Original Article

Histomorphometric effects of calcitonin on pharyngeal bone in fed and starved goldfish *Carassius auratus*

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**ABSTRACT:** The effects of salmon calcitonin (sCT) on osteoblasts and osteoclasts were histologically and histomorphometrically investigated in the pharyngeal bone of fed and starved goldfish *Carassius auratus*. The thickness of the osteoid and the height of osteoblasts were also measured. Fish were given sCT intraperitoneally at a dose of 10 ng/g bodyweight four times every other day under either fed or starved conditions. The sCT treatment induced the retraction of osteoclasts and their disengagement from the bone surface. Salmon calcitonin had no effect on the histomorphometric parameters of bone formation activity, but suppressed the parameter (OcP/EP) that showed osteoclast activity. Salmon calcitonin did not affect the thickness of the osteoid, but increased the height of osteoclasts in starved fish. Histological and histomorphometric results demonstrated that osteoclast activity was suppressed when sCT was given to starved estrogenized goldfish five times for 10 days. Overall, results suggest that calcitonin is involved in bone assimilation by suppressing osteoclast activity and by enhancing osteoblast activity in the pharyngeal bone of goldfish.

**KEY WORDS:** bone resorption, calcitonin, estrogen, goldfish, histomorphometry, osteoblast, osteoclast, pharyngeal bone.

**INTRODUCTION**

Calcitonin is widely distributed among fish and mammals, and is well known as a hypocalcemic hormone in mammals. This hormone is secreted from the thyroid gland after a meal and acts to inhibit postprandial hypercalcemia in rats. Its main action is to stimulate the net mobilization of plasma calcium to bone by stimulating the activity of osteoblasts and/or by inhibiting the activity of osteoclasts.

In fish, however, the effect of calcitonin on plasma calcium levels is enigmatic and depends on the fish species and experimental conditions used. The administration of calcitonin induced a hypocalcemic response in freshwater-acclimated eels, but not in seawater-acclimated eels. In rainbow trout, calcitonin elicited either a hypo- or hypercalcemic response depending on the dosage. Calcitonin caused hypercalcemia in *Salmo trutta* and hypocalcemia in *Cyprinus carpio*.

*Sasayama et al.* and *Suzuki et al.* have found that calcium and/or nutrients absorbed via the digestive tract increased plasma calcitonin levels in goldfish, and have suggested that calcitonin plays a hypocalcemic role in calcium absorption via the digestive tract. Okuda *et al.* have found calcitonin-immunoreactive cells in the intestine of goldfish.

Fish scales and bone have been known to function as mineral reservoirs, especially during starvation and female sexual maturation. Lopez *et al.* have reported that synthetic salmon calcitonin (sCT) increased osteoblastic apposition and inhibited osteoclastic resorption in seawater-acclimated eels, and Wendelaar Bonga and Lammers have demonstrated that the hormone increased the size and number of osteoblasts in tilapia. *Shinozaki and Mugiya* have reported that calcitonin increased calcium deposition on the bone and scales of starved goldfish, and Suzuki and colleagues have found that osteoclastic activity is suppressed by calcitonin in the scales of goldfish and nibbler fish. Despite these studies, the effect of calcitonin on bone-related cells in estrogenized and/or starved fish is still unclear.
The present study histologically and histomorphometrically examined the effect of calcitonin on osteoblasts and osteoclasts of the pharyngeal bone in fed and starved goldfish. Calcitonin was also given to starved estrogenized fish to examine the effect of this hormone on these cells during vitellogenesis.

MATERIALS AND METHODS

Thirty of the goldfish *Carassius auratus*, weighing approximately 8 g, were selected from laboratory stocks and kept in aerated 60-L glass aquaria equipped with a filtration system. They were acclimated to experimental conditions (22°C, light/dark cycle 12 h:12 h) for at least 1 week before use. Fish were fed commercial carp pellets at approximately 2% of bodyweight every afternoon. In all experiments, fish were acclimated in a single aquarium and then randomly separated into experimental and control groups. Data from maturing females were excluded after autopsy.

Calcitonin administration

Salmon calcitonin (sCT; Peninsula Laboratories, Inc., CA, USA) was dissolved in 0.01 M acetic acid containing 1% gelatin (pH 5.0), then diluted in 0.6% NaCl, and administered intraperitoneally at a dose of 10 ng per 10 µL/g bodyweight. The sCT was injected four times every other day. Control fish received the same dose of 0.6% NaCl containing the acid and gelatin. During the eight-day treatment, half (n=5) of the experimental group and half (n=5) of the control group were starved, while the remaining fish (n=10) were fed every day.

Histological and histomorphometric observations

Pharyngeal bones, which are known to be metabolically active, were fixed in 10% formalin, dehydrated through a graded ethyl alcohol series, and embedded in JB-4 (Polysciences Inc., Warrington, UK). Cross-sections measuring 5µm were cut from the ventral and dorsal lobes (Fig. 1) of the bone with a microtome (Supercut 2050; Reichert-Jung, Vienna, Austria). Sections were mounted on glass slides and stained with 1% silver nitrate, and then with 1% toluidine blue and 1% azur II in 1% sodium borate solution for histological observation.

For histomorphometric observations, the following perimeters were measured using Image Analyzing Software (National Institutes of Health) and applied to the same sections as those observed for histology: (i) osteoid surface perimeter (OP), bone perimeter with the osteoid; (ii) osteoblast surface perimeter (ObP), osteoid perimeter lined with osteoblasts; (iii) eroded surface perimeter (EP), perimeter with osteoclasts and resorption lacunae; (iv) osteoclast surface perimeter (OcP), eroded surface perimeter lined with osteoclasts; (v) resting perimeter (RP), perimeter other than OP.

Calcitonin administration after estradiol-17β treatment

Estradiol-17β (E2; Sigma Chemical Co., St Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Wako, Osaka, Japan). Fish were administered with estradiol-17β at a dose of 5 mg per 10 µL/g bodyweight, and they were then separated into two groups (day 1). One group (n=5) was given sCT at a dose of 10 ng per 10 µL/g bodyweight on days 2, 3, 4, 7, and 9, and sampled on day 10. The other group (control, n=5) was given the same dose of the sCT solvent only. Fish were starved throughout the experiment.

Fig. 1 Right pharyngeal bone of a goldfish. Bars indicate the position of cutting.
and EP; and (vi) total bone perimeter (BP), a sum of OP, EP, and RP.

Using these measurements, six ratios (%) were calculated and used as parameters of bone formation (OP/BP, ObP/BP, and ObP/OP) and bone resorption (EP/BP, OcP/BP, and OcP/EP). OcP/EP represents the most actively resorbing area.

Histomorphometric observations were conducted using three different sections obtained from a single sample at intervals of approximately 100 μm and the data were averaged. Using the same sections, osteoid thickness and the height of osteoblasts were also measured with a micrometer. The thickest part of the osteoid and the tallest osteoblast were measured in each section. These observations were made in three to five fish for statistical analyses. The osteoid is formed by osteoblasts preceding calcification and its thickness is regarded as an indicator of bone formation activity.

Statistical treatment

Two-way ANOVA was employed to access the significant effects of feeding and calcitonin. Differences among means were subsequently assessed using Scheffe’s F-test. Student’s t-test was also used for analysis of the E2-treated experiment. The level of significance was accepted at P < 0.05.

RESULTS

Histological observations

A photograph of the pharyngeal bone is presented in Fig. 1. It is a small calcareous body, weighing approximately 15 mg. A pair of the bones is present in a single fish.

Cross-sections of the pharyngeal bone clearly showed that osteoblasts form an epithelioid layer of cuboidal or columnar cells (Fig. 2a, b). These lining cells are responsible for the formation of the osteoid, which exists on the bony surface. No marked difference in the bone formation surface was found between the control group (Fig. 2a) and the sCT-treated groups (Fig. 2b) of fish. Similarly, sCT had no effect on these histological features in starved fish except for the poor development of the osteoid (photograph not shown). The effects of sCT on the height of osteoblasts and osteoid thickness will be described later.

Osteoclasts with multinuclei, which are closely associated with bone resorption, were attached tightly to the osseous surface in the fed control group (Fig. 2c). However, sCT treatment retracted osteoclasts and disengaged them from the surface, resulting in the formation of resorption lacunae (Fig. 2d). In starved fish, osteoclasts had numerous varying-sized vacuoles in the cytoplasm and were attached tightly to the osseous surface in the control group (Fig. 2e); however, sCT treatment generated a space between the osteoclasts and the surface, resulting in their disengagement from the bone surface (Fig. 2f).

Histomorphometric analysis

Parameters of bone formation (OP/BP, ObP/BP, and ObP/OP) remained unaffected by sCT treatment in both the fed and starved fish groups (Fig. 3), although starvation reduced ObP/OP significantly in both the fed and starved fish groups (fed P < 0.05; starved P < 0.01). Starvation increased the EP/BP ratio in sCT-treated fish (P < 0.01; Fig. 4).

In the E2-treated fish, sCT had no effect on any parameters of bone formation (OP/BP, ObP/BP, and ObP/OP; Fig. 5) but it did reduce the bone resorption parameter, OcP/EP (P < 0.05; Fig. 6). The other parameters (EP/BP and OcP/BP) of resorption remained unaffected.

Starvation reduced the thickness of the osteoid to approximately half that of the fed fish, but sCT had no effect on osteoid thickness in the fed, starved, and E2-treated fish groups (Table 1). Although the height of osteoblasts was unaffected by sCT in the fed and E2-treated fish groups, height was increased significantly by sCT in the ventral lobe of the bone in starved fish (Table 1). The dorsal lobe was not affected.

Table 1 Effects of salmon calcitonin (sCT) on the thickness of the osteoid layer and the height of osteoblasts in the pharyngeal bone (ventral and dorsal lobes) in fed, starved, and E2-treated goldfish

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fed Ventral</th>
<th>Starved Ventral</th>
<th>Dorsal</th>
<th>Starved + E2 Dorsal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.0 ± 0.9</td>
<td>6.2 ± 0.5</td>
<td>5.9 ± 0.7</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>sCT</td>
<td>11.5 ± 1.9</td>
<td>7.7 ± 2.0</td>
<td>4.9 ± 0.9</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Height (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.9 ± 1.2</td>
<td>6.9 ± 0.2</td>
<td>6.9 ± 0.4</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>sCT</td>
<td>10.1 ± 0.6</td>
<td>8.5 ± 0.5</td>
<td>7.1 ± 0.6</td>
<td>6.2 ± 0.6</td>
</tr>
</tbody>
</table>

*p < 0.05 for the control groups.

†*P < 0.01 and ‡P < 0.05 for the respective fed groups.

Values are the mean ± SE of three to five fish.
DISCUSSION

The role of calcitonin in regulating plasma calcium levels in fish remains unclear. The results are not consistent and are dependent on experimental conditions and fish species used. In the present study, we selected a dose of 10 ng sCT/g bodyweight at which dosage plasma calcium concentrations were reduced significantly in goldfish. Histological observations revealed that this dosage
Fig. 3  Effects of salmon calcitonin (sCT) on the histomorphometric parameters of pharyngeal bone formation in fed and starved goldfish. Data are the mean ± SE of three or four fish. Cont., control fish; OP/BP, osteoid surface perimeter/total bone perimeter; ObP/BP, osteoblast surface perimeter/total bone perimeter.

Fig. 4  Effects of salmon calcitonin (sCT) on the histomorphometric parameters of pharyngeal bone resorption in fed and starved goldfish. Data are the mean ± SE of three or four fish. *P<0.05 and **P<0.01. Cont., control fish; EP/BP, eroded surface perimeter/total bone perimeter; OcP/BP, osteoclast surface perimeter/total bone perimeter; OcP/EP, osteoclast surface perimeter/eroded surface perimeter.
Fig. 5  Effects of salmon calcitonin (sCT) on the histomorphometric parameters of pharyngeal bone formation in E$_2$-treated goldfish. Data are the mean ± SE of three or four fish. OP/BP, osteoid surface perimeter/total bone perimeter; ObP/BP, osteoblast surface perimeter/total bone perimeter; ObP/OP, osteoblast surface perimeter/osteoid surface perimeter.

Fig. 6  Effects of salmon calcitonin (sCT) on the histomorphometric parameters of pharyngeal bone resorption in E$_2$-treated goldfish. Data are the mean ± SE of three or four fish. *$P<0.05$. EP/BP, eroded surface perimeter/total bone perimeter; OcP/BP, osteoclast surface perimeter/total bone perimeter; OcP/EP, osteoclast surface perimeter/eroded surface perimeter.
clearly retracted osteoclasts and isolated them from the bone surface, indicating that calcitonin inhibited osteoclastic bone resorption. This evidence was further supported by the observation that the OcP/EP ratio, an indicator of active bone resorption, was significantly reduced by the hormone. These results coincide with those of Lopez and coworkers, who reported that calcitonin decreased the number of multinucleated osteoclasts in eels. Johannsson et al. have also reported that the activity of osteoclasts was decreased by calcitonin perfusion in rainbow trout. Therefore, calcitonin functions as an inhibitor of osteoclastic bone resorption in fish as well as in mammals. It has been reported previously that, in mammals, osteoclasts have calcitonin receptors on their surfaces.

Shinozaki and Mugiya have reported that sCT (10 ng/g bodyweight) has no effect on calcium release from the 45Ca-prelabeled pharyngeal bone, as well as the rib bone and scales in goldfish. Their results are contradictory with the present study’s findings that sCT inhibited bone resorption by osteoclasts. This discrepancy might have resulted from the fact that bone resorption occurs in two different ways: (i) bone resorption by osteoclasts, which removes both calcium and organic matrix; and (ii) halastasic demineralization (or calciolysis), which reduces calcium only from the bone by a physico-chemical process. The latter is usually dominant and easily reacts to calcium demand. Therefore, in the study by Shinozaki and Mugiya, in which the results were expressed in terms of 45Ca activity, the inhibitory effect of calcitonin on calcium resorption by osteoclasts might be masked by halastasic demineralization.

Lopez et al. and Wendellar Bonga and Lammers have reported that calcitonin increases the number of osteoblasts in eels and tilapia, respectively. In contrast, calcitonin did not affect any of the bone formation parameters (OP/BP, ObP/BP, and ObP/OP) investigated in the present study. However, it increased the height (size) of osteoblasts in starved fish, suggesting that this hormone promotes bone formation by increasing the activity of each osteoblast. These results are supported by a physiological study that demonstrated that calcitonin increased calcium deposition on the pharyngeal bone in starved goldfish, but not in fed fish. Overall, the results suggest that calcitonin has some effect on restoring the starvation-induced suppression of pharyngeal bone formation.

The administration of E2 induces vitellogenesis during which calcium is mobilized from scales to bind with vitellogenin. In such a case, extra calcium needed for the binding seems to be mobilized. In the present experiment, sCT inhibited osteoclastic bone resorption in estrogenized goldfish. Therefore, this hormone is expected to have a preserving effect on the extra calcium release from tissues. Björnsson and colleagues have found that plasma calcitonin levels increase after E2 treatment in salmonid fish and they suggest that it has a role in reproductive physiology rather than in calcium regulation.

In conclusion, calcitonin inhibited the osteoclastic resorption of the pharyngeal bone in starved and estrogenized goldfish, suggesting that this hormone has a bone-preserving role in fish when there is an extra calcium demand.

ACKNOWLEDGMENTS

We thank Dr M Shimizu (Graduate School of Fisheries Sciences, Hokkaido University) for his invaluable advice and support. We are also grateful to Dr N Kagawa for preparing the figures.

REFERENCES