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Comparative Skin Histology of the White New Zealand and Angora Rabbits: Histometrical and Immunohistochemical Evaluations

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Abstract: In this study, thickness of the skin sub layers and the number, absolute and relative areas of the nuclear Argyrophilic Nucleolar Organiser Regions (AgNORs) and the number of Proliferating Cell Nuclear Antigen (PCNA) positive nuclei of the germinal matrix epithelial cells of the Angora and white New Zealand rabbits were compared. Skin samples were taken from 40 adult healthy rabbits. General histological features of the skin in both Angora and white New Zealand rabbits were quite similar and displayed the characteristics of thin skin. Angora rabbit skin was significantly ($p<0.05$) thicker than that of the white New Zealand rabbit and the difference mainly originated from the thicker papillary layer of the dermis. The Angora rabbit had the greatest hair follicle number in the unit area of the dermis. The shape, dimension and distribution pattern of the AgNORs were similar in both rabbit strains. The number, absolute and relative areas of the AgNORs and PCNA immunoreactivity of the Angora rabbits were higher than those of the White New Zealand rabbits. The histological characteristics of the skin in the white New Zealand rabbit were suitable for leather industry, whereas the Angora rabbit had good hair follicle characteristics for wool production. Further studies on a larger population regarding seasonal differences should be done to elucidate the possible association between AgNOR parameters and PCNA immunoreactivity of germinal matrix cells of hair follicles and wool production.

Key words: AgNOR, PCNA, hair follicles, Angora rabbit, New Zealand rabbit

INTRODUCTION

Domestic rabbits are all purpose animals. High quality rabbit skins are used in fur garments and trimmings in medical and cosmetic research. The white New Zealand rabbit is the most popular meat producing and pet strain in the world. The Angora rabbit was selectively bred over hundreds of years, for its long wool, which is often considered one of the noble fibres and also called Angora fibre (Schlink and Liu, 2003; Allain, 2007). The unusual length of Angora hair fibre arises from a prolonged growth (anagen) phase of the hair follicle growth cycle. Anagen of the Angora hair follicle lasts approximately 14 weeks, whereas that of other rabbit strains lasts only 5 weeks (Allain, 2007).

Hair follicles are classified as primary and secondary follicles. The primary follicles have a large diameter and rooted deep in the dermis and usually associated with sebaceous and sweet glands. A hair fibre that emerges from a primary follicle is called a primary hair fibre (Monteiro-Riviere, 1998). The secondary follicles are smaller in diameter; their roots are superficially located and may have a sebaceous gland but lack a sweet gland and arrector pili muscle. Their hair fibres are secondary hair fibres (Monteiro-Riviere, 1998). Many differences exist in the arrangement of the hair follicles among the animal species (Atlee *et al*., 1997; Monteiro-Riviere, 1998; Moore *et al*., 1998). They are either located individually, as in simple follicles, or as in compound follicles, which consists of clusters of several hair follicles located in the dermis and usually contain one primary hair follicle and several secondary follicles (Atlee *et al*., 1997; Monteiro-Riviere, 1998; Broeck *et al*., 2001).

Growth of a hair fibre is a complex event displaying cyclic changes and interactions between dermal papilla and germinal matrix cells. Dermal papilla plays an important role in the growth cycle of the fibre by sending chemical signals to release the germinal matrix cells, which proliferate and elongate the hair shaft and produce an inner root sheath in response to these signals (Stenn and Paus, 2001). A number of factors, such as season of the year, nutrition, age, sex, health and hormonal status of the animal affect growth cycle of the hair (Paus *et al*., 1990; Lanszki *et al*., 2001). Hair growth is a highly regulated cyclical process. Three distinct phases have been defined for the mammalian cycle: anagen (growing phase), catagen (regressing phase) and telogen (resting phase) (Soma *et al*., 1998).

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Nucleolus Organizer Regions (NORs) are the specific DNA regions containing genes encoding the synthesis of rRNA. Transcriptionally active NORs, also known as silver staining nucleolus organizer regions or AgNORs, are associated with specific nonhistone acidic and argyrophilic proteins that can be visualized as small brown dots by silver staining techniques (Ploton *et al*., 1986; Derenzini and Ploton, 1991; Orrea *et al*., 2001). Variations in the number and normal distribution pattern of AgNORs might indicate qualitative and quantitative changes in protein synthesis activity of the cell (Watchler *et al*., 1986). An increase in the number of AgNORs in interphase nuclei would indicate cellular hyperactivity and might give a valuable indicate of the proliferation rate, differentiation process and secretory activity of a given cell. These changes may also be associated with the processes involved in malignant transformation (Crocker and Nar, 1987; Orrea *et al*., 2001; Sur *et al*., 2003; Güler *et al*., 2005).

Proliferating Cell Nuclear Antigen (PCNA), an essential regulator of the cell cycle is 36 kDa molecules, which are highly conserved among species. It has been shown that PCNA serves as a co-factor for DNA polymerase delta in S-phase and is involved in DNA damage repair during DNA synthesis. It starts to accumulate in G1 phase of the cell cycle, reaches the highest level during the S phase and decreases during G2/M phase (Soma *et al*., 1998; Inoue *et al*., 2006).

In this study, histological and morphometric features of the skin and with special reference to AgNOR and PCNA for proliferative activity in germinal matrix epithelial cells were compared in the Angora and white New Zealand rabbits, since the phenotype of the hair coat and growth rate of hair fibres in these strains are quite different.

MATERIALS AND METHODS

Animals and skin samples: Skin samples of 40 adult healthy Angora and white New Zealand rabbits (equal numbers from both sexes for each strain) were used as materials. Because of the high seasonal variation in hair fibre growth of the rabbit, punch skin biopsies were performed in the same month (November). The 3 mm diameter skin samples were taken from the lumbal dorsum of each animal after anaesthesia by local injection with 2% lidocaine and use of disinfectant thereafter.

Histological procedures: The skin samples were fixed in 10% buffered formalin and divided into two pieces. The samples were processed by means of routine histological methods and immersed in paraffin blocks. One piece of the each sample was settled into the block at surface-down position, which enables taking cross sections of the hair follicles, whereas the other one was settled horizontally, in order to obtain vertical sections. Then, 6 μm thick sections were taken and stained with Masson's trichrome, reticular and elastic fibre stains (Culling *et al*., 1985).

AgNORs staining: The AgNORs method was modified as follows: Deparaffinize and hydrate in distilled water. Treat with AgNORs solution for 30 min at 37°C incubator. AgNORs solution: one volume of 1% gelatin (Sigma, USA) in 1% formic acid (Merck, Germany) solution and two volume of 50% silver nitrate solution were mixed before using (Merck, Germany). Wash in warm water using three cycle changes. Treat with 0.2% gold chloride solution for 5 min. Wash in running water. Dehydrate, clear and mount. No counterstain was used (Korek *et al*., 1991; Pich *et al*., 1994). The AgNORs were visualized as intranuclear black dots under the light microscope and the number of AgNOR dots in 100 germinal matrix cells was counted.

Immunohistochemical procedure: For immunohistochemical PCNA staining of the samples, paraffin sections (5 mm thickness) on glass slides coated with poly-l-lysine were deparaffinized in xylene, hydrated and then placed in phosphate buffered saline (PBS; pH 7.6). Antigen retrieval was performed by boiling for 10 min in citrate buffer (0.01 M). Sections were treated with 3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity, rinsed with deionized water and washed with PBS. Sections were incubated first with blocking serum (ScyTek, UHP 125) to reduce non-specific staining and then with a monoclonal antibody against PCNA (Genetex, PC10, Cat. No: GTX71945) at in a moist chamber for 60 min. This antibody was diluted 1:100 with antibody diluent solution (ScyTek, ABB125). Detection of the antibody was performed using a biotin-streptavidin detection system (ScyTek, UHP 125) with 3, 3-Diaminobenzidine tetrahydrochloride (DAB) solution as chromogen (ScyTek, ACK125). Sections were counterstained with Mayer's hematoxylin, dehydrated and then coverslipped with permount. The number of PCNA positive nuclei in 100 germinal matrix cells was counted.

Histological evaluation of the specimens: All specimens were examined under the light microscope (Nikon Eclipse E-400 equipped with a digital camera head (DS-5M) and camera control unit (DS-L1), Nikon, Japan). The histological evaluation and measurements were performed on the digital images. The following parameters were determined.

Thickness of each skin layer, staining density and orientation of the connective tissue fibres, number of the hair follicles in a unit tissue area (1 mm^2). Mean secondary follicle/primary follicle ratio (S/P) of compound follicles, Mean nucleus area of the germinal matrix cells, mean AgNOR area and AgNOR number per nucleus of the germinal matrix cells, relative AgNOR area (percentage of AgNOR area in a given nucleus area) of germinal matrix cells was calculated by using total AgNOR area and nucleus area data. The number of PCNA positive nuclei in germinal matrix cells.

Statistical analysis: Statistical analysis were performed with a standard computer program (Minitab for Windows, Release, 9.2, 1993). The data were analyzed with two sample t-test and the differences between two strains were compared.

RESULTS AND DISCUSSION

Angora and white New Zealand rabbits displayed quite similar structural characteristics of the thin skin. The epidermis was a keratinized, strafied squamous epithelium and composed of 3-4 cell layers in both strains. There were no significant differences in the content, orientation and staining characteristics of collagen, elastic and reticular fibres in the skins between both strains. The elastic fibre framework was more definite and widespread in the white New Zealand rabbit skin (Fig. 1a, b). Hypodermis of the white New Zealand rabbit was rich in adipocytes.

The overall thickness of skin was significantly ($p<0.05$) larger in the Angora rabbit than in the white New Zealand rabbit. This is mainly due to a thicker dermal papillary layer in Angora rabbits, which measured 3 times the thickness of the papillary dermis in the white New Zealand rabbits. Conversely, the reticular dermis was thinner in the Angora than in the white New Zealand rabbits. No statistical differences were found in either the viable epidermis (without keratin layer) or the stratum corneum. No difference was detected between male and female individuals in any strain (Table 1).

The primary follicles were distinguished with their large diameter and their roots reaching the dermis, whereas secondary follicles were smaller and the roots located superficially. Both rabbits had compound hair follicles. Angora compound hair follicles consisted of one primary follicle and multiple smaller secondary follicles located around it. Most of the compound hair follicles of the white New Zealand rabbit constituted of a central primary hair follicle and clusters of 2-4 compound follicles

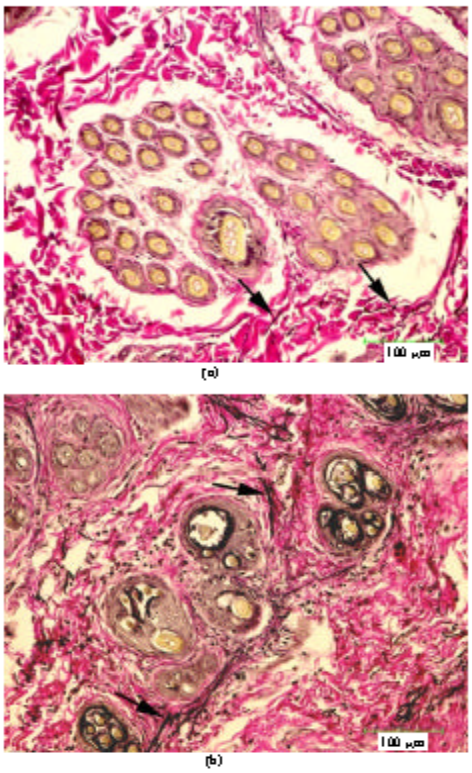


Fig. 1: a): Sections of the skin of the Angora and b): White New Zealand rabbits. Elastic fibre frameworks (arrows) among the compound follicles are more definite in the white New Zealand skin seen. Verhoef's elastic stain. Bar: 100 µm

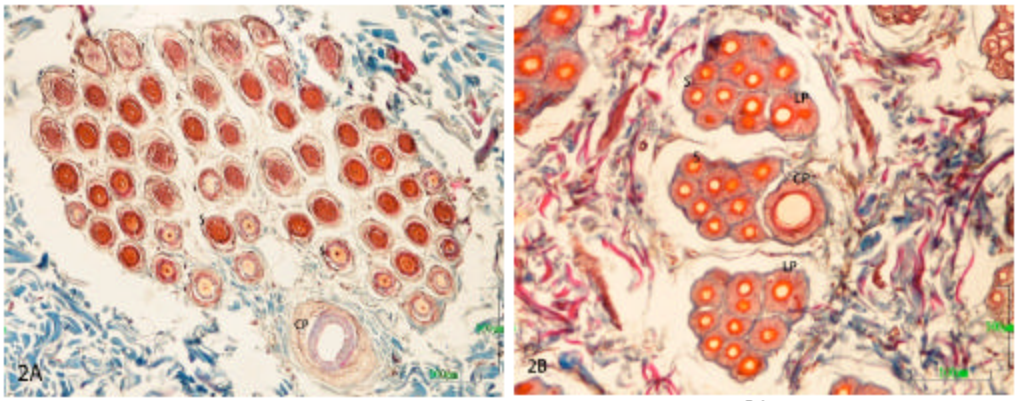
surrounding the central primary follicle. Angora rabbits had significantly ($p<0.05$) more hair follicles per unit area (1 mm^2) than the white New Zealand rabbits. This was primarily due to a larger number of secondary hair follicles (Table 2, Fig. 2a and b).

The nucleus area of germinal matrix cells in the Angora did not differ significantly ($p>0.05$) from those of the white New Zealand rabbits. AgNORs were seen as 1-4 black dots, which were distributed in the nuclei of the germinal matrix cells of the hair follicles, although, some of them were located at close proximity to the nuclear envelope (Fig. 3a, b). There were no significant differences in the shape, dimension and distribution of the AgNORs between the Angora and white New Zealand rabbits. AgNOR number and absolute and relative AgNOR areas of the Angora rabbit were significantly ($p<0.05$) higher than those of the white New Zealand rabbits. There were no significant differences between the male and females in AgNOR parameters given above (Table 3).

Table 1: The thickness of skin layers of the animals

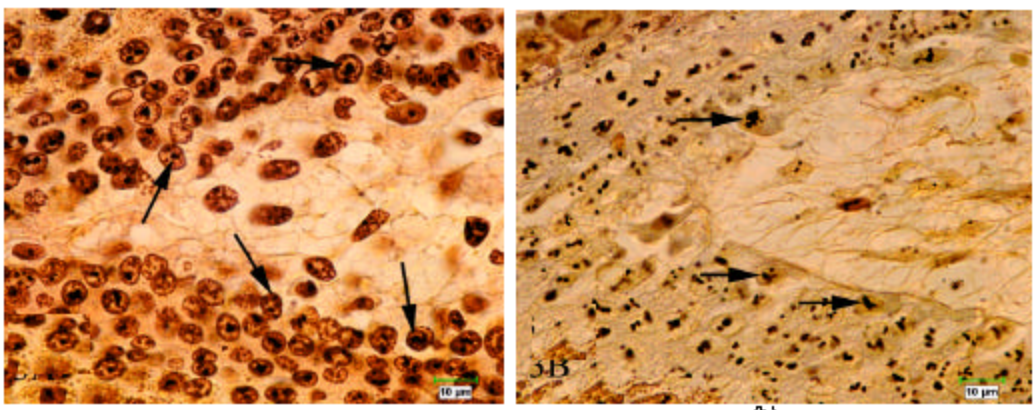
Groups	Epidermis without keratin layer (X±SD)		Keratin layer proper (X±SD)		Papillary layer (X±SD)		Reticular layer (X±SD)		Total skin (X±SD)	
N = 10	Genders	General	Genders	General	Genders	General	Genders	General	Genders	General
ANG♀	21.0±3.8^a	20.6±4.0^a	12.1±3.2^a	11.7±3.6^a	1539.9±439.5^a	1564.1±399.1^a	606.4±172^a	609.9±159.9^a	2179.4±547.6^a	2206.3±494.3^a
ANG♂	20.2±4.3^a		11.3±4.0^a		1588.3±376.7^a		613.3±156.2^a		2233.1±461.9^a	
NZ♀	20.3±5.6^a	19.4±4.8^a	9.3±1.5^a	9.5±1.6^a	589.2±178.3^b	544.5±183.1^b	1187.8±250.4^b	1174.7±199.9^b	1806.6±277.0^b	1748.2±264.9^b
NZ♂	18.5±4.1^a		9.6±1.7^a		499.9±185.8^b		1161.7±145.9^b		1689.7±260.0^b	

ANG: Angora rabbit; NZ: White New Zealand rabbit; a-c) The difference between the values in a column with different superscript is statistically significant ($p<0.05$)



(a) (b)

Fig. 2: a): Cross sections of compound hair follicles of the Angora and white New Zealand, b): Rabbits. Central Primer (CP), Lateral Primer (LP) and Secondary (S) follicles are definite in both section. Trichrome stain. Bar: 100 μm



(a) (b)

Fig. 3: a): Sections of hair bulbs of the Angora and b): White New Zealand rabbits. AgNORs (arrows) are seen as black dots in the nuclei of germinal matrix cells. AgNORs staining. Bar: 10 μm

In anagen hair follicles, germinal matrix cells around dermal papilla were positively stained by anti-PCNA antibody (Fig. 4), indicating that these cells were in the growing phase in the Angora and white New Zealand rabbits. The percentages of PCNA positive cells in the Angora rabbit were significantly ($p<0.05$) higher than those of the white New Zealand rabbits (Table 3).

The histological observations have revealed that both the Angora and white New Zealand rabbit display similar histological characteristics of the thin skin. Both rabbit strains had a thin epidermis constituted of basal, spinous and keratin layers. Similar findings were reported for rabbit skin by Sokolov (1982) and Yagci *et al*. (2006). Morphometrical analysis have revealed that the Angora rabbit skin was significantly thicker (2206.3 μm) than that of the white New Zealand strain (1696.7 μm) and total skin thickness was not significant ($p>0.05$) between the sex of each strain (Table 1). However, Yagci *et al*. (2006) have reported that the male white New Zealand rabbit (2087.6 μm) had thicker skin than the females (1835.4 μm). In the present study, the epidermal thickness of both strains was quite similar ($p>0.05$). Similarly, Yagci *et al*. (2006) did not find any significant difference in the

Table 2: Results of the histomorphometrical measurements of the hair follicles

	Hair follicle numbers in a unit area (1 mm²) and medullary index (X±SD)					
	Primary follicle		Secondary follicle		Total follicle	
Groups (N = 10)	Genders	General	Genders	General	Genders	General
ANG♀	4.1±0.5^{a}	4.1±0.5^{a}	88.4±23.4^{a}	78.1±22.6^{a}	92.6±23.1^{a}	82.1±22.7^{a}
ANG♂	4.0±0.5^{a}		67.8±17.4^{b}		71.8±17.7^{b}	
NZ♀	6.8±0.5^{b}	6.6±0.6^{b}	39.3±4.2^{c}	34.7±6.1^{b}	46.1±4.5^{c}	41.3±6.5^{b}
NZ♂	6.4±0.4^{b}		30.0±3.9^{d}		36.4±4.1^{d}	

ANG: Angora rabbit; NZ: White New Zealand rabbit; a-d): The difference between the values in a column with different superscript is statistically significant ($p<0.05$)

Table 3: Results of the nucleus, AgNOR and PCNA parameters of the germinal matrix cells

	Nucleus and AgNORs area (µm²)									
	Nucleus area (µm²) (X±SD)		AgNOR area (µm²) (X±SD)		AgNOR area/nucleus area ratio (%) (X±SD)		AgNOR number per nucleus (X±SD)		PCNA positive cell number (%) (X±SD)	
Groups (N = 10)	Genders	General	Genders	General	Genders	General	Genders	General	Genders	General
ANG♀	30.9±8.1^{a}	27.7±7.6^{a}	5.5±1.24^{a}	5.3±1.18^{a}	17.8±5.5^{a}	19.1±4.2^{a}	2.4±0.3^{a}	2.5±0.3^{a}	70±4.7^{a}	69.5±3.5^{a}
ANG♂	24.5±6.1^{a}		5.1±1.13^{a}		20.8±2.1^{a}		2.6±0.4^{a}		69.1±2.3^{a}	
NZ♀	27.2±6.9^{a}	28.7±7.9^{a}	3.9±0.96^{b}	3.8±1.15^{b}	14.3±3.6^{b}	13.2±6.8^{b}	1.6±0.5^{b}	1.7±0.5^{b}	55.4±3.5^{b}	54.4±3.9^{b}
NZ♂	30.2±14.7^{a}		3.7±1.35^{b}		12.3±4.4^{b}		1.7±0.6^{b}		53.3±4.4^{b}	

ANG: Angora rabbit; NZ: White New Zealand rabbit; a-b): The difference between the values in a column with different superscript is statistically significant ($p<0.05$)

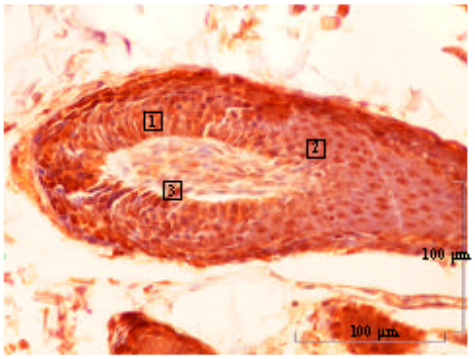


Fig. 4: Immunohistochemical staining for PCNA in the hair bulbs of the Angora rabbit. 1-3: PCNA positive nuclei in the germinal matrix cells. Bar: 100 µm

epidermal thickness of both genders. Dermis was significantly ($p<0.05$) thicker in the Angora rabbit than that of the white New Zealand rabbit and the difference has arisen mainly from the papillary layer, which is the thickest layer of the Angora skin (Table 1). Similarly, Yagci *et al.* (2006) have also reported a lower value for the dermal thickness of the white New Zealand rabbit.

The germinal matrix, which proliferates and produces the hair and the inner root sheath via the signal, has close homologies of the stratum basal of the epidermis (Stenn and Paus, 2001). Moore *et al.* (1998) suggested that a negative but high correlation between fibre diameter and follicle density was previously illustrated and selection efforts on wool-producing activities of skin would predominantly affect follicle density and fibre characteristics. Rafat *et al.* (2007) reported that selection for total fleece weight significantly increased bristle length, the secondary to primary follicle ratio and comfort factor. Atlee *et al.* (1997) reported that the fiber quality of wool-producing species is often expressed in terms of the secondary-to primary-hair ratio (S/P ratio). In this study, the Angora rabbit had significantly ($p<0.05$) greater secondary and total follicle numbers in a unit area and the females had significantly higher number of the secondary follicles (Table 2). The ratio of secondary to primary hair follicles (S/P) was higher in the Angora rabbit (19/1) than the white New Zealand rabbit (5/1). Gul *et al.* (2005) have reported that the race, age, sex and different body regions had significant effects on the number of hair follicle and dermal papilla in the wistar albino rats. However, Yagci *et al.* (2006) reported that there was not any difference derived from sex in both primary and secondary follicle numbers of the white New Zealand rabbit.

The staining intensity, shape and number of AgNORs in interphase cells have been suggested to indicate the cell activity and proliferation levels (Russel *et al.*, 1991; Rosana *et al.*, 2005). Rabbit ribosomal cistrons are located on the secondary constrictions of the 13, 16, 20 and 21 chromosome pairs, which frequently associate to give rise nucleolus (Arruga and Monteagudo, 1989). The amount of AgNORs proteins is used as a marker of proliferation, since it is low in G1 phase and high in the S-G2 phase. Therefore, a higher AgNORs value indicates that the major part of the cells is in the S-G2 phase and correlatively few are in the G1 phase, suggesting a rapid cell cycle (Field *et al.*, 1984; Pession *et al.*, 1991;

Sirri *et al*., 2000). Significant difference was observed in AgNOR number of germinal matrix epithelial cells between the Angora and white New Zealand rabbits. The Angora had the greater AgNORs number, absolute and relative AgNOR area values than that of the white New Zealand rabbit. An increase in AgNOR counts in the Angora rabbits would therefore suggest that the cell cycle of germinal matrix cells is increased compared to the New Zealand rabbits. AgNOR count has been assumed as a reflection of transcriptional activity of intherphase or mitotic cells. Moreover, silver staining has been suggested to reveal transcriptionally active NORs (Orrea *et al*., 2001) and variations in the normal pattern of AgNORs might indicate qualitative and quantitative changes in protein synthesis (Watchler *et al*., 1986). Crocker and Nar (1987), Cabrini *et al*. (1992), Grotto *et al*. (1993) and Orrea *et al*. (2001) have reported that an increase in the number of AgNORs in interphase nuclei indicates cellular hyperactivity.

PCNA is an auxiliary protein of DNA polymerase™ enzymes necessary for DNA synthesis and it is used as a standard marker in proliferating cells (Wood and Shivji, 1997). Expression of PCNA increases during the G1-phase, peaks at the S-phase and declines during G2/M-phases of the cell cycle. These immunostaining characteristics allow the identification of cells in the different phases of the cycle (Celis and Madsen, 1986; Wood and Shivji, 1997; Muskhelishvili *et al*., 2003). In this study, in anagen hair follicles, germinal matrix cells around dermal papilla were positively stained by anti-PCNA antibody and PCNA immunoreactivity were higher in the Angora rabbits.

Although, Allain (2007) suggested has that the unusual length of the Angora hair has arisen from the elongated growth period but from the higher growth rate, the results showing higher number and absolute area of AgNORs and PCNA labelling immunoreactivity might evidence that the germinal matrix cells of hair follicles of the Angora rabbit had higher rate of protein synthesis in addition to mitotic index.

The present study confirms the presence of strain-specific characteristics and breed-dependent variations in the thickness of sub layers and hair follicle number in a unit area of the rabbit skin. The histological characteristics of skin of the white New Zealand rabbit were suitable for leather industry, whereas the Angora rabbit had good hair follicle characteristics for wool production. These findings give some histomorphological evidence for making comparisons of the performance traits between both strains. Because that the AgNOR number, absolute and relative AgNOR area and PCNA immunoreactivity were higher in the Angora rabbit, which is famous for both growth and quality of hair fibre, further studies on a larger population regarding seasonal differences should be done to elucidate the possible association between AgNOR parameters and PCNA immunoreactivity of germinal matrix cells of hair follicles and wool production. Finally, the results suggested that determination of number and distribution of hair follicles, AgNOR parameters and PCNA immunoreactivity in the germinal matrix cell might also be used as significant criteria for fur quality in selection.

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