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Effect of Defective Calcium Metabolism on Otolith Formation in Zebrafish

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Abstract Benign paroxysmal positional vertigo (BPPV) is the most common vertigo disease and is more likely to occur in perimenopausal women, suggesting an association with osteoporosis. Since otoconia are primarily composed of calcium carbonate, abnormal calcium metabolism may lead to otoconia dislocation. However, the detailed mechanism is currently unknown. In this study, we investigated the effects of drugs (cadmium and dexamethasone) that cause abnormal calcium metabolism on otolith formation in zebrafish larvae. Here, otolith size was clearly reduced in the cadmium group, and the calcium content of the larvae was also markedly reduced. In contrast, in the dexamethasone group, which also had a lower calcium content than the control group, otolith size increased. Our results suggest that, as in bone, calcium metabolism influences the repeated dissolution and recrystallization of otoliths and maintains homeostasis in response to calcium concentrations in the endolymphatic fluid.

Key words: otolith, zebrafish, benign paroxysmal positional vertigo, calcium metabolism, bone loss

Introduction

Benign paroxysmal positional vertigo (BPPV) is one of the most common causes of vertigo in humans due to otolith dislocation.[1-4] Previous studies have shown that perimenopausal women are especially susceptible to BPPV because of decreased estrogen levels.[1,5-10] In addition, low estrogen levels affect bone and calcium metabolism, leading to bone loss and osteoporosis.[11,12] Since the otoconia is composed primarily of calcium carbonate,[13-15] abnormal calcium metabolism may lead to otoconia dislocation. However, the detailed mechanism of calcium metabolism in otoconia is currently unknown.

In the present study, we investigated the effects of drugs that cause abnormal calcium metabolism (cadmium and dexamethasone, a type of steroid) on otolith formation.16 We used zebrafish larvae with two otolith organs, the saccule and utricle, which is a suitable animal model for drug screening.

Methods

Animals

All experiments were conducted using AB wild-type zebrafish that were kept in rearing water at 29℃ at the University of Yamaguchi. Fertilized eggs born between adult male and female zebrafish were collected. They were maintained in petri dishes filled with embryo media (1 mM MgSO4, 120 mM KH2PO4, 74 mM Na2HPO4, 1 mM CaCl2, 500 mM KCl, 15 mM NaCl, and 500 mM NaHCO3 in dH2O) at 29℃. The experimental protocol was reviewed and approved by the Committee for the Ethics of Animal Experiments of the Yamaguchi University School of Medicine (No.43-007) and was carried out according to the Guidelines for Medicine and the Law (No.

105) and Notification No. 6 of the Japanese Government.

Drug exposure

Because the otoliths were already morphologically identified on the first day post-fertilization (1 dpf) and hatched from the eggs at 2 or 3 dpf, exposure to the drug was started from 2 dpf. The zebrafish were exposed to solutions of 20 μmol/L cadmium chloride (Cl[Cd]Cl, 99.99% trace metals basis; Sigma Aldrich, St. Louis, MO) or dexamethasone (Sigma Aldrich, St. Louis, MO), and dimethyl sulfoxide (DMSO) as the control group and fixed on 5 and 8 dpf. Three concentrations of cadmium (20 μmol/L, 40 μmol/L, and 100 μmol/L) and dexamethasone solutions (20 μmol/L, 40 μmol/L, and 100 μmol/L) were prepared to analyze the differences in otolith changes due to concentration.

Observation and measurement

Microscopy

The zebrafish were fixed in 4% paraformaldehyde overnight and decolorized for 8-9 minutes in 1% KOH and 1.5% H_2O_2. They were washed three times in phosphate-buffered saline for 10 minutes each, and then stained for 10 minutes with a red dye, "Alizarin red S (Sigma Aldrich, St. Louis, MO)". Alizarin binds to metal groups and stains calcium that is deposited in osteodifferentiated and calcified cells.

After washing for 10 minutes, we enclosed the zebrafish in microscope slides using RapiClear 1.47 (SunJin Lab Co., Hsinchu City, Taiwan). The otoliths were observed under an all-in-one fluorescence microscope (BZ-X810, KEYENCE, Osaka, Japan) with a Plan Apochromat 20x objective (NA0.75, BZ-PA20, KEYENCE, Osaka, Japan) from the dorsal view and their areas were measured using Measurement Application (BZ-H4M, KEYENCE, Osaka, Japan) in the BZ-X Analyzer software (BZ-H4A, KEYENCE, Osaka, Japan).

Calcium concentration measurements

Instead of measuring bone mass, calcium concentration was measured by dissolving the whole larva. One hundred larvae in each group of 8 dpf were dissolved with strong HCl (35%, 11.2 mol, 500 μL) and heated at 60℃ for 150 minutes. The samples were kept for 30 min at room air after Vortexing. The 50 μl resulting supernatant after 10 min of centrifugation at 4℃ was added to 450 μl water and neutralized to adjust the pH (pH 2≧) using 1 mol NaOH. The calcium concentrations were calculated by measuring the absorbance, averaging over four measurements, and the amount of calcium per larva was calculated. We used the Metallo Assay Calcium LS (OCPC) kit (Metallogenics Co., Ltd., Chiba, Japan).

Statistics

Significant differences between the control and exposure groups were evaluated using t-tests. All statistical analyses were performed by using Microsoft Excel for Mac version 16. Statistical difference was considered significant at $p<0.05$. Value was mean ± standard deviation (s.d.) in 6 control and exposure groups each.

Results

Otoliths in the cadmium group were significantly smaller than those in the control group in both the utricle and saccule at 5 and 8 dpf (*** $P<0.001$, n=6) (Fig. 1A-C). Furthermore, when comparing different concentrations in the cadmium group (20 μmol/L, 40 μmol/L, and 100 μmol/L), the otoliths tended to be smaller at higher concentrations of cadmium at 8 dpf (n=6) (Fig. 2A-C).

In contrast, in the dexamethasone group, utricular otoliths were significantly larger compared with those in the control group at both 5 and 8 dpf (*: $P<0.05$ ***: $P<0.001$, n=6); no significant difference was observed in the saccule (n=6), but there was a trend toward slightly larger otoliths at 8 dpf compared to controls (Fig. 3A-C). At different concentrations in the dexamethasone group (20 μmol/L, 40 μmol/L, and 100 μmol/L), the zebrafish died at the higher concentrations and were difficult to evaluate.

Alizarin staining showed that the dexamethasone group appeared inadequate and coarse compared with the control group. The cadmium group appeared even more

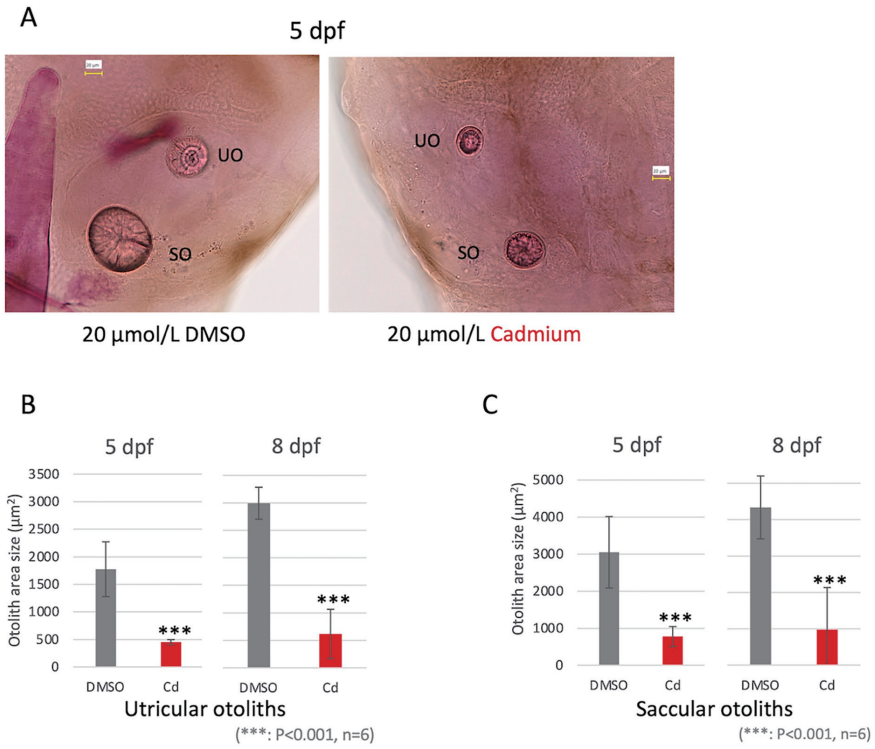

Fig. 1A
Representative figure of 5 dpf zebrafish exposed to a solution of 20 μmol/L DMSO and cadmium. The zebrafish are stained with Alizarin red S. The otoliths' sizes are smaller in the cadmium group than in the DMSO group.
UO, utricle otolith; SO, saccule otolith.

Fig. 1.B, C
The otoliths in the cadmium group were significantly smaller than those in the control group in both the utricle and saccule at 5 and 8 dpf. (***: P<0.001, n=6 t-test)
Cd, cadmium.

significantly inadequate and coarse compared to the control and dexamethasone groups. (Fig. 4A). The calcium concentration per larva in the dexamethasone treated group was 82% of that in the control group (Fig. 4B). On the other hand, the calcium concentration in the cadmium-treated group was 41% of that in the control group (Fig. 4C).

Discussion

The present study demonstrated that the otoliths of zebrafish become smaller with cadmium exposure in a dose-dependent manner and that the calcium content in larvae decreases with higher cadmium exposure. Our results are almost identical to those of the previous reports. Additionally, the calcium supplementation assay has reported that calcium content increased, whereas cadmium decreased.[17,18] Thus, in zebrafish, cadmium might act competitively with calcium to inhibit calcium uptake, which in turn inhibits $CaCO_3$ deposition and prevents otolith growth, resulting in smaller otolith.[15]

On the other hand, in the dexamethasone group, calcium content was also lower than in the control group, otolith size was increased, and otoliths appeared inadequate and coarse. Larger and less dense otoconia have been observed in bilaterally ovariectomized (OVX) rats.[19,20] Moreover, otoconial enlargement has been reported in guinea pigs exposed to streptomycin.[21,22] This is thought to be because decreasing the calcium concentration in the endolymphatic fluid results in the repeated

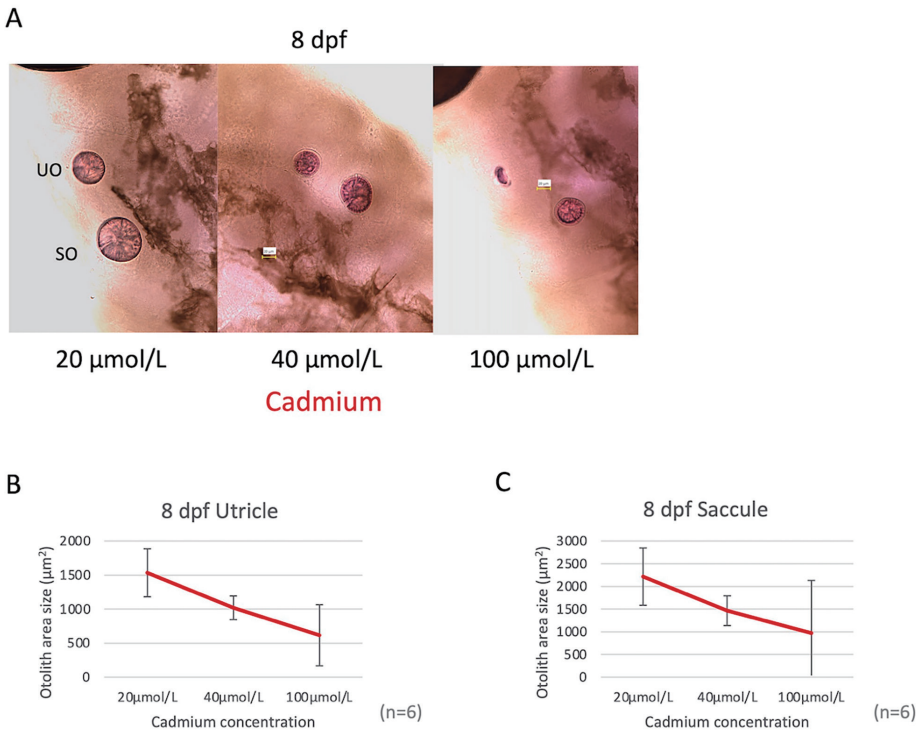

Fig. 2A
Figures of zebrafish at 8 dpf exposed to three different concentrations of cadmium (20 µmol/L, 40 µmol/L, and 100 µmol/L). The otoliths appear smaller in with higher concentrations of cadmium.
UO, utricle otolith; SO, saccule otolith.

Fig. 2B, C
Both utricle and saccule otolith sizes were not significantly different but tended to be smaller at higher concentrations of cadmium at 8 dpf. (n=6)

dissolution and recrystallization of otoliths to maintain homeostasis. In the same manner, such a process might lead to the formation of large otoliths with low density.

Several studies of otoconia have been conducted using animal models,[23-25] and there are several reasons for our use of zebrafish in this study. First, zebrafish are easy to breed, and simultaneous test can be performed on multiple organisms.[26,27] Second, zebrafish have a semicircular canal that is similar to that of humans and two otolith organs, the saccule and utricle. Zebrafish have no cochlea and appear to be receptive to hearing in the saccule and balance in the utricle. Third, the larvae are transparent, so otoliths can be observed from the body surface. zebrafish are widely used as a model for studying bone, and a glucocorticoid-induced osteoporosis (GIOP) model also exists.[28] In the present study, zebrafish in the drug-exposed group also appeared similar to the GIOP model. Since there were changes in the morphology of their otoliths, it is quite possible that some changes are also occurring in the otoconia of osteoporosis patients.

The present study had several limitations. While zebrafish are an excellent animal model for studying otoliths, it is not simple to apply the results to humans. This is because there are several differences between humans and zebrafish. For example, they do not have otoconial membranes, so otolith and hair cells interact directly. Unlike mammals, which have countless “otoconia,” zebrafish have only one “otolith” for each. In addition, while bone mass and bone density are relatively easy to measure in mammals, it was difficult to do

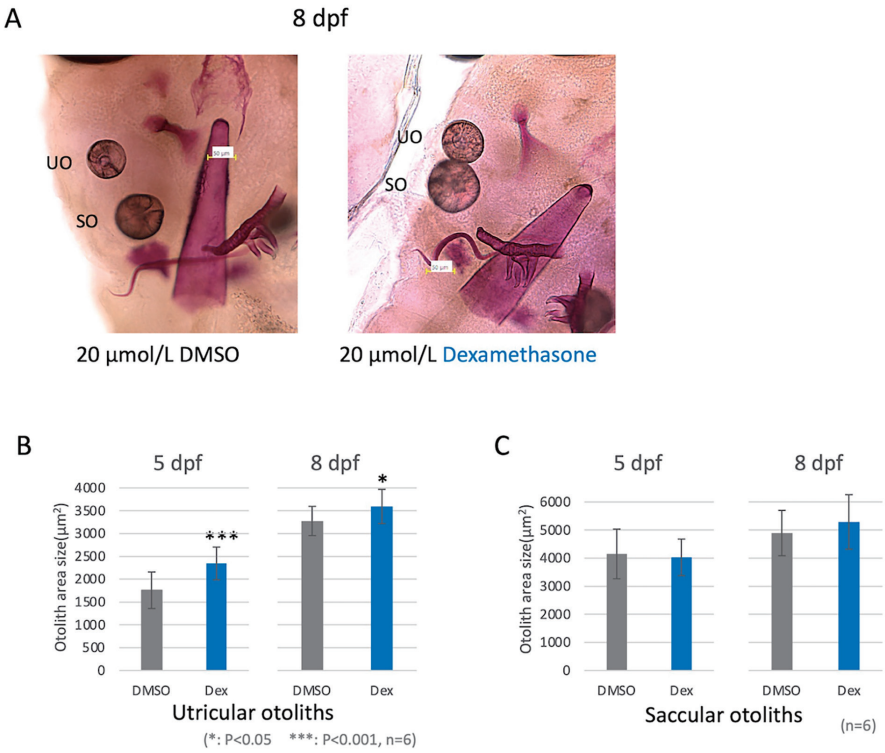

Fig. 3A
8 dpf zebrafish exposed to a solution of 20 μmol/L DMSO and Dexamethasone.
UO, utricle otolith; SO, saccule otolith.

Fig. 3B
In the dexamethasone group, the utricular otoliths were significantly larger compared with those in the control group at both 5 and 8 dpf (*: P<0.05 ***: P<0.001, n=6)
Dex, dexamethasone.

Fig. 3C
No significant difference was observed in saccule (n=6), but there was a trend toward slightly larger otoliths at 8 dpf compared to controls.
Dex, dexamethasone.

so in zebrafish embryos. Therefore, calcium concentrations were instead measured by dissolving whole larvae, but local assessment of bone was not possible.

Conclusion

The pathophysiology of abnormal calcium metabolism affects not only bone loss but also otolith formation. Although zebrafish otoliths are structurally distinct from mammalian otoconia, they are a simple and useful model for observing the relationship between abnormal calcium metabolism and otoliths. In the future, a larger sample size and more detailed evaluation of the effects of osteoporosis medications and whether otolith morphology changes lead to a greater tendency to dislodge from the macula would be necessary.

Conflict of Interest

The authors declare no conflict of interest.

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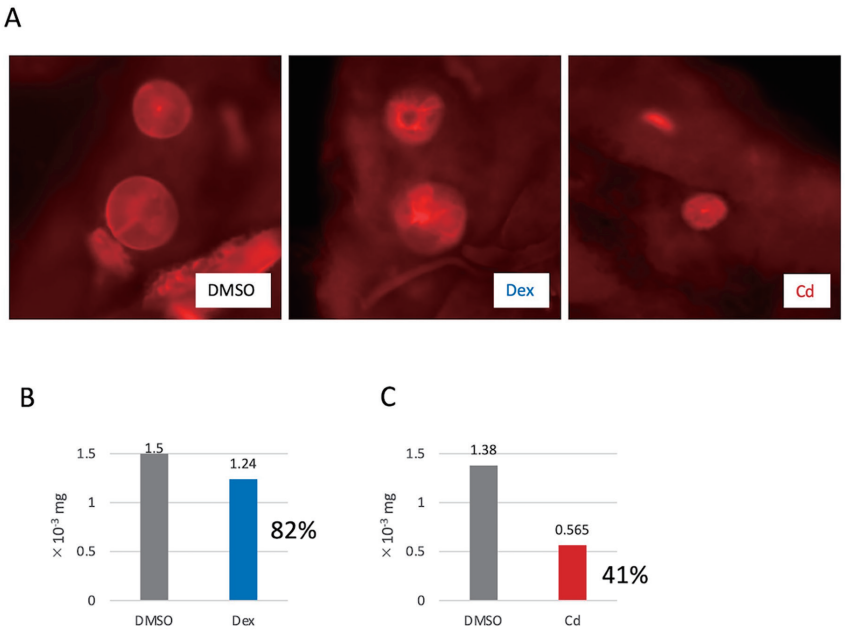

Fig. 4A
The appearance of each group in alizarin staining is compared. The dexamethasone group appeared inadequate and coarse compared to the control group. The cadmium group appeared even more significantly inadequate and coarse compared to the control and dexamethasone groups.
Dex, dexamethasone; Cd, cadmium.

Fig. 4B, C
The graph shows the results of comparing calcium concentration calculated by measuring the absorbance in each group. The calcium concentration per larva in the dexamethasone group was 82% of that in the control group and 41% in the cadmium group.
Dex, dexamethasone; Cd, cadmium.

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