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Cytogenetic Analysis of Meiotic Cells Obtained from Stallion Testes*

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A normal course of meiosis and the associated course of spermatogenesis in males are very significant from the viewpoint of animal breeding, in particular animal reproduction. This takes on special significance when studying late-maturing animals such as horses. The aim of the study was to analyse meiotic cells, with particular consideration of synaptonemal complexes obtained from the testes of young stallions and cryptorchids, based on observations of the X-Y bivalent. The analysis was performed in successive stages of meiotic division using the FISH technique. The greatest diversity and most advanced meiotic stages were observed in the normal testis of a unilateral cryptorchid. No abnormalities were observed that could have caused cryptorchidism in the analysed horses.

Key words: Horses, meiosis, synaptonemal complexes.

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Sex chromosome aneuploidy is the most common chromosomal aberration in the horse (POWER 1990). The incidence of chromosomal aberrations is associated with a particular propensity of this species for disturbances in the meiotic process. An atypical number of sex chromosomes is associated with abnormal gonad formation, which results in disturbances and anomalous development of further reproductive structures. Disturbances in gametogenesis may inhibit the gametogenic process and make animals infertile. Meiotic chromosomes are rarely the subject of analysis because the collection of biological material for *in vivo* (ovarian follicle puncture, testicular puncture or castration) and postmortem study is highly invasive. Studies on the course of meiosis concentrate on three division stages: prophase I pachytene (chromosome pairing), metaphase I (chiasma formation and terminalization) and metaphase II (segregation of homologous chromosomes) (ŚWITOŃSKI & STRANZINGER 1998).

The course of meiosis in spermatocytes can be analysed using conventional techniques based on cell hypotonization, Carnoy fixation and Giemsa staining, as well as molecular techniques based on probe DNA hybridization with a homologous chromosome fragment. Because of accurate chromosome identification, this method is recommended for analysing different aspects of meiotic processes such as recombination, as well as for analysing defects during this process (FROENICKE *et al*. 2002; CODINA-PASCUAL *et al*. 2004; VILLAGÓMEZ & PINTON 2008). The use of the FISH technique with heterosome-specific probes makes it possible to observe the patterns and abnormalities that occur in different stages of meiotic cells during segregation of chromosomes to daughter cells.

Material and Methods

Sections of testes collected after castration or surgery (as in the case of abdominal cryptorchids) from 7 horses, including 4 colts at the age of 34-37 months (nos. 1-4) which were castrated because they failed to meet required standards and were not qualified as breeding stallions; 2 three-year-old abdominal cryptorchids: unilateral (no. 5) and

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bilateral (no. 6); and 1 stallion withdrawn from breeding at the age of 6 years because of poor reproductive parameters (no. 7).

Meiotic preparations

Testicular sections were placed onto a watch glass and ground into small pieces. The homogenate obtained was transferred to a hypotonic solution (1% sodium citrate) and shaken for 2h, collecting 1ml at 15-min intervals. The samples collected (8x per test tube) were centrifuged for 8 min (1500 rpm). The supernatant was then taken off and the pellet was fixed in a mixture of methanol and ice-cold acetic acid (3:1). This procedure was repeated six times. The suspension was dropped onto a slide, the preparations were left to dry, and stained with 5% Giemsa (20 min). The preparations were observed under an Opton Axiophot fluorescence microscope, successive stages of meiosis were counted and saved on a computer using Lucia software. Selected preparations were decolourized in 70% ethyl alcohol and stored in a freezer to subject them to fluorescence *in situ* hybridization with sex chromosome-specific probes.

Synaptonemal complex preparations

Material for preparations with synaptonemtal complexes was obtained from testicular tissue of sexually mature males. Synaptonemal complexes were observed under both electron and light microscopes. The material was basically prepared in the same way. The stages of the procedure included tissue maceration; installment of the cell suspension on a drop of 0.2N sucrose, placed on a glass slide; fixation with 4% paraformaldehyde in 0.2N sucrose for 10 min and rapid washing in detergent solution (0.4% Photo-flo, Kodak), followed by drying in a vertical position. Selected preparations were stored at -20°C until further analysis.

At the beginning of the procedure, the preparations intended for observation under an electron microscope were covered with a fast drying 20% Necoloidine solution in amyl acetate, and left to dry for about 40 min. The other stages were the same as for preparations for light microscopy. After drying and washing in 0.4% Photo-flo, the preparations were stained with 4% phosphotungstic acid (PTA) for 2 min, washed several times in 96% ethanol and redried in a vertical position. The preparations were then examined under a light microscope and regions rich in dispersed pachytene cells were determined. In the next stage, the Necoloidine film was floated off on a water surface and copper grids were applied on predetermined locations. Copper grids were retrieved from the water surface using parafilm. The preparations were analysed with a JEOL JEM 100SX transmission electron microscope (JEOL, Tokyo, Japan) at the Department of Cytology and Histology of the Institute of Zoology, Jagiellonian University in Kraków.

FISH technique

Molecular probes specific for horse X and Y sex chromosomes in hybridization mix (50% formamide, 10% dextran sulphate, 10% 20xSSC, 1% Tween 20 and 29% H_2O) were applied on chromosome preparations. Briefly, the chromosome preparations were denatured in 70% formamide in saline-sodium citrate buffer (2xSSC) for 2.5 min at 70°C. The probe was denatured at 70°C for 10 min. The hybridization was carried out in 37°C for three days. Post-hybridization washes were as follows: three times with 50% formamide in 2xSSC and three times in 2xSSC at 42°C. Hybridization signals were detected by the avidin-FITC (fluorescein isothiocyanate) and anti-avidin system on propidium iodide stained slides. Microscopic evaluation was performed under an Opton Axiophot microscope equipped with camera and Lucia software.

Staining preparations with silver nitrate

After the application of FISH, microscope observation and saving on a computer, the preparations with synaptonemal complexes were washed in equilibration buffer to rinse out the DAPI stain and 50% $AgNO_3$ solution was applied. The preparations were covered with a cover glass, sealed with Fixo Gum and placed in a moist chamber overnight at 60°C. Cover glasses were then removed and preparations stained for 10 sec with 5% Giemsa. The preparations were analysed using Lucia software under an Opton Axiophot microscope to find previously saved cells with synaptonemal complexes (following FISH) and saved again after staining with silver nitrate.

Results

Meiotic preparations

Meiotic preparations, made from testicular tissue of 7 stallions, were analysed under a light microscope to determine the successive stages of meiotic division (Fig. 1). Meiotic cells were counted and archived using Lucia software. The results obtained are given in the form of graphs

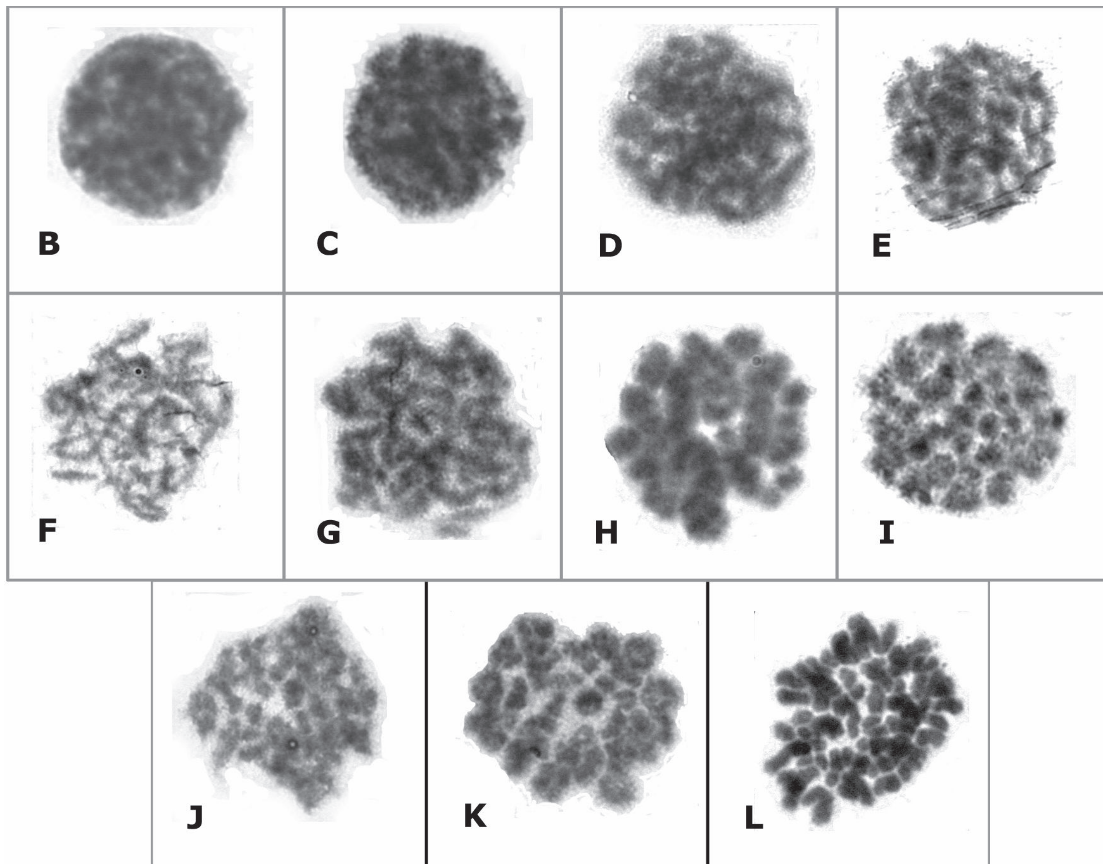

Fig. 1. Different meiotic stages: B,C – leptotene; D,E – early zygotene; F – zygotene; G – pachytene; H,I – diplotene; J – early diakinesis; K – diakinesis; L – late diakinesis.

showing intra- and interspecific differences between the stallions (Fig. 2).

Stallion no. 1 was observed to have 20% of pachytene stage cells in the right testis. No further meiotic stages were found in either left or right testis.

Stallion no. 2 showed further meiotic stages compared to animal no. 1. In the left testis, 0.14% of reproductive cells were classified as early diakinetic. No further stages were observed.

In stallion no. 3, 0.4% of reproductive cells from the right testis were in the diplotene stage and 0.6% of cells from the left testis were classified as early diakinetic. As in the horses described above, no further stages of meiosis were observed.

Stallion no. 4 was characterized by a greater variety of meiotic stages. Although about 80% were leptotene cells, there were some pachytene and diplotene cells and even a small number of diakinesis cells.

The largest diversity of meiotic stages was characteristic of the right testis of the unilateral cryptorchid (no. 5). The above figure shows the diversity of meiotic stages, from the most numerous leptotene to late diakinesis. No spermatocytes were observed in the left testis of this horse, just as in the testes of animal no. 6 (bilateral cryptorchid). In both testes of the oldest horse analysed (no. 7), only a few reproductive cells in early meiotic stages (leptotene and early zygotene) were found.

Fluorescence *in situ* hybridization with probes specific to X and Y sex chromosomes was used to observe heterosomes in meiotic cells, which made it possible to assess the normality of these chromosomes in early meiotic stages (Fig. 3). In the analysed cells, no heterosome aneuploidies were observed.

A sex bivalent was observed in the right bottom corner of Fig. 4, which was obtained using material from stallion no 7 (the entire bivalent is not visible because it was located on the end of the preparation). It is darker than the other synaptonemal complexes. Sex chromosomes are joined together at their ends and, typically, form a loop between non-joined arms.

Sex bivalents in synaptonemal complexes obtained from testicular tissue of stallions were also analysed after fluorescence *in situ* hybridization with X and Y chromosome-specific probes and after staining with silver nitrate (Fig. 5).

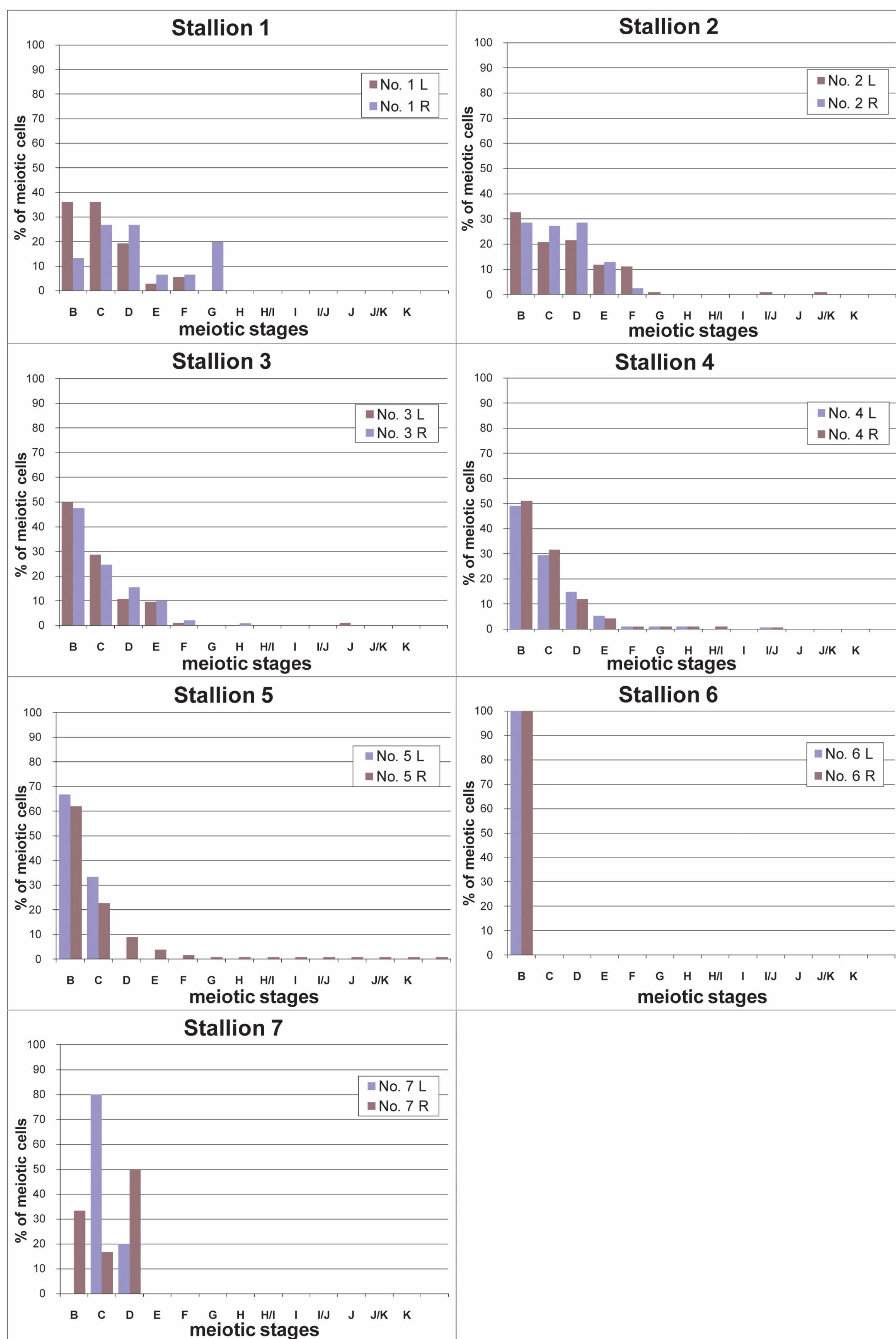

Fig. 2. Number of different meiotic stages as divided into right (R) and left (L) testis of the analysed stallions. Graphs show intra- and interspecific differences between the stallions.

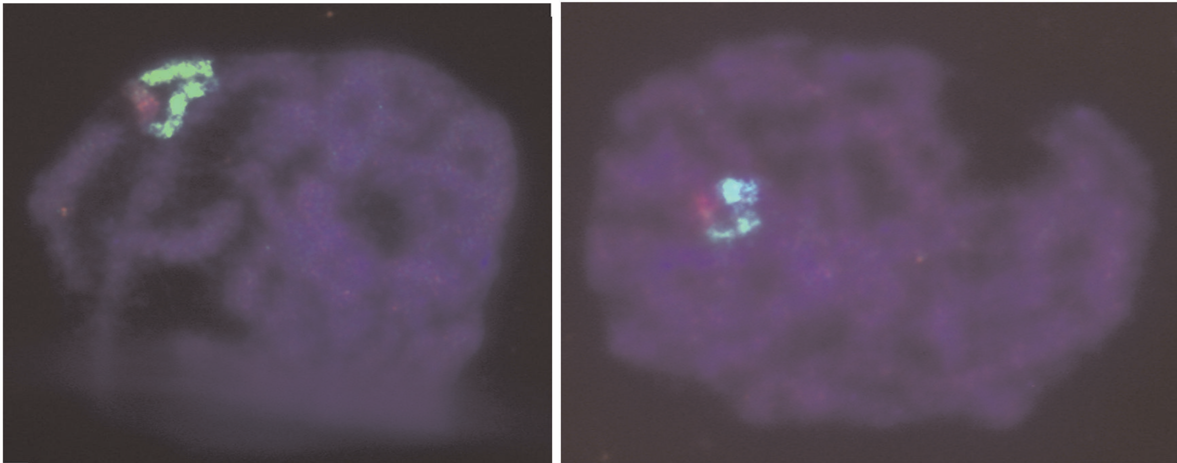

Fig. 3. Fluorescence *in situ* hybridization with meiotic preparations (meiotic cells at the zygotene or pachytene stage) obtained from the testes of castrated stallions; red – signal of Y chromosome painting probe, green – signal of X chromosome painting probe.

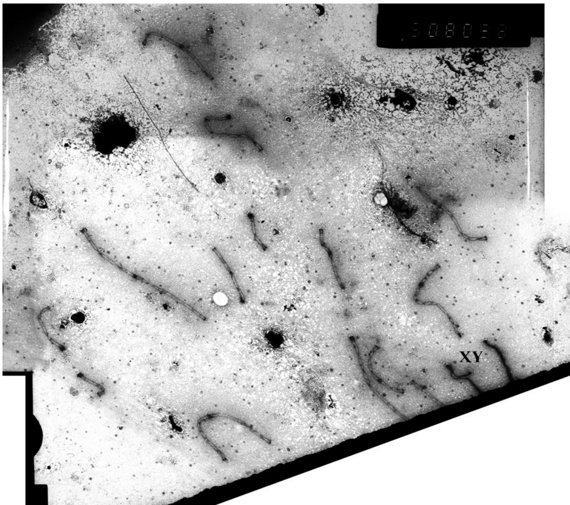

Fig. 4. Synaptonemal complexes from the material obtained from stallion no 7; due to the large size of the photograph, only the part with the visible bivalent is shown (electron microscope photograph taken at the Department of Cytology and Histology of the Jagiellonian University).

The X chromosome was indirectly stained with biotin to give a green colour, and the Y chromosome was directly stained with cyanin (Cy3) to give a red colour on the figures. No early dissociation of the sex bivalent was observed among the analysed cells and all bivalents were paired.

Discussion

In the present study, we observed meiotic stages in cells obtained from testicular tissue of stallions. It should be noted that in adult stallions, testicular parenchyma accounts for almost 90% of total weight, of which only 70% is formed by seminiferous tubules, in which spermatozoa are produced (AMANN 1981; JOHNSON & THOMPSON 1983; KOSINIAK & BITTMAR 1987). Because of the large size of equine testis, we examined randomly chosen sections of testes, in which the number of

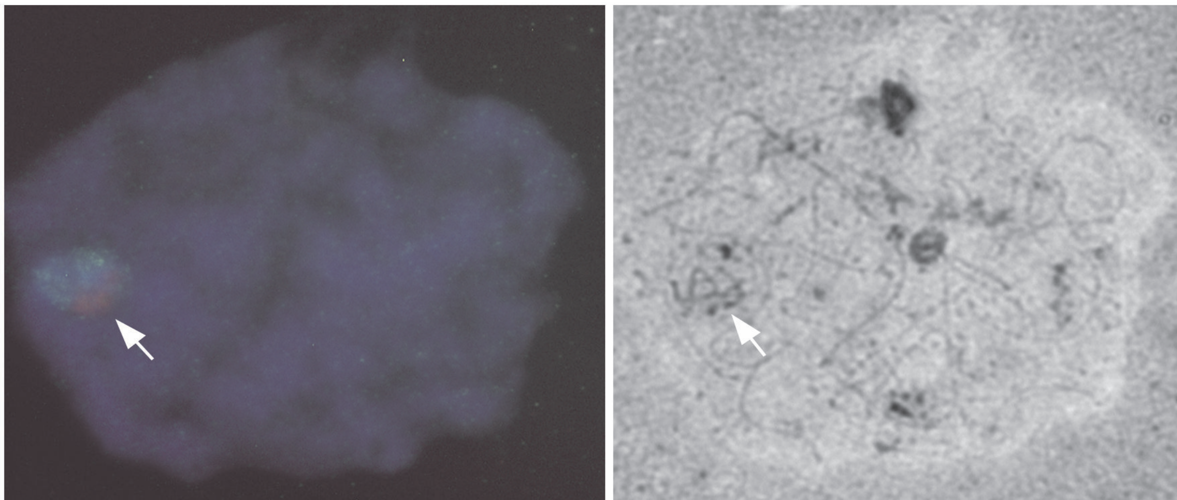

Fig. 5. Synaptonemal complexes (the same cell); the arrows shown the sex chromosomes bivalent: A) FISH technique (green – X chromosome, red – Y chromosome), B) $AgNO_3$ staining.

seminiferous tubules could be severely limited or nonexistent. In addition, more reliable results would require examining a larger group of stallions, including those that had been classified as having high fertility rates. It is pertinent to note that all the horses analysed in this study were castrated because they were not qualified as breeding stock during the revision process.

The analysis of testicular preparations from horse no. 6 (bilateral cryptorchid) revealed no reproductive cells (primary spermatocytes). Appropriate conditions have to be satisfied in the testes to ensure efficient spermatogenesis. A major factor in this process is temperature, which should be 3-4C lower in the scrotum than inside the male body. These conditions cannot be met in cryptorchids, because rather descending into the scrotum, their testes are retained in the abdominal cavity in which the temperature is higher, thus inhibiting the spermatogenesis process. The testes of cryptorchids are of smaller size and results of histological analysis demonstrated that the diameter of seminiferous tubules is up to 60% smaller compared to that in testes located in the scrotum (http://www.acvs.org/AnimalOwners/Health-Conditions/LargeAnimalEquineTopics/UndescendedTesticlesinHorses/).

During castration, stallion no. 7 was aged 80 months and was the oldest horse from which material was tested. Stallions typically reach sexual maturity between 21 and 23 months of age. With age, the number of sperm produced increases by about 30%. In two-year-old stallions, total sperm count per ejaculate is about 3.3 billion. In stallions aged between 2.5 and 3 years, daily sperm production per gram of testicular tissue is almost the same as in adult males. Sexual function peaks between 4 and 5 years of age. In most stallions aged between 4 and 5 years, daily sperm production is stable and remains the same until 20 years old (PICKETT *et al.* 1989; JOHNSON *et al.* 1991). Considering also that from among the analysed animals stallion no. 7 was the only horse approved for mating and producing progeny, later meiotic stages were expected in its histological material. However, analysis of all the preparations revealed in both testes only a few reproductive cells in the early meiotic stages of leptotene and early zygotene. There is no conclusive reason for atrophy of spermatocytes in the testes of stallion no. 7 and the resulting complete loss of breeding ability, although fungal infection or physical injury are the likely cause.

Horses no. 1-4 were of similar age (about 3 years old) when castrated. These colts had the greatest number of early meiotic reproductive cells of all animals analysed.

The largest diversity of meiotic stages was observed in the left-sided cryptorchid no. 5. The right testis was observed to contain both leptotene and late diakinesis cells. In the left testis, which had been located in the abdominal cavity before surgery, no meiotic cells were found. The testis of a unilateral cryptorchid, present in the scrotum, produces spermatozoa. The fertility of such a horse is most often lower but high enough for conception, with the amount of testosterone being comparable with that in other stallions (HOAGLAND *et al.* 1986).

Observations of the X-Y bivalent during prophase I of meiotic division (pachytene substage) involve the analysis of chromosome pairing, with synaptonemal complexes being the test object. Specific morphology of the X-Y bivalent at different stages of pairing makes it possible to distinguish several substages of prophase I: late zygotene – when autosomes are separate and X and Y chromosomes begin to pair in terminal segments; early pachytene – when autosomal chromosomes begin to pair and X and Y chromosomes continue to pair; mid-pachytene – when all chromosomes (both autosomes and heterosomes) are tightly paired; and late pachytene – when autosomes continue to pair and the X-Y bivalent begins to dissociate (VILLAGOMEZ 1993). Detailed analysis of the structure of synaptonemal complexes in farm animals, performed using electron microscopy, showed that the specific course of the X-Y bivalent pairing depends on homology between the heterosomes (ŚWITOŃSKI & STRANZINGER 1998).

Light microscopy observations of chromosomes at prophase I pachytene of meiotic division in boars with centromeric heterochromatin polymorphism (heterozygous forms) did not show any abnormalities resulting from different lengths of polymorphic homologues (SŁOTA 1998). Likewise, electron microscopy (JEOL-JEM 100C) analysis of primary spermatocytes from a boar with centromeric heterochromatin polymorphism in pair 18 revealed no differences in the length of lateral elements from the bivalent of this pair, although early dissociation of the X-Y bivalent was noticed (SŁOTA 1998). Similarly, a low level of early dissociation of the bivalent (at mid-pachytene) was found in bulls carrying 60,XX/60,XY chimerism (REJDUCH *et al.* 2000). In the present experiment, the specific morphology of the X-Y bivalent at different stages of pairing, stained with silver nitrate or identified based on hybridization signals, is evidence that no early dissociation of the sex bivalent took place.

It was found that in case of the cryptorchid, the process of spermatogenesis is disturbed whereas the testicles located in the scrotum work properly.

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