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Pineal concretions in turkey (*Meleagris gallopavo*) as a result of collagen-mediated calcification

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Summary. The intra-pineal calcification is a well-known phenomenon in mammals, however it is almost completely unknown in birds. The aim of the present work was to analyze morphology and genesis of the pineal concretions in the turkey. The studies were performed on the pineals collected from one-year-old turkeys (*Meleagris gallopavo)*. In addition to standard morphological methods, the alizarin red S and potassium pyroantimonate methods were employed for localization of calcium at the light and electron microscopy level. In light microscopy, calcified concretions with diameters from 300 µm to 2 mm and quantities from 3 to 6 per gland were observed in all the examined pineals. They were stained red with alizarin S and showed the presence of collagen in Mallory's staining. Two types of cells were noted inside the concretion: polygonal and elongated ones. Using electron microscopy, three parts were distinguished within the calcification area. The peripheral part contained densely packed collagen fibrils, some elongated cells and numerous pyroantimonate precipitates demonstrating the presence of calcium ions. In the intermediate part, the fibrils were covered by almost continuous sheets of pyroantimonate precipitates and fused side by side. The central part showed an appearance of calcified hard tissue and contained some polygonal (osteocyte-like) cells. The obtained data demonstrated that the formation of the pineal concretions in the turkey is associated with the mineralization of collagen. This process is completely different from the mechanisms responsible for the formation of the concretions in the mammalian pineal.

Key words: Birds, Turkey, Pineal organ, Concretions

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Introduction

Calcification inside the pineal gland is a well-known phenomenon in mammals. Mineral concretions are present in the pineals of many mammalian species (including human), however, their incidence varies considerably between species as well as among individuals (Welsh, 1985; Lewczuk et al., 1994; Vigh et al., 1998). The formation of concretions is considered to be related to the ageing processes, because the size and number of the acervuli usually correlate with the age of the subject (Humbert and Pevet, 1995a,b; Schmid and Raykhtsaum, 1995; Humbert et al., 1997; Vigh et al., 1998; Swietoslawski, 1999). Nevertheless, concretions may be present in young individuals as well.

Two types of calcified concretions are distinguished in mammalian pineals: 1) the meningeal acervuli, which are present in the connective tissue covering the gland and forming the septa; 2) the intraparenchymal acervuli, whose genesis is strongly related to the function of pinealocytes (Welsh, 1984; Krstic, 1986; Vigh et al., 1989, Lewczuk et al., 1994). The mechanisms of pineal acervuli formation are not well-known, although they should be considered as multifarious and multifactor.

The process of calcification in the pineal organ was not observed in lower vertebrates: fish, amphibians and reptiles. Among avian species, calcium concretions located in the choroid plexus have been found in ducks and geese (Fejer et al., 2001). These concretions showed, as in mammals, a prominent concentric lamination. Up till now, the turkey is the only avian species in which calcified concretions have been found inside the pineal organ (see below). Their structure and genesis have not been studied in detail.

The pineal organ of the turkey is a follicular organ consisting of a narrow proximal part and a club-shaped top. In young birds, the follicular wall is formed by elongated cells bordering the lumen and sparse,

peripherally localized round cells. This pseudo-stratified organization is transformed during ontogenesis into a thick, multilayer structure. In addition to the changes in the follicular architecture, a considerable age-dependent increase in the connective tissue septa subdividing the parenchyma and separating the follicles is observed (Przybylska-Gornowicz et al., 2005).

In a previous study concerning the post-hatching development of the turkey pineal organ, it was found that the calcified concretions are present in the pineal organs of 56 week-old birds (Przybylska-Gornowicz et al., 2005). They were never observed in the pineals of younger turkeys. The total number of concretions ranged from 3 to 6 per organ. The concretions were located in the parenchyma. It should be emphasized that they were not observed in the connective tissue of the capsule or in the choroid plexus.

The aims of the present study were to characterize more deeply the morphological features of the mineral concretions in the turkey pineal organ, as well as to recognize the mechanisms of their creation. The investigations performed at the level of light and electron microscopy enabled a description of 1) the unusual internal structure of the concretions, which were composed by calcified collagen fibers and osteocyte-like cells, 2) the process of their formation as an effect of the mineralization of the collagen fibers accumulated inside the follicles.

Materials and methods

Animals

The studies were performed on eight males and eight females of the domestic turkey (*Meleagris gallopavo*) at the age of 56 weeks. The birds were anesthetized with halothane and killed by decapitation between 08:00 and 09:00. The pineal organs with adjacent parts of the brain were immediately removed and prepared for the investigations.

Light microscopy

6 pineal organs were fixed in Bouin's liquid and 6 were fixed in 70% ethanol and prepared for paraffin histology by using standard techniques. The specimens were cut in a sagittal plane into consecutive 7-µm-thick sections using a Reichert microtome. The sections of the tissues fixed in Bouin's liquid were stained with hematoxylin-eosin and with Mallory's trichrome method (Gabe, 1976). The sections of the alcohol-fixed pineals were stained in turns with the Alizarin red S procedure for histochemical demonstration of calcium (McGee-Russell, 1958) and with Mallory's procedure. As a control, the sections decalcified by incubation in 10% solution of EDTA for 5 hours were used. Additionally, the semi-thin sections of Epon-embedded tissue (prepared for electron microscopy) were stained with toluidine blue and examined using light microscopy.

Electron microscopy

The potassium pyroantimonate method (PPA) was used for ultra-structural localization of calcium ions (Wick and Hepler, 1982). Four pineal organs were quickly removed, divided into small pieces and fixed in a solution containing 2% glutaraldehyde, 2% potassium pyroantimonate and 0.735 % potassium acetate for 2 hours at 4°C (pH 7.5). After the first step of fixation, the pieces were rinsed three times (10 min each) with 0.735% potassium acetate in distilled water and incubated for 2 hours in 1% aqueous solution of osmium tetroxide containing 2% potassium pyroantimonate and 0.735% potassium acetate. Next, the pieces were washed in potassium acetate solution to remove any unreacted potassium pyroantimonate and to prevent nonspecific precipitation, they were dehydrated and embedded in Epon 812. Both contrasted with lead citrate and uranyl acetate as well as uncontrasted ultra-thin sections were examined in TEM. As a control for the presence of calcium in precipitates, ultra-thin sections were decalcified by incubation in 13.5% EDTA at 60°C for 2 hours.

Results

Light microscopic study

The pineal organs of one-year-old turkeys were composed of follicles with diameters ranging from 100 to 300 µm. The follicular wall contained one layer of short columnar cells limiting the lumen and several layers of oval cells forming the peripheral part of the follicle. Thick connective tissue septa originated from the pineal capsule and surrounded the follicles (Fig. 4).

Mallory's trichrome stain demonstrated numerous collagen fibres and their bundles in the septa and the capsule. Frequently, thin collagen fibres branching from the bundles located in the septa and penetrating into the follicles were observed. The follicles showed variable density of the collagen fibres. In some cases, the follicles with a high degree of penetration by the collagen fibres were found. Their cellular architecture was destroyed - numerous or almost all cells forming the follicular wall disappeared. The follicles dominated by collagen showed a whorl-like arrangement of the fibres. Some cells, with an appearance atypical for the pineal follicles cells, were noted between the collagen fibres of the whorl-like structures. Two types of these cells were distinguished: 1) polygonal cells randomly distributed in the central part of the whorl and 2) elongated cells usually present in the peripheral part of the whorl (Fig. 1).

Staining with Alizarin red S method revealed the presence of the calcified concretions in all examined pineal organs. They were round in shape and their diameters varied from 300 µm to 2 mm. The number of concretions ranged from 2 to 6 per pineal. They were localized randomly or created aggregates composed of

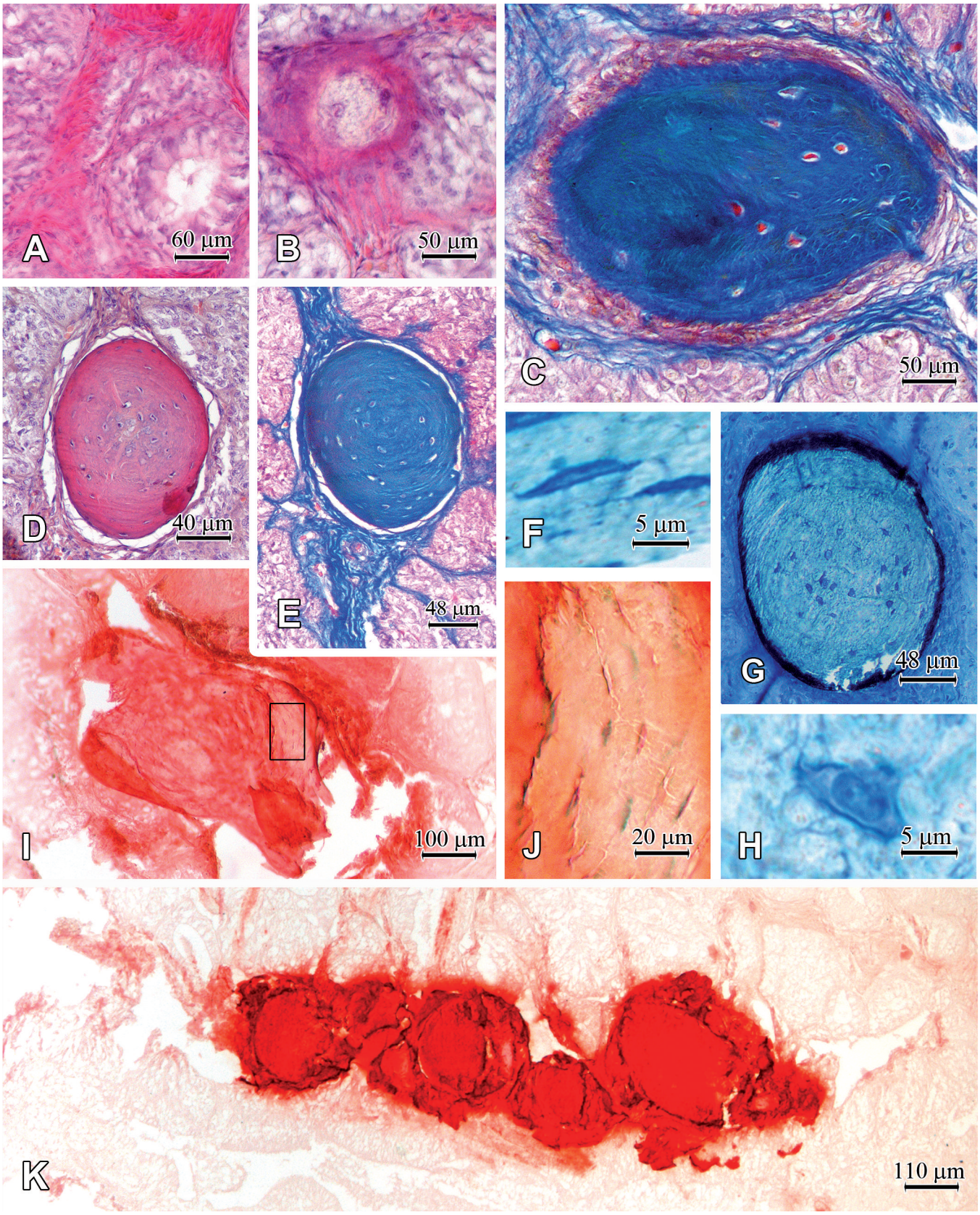

Fig. 1. Histological structure of the concretions in the turkey pineal organ. **A, B.** The early stages of concretion development (HE staining); note the presence of numerous fibers surrounding the follicle and their penetration into the follicle **(A)** as well as the circular arrangement of fibers stepwise replacing the follicle **(B)**. **C.** The late stage of the concretion formation (Mallory staining); note the collagen creating the organic base of the concretion and the presence of cells inside the whorl-like collagen structure and around it. **D, E.** The mature concretions stained with HE **(D)** and Mallory's method **(E)**. **F-H.** Two types of cells occurring in the concretion (semi-thin section stained with toluidine blue): elongated cells **(F)** and polygonal cells **(H)**. **I, J.** The presence of calcium in the concretions demonstrated with Alizarin red S method; note the characteristic appearance of cells located in the concretion (**J** - the part of fig. I in higher magnification) and the damage to the peripheral part of concretion resulting from the fragility of mineralized tissue. **K.** The group of calcified concretions in the pineal gland (Alizarin red S method).

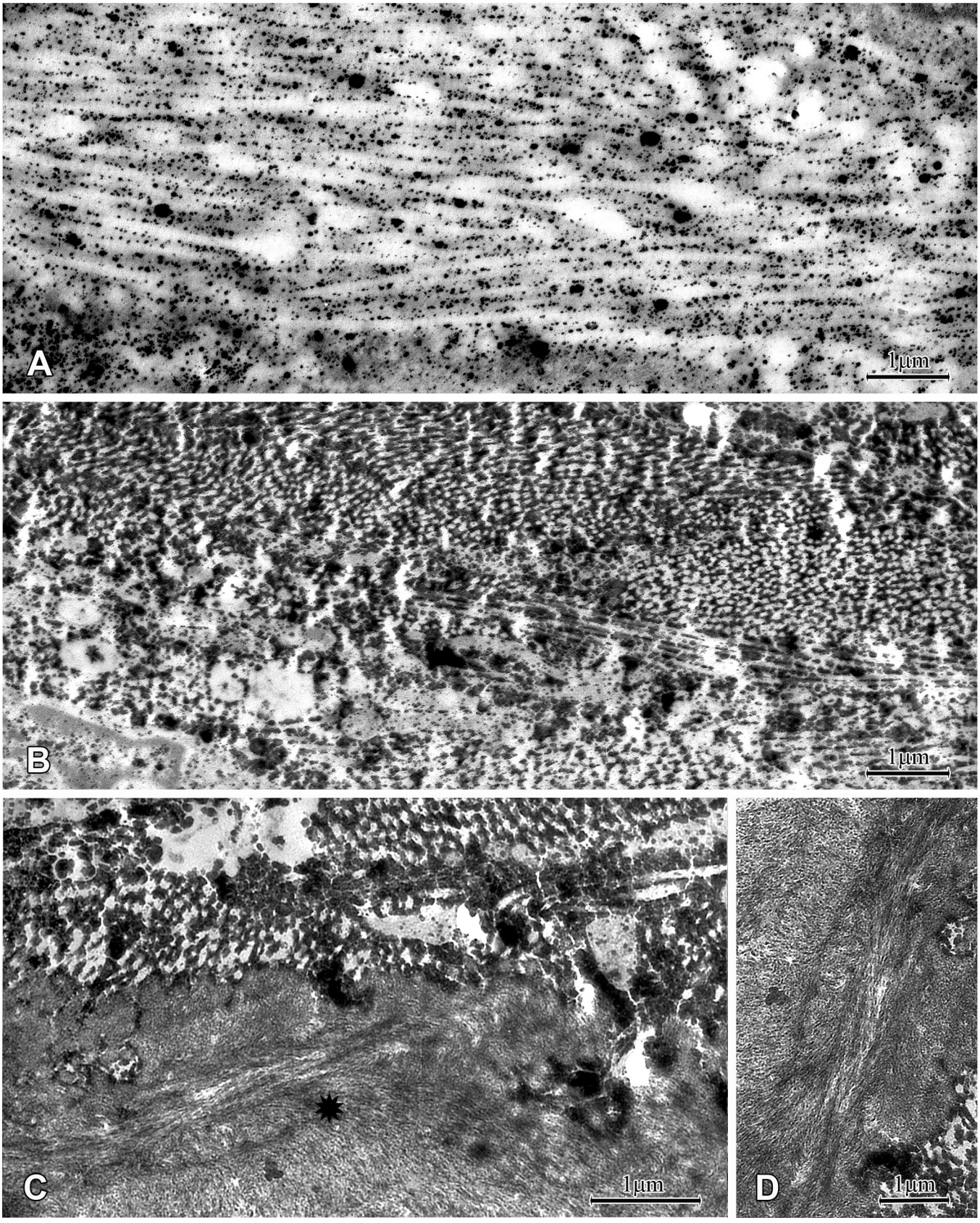

Fig. 2. Electron micrographs of the calcification area. The tissue prepared using the PPA method enabling the location of calcium ions in the form of electron dense precipitates. **A.** The peripheral part of the calcification area. Note collagen fibrils (lacking banding patterns) with numerous pyroantimonate precipitates attached along them. **B.** The intermediate part of the calcification area showing densely packed collagen fibrils distributed in groups running in different directions. Extremely numerous pyroantimonate precipitates created continuous sheets covering the collagen. **C.** The intermediate and central (*) parts of the calcification area. Note the numerous precipitates in the intermediate part and their lack in the central part. **D.** The central part of the calcification area showing mineralized collagen fibrils with an appearance typical of hard tissues.

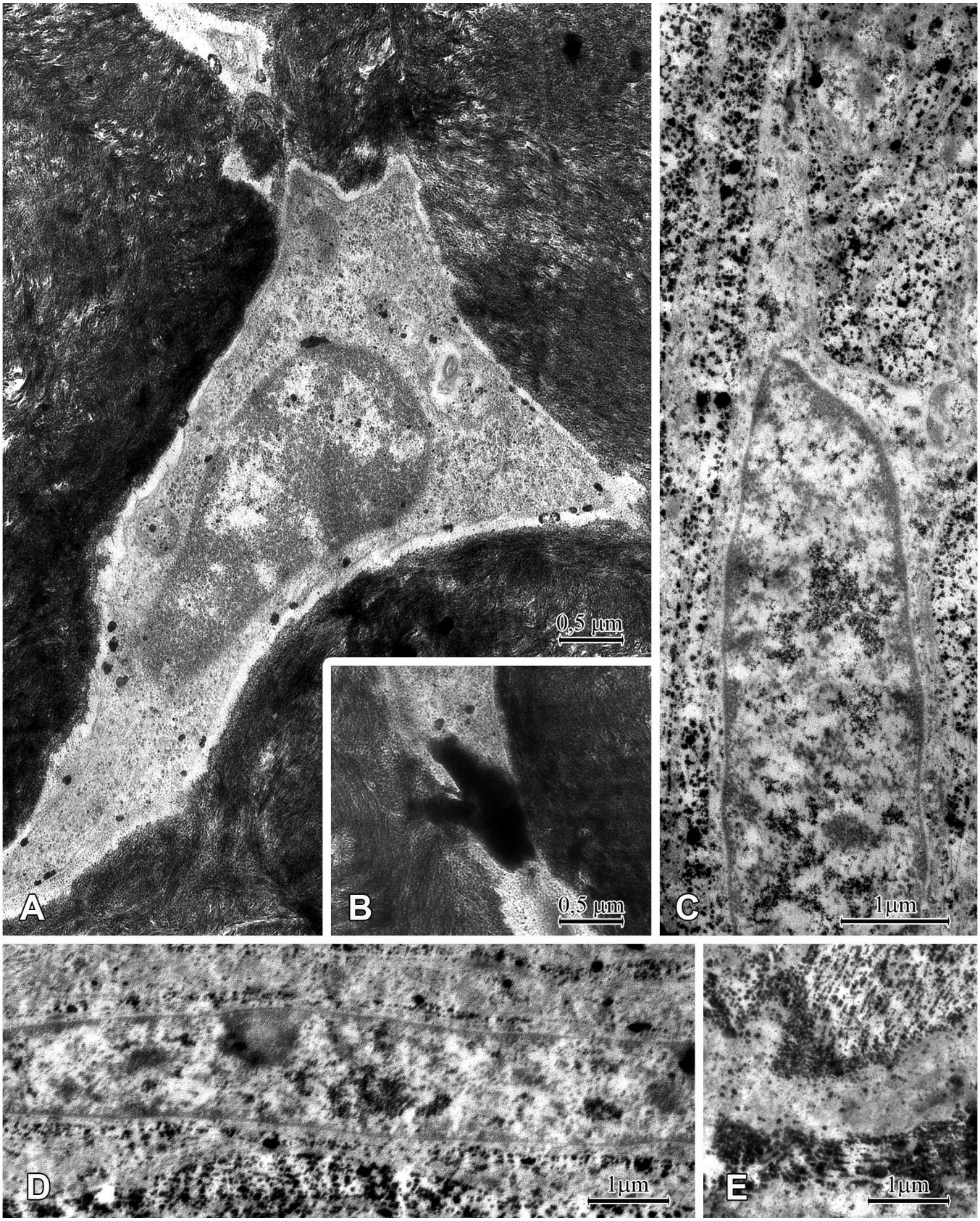

Fig. 3. Ultrastructure of cells located in the calcification area (fixation with the PPA method). **A.** The osteocyte-like cell surrounded by mineralized collagen fibrils in the central part of the calcification area. Note the "halo" around the cell and large pyroantimonate precipitated located mainly beyond the cell membrane. **B.** The junction of processes of osteocyte-like cells. **C.** A cell showing a fibrocyte-like appearance in the peripheral part of the calcification area. Note the extra-cellular matrix containing collagen and calcium deposits. **D.** Numerous calcium precipitates in the intercellular spaces in the peripheral part of the calcification area. **E.** The cell process with scattered deposits in the middle part of the concretion. Note the adjacent extra-cellular matrix rich in collagen and pyroantimonate precipitates.

3-5 connected structures. The intensity of staining with the Alizarin S method varied between concretions (Fig. 1).

An analysis of serial sections, stained by turns with the Alizarin red S method and Mallory's procedure has demonstrated without any doubt that the collagen whorls replacing the follicles are identical to the calcified concretions (Fig. 1).

Electron microscopic studies

The areas of calcification were easily identified due to presence of numerous pyroantimonate precipitates, as well as the electron-dense, mineralized collagen.

The distribution of the collagen fibres and fibrils, cells and pyroantimonate precipitates differed between the peripheral, intermediate and central parts of the concretions.

The peripheral part of the calcification area contained a complex assembly of the collagen fibrils with different directionality. The fibrils tended to fuse side by side, possessed clear contours, but did not show apparent banding patterns. Their diameters varied from 60 to 140 nm. The pyroantimonate precipitates attached to the collagen fibrils along their long axis. The deposits were usually small with diameters of 20-35 nm, but large precipitates, ranging from 70 to 200 nm in diameter were also observed. Small precipitates were distributed with a repeated periodicity of about 70 nm (mostly) or about 140 nm (in some stretches of collagen fibrils). The cross-sections through the collagen demonstrated that small precipitates surrounded the fibrils. Large precipitates were randomly dispersed (Fig. 2).

In the intermediate part of the calcification area, the collagen fibrils closely fused side- by-side. The contours of the fibrils were poorly visible. The pyroantimonate deposits were extremely numerous. They were distributed along the collagen fibrils and created almost continuous strips closely attached to the fibril. A repeated periodicity of deposit distribution did not appear. On the cross sections through the collagen, the distribution pattern of precipitates resembled a dense network with round or oval meshes. The deposits were also present inside the collagen fibrils, but the fibril interiors showed a lower density of precipitates than the exteriors (Fig. 2).

The central part of the calcification area contained densely packed, mineralized collagen with an appearance typical of hard tissues. This area showed high electron density due to the presence of small, needle-like crystals. The pyroantimonate precipitates were infrequently noted in the central part of the calcification area.

Ultra-structural examination of the pineal tissue confirmed the presence of two types of cells in the calcification area (Fig. 3). The first type of cells, separated from the mineralized collagen by a “halo” (80 nm wide), was observed in the central part of this area. These were polygonal in shape and formed thin processes. Cells of this type were characterized by numerous polysomes, few round mitochondria and a system of processes filled by filaments. They contained a small number of pyroantimonate precipitates (with diameter from 40 to 80 nm) present in vesicles or tubules of smooth endoplasmic reticulum located close to the plasma membrane, in mitochondria and the nucleus. The cells of the second type were present in both the intermediate and the peripheral parts of concretions. They were elongated and distributed parallel to the long axes of the collagen fibres. They were not surrounded by

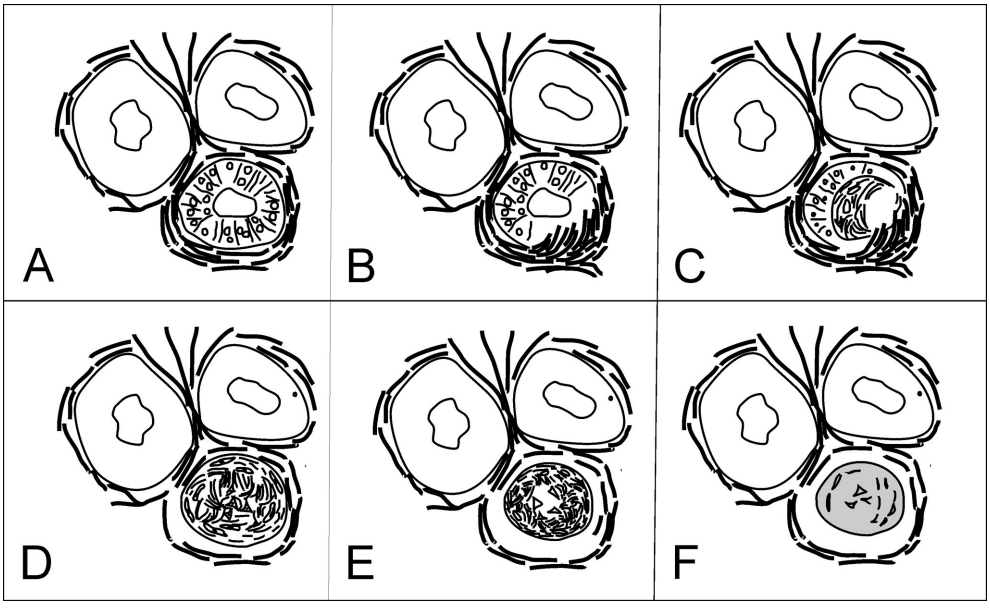

Fig. 4. Schematic representation showing putative steps of the concretion formation in the turkey pineal organ. Notice the increase in the collagen, progressive atrophy of follicle, specific arrangement of the organic matrix as well as the appearance of specific cells. **A.** The thickening of the connective tissue septa around one of the follicles. **B.** The penetration of collagen from the connective tissue septa into the follicle wall. **C.** The increase in the collagen fibers inside the follicle leading to the creation of whorl-like structure; pinealocytes and supporting cell forming the follicular wall degenerate. **D.** The bundles of collagen fibers dominated the follicle; two types of intra-concretion cells are visible. **E, F.** The adult concretion showing the characteristic appearance of collagen in Mallory's staining **(E)** and the positive reaction to calcium in the Alizarin red S method **(F)**.

a “halo”. These cells possessed typical organelles, including well-developed granular endoplasmic reticulum and a variable number of pyroantimonate deposits both in cytoplasm and the nucleus. Some of them displayed signs of degeneration.

Discussion

Previous studies have shown the existence of crucial alternations in the morphology of the turkey pineal organ during the first year of post-hatching life (Przybylska-Gornowicz et al., 2005). The developmental changes affect many aspects of pineal morphology, including organ size, the attachment of the pineal stalk to the intercommissural region, the structure of pineal parenchyma and stroma as well as the appearance of calcium concretions. The current study confirms that the concretions are regularly occurring (2-6 per gland) components of the pineals of one-year-old male and female turkeys. The most important finding of the present investigation is the statement of fundamental differences in structure and genesis between the pineal concretions occurring in the turkey and those described in mammals (Lewczuk et al., 1994; Humbert et al., 1997; Vigh et al., 1998).

The process of calcification inside the mammalian pineal gland has been known and investigated for many years, although the formation of concretions is still poorly understood. Several mechanisms have been proposed as being responsible for pineal calcification (Vigh et al., 1998). A coherent and convincing hypothesis has been proposed concerning the creation of intraparenchymal concretions in the gerbil pineal gland (Welsh, 1984; Krstic, 1986). The suggested mechanism involves the accumulation of calcium ions in vacuoles inside some pinealocytes, which leads to cell degeneration and formation of the concretions. However, the intracellular creation of the pineal acervuli is very rare in mammals or even specific for the gerbils. A much more commonly occurring mechanism involves extra-cellular calcium ions, which may precipitate and form the concretions. This process leads to the creation of concretions in the connective tissue of the pineal gland (Vigh et al., 1989, 1998; Humbert and Pevet, 1991; Lewczuk et al., 1994). However, the detailed mechanisms of this phenomenon are unknown.

It was found that the formation of the pineal concretions in the turkey pineal organ is completely different. The comparison of the sections stained with Mallory's procedure, hematoxylin & eosin and Alizarin methods leads to the conclusion that this process involves two groups of events: 1) formation of organic matrix containing mainly the collagen fibers and 2) accumulation of calcium and the creation of insoluble salts.

The results of light microscopic studies suggest that the creation of organic components of the concretion occurs in a series of steps following in succession. It seems to start with the thickening of the connective tissue surrounding one of the follicles. The next step involves successive degeneration of cells forming the follicular wall and synthesis of collagen fibers inside it. As the amount of collagen fibers increases, they form a structure with an onion-like appearance, which finally replaces the follicle. This area shows positive staining with the alizarin methods, which indicates the occurrence of the calcification process inside it.

A possible role of the collagen fibers in formation of the concretions in the rat pineal gland was studied by Humbert et al. (1997). The authors suggested that the collagen fibers are involved in the genesis and growth of the extra-cellular concretions located in the connective capsule surrounding the pineal gland of ageing rats. Their ultrastructural data showed a close relationship between the collagen fibrils and the periphery of some concretions, as well as the presence of fibrils inside a concretion. The current study on the fox pineal gland has demonstrated no specific relationships between type I and III collagen and the calcium concretions present in parenchyma (Bulc et al., 2006). It should be stressed that the collagen fibers are generally not considered to be a component of the concretions located inside the mammalian pineal gland and in the choroid plexus neighboring this organ in ducks and geese (Fejer et al., 2001).

The potassium pyroantimonate method was used for the study of calcium ion accumulation and mineralization processes in the turkey pineal organ at the level of electron microscopy. This cytochemical method enables localization of free calcium ions in the form of electron dense precipitates. It has been commonly used in studies of many biological samples, including the pineals of various species (Krstic, 1985; Pizzaro et al., 1989; Vigh and Vigh-Teichman, 1989; Humbert et al., 1997). Validation of the specificity and sensitivity of this method was performed and has been previously discussed in detail (Lewczuk et al., 1994, 2007).

Based on ultracytochemical studies, we have found that the loci of concretion formation consists of three parts, reflecting differing stages of the mineralization process. The outer part is composed of numerous collagen fibrils, running in different directions and repeatedly fusing each other. It contains a markedly elevated level of calcium ions, mainly surrounding the fibrils. It could be supposed that this part is the place where the collagen fibrils are formed and accumulated, as well as where calcium ions are concentrated. The intermediate part contains densely packed collagen fibrils, usually fused side-by-side. It should be noted that studies of developed bones have shown that fusion of fibrils is necessary for initiation of the mineralization. The positive reaction for calcium ions was extremely strong in this part and the reaction product was noted both inside and outside of the collagen fibrils, with the highest intensity outside. It is obvious that Ca^{2+} formed almost continuous sheets covering fibrils. The central part is composed of mineralized collagen, with appearance resembling intracellular matrix of newly

formed bone or mineralized tendon. The border between intermediate and central parts of the concretions is probably the place of nucleation of the calcium-phosphate crystals. Therefore, the calcium pyro-antimonate precipitates are almost completely lacking in the central part. Conventional electron microscopy identified the presence of mineral deposits in the forms of thin needles or plates, located between collagen fibrils, although a detailed structural analysis of the deposits was not possible with this technique.

The current state of knowledge of processes of mineral nucleation, growth and development during the calcification of vertebrate tissues derives, in a large degree, from the morphological studies on collagen - mineral interactions (Hoshi et al., 1999, 2000; Boskey et al., 2000; Siperko and Landis, 2001). Normally mineralizing tendons of the domestic turkey represent a commonly used model for study of calcification, mainly due to the simplicity of their organization (Krefting et al., 1980; Landis et al., 1993, 1996; Siperko and Landis, 2001). The coalescence of mineral compounds with collagen, leading finally to the hard mineralized tissue, is a multi-step process (Krefting et al., 1980). In leg tendons, mineral nucleation occurs both outside and inside (in holes and overlap zones) of the collagen fibrils (Arsenault, 1989; Landis and Song 1991; Landis et al., 1991, 1996; Lees et al., 1994; Siperko and Landis, 2001). Subsequently, growth of mineral deposits leads to development of platelets, plates and aggregates of parallel oriented plates (Landis et al., 1993, 1996; Siperko and Landis, 2001). The formation of deposits in holes and overlap zones of the collagen fibrils is responsible for their regular distribution, maintaining a periodicity of 50-70 nm (Siperko and Landis, 2001). Finally, mineral deposits fill all intra- and extra-fibrillar species of a tissue matrix. Analysis of the above presented data about processes leading to mineralization of the turkey tendons and our results concerning formation of pineal concretions in this species undoubtedly demonstrated large similarities between both phenomena.

The presence of two types of cells in the calcification area should be especially emphasized. The multipolar cells with thin processes, occurring in the central part of this area largely resemble tendoblasts of the calcifying leg tendons of the turkey (Landis, 1986; Landis et al., 1996) or even classical osteocytes. Their presence is a strong argument pointing to large similarities between pineal concretion formation and collagen-based mineralization. The elongated cells, located in the intermediate and peripheral parts of the calcification area, did not possess any specific morphological features enabling their unequivocal recognition as a specific cell type. It is reasonable to suspect that they may represent fibrocytes/fibroblasts responsible for the synthesis of intercellular matrix of the concretion.

There are sparse descriptions of the role of cells in the calcification processes occurring in the pineal gland (Humbert and Pevet, 1995a,b; Vigh et al., 1998). Vigh et al. (1998) postulated that the creation of the pineal acervuli in mammals may be regulated by the periacelvular arachnoid cells acting like osteoblasts. According to Humbert and Pevet (1995a,b), dark pinealocytes with notable accumulation of calcium may be involved in the formation of concretions.

Summing up, the genesis and structure of the pineal concretions in the turkey are completely different from all other investigated species. The process of the concretion formation in the turkey pineal organ shows prominent similarity with ossification and other collagen-mediated calcifications, concerning the accumulation of collagen-rich intercellular matrix, side-by-side fusion of collagen fibrils and their mineralization. The presence of osteocyte-like multipolar cells between mineralized collagen fibers also points to such a resemblance. An open question is whether the mechanism observed in the turkey pineal organ also functions in other avian species, or if it exclusively occurs in the turkey, which shows a well known physiological predisposition to the calcification of tendons.

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