Identification of the nucleoli in domestic horse spermatocytes – preliminary investigations

Katarzyna Andraszek, Agata Danielewicz, Elżbieta Smalec, Marian Kaproń

University of Life Sciences and Humanities in Siedlce,
Institute of Bioengineering and Animal Breeding,
Department of Animal Genetics and Horse Breeding,
14 B. Prusa St, 08-110 Siedlce

The aim of the research was to determine the number and shape of the nucleoli during meiosis in cells of the domestic horse. The experiments were conducted on stallions divided into three age groups (2, 3 and 7 years). In the cells of two-year-old animals, predominantly nucleoli with regular shapes were observed, with no fragmented nucleoli detected. The cells of three-year-old horses had an equal share of regular and irregular nucleoli. Seven-year-old horses had an insignificant percentage of regular nucleoli in their cells, whereas fragmented nucleoli were found to be present in them.

KEY WORDS: domestic horse (*Equus caballus)* / meiosis / meiotic chromosomes / spermatocytes / nucleus

Nucleoli are the product of NOR (Nucleolar Organizer Region) activity of certain chromosomes. Their fundamental role is to form ribosomal subunits out of ribosomal protein molecules. The transcription of rRNA-coding genes also takes place in nucleoli. Newly formed rRNA molecules are bound with ribosomal proteins to produce ribosomal subunits [11, 21, 27, 28].

The size of mammalian nucleoli ranges from 5 to 10 μm and varies according to the demand of the cell for ribosomes. Cells that do not intensively synthesize proteins, e.g. skeletal muscle cells, have small nucleoli. Liver cells, which are engaged in more intensive protein synthesis, have more sizeable nucleoli [5, 27]. Fast-growing cells usually contain bigger and more numerous nucleoli [14, 22].

The number of nucleoli in the cells of vertebrates ranges from one to a dozen or so (depending on the cycle duration, genome size or transcription activity). In the cells that are characterized by a particularly long cycle most nucleoli become fused [16, 17]. The cells of species with a small genome normally contain one centrally located nucleolus [6, 13], whereas cells with a high transcription activity may have as many as a thousand nucleoli. This is connected with the amplification of rRNA-coding genes [6, 21, 22].

Nucleoli are structures with various shapes. The shape of nucleoli also reflects their activity. Nucleoli in young cells are smooth and the aging process is associated with a change of their shape – the nucleoli start to assume irregular shapes [13, 28]. The predominantly round shape of nucleoli can turn into lentoid in the zygotene of the first meiotic division. This is accompanied by the displacement from the central to the acrocentric position. Nucleolar shapes also change according to the physiological or pathological state of the organism [6, 11, 17].

The analysis of the structure of nucleoli (NOR products) can provide alternative sources of information on the activity of rRNA-coding genes. Moreover, nucleoli are only degraded at the end of the prophase. They can be easily identified using standard cytogenetic techniques. Nucleolar structure analysis can be conducted with a standard light microscope.

The purpose of the study was to determine the number and shape of nucleoli during spermatocyte meiosis in male domestic horses.

Material and methods

The material was obtained from ten male domestic horses representing the Polish Cold-blooded Horse. The animals had been used on private farms in eastern Masovian Voivodeship. The horses represented three age groups (two-, three- and seven-year-old animals). The nucleoli associated with meiotic chromosomes of first-order spermatocytes were the object of the study.

The meiotic chromosomes were isolated from the testes immediately after castration using the method described by Evans et al. in 1964 [9]. Before isolation, the testes were rinsed in 0.88% sodium citrate. Next, the testicular stroma of connective tissue was removed and fragments of the testicular tissue were sampled (ca. 0.5-1 cm^3). The samples were macerated in a small volume of 0.88% sodium citrate, supplemented with fresh 0.88% citrate up to 10-20 ml, and left for 30 minutes at room temperature. After that time, the liquid was transferred from above the sediment into tubes for 10-minute centrifugation at 1000 rpm. Subsequently, the supernatant was decanted and the sediment in the tubes was supplemented with fresh sodium citrate up to 5 ml. The tubes were then left for 10 minutes at room temperature. The resultant suspension was centrifuged again for 10 minutes at 1000 rpm. The supernatant was decanted and the tubes were replenished with fresh Carnoy fixer up to 7 ml. The fixing procedure was repeated threefold. The fixed cells were suspended in a small amount of fresh Carnoy fixer, spread over degreased slides and dried at room temperature. The preparations for analysis and the remaining cell suspension were stored at 4°C.

The preparations were stained according to the Howell & Black method of 1980, used for staining nucleolar organizer regions of mitotic chromosomes [12]. A 50% $AgNO_3$ solution and a colloidal gelatin solution were applied on the 1-week-old preparations. The preparations were covered with a cover glass and incubated for 15-20 minutes in a thermostat at 60°C, in complete humidity. After the preparations turned brown in colour, the chemical reaction was interrupted and the preparation rinsed several times with distilled water.

The ready preparations were observed using an OLYMPUS BX 50 microscope. The microscopic images were stored in the PC memory using the Multiscan software that made it possible to download the images from the microscope through the Olympus DP25 digi-

tal camcorder connected to the computer. The downloaded images were analysed using graphic display and statistical programs integrated with the Multiscan system.

The preparations were initially examined under the microscope to confirm correct staining. Patchily stained preparations were dyed in a Giemsa solution for 10 seconds. The preparations were rinsed again in distilled water and dried at room temperature.

For each animal, 50 slides with chromosomes in the first meiotic prophase were analysed. The microscopic analysis consisted in determining the number of the nucleoli in a cell and their shapes for all the animals in question. The shapes of the nucleoli were identified as regular, irregular and fragmented. The analysis of the nucleolar numbers and shapes was based on 500 slides with meiotic chromosomes. The results were presented in tables and diagrams.

Results and discussion

The 1964 method of Evans et al. [9] is a standard procedure for obtaining meiotic cells and isolating meiotic chromosomes. Combined with the Howell and Black technique of 1980 [12], the method is also successfully used for the isolation, observation and identification of spermatocyte nucleoli, throughout the first meiotic prophase [1, 2, 3].

In our experiment, silver nitrate staining made it possible to identify nucleoli associated with meiotic chromosomes of first-order spermatocytes in the domestic horse. Regardless of the quality of the preparation, the nucleoli were always distinguishable as distinct structures in: poorly pigmented (Photo. 1a) well-dyed (Photo. 1b) or additionally stained preparations (Giemsa) (Photo. 1c & 1d). In the poorly developed preparations the nucleoli were observed as spots of intensive hue on the background of very light chromatin or

Table – Tabela

The number and shape of nucleoli relative to the age of the horse
Liczba i kształt jąderek w zależności od wieku konia

Age of horse (years) Wiek konia (lata)	No. of nucleoli with a particular shape Liczba jąderek o określonym kształcie		
	regular regularne	irregular nieregularne	fragmented zdefragmentowane
2	32	18	–
2	30	20	–
3	26	24	–
3	27	23	–
3	24	26	–
3	23	27	–
3	25	25	–
7	1	39	10
7	1	29	20
7	1	33	16
Total Łącznie	190	264	46

chromosomes (Photo. 1a). In the well-dyed preparations the nucleoli were visible as dark brown stains on the background of lighter chromatin or chromosomes (Photo. 1b). In the case of re-stained preparations, Giemsa dye facilitated the identification of the nucleoli as brown structures on a blue background of chromatin or chromosomes (Photo. 1c). On the other hand, in the case of the properly silver-dyed preparations, the use of Giemsa dye made it possible to identify the nucleoli as dark, navy blue or purple structures on a lighter background of chromatin or chromosomes (Photo. 1d). Regardless of the intensity of the stain, the nucleoli were unequivocally identified.

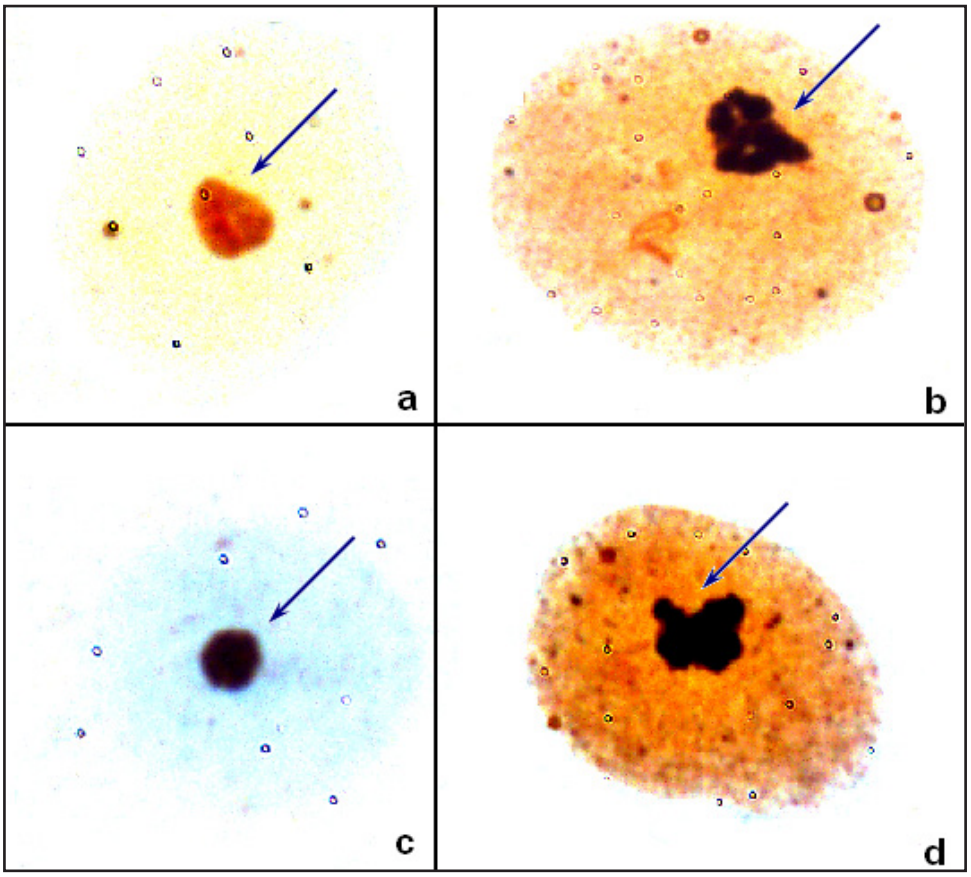

Photo. 1. Nucleoli in domestic horse cells. $AgNO_3$-stained preparation – low staining intensity (a). $AgNO_3$-stained preparation – adequate staining intensity (b). $AgNO_3$-stained preparation + Giemsa – low silver staining intensity (c). $AgNO_3$-stained preparation + Giemsa – adequate silver staining intensity. The nucleoli are marked with arrows.
Fot. 1. Jąderka w komórkach konia domowego. Preparat barwiony $AgNO_3$ – niska intensywność barwienia (a). Preparat barwiony $AgNO_3$ – odpowiednia intensywność barwienia (b). Preparat barwiony $AgNO_3$ + Giemsa – niska intensywność barwienia srebrem (c). Preparat barwiony $AgNO_3$ + Giemsa – odpowiednia intensywność barwienia srebrem. Jąderka zaznaczono strzałką.

One nucleolus was always observed in all analysed preparations. What varied were the shapes of the nucleoli in the cells of particular animals relative to their age. The nucleoli were classified in three groups: regular-shaped – round (Photo. 2a, b), irregular-shaped – oval/ovaloid (Photo. 2c, d) and fragmented (Photo. 2e, f).

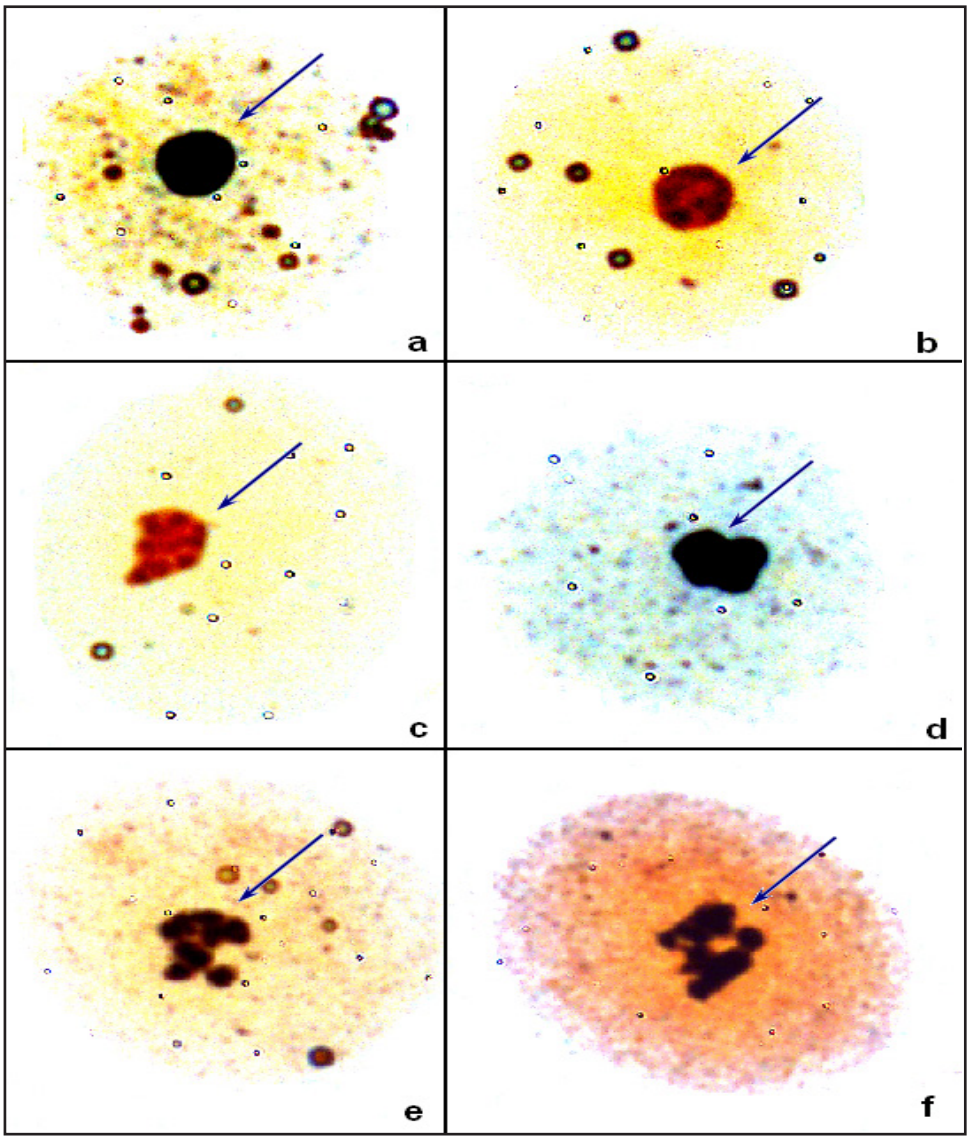

Photo. 2. Shape diversity of nucleoli in domestic horse cells. Regular-shaped nucleoli (a, b). Irregular-shaped nucleoli (c, d). Fragmented nucleoli (e, f). The nucleoli are marked with arrows.
Fot. 2. Zróżnicowany kształt jąderek w komórkach konia domowego. Jąderka o regularnym kształcie (a, b). Jąderka o nieregularnym kształcie (c, d). Jąderka zdefragmentowane (e, f). Jąderka zaznaczono strzałką.

A detailed comparison of the numbers and shapes of nucleoli in particular animals and in the total population under analysis is presented in Table.

It was observed that regular-shaped nucleoli predominated in the cells of the young, two-year-old animals. For the total of 100 nucleoli analysed in the two-year-old horses, 62% were regular-shaped nucleoli, whereas the share of the irregular, second-group nucleoli was 38%. No fragmented nucleoli were observed in the two-year-old horses. As to the cells of the three-year-old horses, we found that the share of regular- and irregular-shaped nucleoli was equal, 50% each. The same as in the case of the two-year-old horses, fragmented nucleoli were not observed in the three-year-old animals. The greatest diversity of shapes was detected in the cells of the seven-year-old horses. Regular-shaped nucleoli constituted a negligible percentage, barely 2% out of the 150 nucleoli analysed. The most numerous were the irregular-shaped nucleoli – 67%. Moreover, in the seven-year-old horses, we also found fragmented nucleoli, which constituted 31% of the total. The percentage share of variously shaped nucleoli for all the age groups is presented in diagram.

The current international standard of the horse karyotype was developed in 1989 in Jouy-Josas, France. This is the only normalised karyotype for *Equus caballus* since the standardization programs for domestic animal karyotypes were established at the First International Conference for the Standardisation of Banded Karyotypes of Domestic Ani-

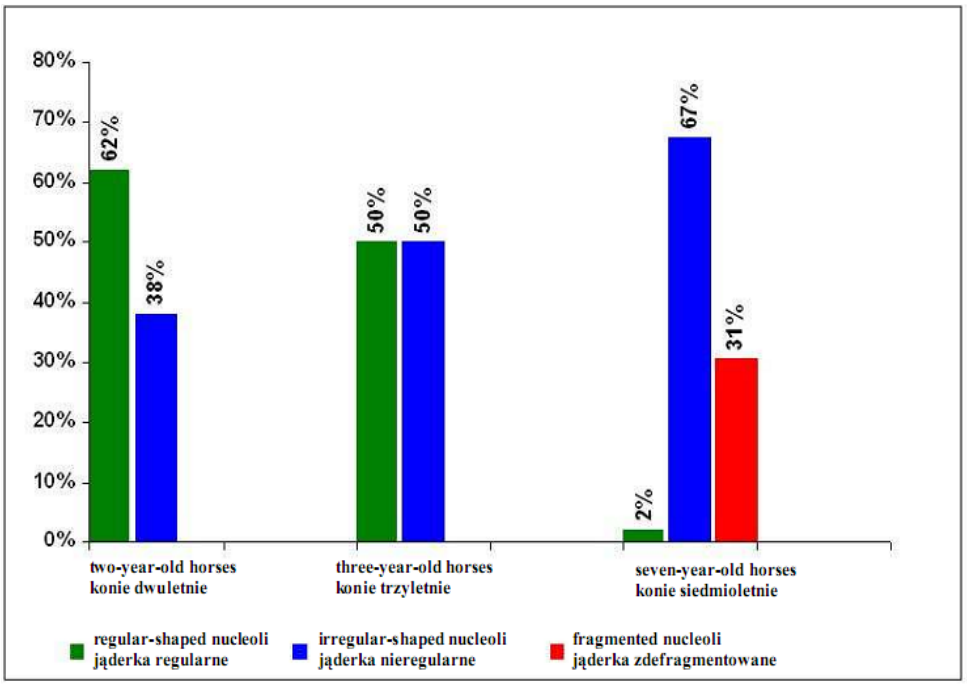

Fig. The proportions of variously shaped nucleoli in domestic horse cells
Rys. Udział jąderek o różnym kształcie w komórkach konia domowego

mals in 1980 in Reading, England [23]. The diploid number of domestic horse (*Equus caballus*) chromosomes is 2n=64. Among the 32 chromosome pairs, 31 are autosomes (13 metacentric and submetacentric pairs, as well as 18 acrocentric pairs). The 32nd pair are sex chromosomes. Chromosome X is one of the largest chromosomes in the karyotype, while chromosome Y is one of the smallest. Nucleolar organizer regions are present in the chromosomes of the 1st, 28th and 31st pair [4, 15, 23].

Nucleoli are found in the nuclei of almost all eukaryotic cells as they contain the genes of primary metabolism. Spermatozoa and mature avian erythrocytes are the exception [18, 22]. The morphology of nucleoli is variable and can undergo sudden changes. An example is provided by non-cycling lymphocytes with negligible ribosomal gene transcription that, following phytohaemagglutinin stimulation, resume transcription. This results in sharp changes in the morphology of the nucleolus [5, 16, 27].

Nucleoli are highly polymorphic and their activity varies depending on the species, cell type, the degree of cell differentiation and the cell cycle phase [6, 13]. Already in late 19th century the close relationship between the size of the nucleolus and cell activity was identified. It was found that the cells that demonstrated high metabolic activity – oocytes, neurons and secretory cells – contained large nucleoli. On the other hand, the cells with less intensive protein synthesis (spermatozoa, blastomeres and myocytes) were found to contain small or no nucleoli [22].

An example of the transformation of nucleolar types accompanied with a change of the nucleolar area is the differentiation of small intestine epithelial cells that belong to the so-called "self-renewing" cell population. In this model of differentiation, the morphology of the nucleoli undergoes dramatic transformations. In stem cells, the nucleoli are small and numerous, whereas in proliferating cells they are large and few. In the final stage of differentiation the nucleoli are fused into a single structure [17, 22].

A nucleolus consists of the primary products of ribosomal RNA genes present in a nucleolar organizer, the related proteins and various enzymes, such as RNA polymerase, RNA methylase and RNA endonuclease [19, 21, 22]. The comparison of the results for the numbers and sizes of meiotic nucleoli with the results for the number of NORs in mitotic chromosomes demonstrated differences between the number of NORs and nucleoli. The reason for the discrepancy may be the fact that NORs of different chromosomes can participate in the creation of one nucleolus [20, 21, 22, 24, 30].

The smaller number of nucleoli in relation to active NORs also results from the fusion of the nucleoli in the interphase nucleus and suppression of the activity of certain rDNA loci [6, 13]. During amphibian organogenesis, the formation of multiple nucleoli is a strategy that makes it possible to increase the number of copies of rDNA genes [21, 22, 29].

In addition, nucleolar morphology is also affected by epigenetic mechanisms of DNA methylation. The rRNA-coding genes occur in two forms. Their "open" conformation is transcriptionally active, whereas the "closed" conformation is associated with the so-called transcription crisis/interruption. Entire NORs or only certain nucleolar organizer genes can be suppressed [10].

The fundamental function of nucleoli is to control ribosomal biogenesis. In recent years, a relationship has been found between nucleolar morphology and cellular aging processes. Research on yeast cells confirmed that damage to rRNA genes causes disintegration of

nucleoli, which is a symptom of aging. This theory was corroborated by investigations of the Werner syndrome – a hereditary disease causing premature aging. In addition, nucleoli play an essential role in carcinogenic mechanisms, telomerase activity regulation and stabilization of the p53 protein [7, 8, 11, 25, 26].

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Katarzyna Andraszek, Agata Danielewicz, Elżbieta Smalec, Marian Kaproń

Identyfikacja jąderek w spermatocytach konia domowego – badania wstępne

S t r e s z c z e n i e

Celem badań było określenie liczby i kształtu jąderek podczas mejozy, w komórkach konia domowego. Badania przeprowadzono na ogierach w trzech grupach wiekowych (2, 3 i 7 lat). W komórkach osobników dwuletnich stwierdzono przede wszystkim jąderka o kształcie regularnym i brak jąderek zdefragmentowanych. W komórkach koni trzyletnich stwierdzono równy udział jąderek regularnych i nieregularnych. Konie siedmioletnie posiadały w komórkach niewielki odsetek jąderek regularnych, stwierdzono natomiast obecność jąderek o zdefragmentowanej strukturze.

SŁOWA KLUCZOWE: koń domowy *(Equus caballus)* / mejoza / chromosomy mejotyczne / spermatocyty / jąderko