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INVESTIGATION OF SOME NUCLEOLI TRAITS IN INTERPHASE LEUKOCYTES OF TWO RABBIT BREEDS AND THEIR HYBRID

*** V. Dsitsiuk [1], O. V. Boiko [2], O. F. Honchar [2], O. M. Havrysh [2], O. Guzevatiy [3]**

[1] M. V. Zubets Institute of Animal Breeding and Genetics the National Academy of Agrarian Sciences of Ukraine, 1, Pohrebniaka Str., Chubynske village, Boryspil District, Kyiv Region, Ukraine, 08321
[2] Cherkasy Experimental Station of Bioresources the National Academy of Agrarian Sciences of Ukraine, 76, Pasterivska Str., Cherkasy, Ukraine, 18036
[3] National Academy of Agrarian Sciences of Ukraine 9, Mykhaila Omelyanovycha-Pavlenka Str., Kyiv, Ukraine, 01010

*E-mail: * valentynadzitsiuk@gmail.com, aleksboy18@meta.ua, of.gonchar@gmail.com, gavrish.olexandr@gmail.com, oleg_guzevatiy@ukr.net*

ORCID: https://orcid.org/0000-0001-9697-4165, https://orcid.org/0000-0002-3917-5583, https://orcid.org/0000-0003-2269-9767, https://orcid.org/0000-0002-8632-6508, https://orcid.org/0000-0002-2470-5430

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Aim. The aim was to study the differences in three traits of nucleoli in interphase leukocytes of two rabbit breeds and a hybrid thereof. **Methods.** Doe rabbits, 4-month -old, from Ukrainian breed Poltavske Sriblo (n = 30), Californian (n = 25), and their hybrid (n = 21) were used in the experiment. The nucleoli in intact blood leukocytes were stained using silver staining according to Howell and Black (1980). Stained cells were observed, and their nucleoli counted in light microscopy at 10×100 oil immersion magnification. 200 leukocytes from each animal were analyzed. The activity of nucleoli was evaluated by the following parameters: the average number of nucleoli in the nucleus (nNO), the total area of nucleoli in the nucleus (ΣS_{NO}, mcm^2), the share of nucleolus area in the lymphocyte nucleus area ($sh\Sigma S_{NO}$, %). Statistical analysis was conducted using the STATISTICA software package (2020). **Results.** The average number of nucleoli per cell varied from – 1.70 ± 0.08 in Californian rabbits to 5.90 ± 0.29 in hybrid animals. A statistically significant difference ($p < 0.05$) was found between the experimental groups of purebred and hybrid rabbits. The variation coefficient for the index of the average number of nucleoli per cell was on the average level of variability: 20.58 % for the rabbits of Poltavske sriblo breed, 19.50 % for Californian rabbits, and 16.49 % for hybrid ones. The total area of nucleoli in the cells of all the investigated animals varied from 5 μm^2 in one Californian rabbit to 12 μm^2 in animals of hybrid origin. The share of the nucleolus area in the nucleus area for rabbits of Poltavske sriblo, Californian, and hybrid breeds was 26.10 ± 1.80 %, 24.30 ± 1.62 and 29.40 ± 2.50, respectively. **Conclusions.** Polymorphism was observed for three nucleolar parameters after silver staining of interphase leukocytes of rabbits of Poltavske sriblo, Californian breed and their hybrid. This concerned 1) the average number of nucleoli ($p < 0.05$); 2) the total area of nucleoli ($p < 0.05$); 3) the average share of the nucleolar area in the nucleus area ($p < 0.05$). The results of our comparative analysis of the investigated nucleolar activity parameters suggest a higher activity of nucleoli in the animals of hybrid origin. In the future, the results of such studies may be used to assess the potential ability of animals to implement productive traits.

Key words: nucleolus, lymphocyte nucleus, Ag-banding, purebred rabbits, and rabbits of hybrid origin.

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INTRODUCTION

Domestic rabbits (*Oryctolagus cuniculus* f. *domesticus)* are considered a valuable farm species due to their high-quality dietic meat, high meat performance and early maturity. The Food and Agriculture Organization (FAO) distinguishes domestic rabbits as one of the "golden five" mammal species which form the basis for the agrarian civilization (FAO, 2015). There are more than 300 breeds and species groups in 70 countries where rabbits are reared, which differ considerably in terms of their productivity directions and breeding conditions (FAO, UN, Domestic Animal Divessity Information System) http://www.fao.org/dad-is/dataexport).

Similar to the global tendency, rabbit breeding in Ukraine is increasing, and it is expected that the demand of rabbit meat will increase and that the production will rise by 2 % (up to 2 million tons by 2025) (https://meat-inform.com/novyny-pro-miaso/u-sviti-zrostaie-popyt-na-kroliatynu.html).

To achieve these goals, it is necessary to introduce modern methods of managing the genetic resources of rabbits based on novel approaches to investigating their genetic structure (Fontanesi, 2021). This involves breeding using molecular marker assisted selection (MAS) involving microsatellites and single nucleotide polymorphisms, whole genome sequencing, quantitative trait locus analysis (QTL analysis), among others (Viryanski et al, 2021).

The cytogenetic study of structures of the cell nucleus, including the functional state of its nucleoli-containing loci, plays a relevant role in comprehending the processes of DNA and RNA synthesis (Yahaya et al,2019; Hirai, 2020).

The nucleoli are structural elements of the cell nucleus and function as ribosome factories. A nucleolus is a dynamic organelle, the structure of which reflects the processes related to the biogenesis of ribosomes. Its sizes and components change depending on the activity of the cell and the stage of the mitotic cycle (Jaecker et al, 2022; Cmarko et al, 2008).

The nucleolus is well discernable in the cell nucleus in the interphase stage. The entire material of the nucleolus is the derivative of nucleolar organizers, so-called NORs (Bersaglieri and Santoro, 2019). The method of *in situ* hybridization was used to demonstrate that the nucleolar organizer is an area of the chromosome containing clusters of ribosomal genes for 18S, 5,8S, and 28S rRNA. The clusters of rDNA occupy some regions in some karyotype chromosomes, which were called "nucleoli-organizing chromosomes" (NO-chromosomes) (McStay, 2016; Cockrell, 2022; Hori et al, 2023). The NORs are associated with acid non-histone proteins (C23, B23, UBF, and RNA-polymerase), which are argentophil and can be stained with silver nitrate ($AgNO_3$) (Pich et al, 1995; Ahmad et al, 2019).

The active state of the nucleolar apparatus in the intact leukocyte cells, including lymphocytes, has also been studied (Iolchiev et al, 2021).

These cells have a nucleus, and they are some of the most complicated and functionally active ones in the blood of mammals. They take signals about any changes in homeostasis, adjusting their activity to maintain biological balance (Moro-García et al, 2018). These cells are at the basis of the immune system response, making leukocytes the indicators of the state of the organism as a whole (Bellingan, 2000). It was shown that the size of the nucleolus reflected the activity of rRNA genes, for instance, it correlated with the activity of cell division (Mosgoeller 2004) and increased synthesis of ribosomes (Nakamoto et al, 2001).

Similar to other species of animals, the nucleolar organizers of the domestic rabbit (*Oryctolagus cuniculus domesticus)* were studied in metaphase chromosomes (Martin-DeLeon, 1980; Monteagudo, Arruga, 1991), to our knowledge only one study was performed on interphase nucleoli staining in rabbits (Ivanov et al, 2009).

Taking this into consideration, in this study, we aimed to investigate the differences in three traits of nucleoli in intact leukocytes in two purebred and one hybrid rabbit reared in Ukraine, viz: the average number of nucleoli in the nucleus of all samples per breed or hybrid, their total area in the nucleus, and the share of the nucleolar area in the leukocyte nucleus.

MATERIALS AND METHODS

The study was conducted at the Department of Genetics and Biotechnology of Animals of the M.V. Zubets Institute of Animal Breeding and Genetics, the NAAS, and the Genetics Laboratory of the Cherkasy Station of Bioresources, the NAAS.

In this study, we used 4-month-old does from Poltavske sriblo (30 animals), Californian (25 animals) breeds and their hybrids (21 animals). The experimental rabbits were kept in clean and dry cages in compliance with the optimal parameters for microclimate, feeding, and care. The combined diet was used to feed the animals, including 70–75 % of concentrated fodder, 10–15 % juicy fodder and 15–20 % rough feed.

Blood samples (3 ml) were collected by puncturing the auricular vein before feeding them in the morning and collected into the tubes with anticoagulant (S-Monovette, Germany).

The nucleoli in leukocytes were studied on smears of peripheral blood fixed with methyl alcohol. The interphase leukocytes were stained according to the method described by Howell and Black (1980) and Ploton et al (1986). The preparations were stained with 150 µl of the 50 % solution of $AgNO_3$ and 100 µl of 1 % solution of formic acid and incubated in the wet chamber of the thermostat at +60° C for 40–60 min. After washing the preparation with distilled water, the preparation was fixed with 70 % methyl alcohol for 10 min and additionally stained with 1 % solution of Giemsa stain (ready-to-use product, Merck, KGaA, Germany). The stained preparations were analyzed using a Zeiss Axiostar plus microscope (Germany) with a magnification of 10 × 100, usding a 100× oil immersion objective with a n.a. of 1.25. The nuclei were stained yellow and nucleoli – dark brown. A limited number of cells, their nuclei and nucleoli were observed and measured, then average values were calculated for each experimental group of animals.

To estimate the possible activated state of nucleoli in interphase nuclei, we calculated (after counting) the average number of nucleoli in the nucleus of all samples per breed or hybrid (nNO), their total area in the nucleus (ΣS_{NO}, µm), and the share of the nucleolar area in the lymphocyte nucleus ($sh\Sigma S_{NO}$, %). The area of nucleoli and nuclei of leukocytes was measured using a calibrated ocular micrometer (Zeiss WF10X).

The statistical processing of the obtained data was conducted using standard programs of variation statistics from the STATISTICA software package (StatSoft Inc. USA, version 14.00.15, 2020). Significance testing was performed using Fishers exact test at a p value <0.05.

RESULTS OF STUDIES

After staining with silver nitrate, two types of cells were distinguished in the leukocytes population of the peripheral blood of rabbits: 1) leukocytes in the state of dormancy, which did not have clearly manifested nucleolar structures, and 2) leukocytes with active nucleoli (**Figure**)

The Figure shows silver stained structures in the leukocytes which, in our opinion, correspond to nucleoli, in mitogen-stimulated chromosomes. The number of active nucleoli in the cell varied from 1 to 9. The size of nucleoli and their staining intensity were different.

Staining with silver nitrate therefore can be used to indicate nucleoli in interphase leukocyte, especially for counting the number of nucleoli in each leukocyte, and the measurement of the area of active nuclear structures.

The main characteristics of nucleoli in the leukocytes of rabbits from the investigated groups are presented in **Table**. It was found that the average number of nucleoli per cell varied from 1.70 ± 0.08 in the group of Californian rabbits to 5.90 ± 0.29 in the group of hybrid animals. The cells with three nucleoli were the most common. No statistically significant difference was found regarding the average number of nucleoli *per cell* between the investigated groups of rabbits, Poltavske sriblo and Californian. The average number of nucleoli in the investigated group of hybrid rabbits statistically significantly exceeded the same parameter for purebred animals of two other groups ($p < 0.05$).

The average number of nucleoli per cell was in the range of 2.0–2.9 (Table).

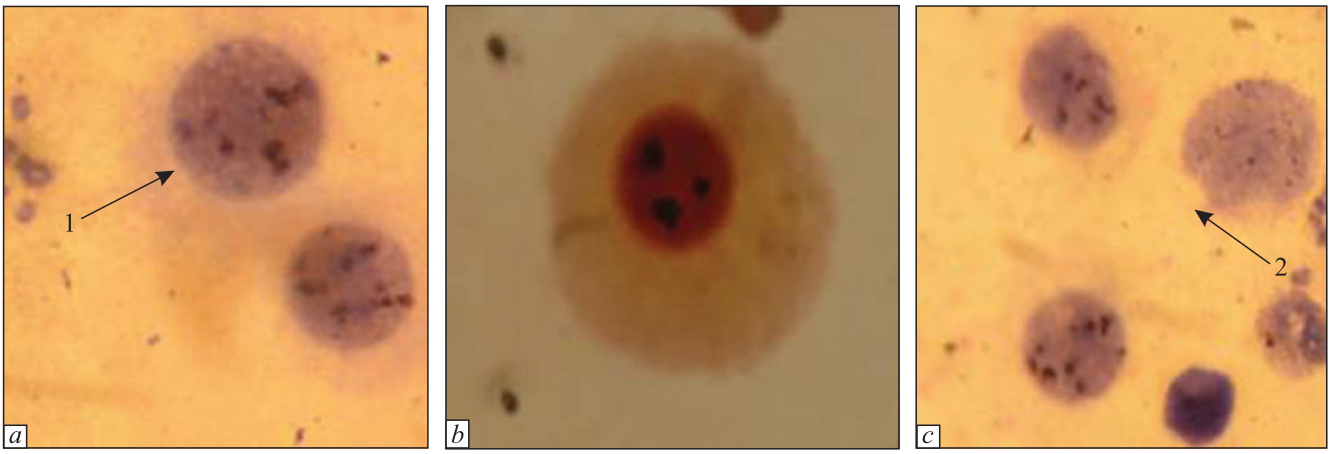
Animals of hybrid origin statistically significantly ($p < 0.05$) exceeded the animals from other investigated groups – purebred rabbits of Poltavske sriblo and Californian breeds regarding the number of cells with nucleoli in an active state.

We found higher values of parameters, characterizing the activity of nucleoli, in the investigated rabbits of interbreed hybrid origin. This suggests a higher level of RNA synthesis in cells.

The index of the total nucleolar area in cells for all the investigated animals was found to be rather variable, from 5 μm^2 in one Californian rabbit to 12 μm^2 in a hybrid animal (Table).

The determined share of the nucleolar surface area in the total surface nucleus area showed that the nucleoli covered about one third of the latter surface. This index fluctuated from 26.1 % for the rabbits of Poltavske sriblo breed to 29.4 % for hybrid rabbits (Table).

Statistically significant higher values of nucleolar parameters were found in the group of rabbits of hybrid origin than in the investigated groups of purebred rabbits from Poltavske sriblo and Californian breeds. This was for the average number of nucleoli ($p < 0.05$), the total area of nucleoli ($p < 0.05$), and the average share of the nucleolar area in the nucleus area ($p < 0.05$) (Table 1). No statistically significant differences were



The nucleoli-containing leukocytes of the peripheral blood of rabbits. Staining with silver nitrate. Magnification: 10 × 100. 1*a* – lymphocytes with active nucleoli; 2*c* – lymphocytes in the state of dormancy, which did not have clearly manifested nucleolar structures

Numerical values for the nucleoli in intact leukocytes of purebred and hybrid rabbits

Measurement parameters	Breed		
	Poltavske sriblo	Californian	Hybrids
Average number of nucleoli per cell, n	2.97 ± 0.48	2.85 ± 0.50	3.51 ± 0.68
Total nucleolar area in the nucleus, μm^2	8.70 ± 1.40	8.32 ± 1.98	9.33 ± 1.90
nucleolar area/ nucleus area ×100, %	26.10 ± 1.80	24.30 ± 1.62	29.40 ± 2.50

found between the investigated groups of purebred animals for these three parameters.

DISCUSSION

In recent years, the study of the physiological activity of NORs is used in medicine for prognostic purposes and diagnostics of diseases (Derenzini et al, 2009; Donizy et al, 2017; Ahmed et al, 2020; Rönnau et al, 2023).

For instance, an index of the number of NORs may be used as a relevant marker in the study of tumors (Sharma, Kumar, 2016). Malignant neoplasms have a higher number of NORs, which reflects the activity of cell division and is a sign of bad prognosis (Cabrini et al, 1992). An efficient and reliable method of quantitative evaluation of the risk of neoplastic transformation of lungs is believed to be the method of *Ag-banding,* used in staining the nucleolar organizers in chromosomes (Ahmed et al, 2020). Some scientific publications also describe the investigations of the association between NORs activity and human aging (McStay, 2016).

The morphology and chromosomal localization of nucleolar organizers was studied in metaphase chromosomes of different species of farm animals: cattle (Jantarat et al, 2012; Dzitsiuk et al, 2021), buffaloes (Iannuzzi et al, 1996; Dzitsiuk et al, 2018), sheep (Di Meo et al, 1993), pigs (Ernst et al, 2009), goats (Di Meo et al, 1991; Andraszek et al, 2009), etc.

In some investigations, the researchers came to the conclusion about the association between the parameters of nucleolar organizers and the features of well-being and performance in farm animals. For instance, Delany et al (1994) studied the polymorphism of the parameters of NORs in different chicken populations and believed that the future investigations on the size of the cluster of rRNA gene, the size of the nucleolus and rRNA synthesis would define the degree of the effect of rDNA variations on such relevant variables as viability, growth, and development in chicken populations, created for different purposes.

In their study of the karyotype of goats, Klenovitskiy et al (2019) demonstrated a positive correlation between the number of clusters of rRNA genes and the number of nucleoli in intact leukocytes. The authors also reported a reliably higher number of nucleoli in the leukocytes of hybrids, obtained from cross-breeding Romanivska goats with *Ovis ammon* argali as com-

pared to the purebred Romanivska goats (Klenovitskiy et al, 2021).

While conducting the cytological study of chinchilla (*Chinchilla lanigera*), Oznurlu (2011) concluded that the increase in the number of nucleoli in the interphase nuclei meant cell hyperactivity, the velocity of proliferation, and the secretory activity of the cell. They believed that the area of argirophile NORs was a prognostic feature of fur quality.

Similar to other species of animals, the study of NORs in domestic rabbits (*Oryctolagus cuniculus domesticus*) was conducted using preparations of metaphase chromosomes (Martin-DeLeon, 1980; Monteagudo, Arruga, 1991). As for the study of nucleoli in the interphase leukocytes of the peripheral blood of rabbits, we have found only one report about them (Ivanov et al, 2009).

CONCLUSIONS

Polymorphism was observed for three nucleolar parameters after silver staining of interphase leukocytes of rabbits of Poltavske sriblo, and Californian breed and their hybrid. This concerned: 1) the average number of nucleoli ($p < 0.05$); 2) the total area of nucleoli ($p < 0.05$); 3) the average share of the nucleolar area in the nucleus area ($p < 0.05$).

The results of our comparative analysis of the investigated nucleolar activity parameters suggest a higher activity of nucleoli in the animals of hybrid origin.

In the future, the results of such studies may be used to assess the potential ability of animals to implement productive traits.

Adherence to ethical principles. The Commission on treatment of animals in scientific research M.V. Zubets Institute of Animal Breeding and Genetics, the National Academy of Agrarian Sciences of Ukraine Minutes No. 4 18.03.2024

Conflict of interests. The authors declare the absence of any confl icts of interests.

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Дослідження окремих ознак ядерець в інтерфазних лейкоцитах двох порід кролів та їх помісей

* В. В. Дзіцюк [1], О. В. Бойко [2], О. Ф. Гончар [2], О. В. Гавриш [2], О. Є. Гузєватий [3]

[1] Інститут розведення і генетики тварин імені М. В. Зубця Національної академії аграрних наук України, вул. Погребняка, 1, Бориспільський район, Київська область, Україна, 08321

[2] Черкаська експериментальна станція біоресурсів Національної академії аграрних наук України, вул. Пастерівська, 76, м. Черкаси, Україна, 18036

[3] Національна академія аграрних наук України, вул. Михайла Омеляновича-Павленка, 9, м. Київ, Україна, 01010

E-mail: * valentynadzitsiuk@gmail.com, aleksboy18@meta.ua, of.gonchar@gmail.com, gavrish.olexandr@gmail.com, oleg_guzevatiy@ukr.net

orcid: https://orcid.org/0000-0001-9697-4165, https://orcid.org/0000-0002-3917-5583, https://orcid.org/0000-0003-2269-9767, https://orcid.org/0000-0002-8632-6508, https://orcid.org/0000-0002-2470-5430

Мета. Дослідження відмінностей трьох ознак ядерець в інтерфазних лейкоцитах двох порід кролів та їх помісей. **Методи.** В експерименті використовували самиць кролів віком 4 місяців українських порід: полтавське срібло (n = 30), каліфорнійської (n = 25) та їх помісей (n = 21). Ядерця в інтактних лейкоцитах крові фарбували азотнокислим сріблом за Howell і Black (1980). Ядерця у ядрах лейкоцитів підраховували під світловим мікроскопом з масляною імерсією за збільшення 10×100. Було проаналізовано 200 лейкоцитів від кожної тварини. Активність ядерець оцінювали за такими показниками: середня кількість ядерець у ядрі (nNO), загальна площа ядерець у ядрі (ΣSNO, мкм^2), частка площі ядерець у площі ядра лімфоцита (shΣSNO, %). Статистичний аналіз проводили за допомогою па-кету програм STATISTICA (2020). **Результати.** Середня кількість ядерець на клітину коливалася від – 1,70 ± ± 0,08 у каліфорнійських кроликів до 5,90 ± 0,29 у помісних тварин. Встановлено статистично значущу різницю ($p < 0,05$) між дослідними групами чистопородних і помісних кролів. Коефіцієнт варіації показника середньої кількості ядерець на клітину був на середньому рівні мінливості: у кролів породи полтавське срібло – 20,58 %, у каліфорнійських – 19,50 %, у помісних – 16,49 %. Загальна площа ядерець у клітинах усіх досліджених тварин коливалася від 5 мкм^2 в одного каліфорнійського кролика до 12 мкм^2 у тварин помісного походження. Частка площі ядерця у площі ядра лейкоцита у кролів порід полтавське срібло, каліфорнійської та помісних становила 26,10 ± 1,80 %, 24,30 ± 1,62 та 29,40 ± 2,50 відповідно. **Висновки.** Після фарбування сріблом інтерфазних лейкоцитів кролів порід полтавське срібло, каліфорнійська та їх помісей спостерігали поліморфізм за трьома параметрами ядерця: 1) середньою кількістю ядерець ($p < < 0,05$); 2) сумарною площею ядерець ($p < 0,05$); 3) середньою часткою площі ядерця в площі ядра лейкоцита ($p < 0,05$). Результати порівняльного аналізу дослідже-

них параметрів ядерцевої активності свідчать про вищу активність ядерець у тварин помісного походження. У перспективі результати таких досліджень можуть бути використані для оцінки потенційної здатності тварин до реалізації продуктивних ознак.

Ключові слова: ядерце, ядро лейкоцита, *Ag-banding*, чистопородні кролі, кролики помісного походження.

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