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The effects of synthetic salmon calcitonin on thyroid C and follicular cells in adult female rats

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Abstract: Structural and morphometric features of thyroid C and follicular cells were studied in adult rat females after treatment with synthetic salmon calcitonin (CT). The animals were chronically treated with either a low (10 IU/kg b.w) or a high (100 IU/kg b.w) dose of CT. A stereological method was applied to determine the volume density and the number of immunoreactive C cells. The height and volume density of follicular epithelium, colloid, interstitium and the follicles (epithelium plus colloid), as well as the index of activation rate were calculated. A significant decrease in body weight, as well as the volume density of immunoreactive C cells and the number of C cells per mm^2, was observed in rats treated with both doses of CT. The height and volume density of follicular epithelium and follicles, as well as the index of activation rate were significantly increased in the animals given the high CT dose, while the volume densities of colloid and interstitium were reduced. No significant changes in the examined morphometric parameters were detected after treatment with the low CT dose. According to these results it can be concluded that the structural features of thyroid C and follicular cells were affected by the high dose CT treatment in the opposite manner, while the low dose CT treatment influenced only C cells.

Key words: Calcitonin - Thyroid gland - C cells - Follicular cells - Rat

Introduction

In mammals, the thyroid gland originates from two initially separate primordia - endodermal cells as the source of follicular cells and ectodermal neural crest as the source of the C cells, which fuse during prenatal life into a unified organ. However, Kameda and Ikeda [14] suggested common precursors for both thyroid gland cells that would, depending on the conditions, differentiate into C or follicular cells.

Salmon CT with a biological potency of some 5000 IU/mg is the most potent calcitonin known among all species [21]. The role of CT in the prevention of bone resorption has already been described in detail. Calcitonin plays a central role in both calcium metabolism and skeletal development and may be a useful and suitable alternative in long-term prevention of postmenopausal osteoporosis in humans, especially in women [3, 26]. This is important since the use of sex hormone replacement therapy has some restrictions due to the potential risks of breast cancer, venous thrombosis, migraine and coronary heart disease [10]. Also, Kavuncu *et al.* [15] observed beneficial effects of calcitonin on ovariectomy-induced bone loss in rats. However, some recent data of Hirsh and Baruch [7] suggest that although CT has been purified and the amino acid sequence determined, its physiological function is still not completely understood.

Kakudo *et al.* [13] showed that high CT doses (30 and 120 IU/kg/day) caused C cell suppression after long-term administration (53 weeks), while low doses (0.075, 0.75 and 7.5 IU/kg/day) expressed no significant effects on the C cell population of adult rats. Mori *et al.* [17] recently reported that short-term (14 days) CT administration suppressed C cell proliferation in rat thyroid, in a dose-dependent manner. A negative feedback mechanism on CT secretion was observed by Morimoto *et al.* [20] after administration of salmon CT to rats.

The close spatial relationship between thyroid C and follicular cells has led to speculations about possible paracrine interaction between them. Thus, the C cell peptides: CT, somatostatin, CGRP and katacalcin seem to be involved as inhibitors of thyroid hormone secretion, while gastrin-releasing peptide, helodermin and

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serotonin stimulate it [1, 6, 12, 25]. Rat thyroid C cells were shown to express the authentic TRH gene, which suggested a potential paracrine regulatory role of TRH gene products on thyroid follicular cells [5]. On the other hand Barash *et al.* [2] reported multiple effects of TSH on C cells, including the induction of 5-HT secretion, what suggests that TSH acts as a C cell secretagogue. Thyroid transcription factor 1 (TTF-1) is a homeodomain protein initially identified as a thyroid-specific factor responsible for thyroglobulin gene transcription. That factor, identified also in rat C cells, was shown to interact with the extracellular Ca-sensing receptor, calmodulin and calcitonin genes in C cells [24].

Thyroid follicular cells affect the synthesis of mRNA for CT and CGRP in rat C cell cultures, and it seems that direct contacts with other cells may be involved in the control of mRNA formation from the CT gene [28].

Keeping in mind that CT treatment is applied in human medicine, as well as the scarce literature data on the influence of CT on thyroid cells, especially thyroid follicular cells, it was of interest to examine immunocytochemical and morphometric features of thyroid C and follicular cells after chronic treatment of adult rat females with two different doses of synthetic salmon CT.

Materials and methods

Eighteen adult (3-month-old) female Wistar rats were divided into three groups each consisting of six animals. They were maintained in a 12/12 h light-dark cycle at 22±2˚C with free access to food and water. The first and the second group received s.c. synthetic salmon calcitonin (ICN, Galenika Pharmaceutical Works, Belgrade, Serbia and Montenegro) at 10 IU and 100 IU per kg body weight, respectively, every second day for 12 weeks. The rats of the third group were treated with physiological saline by the same schedule. The animals were killed under ether anesthesia 24 h after the last injection.

The thyroid tissues were fixed in Bouin's solution at room temperature for two days, embedded routinely in paraffin wax and serially sectioned at 5 μm. Thyroid glands with trachea were oriented perpendicularly to the cut surface and sectioned from the base to the apex.

For localization of CT in the thyroid C cells, the peroxidase-anti-peroxidase (PAP) method described by Sternberger *et al* . [23] was applied. The sections on gelatine-coated slides were deparaffinized and placed in a solution of 0.3% H_2O_2 in absolute methanol for 15 min to inhibit endogenous peroxidase activity. After thorough washing with 0.1 mol/l phosphate-buffered saline (PBS), pH 7.4, the sections were incubated with normal swine serum (1:10) for 45 min to reduce non-specific staining. The sections were further incubated for 45 min with 1:500 rabbit anti-human CT (Dakopatts, Copenhagen, Denmark). This was followed by a wash in PBS, incubation in 1:500 swine anti-rabbit IgG (Dakopatts) for 45 min, another PBS wash, and incubation in 1:100 rabbit PAP serum for 45 min (Dakopatts). After another wash in PBS, the sections were incubated for 5-8 min in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Serva, Heidelberg, Germany) in 0.2 mol/l TRIS-HCl buffer, pH 7.4. To visualize peroxidase activity the 0.03% H_2O_2 was added to DAB solution. The sections were then counterstained with hematoxylin and eosin. After that, they were dehydrated and mounted. Control sections were incubated with PBS instead of the primary antibody.

The sections of thyroid gland with specifically labelled C cells were stereologically analyzed by simple point counting [27]. The mean volumes of the cytoplasm and the nuclei of the C cells and numerical density of their nuclear profiles were determined using the multipurpose M_{42} test system, with 50 test areas and × 1000 magnification. Numerical density of the C cell nuclei/mm^3 (and thus of the cells) was determined according to the formula of Weibel and Gomez. The shape coefficient was assumed to be 1.382 for the C cell nuclei [27]. The number of immunoreactive C cells per mm^2 was also calculated.

Stereological analyses of thyroid follicular tissue was performed on every fifth serial hematoxylin-eosin stained section using multipurpose M_{42} test system, placed in the ocular of Zeiss light microscope, on up to 5 sections per sample and 50 test fields per animal at objective magnification × 40. The volume densities of thyroid follicles (V_{vf}), follicular epithelium (V_{ve}), colloid (V_{vk}) and interfollicular tissue (V_{vi}) expressed in mm^3, as well as the index of activation (ia, the ratio of volume density of follicular epithelium to volume density of colloid) were calculated. The follicular cell height (τ) was determined according to Bogataj *et al.* [4].

All morphometric data for each animal were averaged per group and the standard error of the mean (SEM) was calculated. The significance of the differences was evaluated by Student's t-test.

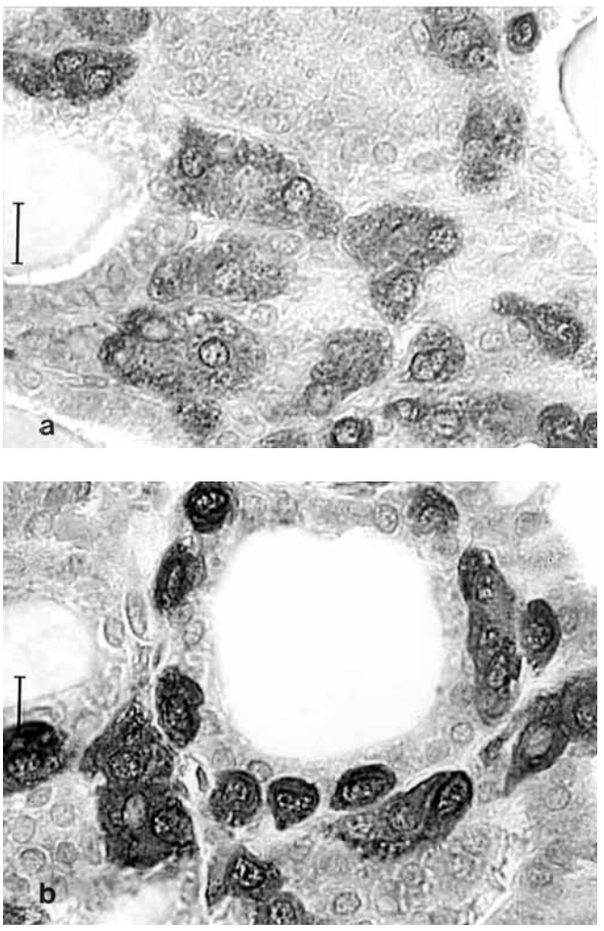

Fig. 1. a. Thyroid C cell clusters in a control adult rat female. Immunoperoxidase staining specific for calcitonin. **b.** Dark immunopositive C cells in the thyroid gland of a rat female chronically treated with calcitonin dose of 100 IU/kg b.w. Bar = 20 μm.

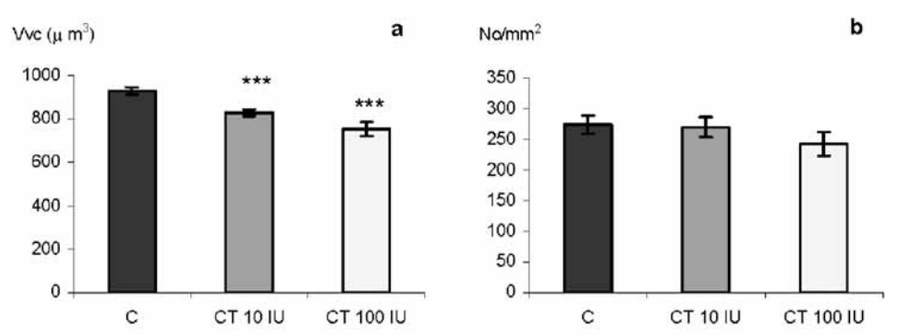

Fig. 2. a. Cellular (V_{vc}) volume (μm^3) of immunoreactive C cells. **b.** The number of C cells (No) per unit area (mm^2); C - control animals; CT 10 IU - animals chronically receiving calcitonin dose of 10 IU/kg b.w. and CT 100 IU - animals chronically treated with calcitonin in the dose of 100 IU/kg b.w. All values are the means±S.E.M. ***$p<0.001$.

Results

In control thyroid glands, C cells were gathered in large groups among the follicles. Their cytoplasm was packed with material immunoreactive for CT (Fig. 1a).

The C cells of animals chronically treated with 10 IU CT/kg b.w. were smaller and their cytoplasm contained dark granular immunoreactive products. A significant decrease in C cell volume by 11% in comparison with control values was observed (Fig. 2a). In addition, the number of C cells per mm^2 was insignificantly changed as compared to the controls (Fig. 2b).

The degree of structural changes in thyroid C cells was more prominent in the animals chronically treated with 100 IU CT / kg b.w. The cytoplasm of the C cells was filled with intensely stained granular immunoreactive CT products (Fig. 1b). The volume of these immunoreactive cells was decreased by 19% in comparison with the corresponding controls (Fig. 2a) and the difference was statistically significant. However, the number of C cells per mm^2 was not significantly changed when comparing to the controls (Fig. 2b).

The most prominent structural features of the thyroid gland in the female rats chronically treated with CT, were a tall columnar follicular epithelium and a lower amount of colloid compared to the controls (Fig. 3a). The whole gland appeared to have a microfollicular structure. These structural features were more expressed after chronic treatment with the higher dose of CT (Fig. 3b) than with the lower dose. The histological structure of the thyroid gland corresponded well with the morphometric values of all the measured stereological parameters.

In females treated with 10 IU CT per kg b.w., the height of the follicular epithelium was significantly increased by 14%, while other stereological parameters were not significantly changed in comparison with the control values (Fig. 4a-d).

In animals receiving 100 IU CT per kg b.w., conspicuously expressed differences in all stereological parameters were found in relation to those of the controls.

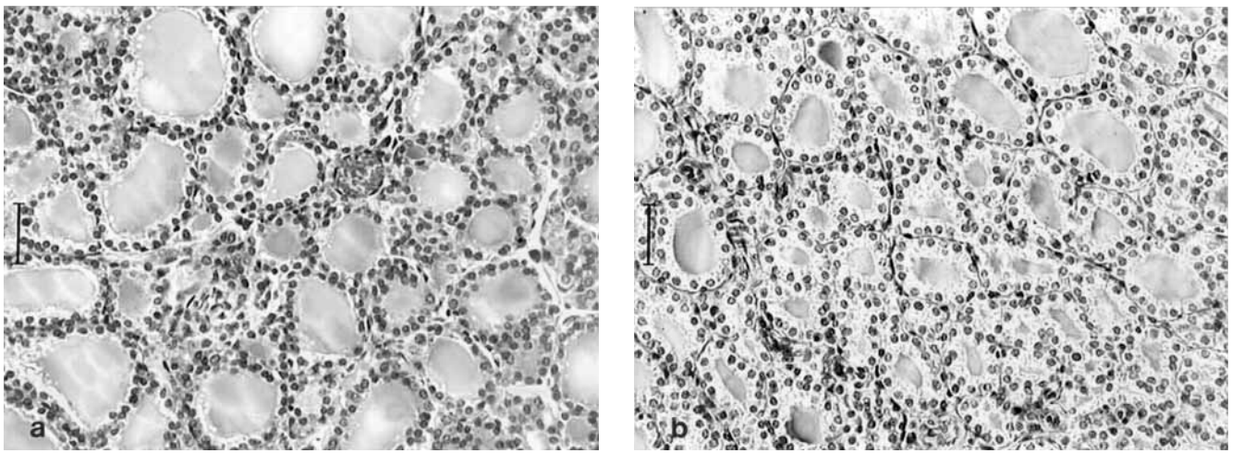

Fig. 3. a. Structure of thyroid gland in a control rat female. Note the follicular epithelium with columnar thyrocytes. **b.** A tall columnar follicular thyroid gland epithelium of a rat female chronically treated with calcitonin in the dose of 100 IU/kg b.w. Bar = 40 μm.

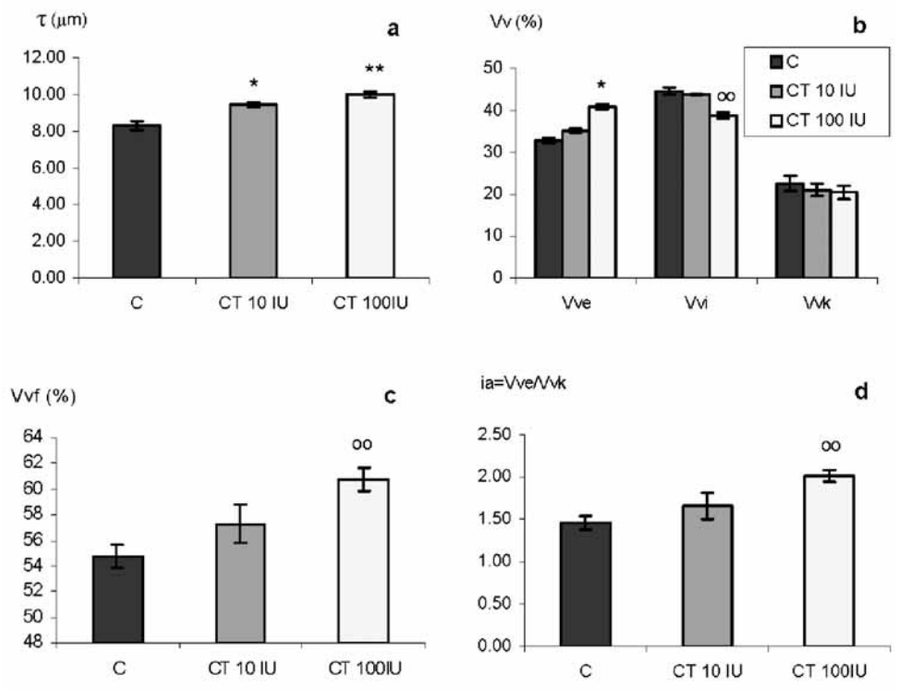

Fig. 4. a. The height of thyroid follicular cells (τ). **b.** Volume densities of thyroid follicular cells (V_{ve}), colloid (V_{vk}) and interstitium (V_{vi}). **c.** Volume density of thyroid follicles (V_{vf}). **d.** Index of activation (ia) rate (V_{ve}/V_{vk}); C-control animals; CT 10 IU - animals chronically treated with calcitonin dose of 10 IU/kg b.w. and CT 100 IU - animals chronically treated with calcitonin dose of 100 IU/kg b.w. All values are the means ± S.E.M. °°p<0.025; *p<0.01; **p<0.005.

There were statistically significant increases in height (by 20%) and volume density (by 24%) of the follicular epithelium, in volume density of thyroid follicles by 11% and in the index of activation (by 38%) in relation to control values (Fig. 4a-d). At the same time, significant decrease in volume density of interstitium (by 13%) was observed (Fig. 4b).

Discussion

Calcitonin is a naturally occurring calciotropic hormone synthesized in thyroid C cells. It plays an important physiological role in mineral and skeletal homeostasis, and for that reason is used in human medicine to prevent osteoporosis.

In contrast to C cells, there are only sparse data in the available literature on the influence of CT on the structure and function of thyroid follicular cells. This prompted us to examine the influence of two different doses of CT chronically applied to adult female rats, on the structure of both thyroid C and follicular cells.

Exogenous CT administration was reported to inhibit CT secretion in rats and therefore CT treatment probably suppresses C cell function due to a negative feedback. Long-term CT administration was also assumed to suppress protein synthesis and hormone secretion by C cells possibly by suppressing their proliferation even in the thyroid of aged rats, and these changes were reversible after 4 weeks of CT withdrawal [11].

Our results showed that the decrease in C cell volume was statisticaly significant and dose-dependent. This is in accordance with previously reported data that CT express autocrine effect on C cells by blocking its own secretion [18]. On the other hand, it is not clear whether exogenous CT induces negative feedback to the proliferation of C cells, through the CT receptor on C cells or indirectly *via* Ca^{2+} or some other factors. The number of C cells was not significanly changed under our experimental conditions, while Kakudo *et al.* [11] found that CT acted by decreasing the incidence of C cell hyperplasia, probably due to a decrease in the number of C cells. Mori *et al.* [17] also observed a decreased S phase fraction of C cells after the application of two different CT doses. Therefore, it would be of a great importance for clinical practice to find out whether CT could control C cell proliferation in advanced C cell carcinoma.

The localization of the C cells in the central regions of the thyroid gland lobes, where thyroid hormone synthesis and secretion seems to be higher than in the periphery of the lobes, has led us to the hypothesis that C cells, in addition to their role in calcium homeostasis, somehow regulate (stimulate and/or suppress) the activity of the follicular cells. The volume density and the height of follicular epithelium, as well as the index of activation rate, were significantly increased in animals treated with the higher CT doses. In rats treated with the lower CT doses, the height of follicular epithelium was significantly higher in comparison to the controls. These results suggest increased activity of the thyroid follicles,

especially in the group of animals chronically treated with the higher CT dose.

Our previous data showed that estradiol affects both thyroid follicular and C cells, and the response of these two cell populations was opposite, *i.e.* stimulation of the former was accompanied by inhibition of the latter cell type [22]. The results presented here support and confirm previously reported data: chronic treatment of adult rat females with two different calcitonin doses lowered the structural parameters of C cells, and enhanced those of thyroid follicular cells in a dose-dependent manner. It seems likely that peptides manufactured by C cells might participate in controlling thyroid activity, by a direct paracrine route.

Some authors concluded that follicular cell activity was positively regulated by peptides released from C cells [6, 13, 19]. Thus, serotonin from C cells could stimulate both endocytosis of thyroglobulin and release of T_4 and T_3 by paracrine action [13]. Moreover, CT and CGRP treatment decreased rat serum TSH level and suppressed the stimulatory effect of TSH on ^{3}H-thymidine incorporation into DNA of thyroid lobes, *i.e.* these neuropeptides acted as modulators of follicular cell proliferation [8, 16, 20]. In that context, it could be hypothesized that thyroid follicular cells indirectly respond to CT: if serum TSH level is low after CT treatment, thyroid follicular cells respond by a known positive feedback mechanism. The changes of all the examined morphometric parameters observed throughout the present study support this hypothesis.

As suggested previously, TRH gene expression by C cells raises the possibility that locally synthesized TRH gene products could be present in sufficient amounts to express paracrine regulatory effects on follicular thyroid hormone secretion, *i.e.* C cells could play a role in the regulation of thyroid hormone homeostasis [5, 9]. Hence, the relationship between the two control mechanisms for thyroid transcription factor (TTF-1) levels and function in both C cells and follicular cells becomes an interesting question, since two signals are functionally interactive in both cell types [24]. Increased intracellular calcium content in C cells was shown to decrease TTF-1 and increase Ca-sensing receptor (CaSR) levels. Conversely, a decreased calcium level acts by increasing TTF-1 and decreasing CaSR levels [24]. These results suggest the possiblility of paracrine interaction between C and follicular cells

Based on the present results, it can be concluded that chronic treatment of female rats with two different doses of salmon CT affects structural features of thyroid C and follicular cells in a dose-dependent and opposite manner.

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