the vaccinated calf, only few Ki-67 positive spermatogonia are present in the basal seminiferous epithelium. (E) Concretions surrounded by vimentin-positive Sertoli cells and small groups of detached vimentin-positive Sertoli cells (asterisks) can be observed in the lumen of some seminiferous tubules from a vaccinated calf. (F) A detail of a intraluminal concretion surrounded by vimentin positive Sertoli cells. S: Sertoli cell; Sg: spermatogonia; Sc: spermatocytes; iC: intraluminal concretions.

respectively), and active spermatogenesis with a high number of proliferating spermatogenic cells in the seminiferous epithelium was observed. One HEAVY-VA animal (the heaviest of the group at the first vaccine dose) exhibited an epididymal spermatozoa concentration that may have indicated fertilization capacity [24]. Apart from the concentration values, the motility results for all VA animals (including the HEAVY animal with active spermatogenesis) tended to be close to zero or were non-existent due to the lack of sperm, which blocks the ability to fertilize via natural breeding.

Regarding testis ultrasound measurements, no differences were found in mediastinum and posterior parenchyma thickness in HEAVY and LIGHT animals. During the testicular growth, the growth rate of the mediastinum is lower than other testicular structures [25]. In our study, the age differences between the LW groups were not large enough to produce statistical differences in the mediastinum thickness between LIGHT and HEAVY animals. Regarding the results for the echogenicity of the testicular parenchyma, no differences in pixel intensity between the groups due to vaccine treatment or LW category were observed. Thus, in the conditions used in this study, testicular parenchyma echotexture analysis could not serve as an effective method for the indirect determination of the cell population in the seminiferous

epithelium or the prediction of seminal quality, which are results that are in concordance with those of other studies [26,27]. However, other authors have described an increase in testicular echogenicity in beef bulls during sexual development [28], as well as a decrease in echogenicity in immunized ram lambs [22].

In accordance with our results, a high degree of individual variation in the restoration of testicular function has been previously observed in boars vaccinated with Improvac® [9,29]. In addition, it has been shown that a proportion of animals do not respond to anti-GnRH vaccination or its effect is not clear [2,11,21,30,31]. The results for individual animals used in the present study suggest that when Improvac® is administered to calves before or after puberty, differences in the immunization effectiveness can be observed. In contrast, D'Occhio et al. [21] found a long-term suppression of reproductive function in a proportion of animals immunized before and after puberty, although a different anti-GnRH vaccine was used in their study. Surprisingly, it has been shown that immunization of boars with the cattle-specific vaccine Bopriva® appears to be more effective than immunization with Improvac® and results in a longer duration of immunity and testicular function suppression [9]. The differences in the effectiveness of vaccination against GnRH may be due to many factors, including the active component and the adjuvant used in vaccine, the number of and interval between doses, individual sensitivity to GnRH and LH and individual immune responses [2,29–32].

An interesting finding in this study was the presence of intraluminal PAS concretions in seminiferous tubules in half of the VA calves. Histologically, concretions are similar to corpora amylacea, glycol-proteinaceous structures that are commonly present in the prostate and brain in elderly persons as well as other organs, such as the lung and uterus [18,33,34]. To our knowledge, this is the first description of concretions that are similar to corpora amylacea in calves' testes.

The origin and role of corpora amylacea still remain unclear [18,35]. Corpora amylacea increase in number with age in physiological conditions, but have also been associated with neurodegenerative disorders in the brain [18] and inflammatory events in the prostate [34,36]. It has been suggested that corpora amylacea may consist of waste elements (Augé et al., 2017) and are thought to be related to epithelial cell desquamation and degeneration in the prostate [34]. In the present study, intraluminal concretions were found only in hypoplastic seminiferous tubules in VA calves. Hypoplastic testes are prone to degeneration; in such testes, when the spermatocyte formation stage is reached, the spermatocytes undergo degeneration and the lumen of the seminiferous tubules can contain cellular debris and multinucleated cells [17]. The results of the present study suggest that endocrine disturbances caused by vaccination during the rapid phase of testes growth may produce a high level of waste elements and detached cells that may result in the formation of corpora amylacea.

During testicular degeneration, small flecks or large areas of mineralization can often be observed, especially in ruminants [17]. In men, calcium deposition within the testes characterises a condition known as testicular microlithiasis [37]. Although we have not observed calcification in our study, the concretions that were observed may reflect the early stages of microlith formation that is similar to that found in prostatic calculi [35,38]. There is no consensus on the clinical significance of testicular microlithiasis and prostatic calculi; however, in humans, testicular microlithiasis has been associated with an increased occurrence of pathological conditions, including infertility and primary testicular neoplasia [37,39,40] and the presence of prostatic calculi has been associated with urological diseases, including urinary retention and prostatitis [34,35,41]. Further studies are required to study the evolution and clinical significance of concretions observed in VA calves.

5. Conclusion

Our results demonstrate that immunization of male calves against GnRH with commercially available Improvac®, which was originally developed for use in boars, severely affects testicular morphology, structure and function. Although the number of animals used in the present study is low, the anti-GnRH vaccine has demonstrated a clear effect on all the variables assessed.

The effect of Improvac® is more pronounced and consistent in calves vaccinated at a low LW than at a heavy LW, which suggests that vaccination is more effective when calves are vaccinated prior to puberty. However, testes from calves vaccinated at a low LW are more prone to the development of intraluminal concretions in the seminiferous tubules.

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