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REPLY TO LETTER TO THE EDITOR

Reply to Artifacts in Electron Microscopic Research

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Dear Editor,

We carefully read the comments on our paper "Pineal Gland Calcification under Hypoxic Conditions" (Physiol Res 68 (Suppl 4): S405-S413, 2019) by Varga *et al*. (this issue); our response is below.

Calcification of the pineal gland has been known for a long time. Our data are in line with the literature and we demonstrated calcification by 3 different techniques: light microscopy, transmission electron microscopy (TEM) and energy-dispersive analysis (EDX) rather than by TEM only, which Varga *et al*. commented upon. The results of all three methods are consistent. The new findings of our study are related to the effects of prenatal hypoxic conditions on the process of pineal gland calcification. We found that prenatal hypoxia can accelerate the process of calcification, although the biological consequences are still unknown.

The application of contrast agents (uranyl acetate and lead citrate) in electron microscopy can cause the presence of artefacts (electron-dense particles) in the investigated samples. To avoid the misinterpretation of observed particles, EDX analysis was performed. We found particles consisting of Ca, P, S, and Na. EDX analysis revealed that the amount of Ca varied from 0.21 to 20.26 weight%, and the amount of U from 0.64 to 8.55 weight%. Some particles did not reveal uranium or lead at all. Moreover, some authors observed that the cytoplasm of pinealocytes contains vacuoles filled with flocculent and fibrous material (Humbert *et al*. 1997, Swietoslavski 1999). From these results, we can conclude that the analyzed electron-dense particles are the result of calcium accumulation and not artefacts.

Biomineralisation (the formation of minerals in the tissue) is a complicated process; the results depend on many factors such as time, the presence of chemical elements and biomolecules, the pH of the surrounding microenvironment, the presence of water, etc. In this case, the structure (concentric lamination) can be strongly influenced by conditions during the hypoxia. The absence of concentric lamination can reflect these conditions.

Of course, the presence of vacuoles can be the result of compression. However, as we mentioned in our article, many authors observed the presence of vacuoles in the pineal gland under physiological conditions (Reiter *et al.* 1976, Humbert and Pevet, 1994, 1995, Schmid and Raykhtsaum 1995) and under hypoxia in other tissues (Zeng *et al*. 2019, Sun *et al*. 2019, Zhuravin *et al*. 2003). In addition, the preparation of all samples was the same for all groups and the control group showed almost no vacuoles. Therefore, we conclude that vacuolisation is not the result of compression during preparation but a reaction to the conditions.

We focused on calcium particles and described the fibrous materials inside the vacuoles in the top right corner of Fig. 4. We agree that the fibrous material outside of the vacuoles in the top right corner could be collagen fibres. Tomonari *et al*. (2012) investigated 383 rats from a histological viewpoint and found focal

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fibrosis on the periphery of the gland; sometimes it was observed in the gland. Therefore, we are convinced that Fig. 4 represents calcification in the parenchyma of the pineal gland.

The whole process of pineal gland calcification is not definitely understood and there are several hypotheses which explain underlying mechanisms. The authors Varga *et al*. mentioned only one hypothesis suggested by Vigh-Teichmann and Vigh (1992) many years ago. Since then, several other hypotheses have been formulated and are reviewed in a recent paper (Tan *et al*. 2018). The paper was either not read or pointedly ignored by Varga *et al.* Our explanation for the increased calcification is based on one of the hypotheses suggested by Tan *et al*. (2018). We did not deal with all possible hypotheses because this was not the aim of our study.

We found this new hypothesis challenging and our data may support it. Hypoxia is generally accompanied by the generation of reactive oxygen species causing oxidative stress, which promotes the production of different cytokines (Jomova *et al*. 2010, Pathipati *et al*. 2013). Cytokines are known to promote the "reprogramming" of mesenchymal cells, as demonstrated many times with different models (Mijiritsky *et al*. 2020, Krampeta 2011, Zimmermann *et al*. 2017). In this context, we discussed a possible mechanism for the calcification of the pineal gland in our study. On the basis of above mentioned arguments we are convinced that conclusions of our paper are valid and cannot be questioned.

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