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LETTER TO THE EDITOR

Artifacts in Electron Microscopic Research

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Comment on:
KOPÁNI M, VRANÍKOVÁ B, KOSNÁČ D, ZEMAN M, ŠIŠOVSKÝ V, POLAKOVIČOVÁ S, BIRÓ C: Pineal gland calcification under hypoxic conditions. *Physiol. Res.* **68** (Suppl 4): S405-S413, 2019.

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Dear Editor,

We have read with great interest the paper "Pineal gland calcification under hypoxic conditions" authored by Kopáni *et al.* (2019). The pineal gland is from the morphological point of view a unique organ. Histologically it represents a nervous tissue (pinealocytes are modified neurons and astrocytes are neuroglial cells) but the official morphological nomenclatures (FIPAT 2008; FIPAT 2019) classify this organ as an endocrine gland (in fact, glands are mostly made up of epithelial tissue). The origin and function of the "brain sand" *(acervulus, corpus arenaceum)* inside the parenchyma of the pineal gland is truly a histological enigma. The degree of calcification is associated with age (more pronounced in elderly patients) and various diseases. However, the presence of calcified concretions seems not to reflect a specific pathological state (Vigh *et al.* 1998). One hypothesis suggests that the formation of pineal acervulus is a result of a high need for calcium exchange of pinealocytes for their supposed receptor and effector function which is similar to cells of the retina (Vigh-Teichmann and Vigh 1992).

The biggest issue of the article by Kopáni *et al.* (2019) is a misinterpretation of the pictures from the transmission electron microscope (TEM), so called electron micrographs. TEM is a significant tool in demonstrating the ultrastructure of cells and tissues both in normal and disease states. However, clinical applications of ultrastructural investigation have fallen out of favor recently in number of laboratories since the implementation of immunocytochemical techniques and the development of commercially available monoclonal antibodies (Gunning and Calomeni 2000). Additionally, electron microscopic examination is methodically a very difficult field of morphological research. Every biological or medical scientist in the field of electron microscopy has to deal with the presence of troublesome artifacts caused by various tissue processing procedures. An artifact is a damage caused by a preparation technique and can easily be confused with a genuine ultrastructural finding (Ayache *et al.* 2010). Unfortunately, these artifacts in electron microscopy and methods of their minimizing have been described only rarely in the scientific literature. Kopáni *et al.* (2019) probably do not have enough experience with ultrastructural research of human or animal cells and tissues, as they described routinely occurring artifacts as their own and unique results. We divided our comments into five principal areas.

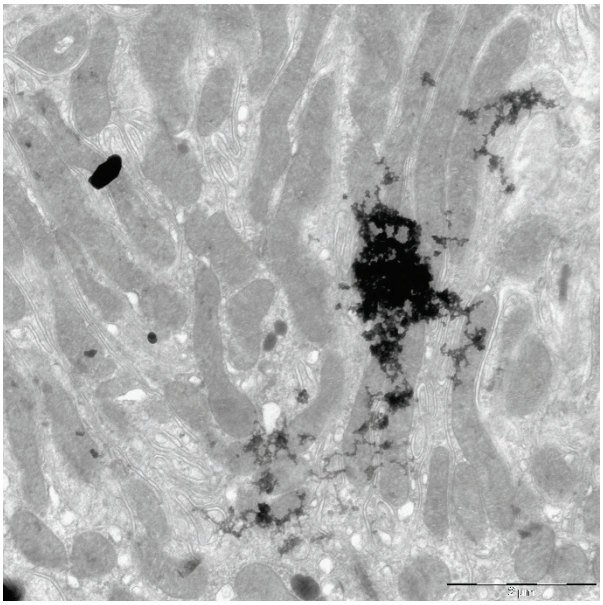

Fig. 1. Example of precipitates of the fixative or contrast agents (lead and uranium artifacts) in a specimen (mitochondria inside a cell of proximal tubule from a kidney are visible).

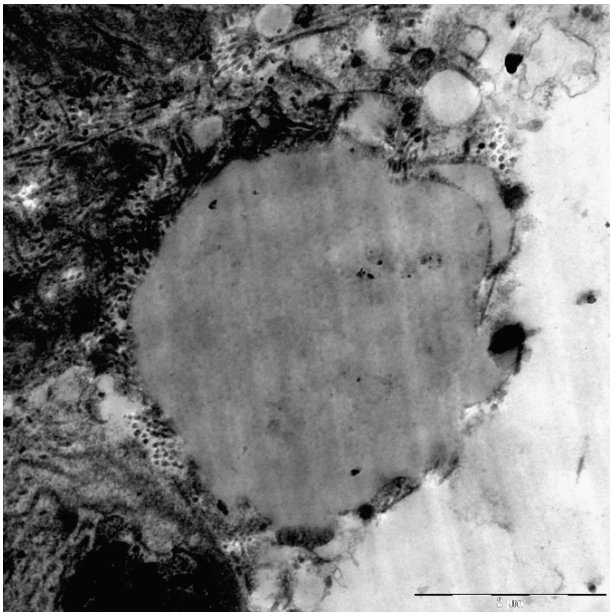

Fig. 2. Example of a "vacuole-like" artifact inside the extracellular matrix of a connective tissue as a result of poorly embedded lipid or triglyceride phases.

1) Lead and uranium stain artefacts

Both presented electron micrographs from the TEM in the article by Kopáni *et al.* (2019) show artifacts – precipitates of the fixative or contrast agents. These particles are highly electron dense, so it is reasonable to suspect that they may result from the use of heavy metal stains during the processing of tissue for ultrastructural examination (Thaete 1979). Nevertheless, Kopáni *et al*. (2019) described these, what we deem common findings in electron microscopy research, as "extracellular accumulation of flocculent material". According to our experience, we are more inclined to think that the presented electron micrographs show lead and uranium stains artifacts (Fig. 1). Additionally, according to Vígh *et al.* (1998), pineal *acervulus* has concentric lamination of alternating dark and light lines and these lines are not visible in electron micrographs by Kopáni *et al.* (2019).

2) Formation of "pseudovacuoles" as a compression phenomenon

Without adequate electron micrographs from the control group of experimental animals (no control results was presented in the discussed electron microscope study), it is too bold to make a conclusion that the cytoplasm of cells contain "vacuoles filled with flocculent and fibrous material". Those vacuoles may represent another artifacts, especially lipid or triglyceride phases that have undergone compression phenomena (during sample preparation the softer phase was poorly embedded). Similar artifact is visible in Figure 2.

3) Lack of experience with ultrastructural description

Every specialist in histology and cell biology is surprised by the controversial description of the "presence of fibrous material inside intracellular vacuoles". According to Figure 4a from the article by Kopáni *et al.* (2019), this fibrous material is outside the (pseudo)vacuoles and these fibers are probably normal collagen fibers. So, the authors described the stroma (the connective tissue septa) instead of the parenchyma of the pineal gland. In high magnification, the collagen fibers have typical and well visible cross striations, which can also be seen in the abovementioned figure by Kopáni *et al.* (2019).

4) Inadequate accelerating voltage

The accelerating voltage of 100-300 kV remains a good choice for the majority of TEM specimens of typical thickness around 100 nm (Egerton 2014). Kopáni *et al.* (2019) used accelerating voltage of 30 kV. This low-voltage TEM is attractive for a very thin (e.g. 10 nm) specimens, but the thickness of Kopáni´s specimens was 100 nm. For this reason the quality of electron micrographs is very poor.

5) Embryological and histological discrepancies

Last but not least, the newly constructed hypothesis of Kopáni *et al*. (2019) is also rather dubious. It postulates that the occurrence of brain sand inside the pineal gland is associated with modified function of osteoblasts and osteocytes. This hypothesis is really

strange. The embryonic origin of the pineal gland is totally different from that of bone cells, so that the process of formation of calcium-rich brain sand is definitely not associated with osteoblasts and osteocytes (these cells are not present inside the pineal gland).

Conclusion

The early work of Johnson (1983) had described the structure of pineal gland in rat using both light and electron microscopy tools. Unfortunately, the recent study of Kopáni *et al. (*2019) did not present any good description of pineal gland and the quality of the results is much less than the quality of the early work 40 years ago.

Electron microscopic research is very useful in morphological sciences - in histology, histopathology, cytology or embryology. However, it is also a methodically demanding approach, which requires not only the experienced laboratory staff, but also highly erudite experts who are able to correctly interpret the ultrastructural findings.

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