

# Pineal Gland Calcification Under Hypoxic Conditions

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## Summary

The pineal gland (*glandula pinealis*) is neuroendocrine gland located at the *epithalamus* of the brain secreting melatonin. The aim of this study was to explore effects of prenatal hypoxia in rats at the age of 33 weeks on the occurrence of pineal gland calcification. Distribution and chemical composition of calcereous material by light, scanning and transmission electron microscopy was investigated. Melatonin concentrations in blood plasma by direct radioimmunoassay were measured. Rats were exposed to prenatal hypoxia for 12 h at day 20 of development and second group to prenatal hypoxia for 2x8 h at days 19 and 20 of development. Vacuoles of intracellular edema in the pineal samples after 12 h hypoxia were found. Their size ranges up to 30 µm. Some of them were filled with the flocculent and fibrous material. Samples of pineal glands after 2 x 8 h hypoxia revealed the pericellular edema of pinealocytes. The amount of calcium rich particles in 2 x 8 h hypoxia group was lower than in 12 h hypoxia group. Plasma melatonin levels did not differ between control and both hypoxia groups. We concluded that calcification is a process induced by osteoblasts and osteocytes with melatonin as a promotor and it is favored under hypoxic conditions.

## Key words

Pineal gland • Calcification • Electron microscopy • Hypoxia

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## Introduction

The pineal gland (*glandula pinealis*) is the neuroendocrine gland localized at the *epithalamus* of the brain and secreting melatonin. This hormone is involved in a control of many physiological processes including control of circadian rhythms, reproductive function in photoperiodic mammals, body temperature, immunity, cardiovascular system and antioxidant activity (Cassone 1990, Reiter *et al.* 2009, 2010a,b, Tan *et al.* 1993, Paradies *et al.* 2010, Chahbouni *et al.* 2010, Dominguez-Rodriguez *et al.* 2010). Environmental light/dark (LD) conditions control pineal gland activity (Reiter 1991, Turgut *et al.* 2008).

Calcium plays an important role in the pineal gland. Neurotransmitter norepinephrine induces both influx and release of Ca<sup>2+</sup> from intracellular stores in rat pinealocytes (Saavedra *et al.* 1973, Saez *et al.* 1994). Genesis of this material (pineal acervuli) in the pineal gland is a natural process associated with ageing (Schmid and Raykhtsaum 1995; Humbert *et al.* 1997), endocrine and neuronal diseases, cerebral infarction, symptomatic intracerebral hemorrhage and neuropathologies (Sandyk 1991, 1992a, b, 1993, Bersani 1999). The relationship between pineal calcification and melatonin production is not clear since several studies showed controversial results (Commentz *et al.* 1986, Bojkowski and Arendt 1990, Kay and Sandyk 1991, Friedland *et al.* 1990, Turgut *et al.* 2008). Although the mechanisms of calcification and its biological significance are not well

understood they are considered to be multifactorial. Two types of calcified granular material are detected in the mammalian pineal gland: 1) the meningeal calcification and 2) the intraparenchymal calcification, which origin relates to the function of pinealocytes (Welsh 1984, Vigh *et al.* 1992, Lewczuk *et al.* 1994).

Hypoxia is a condition of the body in which the tissues and cells are deprived of adequate oxygen supply. Hypoxic brain injury is caused by mechanisms such as calcium influx, free oxygen radical formation and apoptosis activation. It was observed that melatonin production by the pinealocytes is significantly reduced (Schmid *et al.* 1994, Humbert and Pevet 1994, 1995, Coste *et al.* 2004, Kaur *et al.* 2007) and it is not known if the decreased pineal melatonin production is related to calcification of the gland.

The aim of this study was to explore effects of hypoxia on the occurrence of pineal gland calcification. Distribution and chemical composition of calcereous material by light, scanning and transmission electron microscopy was investigated. Melatonin concentrations in blood plasma were measured by direct radioimmunoassay.

## Methods

### Samples

The experiments were performed on 24 adult male Wistar rats (Dobra Voda, Slovakia) at the age of 33 weeks. Rats were housed under standard laboratory conditions at LD 12:12, lights on from 08.00 till 20.00 and fed with standard pellet diet and tap water *ad libitum*. Rats were randomly allocated into three groups. The first group was exposed to prenatal hypoxia for 12 h at day 20 of development (n=9), the second group to prenatal hypoxia for 2x8 h at days 19 and 20 of development (n=5) and the third group was the control (n=10). All procedures were conducted in accordance with the Declaration of Helsinki.

Rats were killed by decapitation during the second half of the darktime (between 3.00 and 5.00). Blood was collected to the heparinized tubes and the pineal glands were gently excised. Samples were fixed in 4 % formaldehyde for 24 h, dehydrated in an ascending series of alcohol, cleared with xylene and embedded in paraffin wax. Serially coronal sections were cut on the rotating microtome (5 µm thick) and mounted on silanized slides. Afterward, slides were stained with hematoxylin and eosin and von Kossa according to standard protocol.

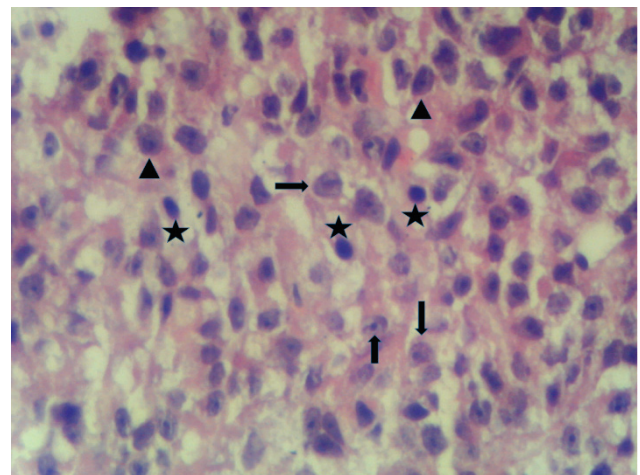
### Radioimmunoassay

Melatonin concentrations in blood plasma were measured by direct radioimmunoassay (Zeman *et al.* 2005). We used melatonin antiserum prepared against N-acetyl-5-methoxy-tryptamine conjugated on bovine thyroglobuline (Stockgrand Ltd., G/S/ 704-8483, University of Surrey, UK) and tritium labelled melatonin ((O-methyl-<sup>3</sup>H) Melatonin, TRK 798, specific activity: 3.07 TBq/mmol (83.0 Ci/mmol), Perkin Elmer, Waltham, MA USA). Sample radioactivity was measured in the scintillating β-counter for liquid samples (Packard Tri-Carb 2900 TR, Packard Instruments, Perkin Elmer, USA). All samples were measured in a single assay, coefficient of intra-assay was 4.3 %.

### Light, scanning and transmission electron microscopy

Samples for SEM + EDX investigation were mounted on carbon block and covered by carbon layer with 30 nm thickness. SEM analysis was performed by scanning electron microscope EVO LS 15 (ZEISS) with the accelerating voltage of 10 kV. Simultaneous EDX line analysis was performed by AMETEK (EDAX) EDS Element Silicon Drift Detector. The time period of spectrum collection was 200 s with the energy range 0.160 to 10 keV.

The samples of pineal gland intended for TEM investigation were fixed in 3 % solution of glutar(di)aldehyde (SERVA, Heidelberg, Germany) for two hours and buffered by phosphate (pH 7.2 – 7.4). After dehydration of the tissue by alcohol, the samples were embedded into Durcupan ACM (Fluka AG, Busch, Switzerland) as recommended by the manufacturer and cut



**Fig. 1.** Pineal gland of rat; the control group. Pinealocytes type 1 are depicted by arrows, pinealocytes type 2 by arrowheads. Glial cells (G) are scattered among pinealocytes and depicted by asterisks. Light microscopy, HE. 40x.

by ultramicrotome with a glass knife (C. Reichert, Wien, Austria). The thickness of the samples was 100 nm. Contrasted sections with uranyl acetate and lead citrate were mounted on copper grids and investigated by scanning electron microscope EVO LS 15 (ZEISS) with the accelerating voltage of 30 kV in transmission mode.

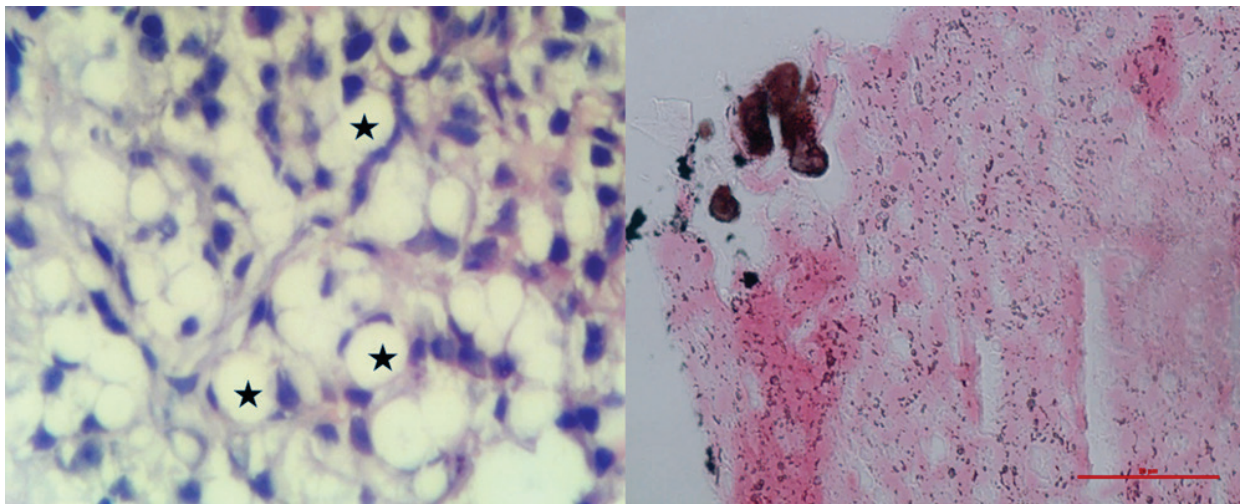
## Results

Histological investigation of the pineal gland under light microscope showed eosinophilic cytoplasm surrounding the large nuclei. These type of cells were ascribed to pinealocytes type 1. Pinealocytes type 2 showed pale cytoplasm and small basophilic nuclei. Glial cells, mainly astrocytes, were scattered among pinealocytes. They contained tiny cytoplasm surrounding

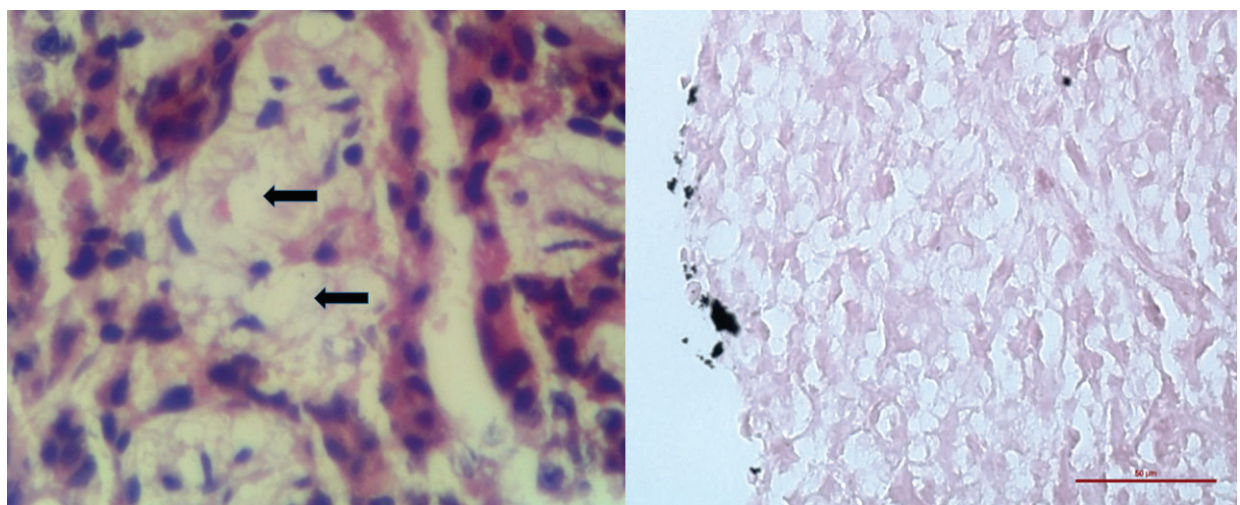
small deeply-stained elongated nuclei (Fig. 1).

Vacuoles were found in the pineal glands after 12 h hypoxia and were classified as intracellular edema. Their size reached up to 30  $\mu\text{m}$ . Some of them were filled with the flocculent and fibrous material. Its size is up to 40  $\mu\text{m}$ . Large focal calcium-rich particles in both meningeal and intraparenchymal part of pineal gland were found. Smaller calcium rich particles were dispersed in intraparenchymal part of pineal gland (Fig. 2).

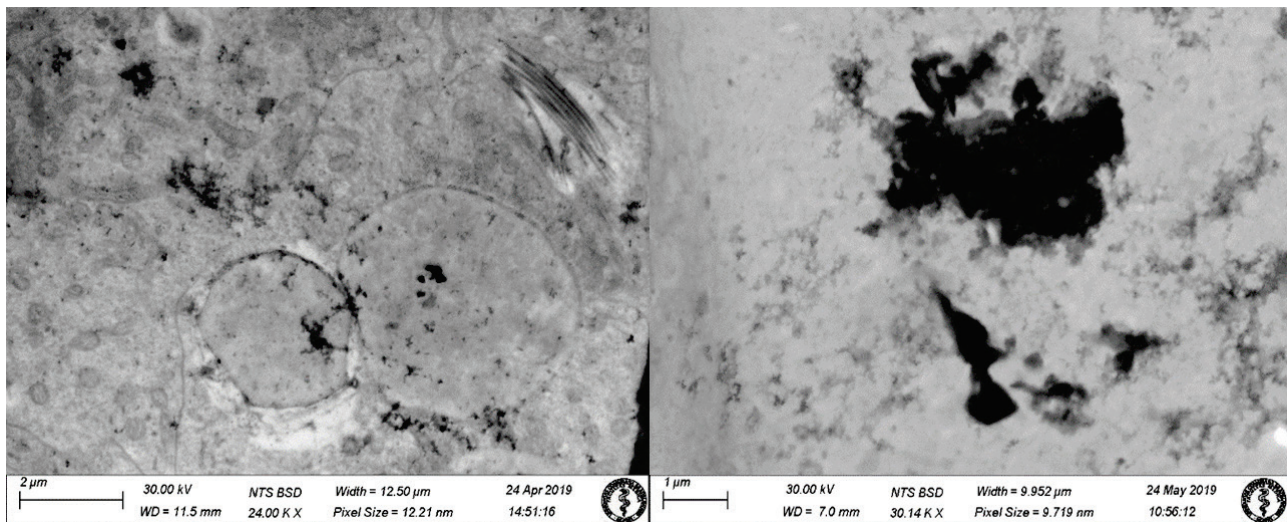
Fig. 3 shows samples of pineal glands after 2 x 8 h hypoxia. The pericellular edema of pinealocytes was observed. Calcium-rich particles mainly in the meningeal part of the pineal gland were observed with size around 20  $\mu\text{m}$ . The amount of calcium rich particles in 2 x 8 h hypoxia group was lower than in 12 h hypoxia group (Fig. 3).



**Fig. 2.** Pineal gland of rat; 12 h hypoxia. Left – many vacuoles of intracellular edema of pinealocytes. Right - black dots represent calcium-rich particles in the rat pineal gland. Light microscopy, HE and von Kossa. 40x.



**Fig. 3.** Pineal gland of rat; 2 x 8 h hypoxia. Left – pericellular edema of pinealocytes (arrows). Black dots represent calcium-rich particles in the rat pineal gland. Light microscopy, HE and von Kossa. 40x.



**Fig. 4.** Pineal gland of rat; 12 h hypoxia. Left – vacuoles filled with flocculent and fibrous material. Right – extracellular accumulation of flocculent material. Transmission electron microscopy.

Fig. 4 shows vacuoles in the cytoplasm of pinealocytes type I filled with both flocculent and fibrous material in 12h hypoxia group. Similar material in intra- and extracellular space was observed. Material consists of Ca, P, S, Na.

Plasma melatonin levels, measured in the second part of the dark phase, did not differ between control (mean±SD: 87.4±24.8 pg/ml) and both hypoxia groups (85.0±16.7 and 110.1±39.7pg/ml for Hypoxia 12h and Hypoxia 2 x 8 h, respectively.). In all groups, the darktime melatonin levels were higher as compared do those measured in the middle of the light phase (24.3±10.9 pg/ml for control group). It is important to note high inter-individual variability in melatonin concentration in animals was observed.

## Discussion

Pineal gland consists of pinealocytes type 1 and type 2 and glial cells – astrocytes and microglia. Pinealocytes type 1 with the clear nucleus and cytoplasm are described as light and pinealocytes type 2 with dark cytoplasm and nucleus are described as dark pinealocytes (Cernuda *et al.* 2003). They form the majority of parenchymal cells and are responsible for melatonin production (Kus *et al.* 2004). Srivastava *et al.* (1999) according to ultrastructural findings proposed that the light and dark pinealocytes in the pineal gland parenchyma represent different phases of the secretory process. This finding is partially in agreement with Khavinson *et al.* (2012) who stated that pinealocytes type 1 contain serotonin and pinealocytes type 2 synthesize

melatonin from serotonin. On the other hand, Al-Hussain (2006) observed besides the light and dark pinealocytes also a very small cell type with thin and elongated cell body and nucleus. The author suggests high secretory activity of these cells.

It is well known that hypoxia can destroy cells and tissues. Our study confirmed alternations of pineal gland morphology – intra- and pericellular edema in the hypoxia groups. Intracellular edema is caused by changes in the activity of ion channels and transport through the cytoplasmic membrane. These changes leads to intracellular accumulation of water and calcium (Dickey *et al.* 2011). Intracellular edema is regarded as a premonitory precursor to extracellular (pericellular) edema. Intracellular edema results in osmotic expansion of the cell and depletion the extracellular space of ions and water. This effect creates a new gradient for movement of molecules across the capillary and formation of pericellular edema (Stokum *et al.* 2015). However, it was observed that the cytoplasm of pinealocytes contains vacuoles filled with flocculent and fibrous material under physiological conditions (Reiter *et al.* 1976, Humbert and Pevet, 1994, 1995, Schmid and Raykhtsaum 1995, Humbert *et al.* 1997, Swietoslavski 1999). It is suggested secretory function of vacuoles (Pevet *et al.* 1977, Haldar and Bishnupuri 2001). Welsh (1984) showed that vacuoles play important role in calcification process. He hypothesized that calcium transported to the vacuoles precipitates into immature concretion. Krstic (1976, 1977, 1986) proposed that vacuoles and other intracellular structures are the initial calcification sites. The cells gradually degenerated and in

turn particles are eliminated from the intracellular to the extracellular space.

The localization of vacuoles containing both flocculent and fibrous material in the cytoplasm of pinealocytes is an open question. Some authors observed the presence of vacuoles in pinealocytes type 1 (Welsh and Reiter 1978, Japha *et al.* 1976). Vacuolation and mineralization are spontaneous morphological changes on the rat pineal gland as a consequence of cytoplasmic calcium, which is elevated in the aged cells. However, they were observed also during seasonal and reproductive cycles, with longer exposure to light, confinement to darkness and time of day (Cernuda-Cernuda *et al.* 2003, Redondo *et al.* 1996).

On the other hand, Humbert and Pevet (1995) found calcium localized mainly in pinealocytes type 2 with many ultrastructural signs of degeneration. Moreover, they also observed a significant increase in the number of pinealocytes type 2 and a decrease in the total number of pinealocytes with ageing. This decrease of total number of pinealocytes can explain the age-related functional decline of the pineal gland activity. Dark pinealocytes revealed dense cytoplasmic matrix and numerous deposits in the cytoplasm and in nucleus (Pizarro *et al.* 1989, Schmid *et al.* 1994, Nakamura *et al.* 1995). The major chemical elements are calcium and phosphorus with traces of magnesium, zinc and sodium (Zimmermann and Bilaniuk 1982, Kodaka *et al.* 1994, Nakamura *et al.* 1995). The calcification process on cell membrane and among pinealocytes was found. Calcium accumulation in the extracellular spaces of the pineal glands of the normal rats were found. In animals sacrificed at midnight accumulations in mitochondrial matrix was observed. After continuous illumination over 7 days, number of these accumulations was lower. In light pinealocytes no intracellular accumulations were found. (Vigh-Teichmann and Vigh, 1992).

Our recent observation revealed in pinealocytes type 1 vacuoles filled with both flocculent and fibrous material consisting of Ca, P, S and Na and sometimes also Mg and Al. We also observed this material in both intra- and extracellular space. These findings confirmed changes in the activity of ion channels with subsequent accumulation in the cells and their elimination from the intracellular to the extracellular space. Our present results from light and transmission electron microscopy reveal that calcification does not alter histology of the pinealocytes what is in agreement with findings of Tapp and Huxley (1972).

Hypoxia generates reactive oxygen species (ROS). General response to ROS generation is a rise of antioxidant defense. Concentration of melatonin, as an endogenous antioxidant enhances to the higher level compared to the normal physiological level (Tan *et al.* 2005), whereas Seifman *et al.* (2008) did not observed this effect. Higher melatonin production and utilization under hypoxic condition could explain why melatonin concentration did not decrease under hypoxic condition in parallel with altered structure of pinealocytes (vacuoles) and extracellular space (pericellular edema). However, it is necessary to mention that melatonin concentrations were measured only in one point during the darktime. More frequent sampling over the whole 24 h cycle is needed to reveal possible smaller changes in amplitude and/or acrophase of the melatonin rhythmicity. In our previous study prenatal hypoxia increased blood pressure in mature offspring, especially during the darktime, but did not affect circadian rhythms in cardiovascular parameters (Svitok *et al.* 2016).

Melatonin may play an essential role in regulating bone growth, bone metabolism, structure and composition. It also regulates production of some proteins and probably prevents from calcium degradation (Ishizu *et al.* 2018, Ikegame *et al.* 2019). Tan *et al.* (2018) review that calcification of pineal gland is process mediated by mesenchymal stem cells localizing in the pineal gland. Hypoxia-inducible factor (HIF)-1 $\alpha$  increases occurrence of mesenchymal stem cells in the hypoxic areas (Li *et al.* 2017, Lee *et al.* 2017, Ciria *et al.* 2017). These cells can differentiate into osteoblasts and osteocytes with melatonin as a promotor. Melatonin supports mineralization of the pineal gland through upregulation of some proteins (osteopontin, bone morphogenetic protein 2, osteocalcin and alkaline phosphatase). Authors concluded that this process favors calcification under hypoxic conditions. However, it should be mentioned that information regarding the mechanisms underlying development of pineal gland particles and their effects on biological processes are still controversial (Sandyk and Awerbuch 1994, Schmid *et al.* 1994, Alcolado *et al.* 1986, Gerasimov *et al.* 2014).

Our previous work showed the presence of calcium carbonate crystals in the pineal gland (Tofail *et al.* 2019). These crystals of the pineal gland have unique properties such as piezoelectricity and can act as an electromechanical biological transducer (Lang *et al.* 1996, Baconnier *et al.* 2002). The interaction of the crystals with electromagnetic radiation could influence

the melatonin production and thus contribute to its circadian control.

## Conclusion

The pineal gland synthesizes melatonin, the important signaling molecule with protective antioxidant activity. The secretory function of the pineal gland can be affected by calcification – deposition of calcium and other ions. The precise mechanism of calcification is still unknown. One theory explains calcification as a process induced by osteoblasts and osteocytes with melatonin as a promotor. Since hypoxia increases occurrence of

mesenchymal stem cells the intensity of calcification should be higher. This explanation of calcification process under hypoxic conditions is in agreement with our results therefore we conclude that prenatal hypoxia can promote calcification of the pineal gland.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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