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Short Communication

# Protective effects of alpha-lipoic acid on hair cell damage in diabetic zebrafish model

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ABSTRACT

Hearing impairment is one of the complications in diabetes mellitus; however, there are very few therapeutic studies on it. In this study, we investigated the protective effect of alpha-lipoic acid (ALA) on hearing loss in diabetic transgenic zebrafish and confirmed that ALA protects the loss of hair cells (HCs) caused by hyperglycemia. The data indicated that ALA has a protective effect on the damage to HCs in diabetic zebrafish.

## 1. Introduction

Diabetes mellitus (DM), characterized by high levels of blood glucose, causes several complications, including hearing loss, neuropathy, cardiomyopathy, and nephropathy. Diabetic hearing loss is a prevalent complication, similar to peripheral neuropathy, and is characterized by changes in cochlear structure and the loss of hair cells (HCs) that be caused by hyperglycemic damage to the vasculature and neural system in the inner ear [1–3]. In addition, hyperglycemia could directly cause HC loss via the glucose toxicity-induced intracellular stress, because HCs use glucose as an energy source and glucose is transported by several glucose transporters (GLUTs), such as GLUT3 and GLUT10; glucose is required for mechanotransduction as well as ATP synthesis [4,5]. However, hearing loss as a diabetes complication is poorly understood in clinical settings, and there are no specific treatment for this condition.

Alpha-lipoic acid (ALA), also known as thioctic acid, is one of the treatments for diabetic neuropathy. It is considered an effective antioxidant [6–8] and can serve as a potential treatment for hearing impairment in patients with diabetes. Several studies have demonstrated that ALA has protective and therapeutic effects against ototoxicity. [9,10]. Therefore, we hypothesized that ALA could protect HCs from damage under diabetic conditions. To test this hypothesis, we generated transgenic zebrafish that exhibit hyperglycemic loss of HCs, using chemogenetic ablation, and examined the protective effects of ALA on HCs.

## 2. Materials and methods

### 2.1. Zebrafish maintenance and ethics

Wild-type (AB strain) and *Tg(ins:nfsB-mCherry)* lines were used in the present study. All experimental procedures were approved by the Korea University Institutional Animal Care and Use Committee and performed in accordance with the animal experiment guidelines of the Korea National Veterinary Research and Quarantine Service.

### 2.2. Generation of Tg(ins:nfsB-mCherry) zebrafish

To establish the *Tg(ins:nfsB-mCherry)* zebrafish line, we used multisite Gateway cloning with 5′-*ins* [11], middle-*nfsb-mcherry* [12], 3′-*polyA* entry clones, and LR clonase II (Invitrogen, CA, USA), according to the manufacturer's instructions [13]. The *Ins:nfsB-mCherry* plasmid was co-injected with transposase mRNA into wild-type embryos at the one-cell

*Abbreviations:* ALA, alpha-lipoic acid; HCs, hair cells; DM, diabetes mellitus; EM, embryo medium.

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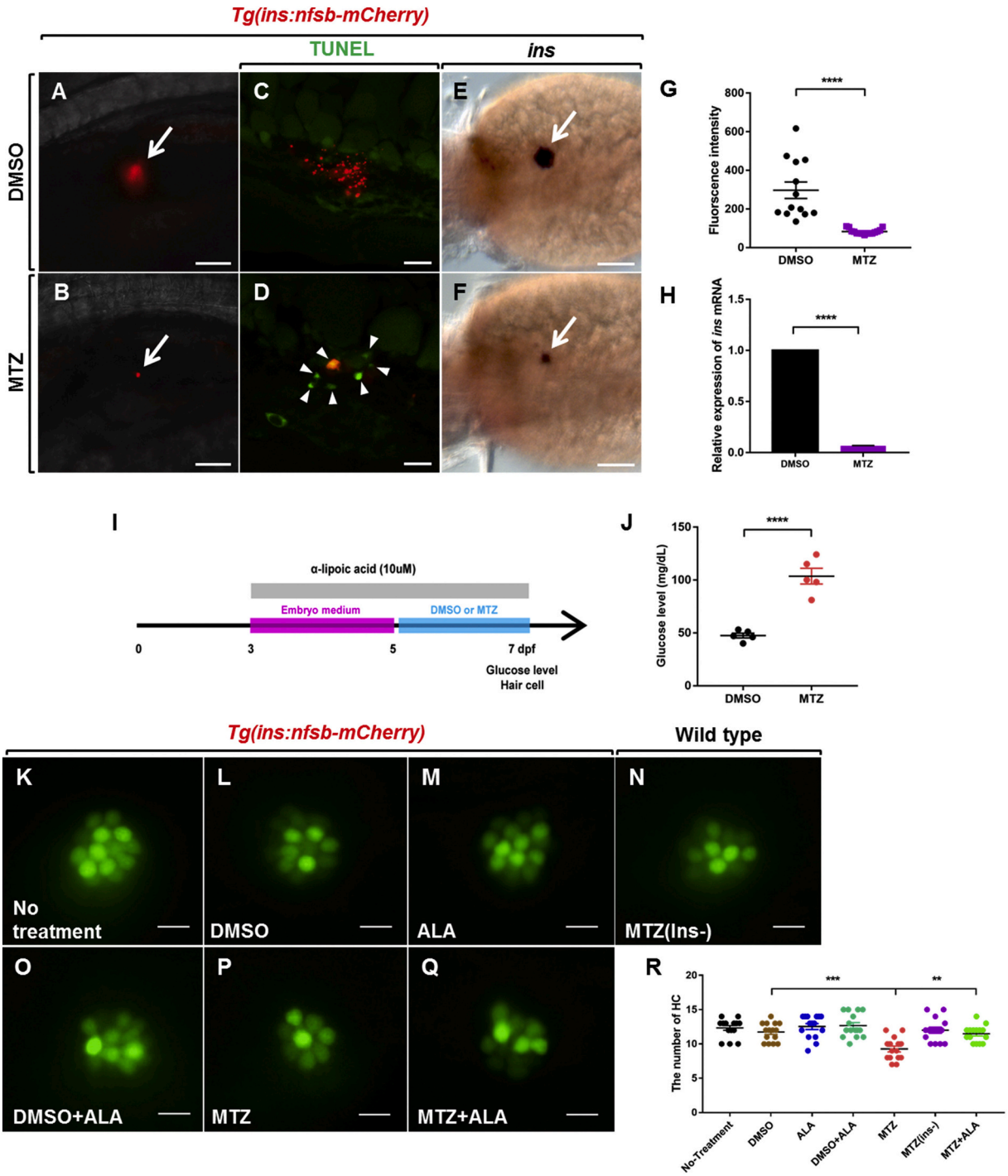


**Fig. 1.** ALA protects against hair cell (HC) loss in hyperglycemia. A-D: Fluorescence images of pancreatic β-cells from DMSO- (A,C) and MTZ-treated *Tg(ins:nfsB-mCherry)* zebrafish (B,D) at 4 dpf. Arrows indicate insulin (mCherry) in pancreatic β-cells (A-B). Arrowheads indicate apoptotic pancreatic β-cells (TUNEL) (C—D). E, F: In situ RNA hybridization with *ins* RNA probe in the pancreas. White arrows indicate *ins* mRNA expression in the pancreas of zebrafish larvae G: Quantification of fluorescence intensity from panel A to B. DMSO vs. MTZ, $^{****}p < 0.0001$; ($n = 13$ larvae in the DMSO group and $n = 15$ larvae in the MTZ group) H: Relative expression of *ins* mRNA from the DMSO- and MTZ-treated *Tg(ins:nfsB-mCherry)* zebrafish. DMSO vs. MTZ, $^{****}p < 0.0001$; ($n = 12$ larvae in the DMSO group and n = 15 larvae in the MTZ group) I: Schematic diagram of the timeline for chemical treatment. J: Glucose levels in DMSO- or MTZ-treated *Tg(ins:nfsB-mCherry)* zebrafish. Black and red dots indicate the respective data points. DMSO vs. MTZ, $^{****}p < 0.0001$; ($n = 20$ larvae in one data point; $n = 100$ larvae per group). K-Q: YO-PRO1 staining in DMSO- or MTZ-treated *Tg(ins:nfsB-mCherry)* zebrafish. R: Quantification of HCs from panel I to L. DMSO vs. MTZ, $^{***}p = 0.0004$; MTZ vs. MTZ + ALA, $^{***}p = 0.0026$; (n = 15 HCs per group). Scale bar: 50 μm in A,B; 10 μm in C,D; 500 μm in E,F; 10 μm in L-R.

stage.

### 2.3. Synthesis of antisense RNA probe and whole-mount in situ RNA hybridization

We amplified the *ins* cDNA using 2–5 days-post-fertilization (dpf) zebrafish cDNA and PCR primers designed from the GenBank sequence

(*preproinsulin (ins)*: NM_131056.1). The amplified cDNA was cloned into the pGEM-T easy vector (Promega, WI, USA). An antisense RNA probe was synthesized using SP6 RNA polymerase. In situ hybridization using *ins* probes was performed using zebrafish embryos, as previously described [14].

### 2.4. Chemical treatment and TUNEL assay

To test the efficiency of pancreatic β-cell ablation, zebrafish larvae were incubated with metronidazole (MTZ, Sigma, MO, USA; 10 mM in 0.2% dimethyl sulfoxide [DMSO]) for 2–4 dpf. To evaluate the effect of ALA, MTZ and ALA (YUHAN; 10 μM in embryo medium (EM)) were used to treat zebrafish larvae for the indicated period. To detect the death of pancreatic β-cells, embryos were fixed with 4% paraformaldehyde overnight, rinsed with PBST (1× PBS with 0.1% Tween-20), treated with 20 μg/mL proteinase K, rinsed again with PBST, and then incubated with TUNEL solution at 37 °C for 1 h; then, apoptosis was detected using an in situ cell death fluorescein detection kit (Roche Diagnostics, Basel, Switzerland).

### 2.5. Measurement of glucose levels and YO-PRO1 staining

Zebrafish larvae ($n = 20$) were homogenized and centrifuged at 12000 rpm for 1 min. Total glucose levels were measured using a blood glucose meter (On Call Extra, ACON Laboratories Inc., CA, USA). To visualize the HCs, embryos were incubated in EM with YO-PRO1 (Invitrogen; 1 μM in 0.1% DMSO) at 28.5 °C for 1 h and then rinsed with EM.

### 2.6. Quantitative reverse transcription PCR (qRT-PCR)

To quantify *ins* mRNA expression, qRT-PCR was performed using a LightCycler 96 Instrument (Roche Diagnostics) and the FastStart SYBR Green Master Mix (Roche Diagnostics).

### 2.7. Imaging and statistical analysis

Fluorescence imaging was performed using an Eclipse Ni—U fluorescence microscope (Nikon Instruments Inc., NY, USA) and an A1Si confocal microscope (Nikon). Unpaired Student's *t*-test was used to analyze differences in fluorescence intensity. For the quantification of HCs, the lateral neuromasts L3-L5 were counted and averaged in larvae at 7 dpf. Kruskal-Wallis one-way analysis of variance (with Dunn's multiple comparisons test) was used to analyze the number of HCs. Statistical graphs were prepared using mean ± standard error of the mean (SEM) data. Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Ablation of pancreatic β-cells causes hyperglycemia in Tg(ins:nfsB-mCherry) zebrafish

To establish a hyperglycemic zebrafish model, we generated *Tg(ins:nfsB-mCherry)* zebrafish that express nitroreductase (nfsB)-fused mCherry proteins in pancreatic β-cells under the control of the *ins* promoter (Fig. 1A). nfsB converts a prodrug MTZ into a cytotoxic product, resulting in specific ablation of pancreatic β-cells. We observed that mCherry$^{+}$ pancreatic β-cells were ablated at 4 dpf in MTZ-treated transgenic larvae compared to those in DMSO-treated transgenic larvae (control) (Fig. 1B, G). Apoptosis of mCherry$^{+}$ pancreatic β-cells was detected using the TUNEL assay (Fig. 1C, D), and the expression of the *ins* mRNA was found to be reduced, as assessed using in situ RNA hybridization (Fig. 1E, F) and qRT-PCR (Fig. 1H). We further confirmed that glucose levels were higher in MTZ-treated transgenic larvae than in controls (Fig. 1J). These data indicate that the impairment of pancreatic β-cells causes hyperglycemia in zebrafish larvae.

### 3.2. ALA protects against HC loss due to hyperglycemia

We observed that the number of HCs in the MTZ-treated transgenic larvae was lower than that in the controls, as evidenced by YO-PRO1 staining results (Fig. 1K, P, R). Conversely, there were no effects on HCs in the DMSO, ALA, DMSO and ALA co-treated transgenic larvae (Fig. 1L, M, R), or MTZ-treated wild-type larvae (Fig. 1N, R). In addition, the number of HCs was higher in larvae co-treated with MTZ and ALA than in those treated with MTZ alone (Fig. 1P, Q, R). These findings indicate that ALA protects HCs from hyperglycemia-induced cell loss.

## 4. Discussion

Hyperglycemia promotes the formation of reactive oxygen species (ROS) through intracellular signaling pathways, such as the polyol pathway [19], and then directly damages the cochlea to induce hearing impairment [1–3]. In the present study, we demonstrated that ALA, a metabolic antioxidant, exerts protective effects against hyperglycemic loss of HCs in a diabetic zebrafish model. Our results are supported by previous studies showing that ALA effectively prevents HC damage from cisplatin-induced ototoxicity in mice as well as in cell cultures by scavenging the ROS [8,9]. In addition, a previous study reported that ALA attenuates kanamycin-induced ototoxicity in mice by inhibiting the expression of p38 and p-JNK involved in apoptosis [15]. Therefore, we suggest that ALA has protective effects against hyperglycemia-induced HC loss and might be effective in clearing ROS and inhibiting apoptotic molecules.

Several studies have reported that ALA can restore the conduction velocity of peripheral nerves following cisplatin-induced peripheral neurotoxicity in rats and reduces the apoptosis of neurons after L-hydroxyglutaric acid-induced neurotoxicity in the zebrafish brain [16,17]. These data indicate that ALA exerts neuroprotective effects. Hong et al. have also shown that the necrosis of pancreatic β-cells causes sensorineural hearing loss and loss of HCs following hyperglycemia in chemical-induced diabetic zebrafish and mouse models [18]. Therefore, ALA could have neuroprotective effects against diabetic hearing loss in zebrafish.

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