ABSTRACT

Key words: *hermaphrodite, pseudohermaphrodite, pig, ovotestis, karyotype, chromatin, citogenetics, electronmicroscopy, SEM, TEM.*

The PhD thesis entitled "***Morphology of the genital tract in intersex swines***" was developed within the Doctoral School of the "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine Iaşi, during four years of study, from October 1st, 2008 - October 1st, 2012 and is structured in accordance with current legal dispositions into two parts: part I - entitled "Literature review" comprises 52 pages and it represents 31% and the second called "Personal researches" is spread over 120 pages and represents 69%.

In Part I, divided into four chapters, there is a summarized version of the information from specialized consulted literature on the subject of the thesis, information that was used to interpret the data obtained in the second part. This part also contains seven figures selected for their suggestively information.

The first chapter presents the "***Swine genital tract morphology***" - the normal morphology of the genital tract in pigs; in morphology of male genitalia subchapter insisting on the structure of testis because at this level, the most important changes occur in intersexuality.

Chapter II - "***The development and differentiation of the genital tract***" discusses the organogenesis of genital tract from morphologically and genetically point of view, pointing out some issues that give rise to developmental abnormalities of organs involved in reproduction - an important subchapter is subchapter 2.3, in this section are presented the chronological stages in the normal development of male and female genitalia in swine.

Chapter III - "***Cytogenetics and normal karyotype in pigs***" presents general information on the structure and role of X chromatin and chromosomes, and normal karyotype in swine, established in 1988 by the *Committee for the Standardized Karyotype of the Domestic Pig* led by Gustavsson.

Chapter IV - "***Swine intersexuality"*** treats the subject of swine intersexuality from morphologically and genetically aspects, in all its forms of expression.

Part II is divided into 7 chapters (Chapter V - XI) and includes: chap. V - "***Aim and Objectives***", chap. VI - "***The morphology of the genital tract in intersex pigs*** ", chap. VII - "***Ultrasound aspects in swine intersexuality***", chap. VIII - "***Histology and histochemistry of the genital tract in swine intersexuality***" chap. IX -" ***Electronmicroscopy aspects in swine intersexuality***", chap. X - "***Cytogenetic aspects in swine intersexuality***" and chap. XI - "***The karyotype of the intersex swines***", the final conclusions ending this part.

The research activity work was carried out on cases offered by private farms and households in Iaşi and Neamţ county, Romania and slaughterhouses in Gent area, Belgium (from 1 March 2011 - 1 June 2011).

In chapter V - "***Aim and Objectives***", the choice of the theme is explained. The main aim of the research is the description of the swine intersex genital morphology in terms of anatomical-clinical, ultrasound morphology, ultrastructure and aspects of cytogenetics. This requirement for the creation of a monograph, came from the lack of complete data on a large number of cases, existing articles either focusing on one case and up to two aspects, either by a large number of cases analyzed from a single point of view-angle.

Thus, the main objectives and corresponding activities of the thesis were preset in agreement with the scientific supervisor of the thesis and were represented by:

- phenotypic description of the animals and the way they can be identified in a herd and remove from breeding;
- trying to establish a rapid and accurate diagnosis of intersexuality by using ultrasound exam;
- description of the genital reproductive system in all forms of manifestation of intersexuality;
- to emphasize certain aspects of the gonads and genital tract structures from histological and histochemical point of view by permanent histological preparations, both in paraffin sections and frozen sections;
- description of some ultrastructural aspects of gonads and some segments of genital tract aspects that were not found in the literature consulted;
- determining the genetic sex of such individuals by quick tests such as chromatin X test that can be conducted in field conditions and by performing the karyotype.

All chapters from part II, begin with a short description of the work material and methods used to conduct the research, and all photos from part II are original, made in the laboratories where the researches were conducted.

For chapter VI - "***The morphology of the genital tract in intersex pigs***" casuistic came

both from pig farms from Neamţ and Iaşi county and slaughterhouses and was represented by live pigs and genital tracts of intersex pigs.

Live pigs were raised in semi-intensive system, in the clinics of "*Reproduction*" and "*Medical Pathology*" of the Faculty of Veterinary Medicine Iaşi. Of the 15 cases of genital tracts, 7 were of true hermaphrodites type and 8 were of male pseudohermafrodites. Of the 7 cases of true hermaphrodites, 4 shown to be bilateral true hermaphrodites and 3 were altern true hermaphrodites.

As working methods it was used the inspection, in which were observed that clinical signs of the animals, and palpation, which consists in perceiving tactile surface of inguinal, scrotal and vulvar areas.

All cases studied anatomically were classified as true hermaphrodites and male pseudohermafrodites type. One subject, true hermaphrodite showed a bilateral hernia and also the vulva and vulvar orifice, visible and functional, and two bags with scrotal median line visible. On scrotum palpation, it was not noticed the presence of testes but a fluctuant mass of liquid, the same fluid being highlighted at ultrasound examination. At slaughtered, it was noticed that the genital consisted of two gonads with a slight look of swine ovaries, attached to the genital tract by two long, thin channels, which would correspond macroscopic to the uterine horns, uterus and cervix.

The following cases of bilateral true hermaphrodites were represented by the genital systems from two cases, from private farms in Belgium and the abnormalities were observed at slaughter. The last case of bilateral true hermaphrodite is represented by the gonads and some segments of genitalia from a seven months old case, collected in field conditions only for histopathology test, macroscopic appearance cannot be shown. All genitals collected from slaughterhouses consisted of gonads, specific uterine horns for sows, uterus, cervix, vagina and clitoris vaginal vestibule more or less developed.

Regarding the altern true hermaphrodites cases, they were represented only by genital tracts from slaughterhouses. They were composed of specific female genital tract and as for the gonads, it was present the ovary on one side and on the other it was the testis, with different sizes and shapes. The connection between testes and genital system was realized at the epididymis level with the fallopian tube.

The first three cases of swine male pseudohermafrodites type were represented by animals coming from swine farms within the county of Iaşi. On them were conducted anatomoclinical studies, ultrasonographic, genetic and histopathological tests. The first case was a classic male pseudohermafrodite for this type of intersexuality - gonads as testes and female

genital tract and the other two cases were atypical. One presented two small testes covered by epididimys, very developed compared to the testicular tissue. From the testes proceeded two deferent ducts which decrease in diameter, and as they approached the urinary vestibule tended to merge. In this case it was noted a rudimentary male accessory gland. The other case had a different phenotype compared with the previous cases of live animals - the scrotum on the outside, in normal position, and on palpation it was noticed the presence of testes like structures but between the two testicles, the scrotum presented on the median line the vulvar orifice, with soft and wrinkled skin, with a penis like appearance and the externalized clitoris was missing.

Next cases of male pseudohermafrodites were analyzed histologically at the Faculty of Veterinary Medicine, Ghent, Belgium and were represented by genital tracts from slaughterhouses, structured of testes as gonads and female genital tract, in some cases there also rudiments of male accessory glands.

The case of young male pseudohermaphrodite pig was obtained from a pig farm of Iaşi County. The gilt had the same phenotype as the adult cases. The genital tract was composed out of short and straight female genitals that merged into a short uterus and the gonads were represented by two oval structures, covered by connective tissue, assembling to the epididimys, not possible for a clean demarcation between it and the testis.

Chapter VII - "***Ultrasound aspects in swine intersexuality***" was made in an effort to determine the type intersexuality, to see the ultrasound aspects of the genital tracts in swine intersexuality and also their topography development in young intersex pigs.

The ultrasound was performed on three cases, using *Aloka Prosound 2* ultrasound device in "*Medical Pathology*" Clinic from Faculty of Veterinary Medicine, Iaşi.

The case that presented the abdominal hernia, on the ultrasound examination, showed the same inguinal fluid as in the testicular bags, probably as secondary lesion from the hernia. The same amount of liquid was reported instead of the testes in the scrotum, as an anechoic image. In the abdominal hernia were observed two formations with a structure similar to ovarian echographic images.

In all other cases, at the ultrasound exam, testicular parenchyma appears homogeneously, giving an echogenic image of mediastinum testis. Testes appeared surrounded by a hyperechogenic line representing the tunica albugineea. Also on ultrasound exam of the gonads it was observed the presence of the pampiniform plexus near the testicle, attached to it but also attached to the uterine horns.

For chapter VIII – "***Histology and histochemistry of the genital tract in swine intersexuality***" the investigations have been conducted on the genital tracts of 13 cases of swine

adults intersex. Research was conducted in the laboratory "*Cell Biology, histology and embryology*", Faculty of Veterinary Medicine, Iaşi and "*Department of Morphology*", Faculty of Veterinary Medicine, Gent, Belgium.

The slides were examined in the research laboratory "Victoria Zinca" of the Department of "Cell Biology, histology and embryology ", from Faculty of Veterinary Medicine, at the optical microscope *Motic B1-211A* with video *Moticam 1000* camera attached, and *Olympus BX61* microscope with *Olympus DP50* attached camera in the "Department of Morphology" from the Faculty of Veterinary Medicine, UGent, Belgium.

In the alternate true hermaphroditism and bilateral, was observed that the ovary is covered on the outside by a thin tunica albugineea, represented by delicate connective tissue and that it fulfills its exocrine function, through the presence of ovarian follicles in various stages of development, in the cortical area.

Ovarian medulla is composed out of connective tissue, rich in elastic and reticulin fibers and contains blood vessels of different sizes. Follicular cavities were observed filled with a small amount of fluid - so that follicular walls are not kept under tension, thus creating pleated or folding follicles. They appear with an irregular lumen, with internal cells layer hypertrophied and well defined space between internal and external layers.

As signs of follicular atresia the observed follicles remained in various stages of development. After ovulation, the cortical area outlines the corpus luteum with glandular appearance.

Histological, the testis of alternate true hermaphrodites and male pseudohermaphrodites is surrounded by a thick tunica albugineea, vascularized, composed of connective tissue that sends fibrous septa inside and divides the testes into lobes and lobules. The testes contains both exocrine and endocrine compartments.

Exocrine compartment is represented by the seminiferous tubules of various sizes and shapes in histological section. Regarding the structure of seminal epithelium this is composed only of Sertoli cells with oval or round nucleus. To prove this, were used two specific stains (Feulgen and Lillie `s) indicating mitotic activity of cells in division. In these stains, seminal epithelium reaction was negative, have not been seen these germinal line cells, highlighting only the Sertoli cell nucleus on the basal lamina. Due to the lack of seminal cells of seminiferous tubules, they have a three-dimensional space, where cytoplasm of Sertoli cells appears vacuolated.

The endocrine compartment was represented by the Leydig gland, between the seminiferous tubules, composed out of polygonal cells with vesicular nuclei, and sinusoid capillaries among the cells.

Referring to case VII, there were observed, both macroscopic and microscopic, portions of erratic testicular areas. These were represented by developed areas outside the tunica albugineea and were also covered by a thin capsule of connective tissue. Seminiferous tubules were situated immediately below the tunica albungineea and were structured by only Sertoli cells.

To confirm that the seminiferous tubules containing only Sertoli cells, in the "*Cell Biology, histology and embryology"* Research Laboratory from Faculty of Veterinary Medicine Iaşi, frozen sections with 10 μm thickness were performed. Lipids secreted by Sertoli cells are the most important product for nutrition of seminal line cells and in the process of spermatogenesis. Lipids were found in all three stains used (Sudan IV, Red Oil and Nile Blue).

In cases of bilateral true hermaphrodites, the testicular and the ovarian area is separated by a thick common albugineea. In all cases the testicular area was clearly more developed than the ovarian area.

In the seminiferous tubules from the testicular area of ovotestes was observed an intense vacuolation of Sertoli cell cytoplasm. Leydig cells have a pronounced polymorphism and are located as defined groups in the angular space of seminiferous tubules. In these cells, there was noticed for the first time the Barr body on the inner surface of the nuclear membrane, which provides clues on genetic sex of these individuals.

In all studied cases of intersexuality, where instead of ovaries were testes or ovotestes, the junction between the gonads and the genital tract was done by the epididymis and fallopian tube and from the tail of the epididimys started the deferent duct with a parallel path along with uterine horns. The size of the deferent duct, both macroscopic and microscopic was noted of different sizes. Its lining epithelium consisted of pseudostratified ciliated epithelium on the basal lamina.

Uterine horns, looking normally for females pigs, had a thick endometrium of which the epithelium was structured by secreting cells and fewer ciliated cells.

Cervical mucosa appeared with folds of different heights, printing into the lumen a laced appearance. Inside the lumen there were no signs of filaments of mucus or any sign of secretion due to a reduced secretory activity of the goblet cells. Cervical glands of the lamina propria were rudimentary, without secretory activity.

The clitoris was structured by two well-defined areas, a vascular one with numerous blood vessels that irrigate the clitoris and a sensitive one with and autonomic nerve endings and genital sensitive corpuscles (Meissner, Krause, Vater Paccini). Clitoral hoof surrounds and protects the clitoral gland and contains genital sensitive corpuscles and lymph nodes.

Seminal glands were observed macroscopically and histologically in only two cases in thickness of the cervical and vaginal wall. The secretory cells have a vesicular nucleous and the connective tissue between the glandular cavities is intensely vascularized dividing the gland into lobes. Cell cytoplasm is clear, sign of low secretory activity.

Of the 13 cases examined histologically, the prostate gland was seen macroscopically as a rudiment of the normal male gland in two cases (case IX and X). Histological, the gland was divided into lobules by connective tissue septa and the angular space between them appeared highly vascularized. The prostatic acini were found with large lumen and irregular, formed by simple basal cell epithelium (replacement cells) and prismatic secretory cells.

Bulbourethral glands were observed macroscopically only in case X surrounding the vaginal vestibule and being surrounded by bulbourethral muscle. The gland was divided into lobules by thin connective tissue septa. The glandular acini were in various stages of secretion and thus of different sizes. The second case where the bulbourethral gland was noticed was a male pseudohermafrodite - case VIII, and histological it was shown in thickness of vaginal wall, divided into lobes of glandular acini of various sizes that would indicate different secretions phases.

For an accurate morphological study, two cases of true hermaphroditism and male pseudohermaphroditism (VI and IX) were analyzed in electron microscopy in order to observe the ultrastructure of the genital tract and gonads, the results being presented in chapter IX- "***Electronmiscroscopy aspects in swine intersexuality***". The samples for electron microscopy investigations were processed in the laboratory of "*Pathology*" of the Faculty of Veterinary Medicine Iaşi.

Electronmicroscopy investigations were conducted with the scanning microscope *Jeol JSM 5600 - Scanning Electron Microscope* in the "Department of Morphology" of the Faculty of Veterinary Medicine, Gent, Belgium and transmission electron microscope *Jeol Jem 1010 - Electron Microscope* at the "Centre of Electron Microscopy", UBB, Cluj-Napoca and retrieved using *AnalySIS Soft Imaging Software* program.

In scanning microscopy on true hermaphrodites showed that the muscle layer is more developed in the uterine horns than in the uterus. The mucosa layers of the two structures are composed of ciliated cells and secretory cells, oviduct mucosal surface being richer in ciliated cells and secretory cells compared to the uterine mucosal surface. Some of the secretory cells of uterus were captured in different phases of secretion, so the surface of the cells showed a decapitation and elimination of the secretory vesicles.

Electronmicroscopy images through the testes showed that the Leydig gland is developed, at the expense of seminiferous tubules. Interstitial gland is composed of polygonal

cells with nuclei in different phases. In normal Leydig cells, in semithin sections, it was observed an abundant smooth (agranular) endoplasmic reticulum covering all cytoplasmic mass appearing as anastomosis between tubular and vesicular formations.

Ultrastructural, it reconfirms that seminiferous tubules in pigs intersexuality consist of a single type of cell - Sertoli cells. The cytoplasm appears vacuolated, with vacuoles of various sizes - sign of cellular degeneration. As cell organelles were observed rough endoplasmic reticulum that provides necessary circulating substances and demonstrates the trophic role of Sertoli cells. Between two Sertoli cells were observed microfilaments of actin, providing strong links between cells and usually occurs on the periphery of cells where are anchored by different proteins so that the junction between cells can exist.

To determine the genetic sex of such individuals it was made a quick test - chromatin X test - chapter X - "***Cytogenetic aspects in swine intersexuality***". Chromatin X test provides information on the number of sex chromosomes in order to determine sex genetic or numerical abnormalities. Determination of X chromatin was performed on both oral and vaginal mucosa from live animals and by making blood smears to determine the drumstick in neutrophils. Staining and interpretation of slides took place in the laboratory of "*Cell Biology, histology and embryology*", Faculty of Veterinary Medicine, Iaşi and "*Department of Morphology*", Faculty of Veterinary Medicine, Gent, Belgium. In all cases, X chromatin test, came out positive, both on oral and vaginal mucosal cells but also in leukocytes from blood smears - which suggests the existence of two X chromosomes but does not exclude the existence of a Y chromosome.

To confirm that there is no Y chromosome in addition to the normal female genetic constitution, in chapter X - "***The karyotype of the intersex swines***", the karyotype was performed - both conventional staining methods of chromosome as G banding method. This test proved that all cases of sex were females with chromosome constitution of 38, XX.