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Study of melanin distribution in the hair cells of Karakul lambs of different colours

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ABSTRACT

The relevance of this study is explained by the need to have a more accurate instrument for assessing the phenotype of Karakul lambs by colour. Despite the fact that the colouring of Karakul lambs is an important sign for obtaining a high-quality krimmer skin, its determination occurs only visually, which leads to large errors. This fact indicates the need for a more objective method for assessing the colour of Karakul lambs. In this regard, this article is aimed at studying melanin distribution type in cortical layer cells of Karakul lambs' hair of different colours and evaluating the possibility of using this trait as an additional criterion for identifying the phenotype by lear. After the preparation of smears from hair macerates, light microscopy was performed with a visual count of cells with a particular distribution pattern of melanosomes in cells, according to the chosen classification. The absence of class zero cells (complete absence of melanin) for black lambs was revealed, however, there were unexpectedly quite few cells of the fifth class (with superpigmentation). The largest number of cells of grey lambs were assigned to the third or zero classes. With an increase in the cells of the zero class, the colour intensity of the taken hair also decreases. In lambs of medium-blue and dark-blue colours, cells of the third class prevailed. The materials of the article are of practical value for breeding work with Karakul sheep, providing more accurate control of the selection of sheep to achieve the desired result using a quantitative parameter.

1. Introduction

The Karakul breed of sheep is an important breed in the production of lambskins of various colours, which are then used in the fashion industry. There is a variety of colouring of skins from black to sur-shade. The colour shades are the result of the interaction of a dozen different genes and conditions, which ultimately determines the colour of the lambs' skin (Lakhanova, 2015; Lakhanova et al., 2008, 2007). Karakul sheep do not require difficult conditions for their breeding and maintenance, but an increase in the percentage of individuals with more valuable traits for sale is a problematic issue (Wei et al., 2015). One of these signs is krimmer skin – curls of the hair covering during the first days after birth, as well as during foetal development.

Morphologically, the hair is divided into two parts – the root (subcutaneous part) and the stem (supercutaneous part). The stem of each hair consists of three layers: medulla, cortex and cuticle (Verma and Ali, 2013). The cortex gives the hair rigidity, it is in this layer that keratinocytes containing melanin are located, which provides the colour of the hair (Park et al., 2018). Melanin is transmitted to keratinocytes by melanocytes located in the matrix of the hair follicle bulb (Tadokoro et al., 2019; Ohbayashi and Mitsunori, 2020). The amount of melanin formed depends on many factors: on the interaction of a dozen different genes, but also on environmental conditions. The colour of the skin is also affected by both natural and artificial selection (Lakhanova, 2015; Lakhanova et al., 2008, 2007).

In the 20th century, many studies were conducted linking the quality of krimmer skin with other phenotypic features, including the histological parameters of the skin and hair covering: the structure of the skin, the nature of the cover, the density, depth and group arrangement of the hair, including the direction of the collagen fibres of the hair

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(Kiamov, 1961; Diachkov, 1960; Vinogradova, 1978; Shulov, 1974). More recent studies have confirmed the previously obtained conclusions. In 2017, the relationship between the density of the skin and the quality of the fur was also shown – thinner skin was also associated with softer fur in the Merino breed (Zhang et al., 2017a). In 2021, it was shown that the Minxian breed of sheep with black fur colour has relatively thin skin and therewith both a higher number of melanin granules and a larger area of their distribution in the skin and hair. The comparison was made with the short-tailed Han breed with white or brown fur colour (Lakhanova et al., 2007).

From the above, it can be concluded that to produce as much as possible of a more valuable type of krimmer skin, it is necessary to effectively select individuals of the closest to black or another preferred colour for their further breeding (Kotsiubenko et al., 2021; Vinichenko et al., 2021). At the moment, for a more accurate assessment of the phenotype of Karakul lambs by colour, it is necessary to develop a quantitative method that allows differentiating lambs more accurately and under technically limited conditions into groups for the possibility of breeding the most suitable individuals for further breeding of the preferred phenotype, which determines the significance of the study. In this article, the authors analysed melanin distribution types cortical layer sells in the hair samples of newborn Karakul lambs of black and grey colours of different intensity taken from the dorsal surface in the sacrum area and determined the selection parameters that, when applied in practice, will provide more efficient breeding of individuals of the preferred phenotype and thus contribute to an increase in the volume of products obtained during karakul breeding.

2. Materials and methods

The experimental part of the work was carried out on the farms of the South Kazakhstan region (Shymkent, Aktobe, Arys) during April-September 2021. 103 newborn Karakul lambs of black colour, and 105 newborn Karakul lambs of grey colour were researched. The study was conducted on hair samples taken from newborn Karakul lambs from the dorsal surface in the sacrum area. The ratio of lambs to a particular colour and the intensity of the colour were determined visually. One of the methods for characterising melanin distribution in the hair volume can be an analysis of the frequency of cells located in the hair cortex with different degrees of pigmentation.

Hair smears were prepared according to the established protocol (Vsevolodov et al., 1974). Hair samples of each individual were subjected to acid hydrolysis (6 H HCl) for one hour. After ten times in distilled water, the resulting hydrolysis product was stirred until obtaining a thick suspension. Then a smear from the suspension was applied to the smear glass, after which it was left to dry out and at the final stage it was enclosed in a balm or glycerin.

Conditioned upon the deep dark colour of the melanosome, it can be observed using different methods of light microscopy. However, the study also requires the identification of keratinocytes that do not contain melanosomes. Keratin fibrils have the property of anisotropy or double refraction (separation of a light beam after its passage through an anisotropic substance). Based on this property, the method of polarisation microscopy using immersion was chosen (Ohbayashi and Mitsunori, 2020; Benito-Martínez et al., 2020). 100 cells were viewed on each smear related to an individual. A visual count of cells with a particular pattern of melanosome distribution in cells was carried out (Table 1), according to the chosen classification (Vorobyovsky et al., 1983).

The obtained data on cells taken from black lambs and on cells taken from grey lambs with a blue colour tone were processed separately. The average number of cells belonging to a particular class was calculated, including the percentage of cells of each class relative to all registered cells of lambs of the same colour intensity; the relative total number of all cells viewed on the smears belonging to lambs of the same colour (black or grey with blue colour tone). After that, a modal class of cells was determined for each colour type studied.

Table 1
Classification of cell classes by the distribution nature of melanosomes.

Cell class	The distribution nature of melanosomes in the cell
0	there are no melanosomes in the cell
1	there are no more than 20 individual melanosomes in a cell
2	there are more than 20 individual melanosomes in the cell, the number of which, however, can be counted
3	a) only individual melanosomes ("scattering" of melanosomes) are observed in the cell, but there are so many of them that it is not possible to count them without special equipment; b) there are 1–3 "lumps" (compact clusters of melanosomes) of melanin in the cell, the diameter of which does not exceed 1/2 the diameter of the cell
4	a) there is one large lump of pigment in the cell, with a diameter of more than 1/2 the diameter of the cell; b) there are 4 or more lumps of pigment in the cell, the diameter of which does not exceed 1/2 the diameter of the cell
5	there is so much pigment in the cell that its clusters shield each other and it is impossible to count them.

3. Results

In the course of the study, data were obtained that allow determining the frequency of the presence of cells of a certain class by the nature of the presence of melanosomes in black lambs (Fig. 1, Table 2) and lambs of grey shades (Fig. 2, Table 3).

Counting and processing the data of black lambs of the Karakul breed showed that all lambs had no hair cells of the zero class - cells that do not contain melanin. The frequency of the presence of cells of the first and second classes was also low. The cells of the third class had a high frequency of occurrence, followed by the fourth and fifth in descending order of frequency. The largest number of cells was assigned to the third class, with a large number in lambs with a weakened black colour. Thus, the modal class of cells taken from black lambs should be considered the third for all colour severity.

In relation to colour intensity, the number of cells of the first, second and third classes decreased along with an increase in colour intensity, and the number of cells of the fourth and fifth classes increased. Thus, it is possible to trace the inverse proportionality of the indicators in the

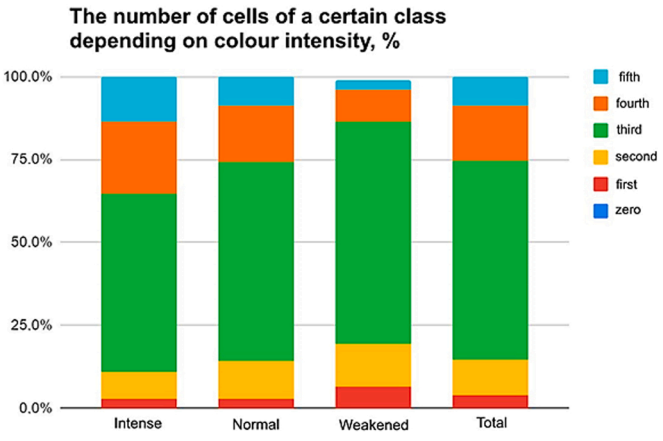

Fig. 1. The frequency of cortical cells of different pigmentation classes in the hair macerates of Karakul lambs of black colour, depending on colour intensity, bar chart.

Table 2
The frequency of cortical cells of different pigmentation classes in the hair macerates of Karakul lambs of black colour, %.

The colour intensity	Lambs counted	The number of cells of a certain class, %					
		Zero	First	Second	Third	Fourth	Fifth
Intensive	37	–	2.7 ± 2.66	8.1 ± 4.48	54.1 ± 8.19	21.6 ± 6.76	13.5 ± 5.62
Normal	35	–	2.9 ± 2.84	11.4 ± 5.37	60.0 ± 8.28	17.1 ± 6.36	8.6 ± 4.74
Weakened	31	–	6.4 ± 4.40	12.9 ± 6.02	67.7 ± 8.40	9.6 ± 5.29	3.2 ± 3.16
Total	103	–	3.9 ± 3.64	10.7 ± 3.05	60.2 ± 4.82	16.5 ± 3.66	8.7 ± 2.78

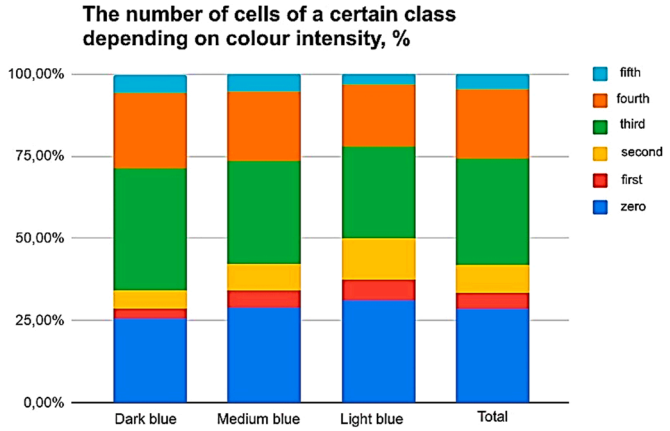

Fig. 2. The frequency of cortical cells of different pigmentation classes in the hair macerates of Karakul lambs of grey colour, depending on colour intensity, bar chart.

cells of intense and weakened black colours according to these classes of cells. Lambs of normal colour intensity have average indicators relative to all classes. Lambs' cells that had a weakened black colour were most represented by the third class (67.7 ± 8.40), followed by the second (12.9 ± 6.02%) and fourth (9.6 ± 5.29%) classes, and the smallest number of cells were assigned to the first (6.4 ± 4.40%) and fifth (3.2 ± 3.16%) classes. Therewith, notably, the number of cells of the third class exceeds even the total number of cells of the remaining classes by almost two and a half times. Thus, with the increase in colour, there is an increase in the percentage of cells of the fifth and fourth classes, and a decrease in the cells of the third, second and first classes.

The processing of data on the cells of grey lambs showed slightly different results. The cells of the cortical layer of the hair of Karakul lambs of grey colour and blue colouring according to the intensity and nature of pigmentation were classified into classes with the corresponding intensity of colours: dark blue, medium blue and light blue. The largest number of cells of grey lambs were assigned to the third (32.3 ± 4.56%), zero (28.6 ± 4.47%) and fourth (20.9 ± 3.97%) classes, followed in descending order by the second (8.6 ± 2.74%), first (4.8 ± 2.09%) and fifth (4.7 ± 3.54%), and the difference in the number of cells of the first and fifth class is insignificant.

With an increase in the cells of the zero class, and hence the number of white hair, the severity of the colour of the taken hair decreases, which leads to a zero modal class for the Karakul lambs, which have a light blue shade of grey colour. In lambs of medium-blue and dark blue shades, the cells of the third class prevailed, the number of which was directly proportional to the increase in colour intensity. There is also an inverse proportionality of the number of cells of the first and second classes with respect to the increase in colour intensity. Thus, in the group of Karakul lambs with grey hair colour, with an increase in the colour intensity, there is an increase in the percentage of cells of the fifth, fourth and third classes, and a decrease in keratinocytes of the second, first and zero classes.

The collected data shows that in both groups a lighter shade of colour is achieved by increasing the percentage of keratinocytes of the lower classes, while simultaneously reducing the percentage of keratinocytes belonging to higher classes. Accordingly, with an increase in colour intensity of hair covering, the percentage of cells of these classes becomes directly opposite. It is also worth noting that in all variants, in terms of colour intensity of black fur, the predominant class of cells is the third, it also follows the zero class of keratinocytes in the case of the wool of lambs of grey colour with a blue colour tone, with the exception of lambs with a light blue colour, where the influence of the zero class becomes stronger.

4. Discussion

Initially, melanocyte stem cells (MSC) and hair follicle stem cells (HFC) are located near the arrector pili muscle. The development of the hair is divided into three time intervals: anagen, catagen and telogen. During the development of new hair, MSC and HFSC act synchronously. At the anagen stage, MSC and HFSC are coordinated to activate, proliferate, differentiate and move to the hair papilla. During catagen, mature melanocytes and keratinocytes go into apoptosis, located in the matrix of the stem. MSC and HFSC that have not gone into apoptosis pass into the telogen-resting stage (Qiu et al., 2019). It is the synchronisation of the work of the MSC and the SQUF that allows maximum efficiency in the development of hair colouring. In case of loss of MSC or the absence of their work, unpigmented hair is formed (Qiu et al., 2019; Mort et al., 2015).

The MSCs are activated synchronously with the HFSC during anagen. Next, they migrate along the ERS to the hair follicle and differentiate into mature melanocytes. In the catagen, mature melanocytes go into apoptosis. It is unknown whether MSCs that have not gone into apoptosis migrate back. During the telogen, the MSCs are at rest until the next anagen is initiated. Epi is the epidermis, HM is the hair matrix, ERS is the external root sheath, SG is the sebaceous gland (Qiu et al., 2019). Melanin is a pigment with a polymer structure, which is found both in the cells of the skin and hair and in many other tissues. The most important functions of melanin are the maintenance of skin homoeostasis and protection from ultraviolet radiation (Boo, 2020, 2021). Two

Table 3
The frequency of cortical cells of different pigmentation classes in the hair macerates of Karakul lambs of grey colour, %.

The colour intensity	Lambs counted	The number of cells of a certain class, %					
		Zero	First	Second	Third	Fourth	Fifth
Dark blue	35	25.7 ± 7.39	2.9 ± 2.84	5.7 ± 3.92	37.1 ± 8.16	22.9 ± 7.10	5.7 ± 3.92
Medium blue	38	28.9 ± 7.35	5.3 ± 3.63	7.9 ± 4.38	31.6 ± 7.54	21.0 ± 6.61	5.3 ± 3.63
Light blue	32	31.3 ± 8.20	6.2 ± 4.26	12.5 ± 5.85	28.1 ± 7.95	18.8 ± 6.91	3.1 ± 3.06
Total	105	28.6 ± 4.47	4.8 ± 2.09	8.6 ± 2.74	32.3 ± 4.56	20.9 ± 3.97	4.7 ± 3.54

types of melanin, eumelanin and pheomelanin, were found in the mammalian body. It is worth noting that the fur colour does not always depend only on the concentration of melanin, but also on the ratio of eumelanin and pheomelanin also has its influence, which varies from species to species (Ohbayashi and Mitsunori, 2020; Crossley et al., 2017).

Strictly speaking, the term "melanogenesis" refers only to the synthesis of pigment, but it also includes the development processes of melanosomes, their transport, transfer to keratinocytes and melanin metabolism (Ohbayashi and Mitsunori, 2020). Melanin is formed inside lysosome-like organelles - melanosomes. A separate compartment for the synthesis of melanin is necessary since this process is accompanied by increased redox toxicity (Ohbayashi and Mitsunori, 2020; Bowman et al., 2019). The development of melanosomes is divided into four stages (Benito-Martínez et al., 2020), Wu and Hammer (2014). The melanosomes of the first stage are early endosomes in which amyloid fibres are developed, giving the melanosome an ellipsoid appearance and serving as a scaffold for the subsequent planting of melanin. At the second stage, the organelle receives tyrosinase, dopachrome-tautomerase and other components necessary for the development of melanin. At the same stage, its loading onto the amyloid fibres begins. Melanosomes of the third and fourth stages are differentiated by the degree of pigmentation (Ando et al., 2020; Bowman et al., 2019). During maturation, melanosomes approach the endings of melanocyte dendrites more and more closely (Benito-Martínez et al., 2020). At the moment, the exact mechanism of melanosome transport from melanocytes to keratinocytes has not been established. However, four main models were suggested (Tadokoro et al., 2019; Ohbayashi and Mitsunori, 2020):

Model 1development of a melanocyte dendrite end rich in melanosomes, phagocytic keratinocyte;.

Model 2fusion of melanocyte and keratinocyte membranes and transfer of melanosomes through the formed "bridge".

Model 3development of a vacuole with a large number of melanosomes by dendrites of melanosomes, which after separation from the melanocyte is absorbed by the keratinocyte;.

Model 4exocytosis-endocytosis model: after exocytosis of individual melanosomes, it is endocytosed by a keratinocyte.

In 2013, a study was conducted in which transcriptomes of black-coloured sheep and white-coloured sheep were compared. The group studied almost 900 genes. Of the 49 genes associated with pigmentation, 13 genes had significantly higher expression in black sheep. Many of these genes are involved in the development of melanosomes and their precursors: for example, DCT, MATP, TYR and TYRP1. Of the other new 107 genes with increased expression in black sheep: 16 were expressed exclusively in black sheep and 26 genes were expressed 8–256 times higher than the same genes in white sheep. The expression of tyrosinase was the most strongly increased of all the mentioned genes. In addition, all the genes encoding the components of melanosomes and their precursors also had a higher expression in all black sheep, but the genes involved in the development of melanosomes from the components showed a reduced expression (Fan et al., 2013).

Interesting data were obtained in the study of Tibetan sheep, which have wide variability in the colour phenotype. The agouti-signalling protein gene (ASIP) is a possible candidate that affects this trait. The study showed a difference in the copy number of the ASIP gene in genomic DNA in sheep of different colours (from 1 to 8). The presence of only one copy gave an absolutely black colour, almost in 100% of cases, which suggests that this trait does not depend on this gene alone. The increase in the copies caused various variations in the pigmentation of the fur. The white colour of the skin is a dominant feature and is caused by disturbances in the regulation of ASIP expression (Han et al., 2015). Studies on Nelluru cattle also showed dependence on the ASIP gene, but in this case, the relationship between the SNP in this gene and the skin colour was investigated (Trigo et al., 2021).

In 2015, a group of scientists from China conducted a genome-wide analysis of 42 groups of sheep, some of which belong to the Kazakh group of sheep. Based on the analysis of the detected single nucleotide polymorphisms (SNPs), a phylogenetic tree was obtained, according to which it is possible to track the signs of selection. Strong signs of breeding were found in the Kazakh group, including genes associated with the development of sheep fur colouring, KIT, MC1R, and FRY, which is also associated with the quality of hair. In 2019, the genomes of 15 Russian sheep breeds, including the Karakul breed, were examined for signs of selection - density of existing SNPs. A group of scientists discovered key genes in possible regions of the genome with signs of selection associated in humans with many disorders and the inability to feel pain and temperature, which can be considered an adaptation of breeds to severe climatic conditions. The melanocortin 1 receptor (MC1R) genes were also noted as a sign of selection for the subgroup to which the Karakul breed belongs. However, the most pronounced gene in the Karakul breed was the microphthalmia transcription factor (MITF), the expression product of which is a development regulator of melanocytes. In the mentioned subgroup, genes related to the quality of fur, for example, the KRT gene cluster, are also marked as a strong sign of selection (Yurchenko et al., 2019).

The diversity of sheep breeds is based on the influence of environmental factors and artificial selection, in which there is a shift towards improving the most favourable economic characteristics, including the shades of skin colour and the quality of fur. Thus, it can be assumed that by changing environmental factors or acting directly on genes, it is possible to manipulate the manifestation of phenotypic traits, including the colour of fur. In 2017, a direct relationship was shown between colouration and the amount of tyrosine in the diet of black-coloured cat breeds. The same relationship was observed with the concentration of phenylalanine in blood plasma (Crossley et al., 2017). In 2020, it was shown that the concentration of cortisol in dogs correlates with a lighter colour (Bowland et al., 2020). In a 2017 study, it was shown that a violation of the ASIP gene using the CRISPR/Cas9 method changed the colour of lambs, a deletion of 4 bases gave a darkening of the colour, where additional deletions of 2 and 6 bases gave a black body colour with a certain badger pattern of head colouring. The deletion caused a shift in the reading frame and a premature stop of transcription with the development of a truncated protein (Zhang et al., 2017b). In 2019, using the method of directed imitation of short tandems (STTM), a group from China caused a block of miR-143-5p expression, which lowered the expression of genes associated with melanogenesis. In turn, overexpression of this micro-RNA caused increased expression, including of the tyrosinase gene (Qi et al., 2019). Accordingly, it is possible to change the colour in the direction of a more preferable option not only based on artificial selection as such but also conditioned upon higher temperature in the living conditions of sheep, an increase in tyrosine in their diet. However, it is worth noting that without fixing the cause of the colour change through the genome, inheritance of the desired skin colour will also not happen.

5. Conclusions

In the course of the study, the authors revealed a direct relationship between an increase in the intensity of black colour in hair covering of Karakul sheep and an increase in the percentage of cells of the fifth class according to the Vorobyevsky classification and cells of the fourth class in the cortex of the studied hair, while the content of keratinocytes of the other classes fell. In lambs with grey and blue skin colour, in addition to keratinocytes of the fifth and fourth classes, the percentage of cells of the third class also increased. Therewith, when counting keratinocytes from black lambs with different levels of intensity, no zero-class cells were found – cells where melanin is absent.

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Conflict of Interest

The authors declare no conflicts of interest.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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