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ORIGINAL ARTICLE

Qualitative and quantitative studies on the ultrastructure of ovine pinealocytes during postnatal development

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Abstract

OBJECTIVE: The study was performed to analyze the ultrastructure of ovine pinealocytes during the period of postnatal development.
MATERIAL AND METHODS: The pineals of newborn, 10-week and one-year old females of the domestic sheep were prepared for ultrastructural investigations. The point count analysis was used in quantitative studies of the pinealocyte substructures.
RESULTS: The prominent feature of pinealocytes in the newborns was the presence of well developed rough endoplasmic reticulum and numerous polysomes. The pinealocyte cytoplasm contained also smooth endoplasmic reticulum, mitochondria, Golgi apparatus, lysosomes, dense core vesicles, multivesicular bodies and lipid droplets. Pinealocytes of the 10-week and 1-year old sheep were characterized by the occurrence of numerous vesicles and short cisterns of smooth endoplasmic reticulum, abundant microtubules and lipid droplets. Pinealocytes of the adult sheep were distinguished by well developed Golgi apparatus, numerous dense core vesicles and multivesicular bodies.
The relative volume of rough endoplasmic reticulum in pinealocytes was significantly higher in the newborn sheep than in two other groups. The relative volume of mitochondria was significantly higher in pinealocytes of the 10-week old sheep than the newborns and one-year old animals. The relative volume of Golgi apparatus was significantly higher in the one-year old animals than in two other groups. No differences concern lysosomes. The relative volume of lipid droplets as well as the numerical density of dense core vesicles and multivesicular bodies increased significantly with age.
CONCLUSION: The ultrastructure of ovine pinealocytes undergoes the marked changes during postnatal development. The changes concern mainly substructures involved in secretory activity.

Introduction

The mammalian pineal gland undergoes both morphological [1, 2, 3, 4, 5, 6, 7, 8] and functional changes [8, 9, 10, 11, 12, 13, 14, 15] during the period of postnatal life. The present knowledge about postnatal changes in the pineal ultrastructure is limited. According to our data, quantitative ultrastructural studies on the developmental processes occurring in pinealocytes from birth to maturity have been performed till now in two mammalian species only - the rat [1] and the domestic pig [2].

A special interest in studies on the ovine pineal gland derives at least from two reasons. Firstly, the pineal gland, via its main hormone – melatonin, is involved in regulation of the seasonal changes in reproductive activity, sexual maturation and pelage in many mammals including the sheep [16, 17, 18, 19]. The annual rhythm of reproduction is much more prominent in ewes and rams than in other domestic animals. Therefore the sheep is the subject of several studies concerning the role of the pineal gland in processes governing the seasonal breeding [17, 19, 20, 21, 22, 23]. Secondly, the fundamental differences have been recently demonstrated in the mechanisms regulating the melatonin secretion between ovine and rat pinealocytes, while the last ones were predominantly used in the pineal cytophysiological research, so far. In contrast to the rat pineal gland, the melatonin synthesis in ovine pinealocytes is regulated mainly at posttranslational level [24] via phosphorylation of arylalkyamine N-acetyltransferase and binding of this enzyme to 14-3-3 proteins [25]. It seems to be very probable, that the regulation of the pineal melatonin secretion in the sheep shows many similarities with the primates including the human [26, 27]. In the view of this fact, the ovine pineal gland represents an important subject of comparative studies concerning the intracellular mechanisms controlling melatonin synthesis in mammals and may be an appropriate model for investigations on regulation of the human pineal activity.

The prenatal development of the ovine pineal gland was a subject of several studies, that provided data about the formation of parenchyma and stroma, the differentiation of pinealocytes and glial cells as well as the presence of pigment granules [28, 29, 30, 31, 32]. On the contrary, the postnatal maturation of ovine pinealocytes was not investigated in details. Regodon and co-workers [7] compared the pineal ultrastructure in three groups of sheep formed according to their developmental stages, however with large age-diversity within a group: 1) 1 – 6 months old, 2) 9 months – 2 years old and 3) more than 2 years old. The authors reported important changes in the pinealocyte ultrastructure during the postnatal life, but they did not support these observations by the use of quantitative analyses. More recently, the pineals from the same groups of sheep have been studied using immunohistochemical methods with an idea to characterize the postnatal changes in the general structure of the gland [8].

The aim of the present study was to investigate the ultrastructure of pinealocytes in newborn, 10-weeks old (immature) and one-year-old (sexually mature) female sheep using qualitative and quantitative methods. The studies performed on the rat and the pig showed that the pinealocyte ultrastructure underwent important transformations from birth to puberty time, however the structural changes were the most intensive during the first weeks of life [1, 2]. Moreover, the first 10 weeks of postnatal life were reported as a period of development of the diurnal rhythm in melatonin secretion in the sheep [9, 10, 11, 33]. Due to the importance of the pineal gland in sexual maturation of the sheep [17, 19], we decided also to compare the pinealocyte ultrastructure in immature (10-week old) and mature (one-year old) animals.

Material and methods

The study was performed on the pineal glands of newborn, 10-week old and one-year old females of the domestic sheep. The animals (four in each group) were killed between 8:00 and 12:00 during spring in Poland and the pineal glands were removed no later than 3 min after the heart stopped beating.

The pineals were cut into several parts, immersion fixed (2 hrs, 4°C) in a mixture of 1 % paraformaldehyde and 2.5 % glutaraldehyde in 0.2 M phosphate buffer (pH 7.4), washed, postfixed in 2 % osmium tetroxide (2 hrs, room temperature) and embedded in Epon 812. Four blocks from each animal were selected at random for sectioning. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a transmission electron microscope.

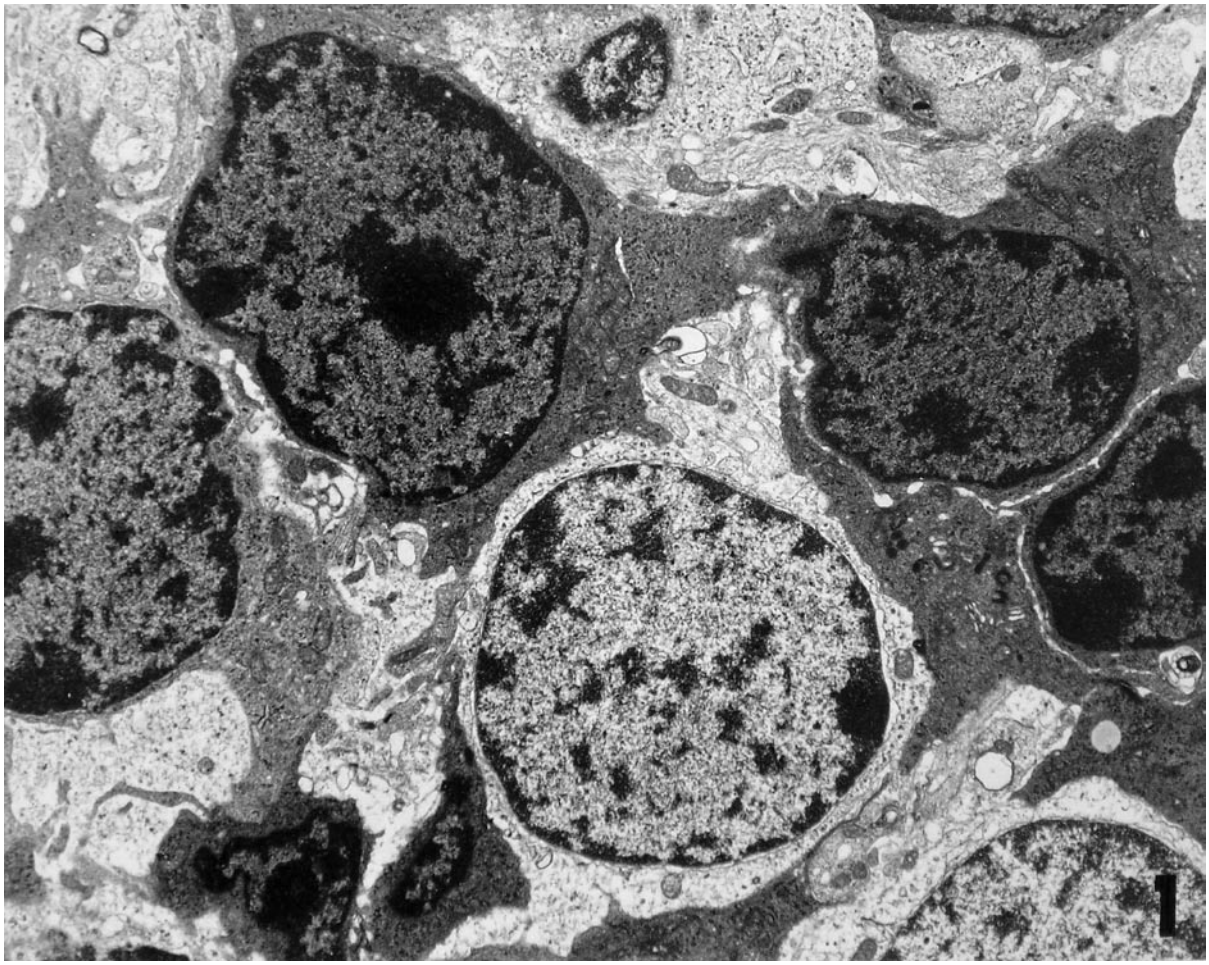
Based on their electron density, dark and light pinealocytes were counted up to the total number of 1000 per one tissue block. Ten micrographs at a magnification of x 8 000 were taken from each block (i.e. 40 micrographs per animal) using systematic random sampling, photographically enlarged to x 20 000 and used for quantitative study [34]. Point count analysis was employed to estimate the relative volume (expressed as the percentage of the cytoplasm of the pinealocyte cell body) of the following cell components: mitochondria, Golgi apparatus, rough endoplasmic reticulum, lysosomes, and lipid droplets [34]. The numerical density (expressed as the number per 500 μm^2 of the cytoplasm surface of the pinealocyte cell body) of dense core vesicles and multivesicular bodies was also estimated.

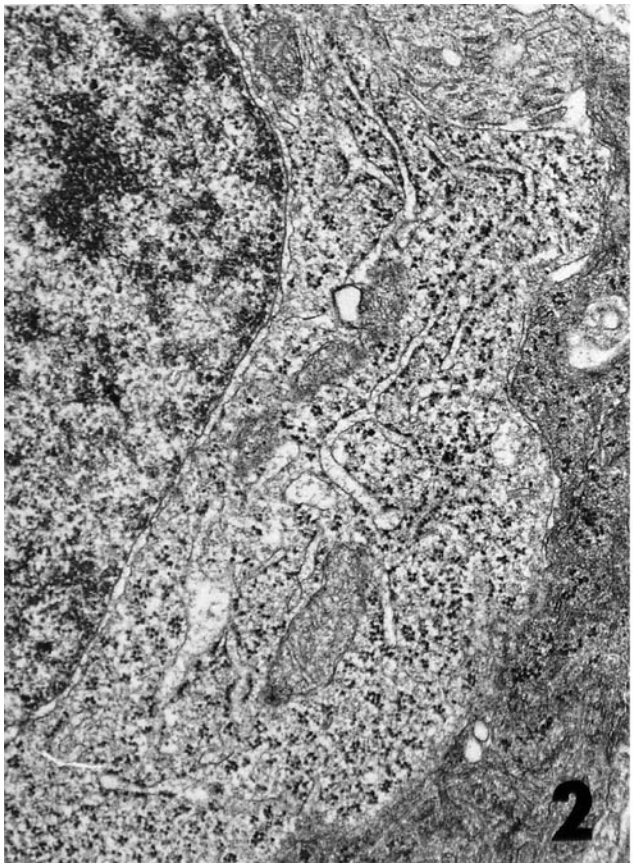
Statistical analysis was performed using one-way analysis of variance and Duncan test.

Results

Qualitative study

Newborn sheep. In the pineal glands of the newborn sheep, two types of pinealocytes could be distinguished based on their electron density – light (46.6% of pinealocytes) and dark (53.4% of pinealocytes) cells (Fig. 1). Regardless of the electron density the ultrastructure of both cell types was similar. The pinealocyte nuclei were oval or irregular in shape. The





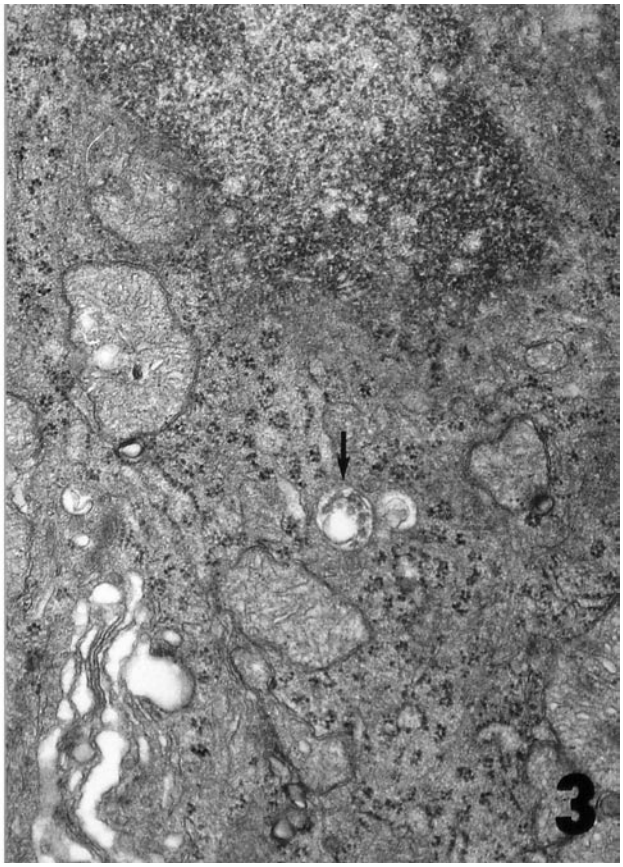


Fig. 1. Pineal parenchyma of the newborn lamb. Presence of light and dark pinealocytes. x 9,000.
Fig. 2. Part of pinealocyte cell body of the newborn lamb. Presence of very well developed granular endoplasmic reticulum and polysomes. x 20,300.
Fig. 3. Part of pinealocyte cell body of the newborn lamb. Presence of well developed Golgi apparatus, mitochondria and numerous polysomes. Arrow – multivesicular body. x 20,300.
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Fig. 4. Part of pinealocyte cell body of the 10-week old sheep. The cytoplasm with numerous vesicles and short cisterns of smooth endoplasmic reticulum as well as abundant microtubules. x 20,300.

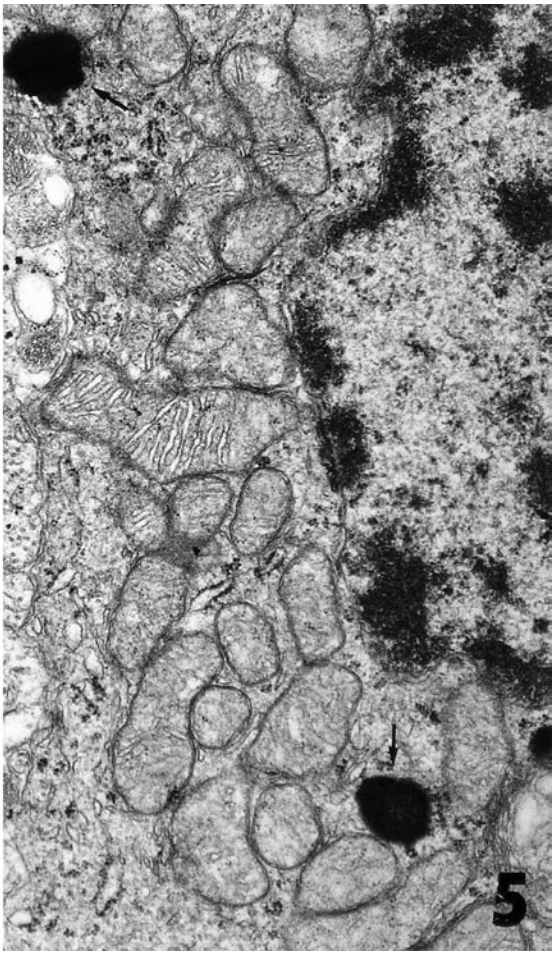

Fig. 5. Part of pinealocyte cell body of the 10-week old sheep. Accumulation of mitochondria in the close vicinity of the pinealocyte nucleus. Lipid droplets – arrows. x 20,300.

cytoplasm surrounded the nucleus in a form of thin rim as well as usually formed one or two accumulations. The characteristic feature of the pinealocyte cytoplasm in the newborn sheep was the presence of well developed rough endoplasmic reticulum and numerous polysomes (Fig. 2, 3). The cytoplasm contained also vesicles and cisterns of smooth endoplasmic reticulum, one, two or three profiles of Golgi apparatus, mitochondria with well developed cristae, lysosomes and microtubules. The lipid droplets, dense core vesicles and multivesicular bodies were sporadically observed.

10-week old (immature) sheep Like in the newborn animals, the parenchyma of the pineal gland in the 10-week old sheep was formed by light (40.1%) and dark (59.9%) pinealocytes. The pinealocytes were irregular in shape, showing cytoplasmic processes emerging from their perikarya. The pinealocyte nuclei were oval with sparse heterochromatin usually clamped around the nuclear envelope. The well developed cytoplasm was characterized by the presence of numerous vesicles and short cisterns of smooth endoplasmic reticulum as well as numerous microtubules (Fig. 4). The rough endoplasmic reticulum was not very prominent. The pinealocytes contained also numerous mitochondria, whose shape and density of matrix varied between cells, well developed Golgi apparatus (2-3 profiles) with moderate number of small electron-lucent vesicles and single lysosomes. The accumulations of mitochondria were often observed in close vicinity to the nuclear envelope (Fig. 5). The lipid droplets with a diameter of 0.5 - 1 μm were frequently noted. The pinealocytes contained also dense core vesicles and multivesicular bodies.

One-year old (sexually mature) sheep The parenchyma of the pineals in the one-year old sheep contained light (43.5 %) and dark (56.5 %) pinealocytes. General appearance of pinealocytes was similar to that in the 10-week-old animals (Fig. 6, 7). The characteristic feature of the pinealocytes in the adult sheep was a very well developed Golgi apparatus (4 - 6 profiles) with a large number of small electron-lucent vesicles (Fig. 8). The pinealocytes contained also numerous dense core vesicles and lipid droplets with a diameter of 1 - 3μm.

Quantitative study

The relative volume of mitochondria in pinealocytes was significantly higher in the 10-week old sheep than in the newborn and sexually mature animals (Fig. 9). The relative volume of rough endoplasmic reticulum was significantly higher in pinealocytes of the newborn sheep than two other investigated groups (Fig. 9). The relative volume of Golgi apparatus in pinealocytes was significantly higher in the one-year old animals

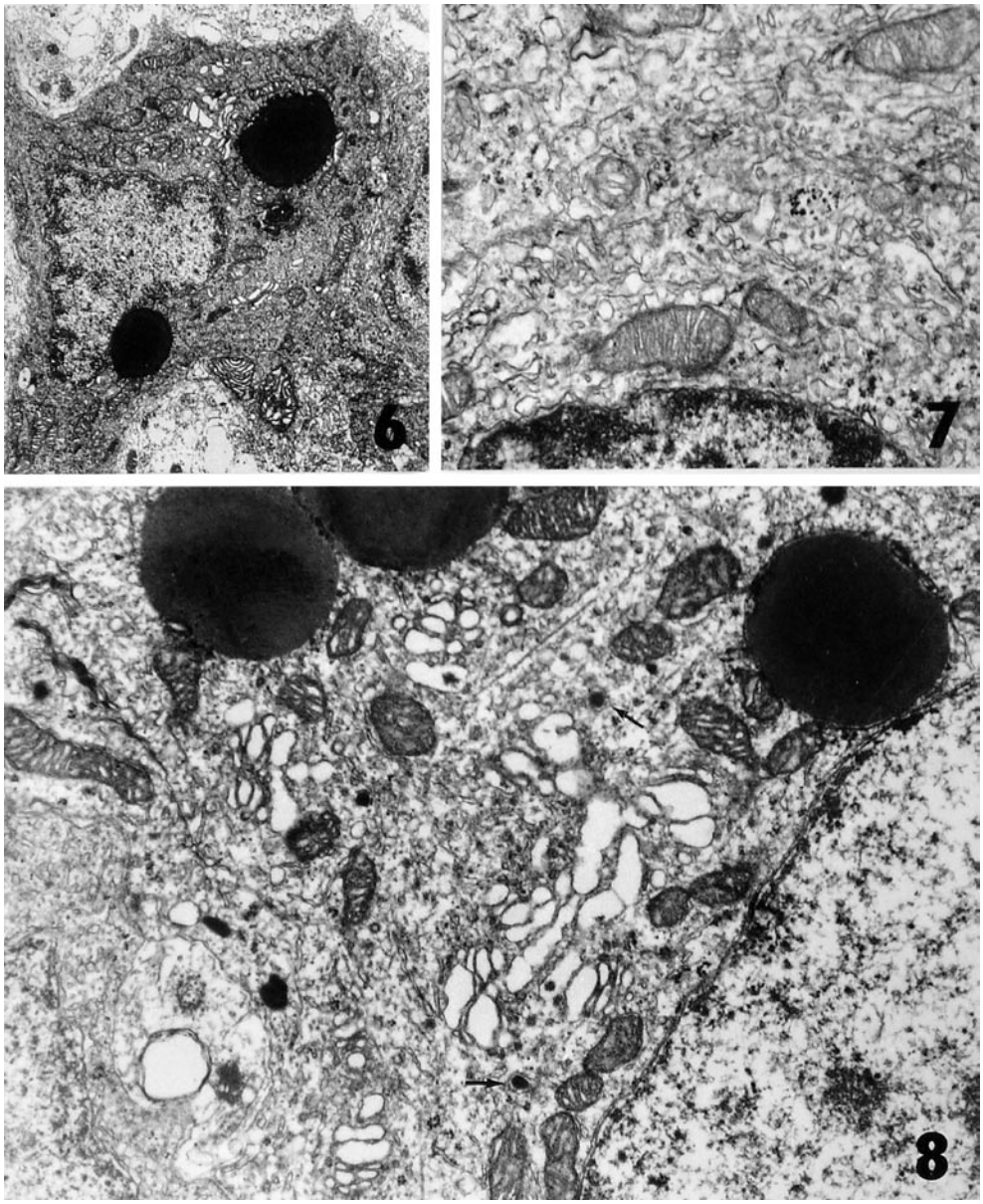

Fig. 6. Pinealocyte of the one-year old sheep. Irregularly shaped, cytoplasm-rich cell body with outgrowing processes. x 3,000.
Fig. 7. Part of pinealocyte cell body of the one-year old sheep. The cytoplasm with numerous vesicles and short cisterns of smooth endoplasmic reticulum as well as sparse polysomes. x 30,000.
Fig. 8. Part of pinealocyte cell body of the one-year old sheep. Presence of very well developed Golgi apparatus, large lipid droplets and dense core vesicles (arrows). x 20,300.

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than in the newborn and 10-week old sheep (Fig. 9). No significant differences were observed in the relative volume of lysosomes in pinealocytes (Fig. 9). The relative volume of lipid droplets as well as the numerical density of multivesicular bodies and dense core vesicles increased significantly with age (Fig. 9).

Discussion

The obtained results indicate that the ultrastructure of ovine pinealocytes changes markedly during the investigated period of the postnatal development. The important changes concern the majority of cytoplasmic components including rough and smooth endoplasmic reticulum, mitochondria, Golgi apparatus, dense core vesicles, multivesicular bodies, microtubules and lipid droplets.

The rough endoplasmic reticulum was the predominating structure in the cytoplasm of pinealocytes in the newborn lambs. In older sheep the relative volume of the rough endoplasmic reticulum in pinealocytes was significantly lower than in the newborn ones and did not differ between the 10-week old and adult animals. The presence of well developed rough endoplasmic reticulum in pinealocytes of the newborns is probably related to the intensive synthesis of membrane proteins during the cell growth and formation of other cell organelles including the smooth form of endoplasmic reticulum. Abrams et al. [35] reported very intensive leucine incorporation into proteins in the pineal body, brain stem and hypothalamic nuclei comparing to the other parts of brain in the newborn lambs (1 - 5 days of age). Similarly, very well developed rough endoplasmic reticulum in pinealocytes of newborns as well as the decrease in its relative volume in older animals were also observed in the domestic pig [2]. Bayerova and Malinsky [1] found in rat pinealocytes that the relative amount of rough endoplasmic reticulum and ribosomes increased strikingly between birth and 10 days, and then remained fairly constant to the adulthood.

According to our results, the developmental changes of the smooth endoplasmic reticulum in ovine pinealocytes show opposite course to those described for the rough form. The smooth endoplasmic reticulum was sparse in the newborn lambs and very

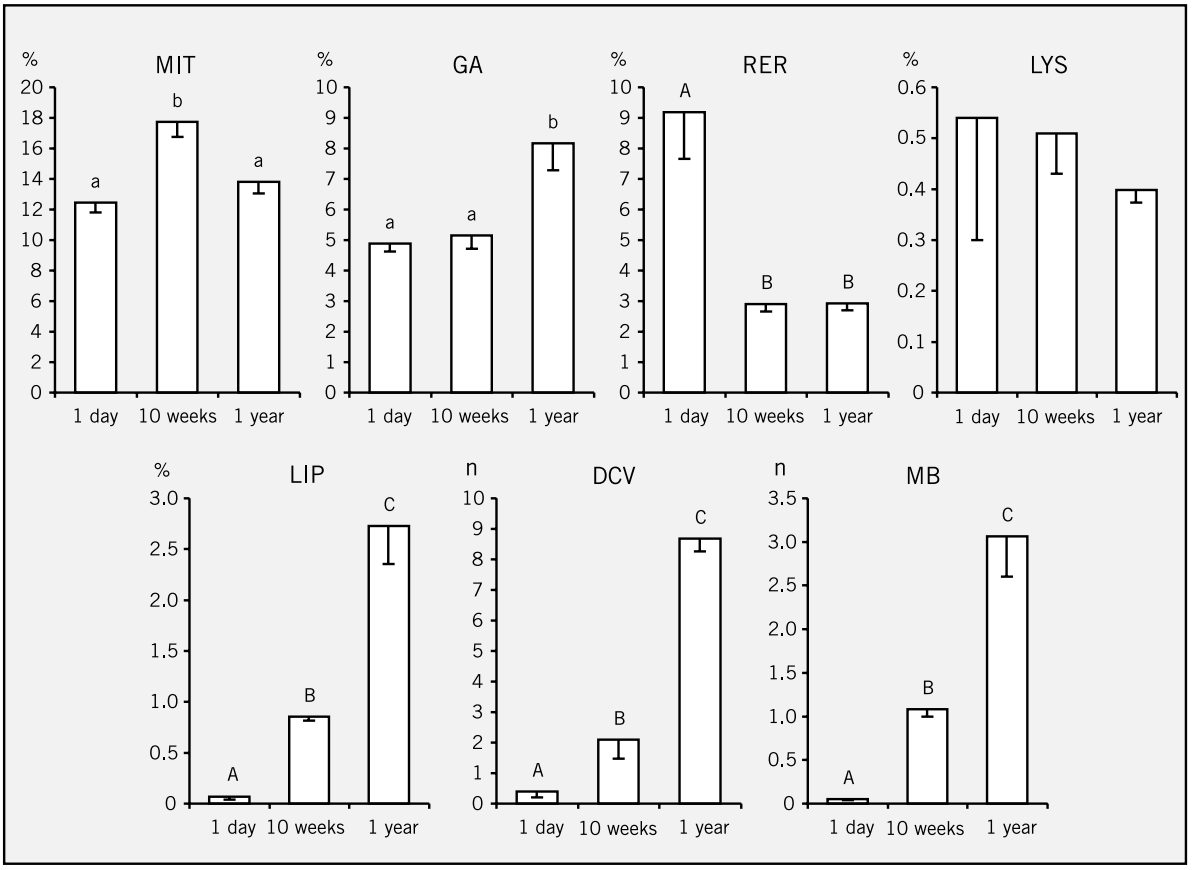

Fig.9. The mean (±SEM) relative volume of mitochondria (MIT), Golgi apparatus (GA), rough endoplasmic reticulum (RER), lysosomes (LYS), lipid droplets (LIP) as well as the mean numerical density (±SEM) of multivesicular bodies (MB) and dense core vesicles (DCV) in the pinealocyte cell bodies of the sheep at the age of 1 day, 10 weeks and 1 year. The values signed with different letters are significantly different (small letters - at $p \leq 0.01$, capital letters - at $p \leq 0.001$) from each other.

well developed in the pinealocytes of the 10-week old and mature sheep. The obtained results agree with the observations of Anderson [36], who described the pinealocyte of adult sheep as the cell having more developed smooth endoplasmic reticulum than the rough one. Moreover, in our previous study concerning the ultrastructure of pinealocytes during breeding season in adult rams, we observed well developed smooth endoplasmic reticulum and only small amount of single cisterns of the rough one [37]. In contrast, the opposite data were reported by Regodon et al. [7]. They described more abundant rough form of endoplasmic reticulum than the smooth one in pinealocytes of sheep at the age from 9 months to 2 years as well as more developed rough reticulum in this group comparing to animals from 1 to 6 months. However, the authors did not apply quantitative methods to objective these observations.

During the postnatal development of ovine pinealocytes the relative volume and distribution of mitochondria undergo age-dependent changes, whereas the mitochondrial inner structure and the matrix electron density do not change. The relative volume of mitochondria in pinealocytes was significantly higher in the 10-week old sheep than in two other investigated groups. A prominent feature of pinealocytes in the 10-week old sheep was the presence of mitochondria accumulation near-by the pinealocyte nucleus. The studies dealing with the developmental aspect of the mitochondria in mammalian pinealocytes have been performed in few species. In rat pinealocytes mitochondria increased up to 28 days of age and then slightly decreased [1]. On the other hand, in the domestic pig no significant changes in the relative volume of mitochondria in pinealocytes were observed from birth to the age of 7 months [2]. Then, a decrease in the relative volume was observed in 8-month old pigs, what was correlated with puberty of the animals [2]. Regodon et al. [7] in the paper dealing with postnatal changes in the pinealocyte ultrastructure noted well developed mitochondria in a group of sheep in age from 9 months to 2 years.

Our results showed the significantly higher relative volume of Golgi apparatus in the one-year old sheep compared to the 10-week old animals and the newborn ones. A more developed Golgi apparatus was also noted by Regodon et al. [7] in the adult sheep (9 months to 2 years of postnatal live) in comparison to the younger ones (1 to 6 months of postnatal life), however no quantitative methods were used in this study. An increase in the relative amount of Golgi apparatus in the pinealocytes during sexual maturation was not observed both in the rat [1] and the domestic pig [2].

The significant increase was noted in the numerical density of dense core vesicles during the postnatal development of ovine pinealocytes. Dense core vesicles were intensively studied in many species, because they are considered as the structures formed by Golgi appa-

ratus and involved in one (called neurosecretory-like) of the secretory processes occurring in pinealocytes [38, 39, 40]. The number of dense core vesicles in mammalian pinealocytes differs significantly among species [38, 39, 40, 41, 42]. In rodents as well as in the domestic pig the formation and/or transformations of dense core vesicles seem to be controlled by light conditions as well as by the sympathetic innervation [41, 43, 44, 45, 46, 47]. The study of quantitative changes of dense core vesicles in pinealocytes during postnatal development has been performed in the domestic pig [2]. Its results showed the highest numerical density of dense core vesicles on the first day of postnatal life and then the decreased, relatively stable value up to the age of 8 months.

Multivesicular bodies were another component of ovine pinealocytes, which showed changes during the investigated period of sheep life. The increase in their numerical density with age was observed. Hitherto, multivesicular bodies of mammals pinealocytes have not been intensively investigated. These structures are commonly observed in pinealocytes of the rat, the hamster, the ground squirrel and rarely in the guinea pig [41]. The quantitative changes of multivesicular bodies under experimental conditions were reported in the hamster [45] and the domestic pig [46]. In the last species the number of multivesicular bodies decreases after the administration of sympathicolytic drugs [46] and the exposition to continuous illumination [47] and it is negatively correlated with the relative volume of membrane bound bodies, which are specific structures of swine pinealocytes [46, 47]. Moreover, multivesicular bodies showed also some changes during the postnatal development of the pig, with the decrease at the time of puberty [2]. The present data point to the opposite tendency in the ovine pineal gland.

The lipid droplets are typical structures of ovine pinealocytes [7, 36, 37, 42]. In the present study the lipid droplets were observed in ovine pinealocytes from the first day of postnatal life. Their relative volume in pinealocytes increased with age. The amount of lipid droplets differs significantly among mammalian pinealocytes being high in the rat, the ruminants and the horse, moderate - in the mouse and the Syrian hamster, very low - in many other species including the gerbil and the pig [2, 41, 42]. The functional significance of the lipid droplets in mammalian pinealocytes remains unknown, but in some species these inclusions were suspected of being involved in the secretory activity of these cells [41].

In the present study no significant differences between the groups were noted in the relative volume of lysosomes in pinealocytes. According to our best knowledge, quantitative studies of lysosomes during the postnatal development of pinealocytes have been performed only in the domestic pig so far [2]. Their results showed some fluctuations of the relative volume of these structures during the postnatal life.

The population of pinealocytes in investigated sheep consisted of light and dark cells due to the differences in the electron density of the cytoplasm. The percentage of light and dark pinealocytes did not change markedly during the postnatal development of the sheep pineal gland. The existence of these two cell forms was observed in pineals of several mammalian species and it was widely discussed [48]. The most often, the presence of light and dark pinealocytes was interpreted as a morphological picture of two different functional stages of pinealocytes. Our earlier studies revealed only slight differences in subcellular organization of both cell types in gilts [48] and rams [49].

The *in vitro* studies have demonstrated that the ovine pineals taken from the fetuses during the late gestation as well as from the lambs at the age of 4 – 8 days secrete melatonin and response with increased release of this hormone to adrenergic stimulation [11]. However, the pineals from the lambs at the age of 4 – 8 days produce 9–10-fold more melatonin before and during the adrenergic stimulation than the fetal glands. *In vivo*, during the first two weeks of postnatal life the nocturnal increase in plasma melatonin level in the sheep is very low and occurs not in all individuals [9, 10]. The diurnal rhythm of melatonin secretion develops during the following weeks of life, what is visible both in the marked increase in the nocturnal secretion of this hormone and in the decrease of daytime melatonin level in blood plasma [9, 10]. The first 10 weeks of postnatal life are considered as a period of stabilization of the diurnal rhythm of melatonin secretion in the sheep [9, 10, 33]. Redondo et al. [9] showed significantly higher plasma melatonin level (both during day and night) in the group of animals at the age of 9 - 24 months than in the group of 1 - 6 months old sheep. In agreement with the data concerning melatonin secretion, the results of electron microscopic studies show the presence of marked differences in structure of pinealocytes between the newborn and 10-week old sheep. Our observations point to this period as the time of intensive developmental changes, concerning structures connected with both general and secretory activity of the pinealocytes. There are also some modifications of the pinealocyte ultrastructure, which suggest enhancement of their secretory activity between the age of 10 weeks and one year. These changes may be related to the sexual maturation and the role of the pineal gland in this process.

Summing up, our study described the qualitative and quantitative changes in pinealocyte ultrastructure occurring during the postnatal development of the ovine pineal gland. Between the birth and the age of 10 weeks we noted the increase in smooth endoplasmic reticulum, mitochondria, Golgi apparatus (only qualitative changes), dense core vesicles, multivesicular bodies, microtubules and lipid droplets. Simultaneously the reduction of rough endoplasmic reticulum was found. From the age of 10 weeks to the sexual maturity an intensive development of Golgi apparatus, slight decrease in the relative volume of mitochondria as well as further increase in dense core vesicles, multivesicular bodies and lipid droplets took place. The ultrastructural modifications are somehow related to the changes in melatonin secretion reported

during the ovine postnatal development. The comparison of the results obtained in the sheep to those found in other previously investigated mammals shows also the existence of some interspecies differences in the developmental processes occurring in pinealocytes.

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