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Immunohistochemical characteristic of C cells in European bison thyroid gland

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Abstract

Introduction. C cells constitute a small percentage of thyroid gland parenchyma. The number, morphology and distribution of C cells differ among species; however, data regarding their characteristics in European bison are sparse. The aim of this study was to evaluate the morphology, distribution pattern and percentage of C cells in European bison thyroid gland together with morphometric analysis.

Material and methods. Thyroid glands from 28 European bisons of different sex and age were collected either in autumn-winter (13/28) or in spring-summer (15/28) periods and analyzed by immunohistochemistry.

Results. The mean total C cell number per all endocrine (follicular and C cells) cell number (C cell concentration) was 7.33%. The tendency to increase the C cell number from periphery to the central region of thyroid lobe was observed with the mean C cell concentration of 3.95%, 7.89% and 9.97% in peripheral, intermediate and central areas, respectively. Most frequently, C cells were situated intrafolliculary whereas epifollicular and interfollicular positions were observed less often. C cells were polymorphic with long cytoplasmic processes. The mean C cell area was 61.97 μm^2 and the mean C cell perimeter, length and width were: 34.92 μm, 12.85 μm and 4.91 μm, respectively. In the majority of C cells, strong immunohistochemical cytoplasmic reaction was observed with the mean color intensity of 78.32. In autumn-winter period, C cells were significantly larger with lower color intensity than during spring and summer.

Conclusions. This study leads to deeper characteristics of thyroid gland C cells in European bison. The histomorphometric data suggest that in European bison production of calcitonin by C cells may differ depending on the time of the year. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 4, 222–230)*

Key words: C cells; European bison; histology; immunohistochemistry; morphometry; thyroid gland

Introduction

The European bison (*Bison bonasus*) had been threatened with extinction in the early 20th century. Despite successful restitution it still belongs to endangered species. Hence, many investigations have been undertaken on this species. Most of them are anatomical [1] and pathomorphological [2] studies. However, histological studies of various organs including endocrine glands are not numerous [3, 4]. Until now, little is known about the histological structure of the European bison thyroid gland [5, 6].

The mammalian thyroid gland parenchyma consists of two cell types: follicular and parafollicular. Follicular cells (thyrocytes) are the prevailing element of thyroid parenchyma forming walls of thyroid follicles and producing triiodothyronine and thyroxine. Parafollicular cells, also called as C cells, represent the second type of the endocrine cells in thyroid gland

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and constitute a small percentage of its parenchyma. They produce calcitonin and endocrine peptides such calcitonin gene-related peptide, somatostatin and helodermin [7]. It is widely known that distribution of C cells within the thyroid lobes as well as their number and morphology vary [7–9] and some of these features are species-specific [9]; however, studies regarding this subject are not numerous. Most of them focus on human [10] and laboratory animals [9, 11, 12] and less often on other domestic animals [12–14].

According to our knowledge, there are only four papers presenting some data regarding C cells in European bison thyroid gland. Two of them describe microscopic structure of thyroid follicles [5] and remnants of ultimobranchial bodies within thyroid parenchyma [6], so they contain only sparse information regarding C cells. Among another two papers intended to analyze C cells in bison's thyroid gland, one focused on expression of neuroendocrine markers and regulatory peptides in C cells and their distribution pattern in normal thyroid gland and ultimobranchial remnants [15]. Only one paper [16] provided more detailed data on distribution of C cells in European bison thyroid gland. However, in available literature there is no information regarding the proportion of C cells to either all endocrine cell (follicular and C cells) number or follicular cells, although such studies were conducted on thyroids of humans and various mammalian species, including laboratory and domestic animals [7, 8, 13, 14]. Moreover, no morphometric studies of European bison C cells were conducted.

Thus, the aim of this study was evaluation of the morphology, distribution pattern and percentage of immunohistochemically detected C cells in different areas of the middle part of European bison thyroid gland together with their morphometric analysis.

Material and methods

Animals and tissue collection. Thyroid glands collected from lowland European bison of both sexes (16 males and 12 females), aged from 3 months to 24 years were included in this study. Thirteen animals were culled during autumn-winter period and the remaining 15 animals were eliminated during spring-summer period. The Bialowieza National Park (Poland) was responsible for culling of all European bison examined. The most common reasons for culling the animals were population control, car accidents and bone fractures. None of animals was killed for the purpose of this study. The culling was carried out with the permission of the Ministry of Environment and the General Director for Environmental Protection in Poland (decision number: DOP-OZGIZ. 6401.06.7.2012.ls, DOPOZ.6401.06.7.2012.ls.1 and DLP-III-4102-459/36490/14/ZK). Immediately after culling, the thyroid glands and other organs were collected during section. The autopsy protocols are available in Bialowieza National Park. According to the Polish law, tests on tissues obtained post-mortem do not require an approval of the Ethics Committee (Parliament of the Republic of Poland, 1997).

Only grossly normal glands were included. For the study purposes, the medial part of the left thyroid lobe of each animal sectioned transversely was collected. Tissue specimens were fixed in 10% neutral buffered formalin, processed by common paraffin technique and cut into 3 μm slides. In each case, two specimens located at the distance of about 100 μm were collected.

Immunohistochemistry. C cells were visualized by the immunohistochemical (IHC) method with anti-calcitonin rabbit polyclonal antibody (Dako, Glostrup, Denmark). The detection of C cells in European bison and bovine tissues with the polyclonal anti-human calcitonin antibody have been published previously [15, 17]. All IHC procedures were performed according to the manufacturer's protocols. Microwaving (7 and 5 min, 700 W, in citrate buffer pH 6.0) was used for antigen unmasking. Then, the slides were incubated with primary antibody diluted 1:400 for 1 h at room temperature. The REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako) visualization system was used for antigen detection. The sections were counterstained with Erlich's hematoxylin. Swine, equine and bovine thyroid glands were used as positive controls. Substitution of primary antibody by TBST buffer (Dako) was employed for negative controls. European bison salivary gland was used to exclude cross-reactivity with glandular tissue.

C cell scoring. Quantification of C cells was performed manually by two independent observers in three areas of thyroid lobe in transverse plane: superficial, central and intermediate. In each region, cell calculation was made in 14 randomly selected visual fields at total microscope magnification 600× (154.89 × 193.61 μm) by counting all C cells and follicular cells present in the microscopic field (Nikon Eclipse 80i; Nikon, Tokyo, Japan). Then, results obtained from each region were summarized and the proportion of C cells to total endocrine cell population (C cell concentration) was calculated as follows:

Concentration of C cells [%] = number of C cells/number of all endocrine cells (C cells and follicular cells) × 100.

In each case, the C cell concentration was scored in both tissue sections separately and then, as final results, the mean values were calculated. The quantification was made for each region of thyroid gland and then the C cell concentration in whole gland was calculated.

Morphometric analysis of C cells. Sections were imaged using brightfield optics on a Nikon Eclipse 80i microscope. Individual images were acquired using a Nikon DS-Ri1

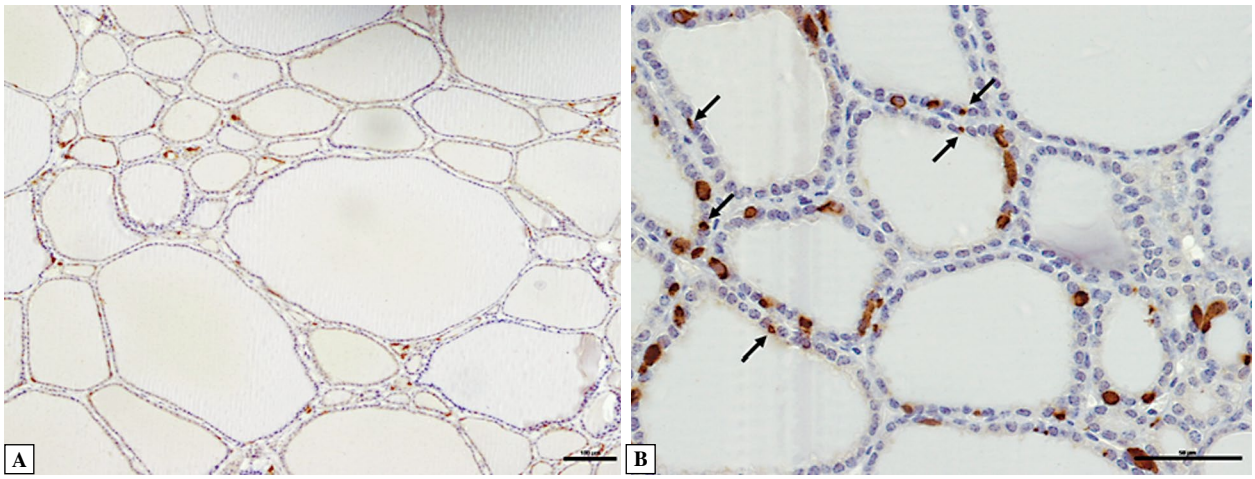

Figure 1. Section of thyroid gland parenchyma. **(A)** C cells arranged in small groups. **(B)** Group of C cells located intrafollicularly, individual C cells are scattered between thyrocytes, cross-sections thorough cytoplasmic processes are visible (arrows). Immunoperoxidase staining method with anti-calcitonin antibody, scale bars: A = 100 μm; B = 50 μm.

camera. NIS-Elements BR 3.22.15 microscope imaging software (Nikon, Tokyo, Japan) was used to capture the images. Images were sampled randomly throughout thyroid sections, but areas that contained preparation artifacts, cell debris, nonspecific staining, groups of C cells as well as tissue peripheral regions were avoided. Acquired images (24-bit RGB, 8 bits/color) had a resolution of 3840 × 3072 pixels and were stored in TIFF format.

To distinguish C cells from background the color threshold was performed on RGB images by defining the limit values for all channels leading to the selection of the "blue" background pixels from the "brown" pixels of stained C cells. Careful selection of conditions and the method of taking pictures caused clear discrimination between background and cells stained immunohistochemically. All measurements were performed by the NIS Elements software in an automatic way. The following parameters were calculated: cell area, length, width and mean color intensity. Overall, in each thyroid 40 randomly selected C cells were measured and the mean value was calculated for each parameter.

Statistical analysis

Data, presented as mean values ± SD, were analyzed using Statistica 13.3 for Windows (Tibco Software Inc. Palo Alto, CA, USA). Statistical comparisons were made using the Mann-Whitney U-test. $P \leq 0.05$ was considered significant.

Results

The distribution and concentration of C cells in the thyroid gland of European bison

In all analyzed specimens, C cells were unevenly distributed thorough the thyroid gland parenchyma with some inter-individual variations in their distribution patterns.

Microscopic analysis showed that in European bison thyroid gland C cells were located in groups surrounded by parenchyma containing either individually located C cells or only follicular cells (Fig. 1). Generally, together with the increase in total C cells number, the groups of parafollicular cells became larger and sometimes clustered. Only in the narrow zone of thyroid parenchyma lying directly under the capsule, C cells were sparse and the areas devoid of them were observed.

In 13/26 cases C cells hyperplasia was found. Usually it was focal, however, in 5/13 cases it occupied large areas of thyroid parenchyma. In all 13 cases, C cell hyperplasia occurred in medial part of the thyroid lobe. Areas of C cell hyperplasia were located peripherally; however, in cases of massive hyperplasia they extended into the central part of thyroid lobe.

Analysis of C cell concentration in three different regions of thyroid lobe in cross-section showed that in 4/28 cases any significant differences in percentage of C cells presented in these areas were found. Among remaining 24 cases, the significant differences in C cell concentration between peripheral and central areas were found in 23 cases and between peripheral and intermediate areas in 15 cases. The differences in percentage of C cells in central and intermediate areas of the thyroid lobe were statistically significant only in 9/24 cases. The mean C cell concentration in central area was 9.97% ± 6.42 (range 4.8–19%) followed by intermediate and peripheral regions with the mean C cell concentration of 7.89% ± ± 6.11 (range 2.43–14.43%) and 3.95% ± 4.92 (range

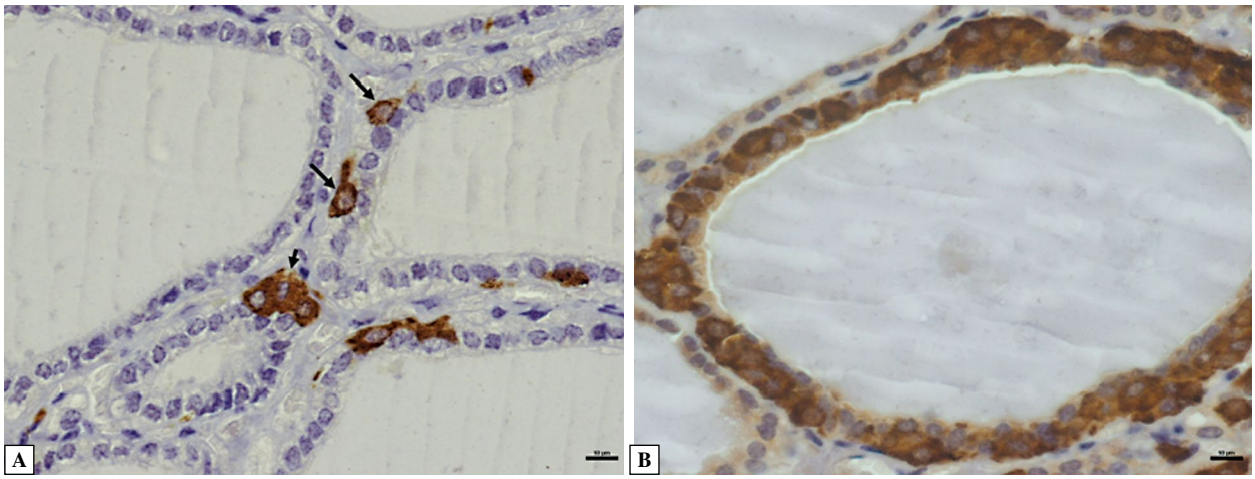

Figure 2. C cells in epifollicular position. **(A)** C cells lying either individually (arrows) or in small group (arrowhead). **(B)** C cells forming ring around thyroid follicle. Immunoperoxidase staining method with anti-calcitonin antibody, scale bar = 10 μm.

0.83–7.95%), respectively. These differences were statistically significant ($p \leq 0.001$). The mean total C cell concentration in the medial part of European bison thyroid gland was 7.33% ± 6.34. Detailed data on C cell concentration in particular areas of thyroid glands and in whole organ in individual cases are given in Supplementary Table 1.

In European bison thyroid glands, C cells were situated in three positions: intrafollicular, epifollicular and interfollicular. C cells located intrafollicularly were presented between thyrocytes in the follicular epithelium (Fig. 1B). C cells situated epifollicularly were located below follicular wall, but had direct contact with basal surface of thyrocytes (Fig. 2). Interfollicular C cells were located in connective tissue stroma without contact with follicular walls (Fig. 3).

C cells lying intrafollicularly were seen most frequently. In cases characterized by small number of C cells, they were individually scattered between follicular cells (Fig. 1B). However, they became more numerous within the follicular wall as the number of C cells increased. Usually, C cells presented in follicular wall did not contact with themselves, with the exception of areas of hyperplasia were the C cells were numerous and some follicles consisting almost exclusively of C cells were observed (Fig. 4). C cells located in epifollicular and interfollicular positions occurred less often. In the areas of European bison thyroid glands with relatively small number of parafollicular cells, C cells in epifollicular position were observed most often individually. Sometimes, they formed small groups consisting of 2–3 cells (Fig. 2A). In areas with higher number of C cells, parafollicular cells located in epifollicular position, either individually or in cell clusters, became more numerous. Sometimes, in hyperplastic areas, they encircled whole follicles in form of a solid or discontinuous rim (Fig. 2B). C cells located interfollicularly were observed the most rarely. They behaved similarly to C cells in remaining two localizations, *i.e.* they were presented individually throughout thyroid gland in areas with small number of C cells (Fig. 3A) and formed groups consisting of a few cells in regions with higher numbers of C cells (Fig. 3B).

Morphology of C cells

C cells of European bison were characterized by polymorphism. They were polygonal, oval or irregular in shape and in some of them long cytoplasmic processes were visible (Fig. 5). The processes were the most apparent in C cells lying individually, especially in follicles visible in tangential sections. Moreover, cross-sections thorough the processes were also visible between follicular cells within walls of thyroid follicles (Fig. 1B). The cellular borders of individual C cells were apparent, but within C cell clusters cellular outlines were not always clearly visible. The nuclei were oval or round usually located eccentrically; however, they were not always visible.

Morphometry of C cells

European bison C cells constituted a heterogeneous population in terms of their size and shape. Generally, they were large but some variations in C cell size were observed. The mean C cell area was 61.97 ± 23.98 μm^2 (range 12.19–190.62 μm^2) and the mean C cell perimeter, length and width were: 34.92 ± 9.41 μm (range 10.83–99.23 μm), 12.85 ± 4.63 μm (range 3.76–46.45 μm) and 4.91 ± 1.24 μm (range 1.53–9.8 μm), respectively.

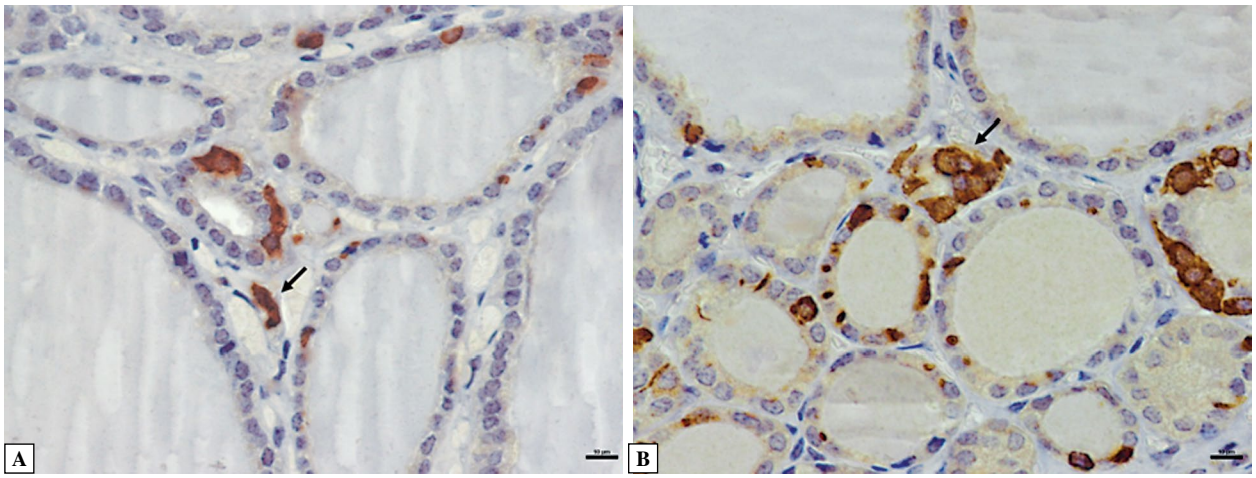

Figure 3. C cells in interfollicular position. **(A)** C cells lying individually between capillaries (arrow) and **(B)** in small group (arrow). Immunoperoxidase staining method with anti-calcitonin antibody, scale bar = 10 μm.

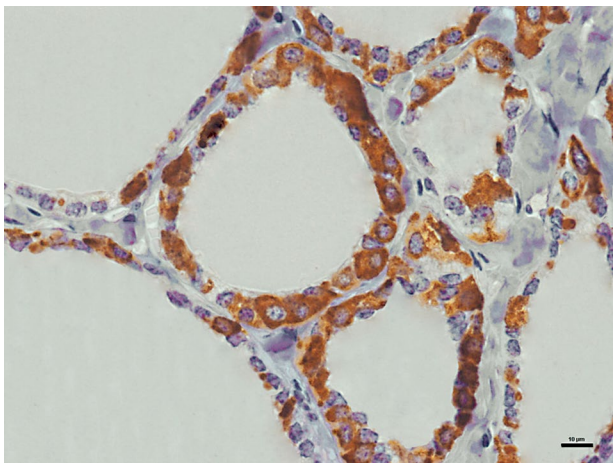

Figure 4. Thyroid follicle wall composed predominantly of C cells. Immunoperoxidase staining method with anti-calcitonin antibody, scale bar = 10 μm.

In the majority of C cells, strong cytoplasmic immunoreactivity was observed; however, in single cases some differences in staining intensity among cells were found. The mean color intensity was 78.32 ± ± 31.02 (range 27.03–180.07). Detailed data on C cell morphometric parameters in individual cases are given in Supplementary Table 2.

We also compared the mean values of C cells morphometric parameters obtained from European bison culled in autumn-winter and spring-summer periods. C cells of animals eliminated during autumn and winter were significantly larger with lower intensity of immunohistochemical staining compared to European bison culled during spring and summer (Table 1).

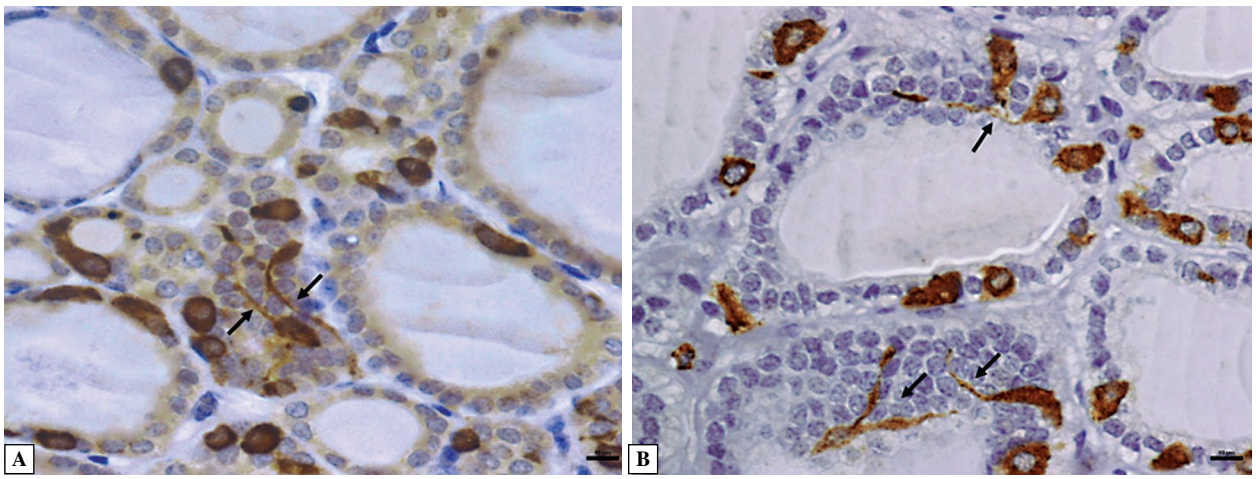

Figure 5. C cells with long cytoplasmic processes (arrows) **(A, B)**. Immunoperoxidase staining method with anti-calcitonin antibody, scale bar = 10 μm.

Table 1. Comparison of the mean values of the morphometric C cell parameters in European bison culled during autumn-winter and spring-summer periods

Culling period	Area [μm^2]	Perimeter [μm]	Length [μm]	Width [μm]	Mean color intensity (arbitrary units)
	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)
Autumn-winter	67.83 ± 25.08[b] (21.16–182.07)	37.24 ± 9.77[b] (19.86–72.45)	13.89 ± 4.89[b] (5.83–32.26)	5 ± 1.23[a] (2.12–9.22)	60.54 ± 15.67[b] (27.03–121.62)
Spring-summer	57.57 ± 22.14[b] (12.19–190.62)	33.18 ± 8.74[b] (10.84–99.23)	12.06 ± 4.26[b] (3.76–46.45)	4.84 ± 1.24[a] (1.53–9.8)	91.65 ± 32.95[b] (28.46–180.07)

Identical letters in the same column indicate statistically significant differences: [a]the difference significant ($p \leq 0.05$); [b]the difference very highly significant ($p \leq 0.001$)

Discussion

We found that European bison C cells are polymorphic with cytoplasmic processes. According to our knowledge, this morphological feature of C cells was not reported in available literature neither in European bison [5, 6] nor domestic cattle [17, 18], but it was observed in some other species as rat, field vole [11] and pig [12, 14]. However, to confirm the presence of cytoplasmic processes in European bison C cells further transmission electron microscopy studies are needed. They would not only allow describing their morphology and exact localization relative to thyrocytes and follicular basement membrane, but also give the opportunity to characterize the secretory granules in terms of their subcellular localization, size, morphology as well as cell secretion stage.

Our results indicated that on average C cells consist 7.33% of all endocrine cell population presented in European bison thyroid gland. It falls within the range of C cells reported for other animal species. The lowest C cell concentration (less than 1%) was found in humans [7] followed by pig (3.8%) [14], some laboratory animals (mice, guinea pigs, rabbits, approximately 10–13% [9, 13], rats (23.1%) [13] and dogs (20–45%) [13]). It should be noticed, that in cases of animal species in which parafollicular cells possess cytoplasmic processes, the number of C cells could vary depending on the way of C cell definition in histological sections, *i.e.* counting only C cell bodies or C cell bodies and processes. The problem of underestimation of C cell number in such cases was raised by Sawicki and Kuczynski [11]. Taking into account this issue and to more precisely determine the number of C cells, we counted both cells bodies and positively stained cytoplasmic processes.

The results of our study confirmed the observation, that C cells are the most numerous in the central areas of thyroid lobes. Differences in C cell distribution within the thyroid parenchyma were reported in humans, various rodents and pigs [7, 11, 12, 14]. This phenomenon was observed also in European bison [16] and we made the attempt to confirm it quantitatively. To precisely analyze the distribution of C cells within the thyroid parenchyma, we counted their numbers in three regions of the middle part of thyroid lobes in transverse section. We found that the mean number of C cells gradually increases from periphery to central part of thyroid parenchyma. This observation was confirmed statistically, when we analyzed all cases together. However, this tendency was not so evident when we analyzed particular cases. In general, the difference in C cell concentration between peripheral and central areas of thyroid lobe was the most consistently observed feature (82%), but statistically significant variations in C cell concentration in neighboring areas of individual thyroid lobes (*i.e.* peripheral *vs*. intermediate, and, especially, intermediate *vs.* central) were found less often (in 53% and 32%, respectively). Moreover, in some cases (14%) no significant differences in C cell concentration among all analyzed areas of thyroid lobe were found. This lack of statistically significant differences between percentages of C cells in particular regions of thyroid tissue sections observed in these cases can result from two reasons. Firstly, it could result from C cell hyperplasia which frequently occurred in the examined cases. The foci of C cell hyperplasia occupying periphery of middle part of thyroid lobes and expanding into central areas, influenced the mean number of C cells in intermediate and peripheral regions in some of randomly chosen visual fields. Secondly, the characteristics of C cell distribution pattern in European bison thyroid gland, with presence of groups of parafollicular cells of various size surrounded by areas more or less devoid of them, created additional differences in C cell numbers among visual fields in each analyzed areas.

In our study, the quantitative analysis of C cells was performed only in the medial part of left thyroid lobes

in cross-sections. We chose this region for two reasons. Firstly, because it has been shown that in most species C cells are most abundant in the central region of thyroid lobes [7, 8, 14]. Moreover, this method was applied in histological studies conducted on European bison thyroid gland, so we use the same method for comparison purposes [5, 6, 15, 16]. However, to fully analyze the C cell distribution thorough parenchyma of European bison thyroid gland and especially the C cell concentration further studies on other regions of thyroid lobes are needed.

C cell hyperplasia was a frequent finding in our study. It was found in approximately 45% of cases. C cell hyperplasia may represent a physiological or reactive proliferation of calcitonin-producing cells in response to different physiological and/or pathological endocrine stimuli (*e.g.* TSH overstimulation, hypercalcemia, paracrine factors). In humans, it was reported in newborns, elderly as well as in patients with hyperparathyroidism, hypergastrinemia due to Zollinger-Ellison syndrome, exogenous estrogen administration and in some thyroid diseases [19, 20]. It was also described in tissue adjacent to thyroid tumors and as a preneoplastic condition [20]. However, without knowledge about serum calcium level and hormonal status it is impossible to rationally explain causes of C cell hyperplasia observed in our study. We cannot also exclude that, at least in some of these cases, increased number of C cells was related to the presence of ultimobranchial remnants. Sawicki [6] observed ultimobranchial follicles in medial parts of European bison thyroid glands. We did not found histological features of ultimobranchial remnants in examined tissue sections; however, taking into account that in the vicinity of these structures an increased number of C cells was reported [21, 22], we cannot exclude that in some of our cases C cells hyperplasia was related to presence of ultimobranchial remnants. Analysis of serial sections is necessary to confirm this hypothesis.

We found that in European bison thyroid parenchyma C cells were localized in three different positions: intrafollicular, epifollicular and interfollicular. According to Sawicki and Kuczynski [11], the term "interfollicular position" should be used in relation to C cells clearly located within the follicular epithelium, irrespective of whether they have visible contact with colloid or not and we observed C cells located between follicular cells in all analyzed cases. Moreover, this C cells distribution pattern was frequent. However, in the available literature information regarding C cells in interfollicular position in European bison thyroid gland are sparse. Studies conducted on parafollicular cells in this species [5, 6] indicate that typically European bison C cells occupy the parafollicular position. This is not consistent with our observations as we found C cells presented within walls of thyroid follicles with comparable or sometimes higher frequency than in parafollicular position. C cells lying parafollicularly became more apparent as the number of C cells increased.

We found that European bison C cells localized outside of the follicular wall can occur in two positions: epifollicular and interfollicular. Classifying distribution pattern of C cells lying interstitially we applied criteria proposed by Sawicki and Kuczynski [11]. We agree with these authors that sometimes it is difficult to state unequivocally whether the observed position of C cells corresponds to the true interstitial or epifollicular position. However, we observed C cells lying individually or in groups which clearly adhered to the basal surfaces of follicular cells and we treated these cells as located in epifollicular position. Other C cells presented within connective tissue stroma without direct contact with thyroid follicles were considered as truly interfollicular. However, we cannot exclude that in some cases, especially when C cells were arranged in clusters, they could represent the epifollicularly located group of C cells in tangential section. In our opinion, both distribution patterns of C cells in European bison thyroid stroma are possible; however, this could be confirmed by further transmission electron microscopy studies. Moreover, we found that in hyperplastic areas some of C cells in epifollicular position form rings around thyroid follicles. Such distribution pattern of C cells was reported in rat [11], but to date has not been described in European bison.

It is widely known that microscopic appearance of thyroid gland reflects its functional state. It has been shown that in animals very well adapted to low temperatures, hormonal activity of the thyroid gland undergoes seasonal changes and is significantly lower in winter than in summer. This phenomenon was confirmed by microscopic examinations and analysis of serum thyroid hormones levels in different animal species, including deer and cattle [23, 24]. Similar changes in thyroid gland activity were reported also in European bison. Sawicki *et al.* [5] have observed microscopically the seasonal changes in height of epithelial cells lining the follicles. In summer and early autumn, the follicular cells were higher than in winter. However, all these observations are related to thyrocytes and our study design gave also the opportunity to elucidate whether any morphological features could indicate that European bison C cells also undergo seasonal changes. Such changes have been reported in hibernating animals, including woodchucks [25] and bats [26]. Ultrastructural studies conducted on these

animals have indicated increased synthetic activity of C cells during spring, summer and autumn, which is reflected by progressive accumulation of secretory granules and increase length of C cells. In winter, the granules are stored and undergone progressive degranulation [25]. Moreover, it has been found that ultrastructure and size of C cells granules also differ depending on annual cycle of physiological activity of hibernating animals [26]. It could not be excluded that such changes in C cell activity; however, less pronounced, can also occur in non-hibernating animals, especially that seasonal changes in serum calcium level were described in some species [27, 28]. Moreover, seasonal changes of serum calcitonin level were reported in deer [29, 30] with the lowest levels in winter and the highest in the summer-autumn period [30]. Unfortunately, the technical conditions of obtaining material from European bison autopsies did not allow analyzing blood serum.

Morphometrical analysis in our study showed that in spring-summer period European bison C cells are smaller but of higher immunoreactivity of C cells than in autumn and winter. As the IHC color intensity parameter reflects the amount of calcitonin accumulated in C cells, our results suggest that in European bison, production of calcitonin by C cells may differ depending on the time of the year. Increased size of C cells in autumn and winter together with their lower immunoreactivity could be the effect of more extensive synthesis of regulatory peptides instead of calcitonin by C cells at this period. It is widely known that apart their role in calcium homeostasis, C cells are source of many regulatory peptides and are involved in the local regulation of thyrocytes activity. Some of these peptides display an inhibiting action, whereas others act as stimulators of thyroid hormones synthesis [7]. We cannot excluded that in an autumn-winter period C cells are much more involved in regulation of follicular cell activity than in calcitonin synthesis and presence in their cytoplasm more granules containing regulatory peptides results in increase of their size. Somatostatin is an example of such inhibitory peptide which is synthesized by C cells together with calcitonin [7] and its presence in European bison parafollicular cells was confirmed immunohistochemically [15]. However, to confirm this hypothesis further studies of the expression of various regulatory peptides by European bison C cells are needed.

In the present study we described in details the morphology and distribution pattern of C cells in European bison thyroid gland. We also performed their morphometric analysis. However, to fully verify some of our current findings further studies are needed. Transmission electron microscope analysis will allow confirming the shape and exact localization of C cells relative to thyrocytes and characterization of the secretory granules. To validate our hypothesis of seasonal changes in C cell activity, analysis of levels of calcitonin as well as other regulatory peptides with western blot assays together with immunohistochemical characteristic of C cells expressing these peptides should be performed. Moreover, measurements of serum calcitonin levels in particular seasons are needed to confirm or exclude some of the above presented hypotheses.

Authors' contributions

JS participated in the design of the study, carried out microscope examination, morphometric analysis, interpreted of the results and drafted the manuscript; JB, AP and DR carried out microscope examination and morphometric analysis; KO collected material, performed macroscopic classification of obtained thyroid glands for study purposes; KU participated in immunohistochemical analysis and helped to draft the manuscript.

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