Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Screening for protective effects of herbal medicine on sensory hair cells using the zebrafish lateral line

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Article history:
Received: May 13, 2025
Received in revised form: Nov 12, 2025
Accepted: Nov 14, 2025
Epub ahead of print

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Keywords:
Zebrafish
Screening
Inner ear protection
Oxidation
Herbal medicine

Abstract

Objective: Mammalian sensory hair cells do not regenerate. Therefore, developing therapeutic agents to protect the inner ear is essential. Eight herbal medicines (Shosaikoto, Orengedokuto, Tokishakuyakusan, Shigyakusan, Hochuekkito, Juzentaihoto, Unseiin, and Shimotsuto) were screened using a zebrafish lateral line to determine their possible protective effects against neomycin insult in sensory hair cells.

Materials and Methods: Zebrafish larvae were divided into four groups: untreated control, neomycin-treated, herbal medicine–treated only, and herbal medicine + neomycin–treated groups. Each herbal medicine was administered 1 hr before exposure to 200 µM neomycin, and the larvae were fixed with 4% paraformaldehyde 1 hr later. Hair cell survival in the lateral line neuromasts was quantified to generate dose–response curves.

Results: All eight herbal preparations demonstrated protective effects against neomycin-induced hair cell injury in the zebrafish lateral line. Within 3 min of neomycin exposure, oxidative stress markers indicated a pronounced increase in reactive oxygen species within lateral line hair cells, whereas pretreatment with Shimotsuto effectively suppressed this response. Among the tested formulations, Shimotsuto exhibited the strongest otoprotective effect. Furthermore, its protective potential was confirmed in mammalian vestibular epithelia where the survival of hair cells was significantly higher in the Shimotsuto plus neomycin group compared with the neomycin-only group.

Conclusion: These findings suggest that zebrafish-based drug screening provides a rapid and reliable platform for identifying herbal formulations with inner ear protective properties, thereby contributing to determine their possible protective effects.

Please cite this paper as:
Takemoto Y, Tsuda J, Hirose Y, Yamamoto Y, Okinaka Y, Nishimura S, Sakamoto M, Hashimoto M, Sugahara K. Screening for protective effects of herbal medicine on sensory hair cells using the zebrafish lateral line. Avicenna J Phytomed, 2025. Epub ahead of print.

Introduction

Hearing loss is an increasingly common problem caused by noise, aging, and diseases. Sensory hair cells in the cochlea convert mechanical sound vibrations into neural signals. Once these cells are damaged or lost, they do not regenerate in mammals, resulting in irreversible

sensorineural hearing loss. Although hearing aids and cochlear implants have been developed, preventing hearing loss is of utmost importance. If methods to prevent hair cell death are successfully developed, fewer people will have hearing loss worldwide. Therefore, developing therapeutic agents to protect the inner ear is essential.

Drugs such as vitamin E and coenzyme Q10 are known to protect the inner ear in animal models (Fetoni et al. 2008). Certain herbal medicines have also been shown to protect hair cells in animal models (Niwa et al. 2016). Although many drugs have been evaluated for inner ear protection, there have been few reports on the screening of herbal medicines (Hirose et al. 2016).

Inner ear hair cells evolve from lateral-line hair cells (Ghysen and Dambly-Chaudiere 2004). Hence, zebrafish lateral line hair cells are physiologically and morphologically similar to the inner ear hair cells. As the zebrafish lateral line is located on the body surface, damage to hair cells can be rapidly assessed. Therefore, the zebrafish lateral line effectively evaluates drugs that damage or protect hair cells (Owens et al. 2007). Additionally, the low cost and availability of large quantities of zebrafish fry allow researchers to use them in experiments to evaluate multiple drugs simultaneously.

Moreover, various phytochemical compounds have been reported to protect cells from oxidative stress–mediated toxicity through antioxidant and anti-apoptotic mechanisms (Ajibade et al. 2024; Khordad et al. 2024). These findings suggest that herbal medicines may also provide inner-ear protection via similar mechanisms.

In a previous study, zebrafish were used to screen the ototoxic effects of numerous anti-cancer drugs and the protective effects of supplement drugs (Hirose et al. 2016; Ou et al. 2007). In this study, we used the zebrafish lateral-line hair cells to screen herbal medicines for their protective effects against neomycin.

Materials and Methods

Animals

Adult zebrafish (*Danio rerio*, AB wild-type strain) were maintained under standard laboratory conditions at 28.5°C in the facilities of the University of Yamaguchi. Fertilized embryos were obtained by natural pairwise mating. The embryos were reared in embryo medium composed of 1 mM $MgSO_4$, 120 mM KH_2PO_4, 74 mM Na_2HPO_4, 1 mM $CaCl_2$, 500 mM KCl, 15 mM NaCl, and 500 mM $NaHCO_3$ in distilled water, at a density of 50 embryos per 100 mm^2 Petri dish.

Six-week-old CBA/N mice exhibiting normal Preyer reflexes were purchased from Kyushu Animal Corporation (Fukuoka, Japan). Mice were deeply anesthetized with pentobarbital and immediately decapitated prior to tissue collection. All animal experiments were approved by the Committee for the Ethics of Animal Experiments of Yamaguchi University and conducted in accordance with the Japanese Government Guidelines for Medicine and the Law (No. 105) and Notification No. 6.

Screening of eight herbal medicines for hair cell survival

This study evaluated eight medicines (Shosaikoto (Shimizu 2000), Orengedokuto (Miura et al. 2007), Tokishakuyakusan (Irahara et al. 2000), Shigyakusan (Tanaka et al. 2013), Hochuekkito (Minami et al. 2018), Juzentaihoto (Takeno et al. 2015), Unseiin (Andoh et al. 2004), and Shimotsuto (Takiyama et al. 2021)), which will be referred to as herbal medicine. These medicines were gifted by Tsumura Co., Ltd. (Osaka, Japan). Each extract powder was produced by hot-water extraction, which is the standard manufacturing process for Kampo medicines by Tsumura Co. following the Japanese Pharmacopoeia. For experimental use, each extract powder was dissolved in distilled water and filtered through a 0.22-µm membrane before administration to zebrafish. Water was chosen instead of

ethanol to reproduce the clinical Kampo formulation and to avoid potential toxicity or confounding effects of alcohol on zebrafish larvae. The constituent crude drugs and their botanical (scientific) names for each herbal medicine are listed in Table 1.

Table 1. Constituent crude drugs and botanical origins of eight herbal medicines

Herbal Medicine	Herb Name	Scientific Name	Reference
Shosaikoto	Bupleurum Root	*Bupleurum falcatum*	Shimizu, 2000
	Scutellaria Root	*Scutellaria baicalensis*	
	Ginseng	*Panax ginseng*	
	Pinellia Tuber	*Pinellia ternata*	
	Licorice Root	*Glycyrrhiza uralensis*	
	Ginger	*Zingiber officinale*	
	Jujube	*Ziziphus jujuba*	
Orengedokuto	Scutellaria Root	*Scutellaria baicalensis*	Miura et al., 2007
	Coptis Rhizome	*Coptis japonica*	
	Phellodendron Bark	*Phellodendron amurense*	
	Gardenia Fruit	*Gardenia jasminoides*	
Tokishakuyakusan	Peony Root	*Paeonia lactiflora*	Irahara et al., 2000
	Cnidium Rhizome	*Cnidium officinale*	
	Angelica Root	*Angelica acutiloba*	
	Atractylodes Rhizome	*Atractylodes lancea*	
	Alisma Rhizome	*Alisma orientale*	
	Poria	*Poria cocos*	
Shigyakusan	Bupleurum Root	*Bupleurum falcatum*	Tanaka et al., 2013
	Peony Root	*Paeonia lactiflora*	
	Immature Orange Fruit	*Citrus aurantium*	
	Licorice Root	*Glycyrrhiza uralensis*	
Hochuekkito	Astragalus Root	*Astragalus membranaceus*	Minami et al., 2018
	Ginseng	*Panax ginseng*	
	Angelica Root	*Angelica acutiloba*	
	Atractylodes Rhizome	*Atractylodes lancea*	
	Bupleurum Root	*Bupleurum falcatum*	
	Cimicifuga Rhizome	*Cimicifuga simplex*	
	Jujube	*Ziziphus jujuba*	
	Ginger	*Zingiber officinale*	
	Licorice Root	*Glycyrrhiza uralensis*	
	Citrus Unshiu Peel	*Citrus unshiu*	
Juzentaihoto	Rehmannia Root	*Rehmannia glutinosa*	Takeno et al., 2015
	Peony Root	*Paeonia lactiflora*	
	Cnidium Rhizome	*Cnidium officinale*	
	Angelica Root	*Angelica acutiloba*	
	Atractylodes Rhizome	*Atractylodes lancea*	
	Poria	*Poria cocos*	
	Ginseng	*Panax ginseng*	
	Cinnamon Bark	*Cinnamomum cassia*	
	Astragalus Root	*Astragalus membranaceus*	
	Licorice Root	*Glycyrrhiza uralensis*	
Unseiin	Rehmannia Root	*Rehmannia glutinosa*	Andoh et al., 2004
	Peony Root	*Paeonia lactiflora*	
	Angelica Root	*Angelica acutiloba*	
	Cnidium Rhizome	*Cnidium officinale*	
	Scutellaria Root	*Scutellaria baicalensis*	
	Phellodendron Bark	*Phellodendron amurense*	
	Coptis Rhizome	*Coptis japonica*	
	Gardenia Fruit	*Gardenia jasminoides*	
Shimotsuto	Rehmannia Root	*Rehmannia glutinosa*	Takiyama et al., 2021
	Peony Root	*Paeonia lactiflora*	
	Cnidium Rhizome	*Cnidium officinale*	
	Angelica Root	*Angelica acutiloba*	

The listed herbal medicines are derived from traditional Japanese Kampo formulas.

Five-day-post-fertilization (dpf) zebrafish larvae were exposed to each herbal extract at concentrations of 0, 1, 10, 100, and 1000 µg/ml for 1 hr prior to neomycin treatment. Subsequently, neomycin was added to the embryo medium to reach a final concentration of 200 µM, and the larvae were incubated for another 1 hr. After drug exposure, larvae were immersed in 4% paraformaldehyde at 4°C and fixed overnight. The fixed specimens were subsequently subjected to immunohistochemical labeling of hair cells, followed by quantitative analysis of hair cell survival.

Zebrafish immunohistochemistry and hair cell counts

Zebrafish were placed in a blocking solution (1% Triton-X, 5% normal goat serum [NGS] in PBS (Phosphate-Buffered Saline)) for 1 hr at room temperature after being fixed in 4% paraformaldehyde and three PBS rinses. The next step was an overnight incubation at 4°C with a monoclonal anti-parvalbumin antibody solution containing 1% Triton-X, 1% NGS, and PBS. Zebrafish were then treated with an Alexa 488 goat anti-mouse fluorescent antibody (1:500, in 1% Triton-X, 1% NGS, in PBS) for 4 hr before being rinsed three times in 1% Triton-X in PBS (PBS-T). Zebrafish were labeled with secondary antibodies, rinsed in PBS-T and PBS, and then mounted between two coverslips in Fluoromount-G (Southern Biotech, Birmingham, AL, USA) for imaging.

An FITC filter with a 60x magnification setting was used to view the mounted specimens under a fluorescent microscope (Keyence, BZ-8100, Osaka, Japan). The SO1, SO2, O1, and OC1 neuromasts' hair cells were counted (Raible and Kruse 2000). For each dose, ten fish were counted. The average hair cell survival as a proportion of the control was used to determine the results.

ROS (Reactive Oxygen Species) detection with 2',7'-dichlorofluorescein (H_2DCFDA)

The generation of reactive oxygen species (ROS) was assessed using 2',7'-dichlorofluorescein diacetate (H_2DCFDA). This compound readily permeates cell membranes and is deacetylated by intracellular esterases, yielding non-fluorescent H_2DCF which is subsequently oxidized to a green fluorescent product upon exposure to ROS.

Zebrafish larvae were either untreated or preincubated with Shimotsuto (100 µg/ml) for 1 hr. They were then exposed to 10 µM H_2DCFDA (prepared in embryo medium) for 5 min, followed by washing with fresh embryo medium. Hair cell nuclei in neuromasts were counterstained with DAPI (0.1 µg/mL; Sigma D-9542). The larvae were subsequently treated with 200 µM neomycin sulfate (N-1876; Sigma) and anesthetized before mounting for fluorescence microscopy.

Semi-quantitative analysis of ROS fluorescence was performed using ImageJ software (NIH, USA). For each experimental group (n = 6 larvae), fluorescence intensity was measured in randomly selected neuromasts. The mean gray value of each neuromast was determined after background subtraction. The relative fluorescence intensity was calculated by normalizing each value to the mean fluorescence intensity of the control group which was defined as 1.

Culture of vestibular organs

Six-week-old CBA/N mice with normal Preyer reflexes were obtained from Kyushu Animal Corporation (Japan). Deep anesthesia was induced by intraperitoneal administration of pentobarbital (100 mg/kg), followed by rapid decapitation. The temporal bones were immediately removed, and the vestibular organs were dissected in a solution consisting of Earle's balanced salt solution and Eagle's basal medium mixed at a 2:1 (v/v) ratio (Invitrogen, Carlsbad, CA, USA).

Only the utricles were used for culture. Each specimen was maintained in a medium composed of Earle's balanced salt solution (2:1, v/v) supplemented with 5% fetal bovine serum and Eagle's basal medium. The utricles were cultured free-floating in 24-well plates at 37°C in a humidified atmosphere of 5% CO_2. For each experimental condition, six or more utricles derived from at least three animals were analyzed. The general procedures followed the method originally described by Cunningham (Cunningham 2006).

Immunohistochemistry in CBA/N mice

Utricular specimens were incubated at room temperature for 3 hr in a blocking solution containing 1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, and 0.4% Triton X-100 in PBS. Hair cells in the whole-mounted preparations were identified using a mouse monoclonal anti-calmodulin antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:150 in blocking solution. The specimens were incubated with the primary antibody overnight at 4°C, followed by incubation with Alexa Fluor 488–conjugated goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) after thorough washing with blocking buffer. The stained utricles were rinsed again, mounted using DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA), and covered with a glass coverslip for imaging.

Hair cell counts in utricles

Hair cell survival was evaluated using a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan). Calmodulin-positive cells in the extrastriolar region were identified and counted as hair cells. For each utricle, four randomly selected square areas (each 20 μm × 20 μm) were analyzed, and the average number of labeled hair cells was calculated to determine the hair cell density per utricle. At least six utricles were examined for each experimental condition.

Statistics

All data are presented as the mean ± standard error (SE) obtained from three independent experiments (n = 5 larvae per group). Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple-comparison test, using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered to indicate statistical significance.

Results

Protective effect of herbal medicines against aminoglycoside toxicity

The dose-response relationships of the eight herbal medicines (the relationship between the concentration of the drug and the survival rate of hair cells) are shown in Table 2. All herbal medicines exerted protective effects against neomycin in this experiment.

The results of the Shimotsuto group, which showed the greatest protective effect, are shown in Figure 1. Although neomycin caused severe hair cell damage (Figure 1A), increased hair cell survival was observed in the Shimotsuto group (Figure 1B). We performed a dose-response experiment using Shimotsuto (Figure 1C). Shimotsuto significantly protected hair cells at concentrations of 10 μg/ml ($p<0.05$) and 100 μg/ml ($p<0.01$) in the zebrafish lateral line.

Table 2. Protective effect of eight herbal medicines against aminoglycoside toxicity (hair cell survival rate, %)

Herbal Medicine	0 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml	1000 μg/ml
Shosaikoto	22.4±1.8	17.9±2.6	16.4±3.4	24.7±4.1	40.1±5.2**
Orengedokuto	22.4±1.8	25.4±4.6	24.9±4.4	28.2±4.8	38.0±4.5**
Tokishakuyakusan	22.4±1.8	21.4±3.4	19.6±3.5	22.2±2.8	36.0±2.4**
Shigyakusan	22.4±1.8	28.2±3.3	28.0±2.6	30.5±3.5*	41.1±3.7**
Hochuekkito	18.5±2.7	15.7±3.0	23.0±7.0	20.1±3.5	36.4±3.5**
Juzentaihoto	18.5±2.7	16.6±2.1	11.9±3.3	24.5±2.2*	34.7±3.5**
Unseiin	18.5±2.7	14.5±2.6	17.7±3.9	26.3±5.6	61.3±6.8**
Shimotsuto	18.5±2.7	23.6±2.6	21±3.3	28.7±3.1*	65.1±6.7**

The screening was conducted in two separate experimental batches because of the large number of herbal medicines tested. As a result, two independent control values (0 μg/ml herbal medicine, with 200 μM neomycin exposure) values appear in the Table, each corresponding to the control group of one experimental batch. Data are expressed as mean ± SD (n = 5), and represent the percentage survival of lateral line hair cells. The survival rate in the untreated control group (not exposed to either neomycin or herbal medicines) was defined as 100%. *p<0.05 and **p<0.01.

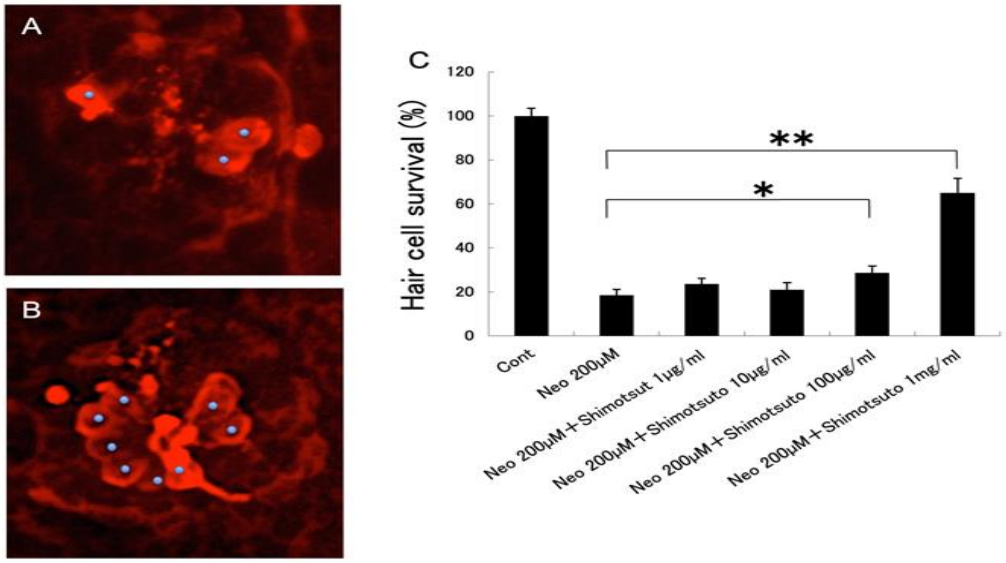

Figure 1. Protective effect of Shimotsuto against aminoglycoside-induced hair cell loss. (A) Neomycin caused severe hair cell damage. (B) Shimotsuto (100 μg/ml) protected hair cells against aminoglycoside ototoxicity. (C) Shimotsuto significantly protected hair cells at 100 μg/ml (p<0.05) and 1 mg/ml (p<0.01). *p<0.05; **p<0.01.

ROS detection with H_2DCFDA

In untreated control larvae, only faint H_2DCFDA fluorescence was detected (Figure 2A–C). In contrast, exposure to neomycin for 3 min markedly increased fluorescence intensity in lateral line hair cells (Figure 2D–F), indicating elevated ROS production. Pretreatment with Shimotsuto, however, noticeably attenuated this fluorescence enhancement (Figure 2G–I). These findings suggest that Shimotsuto suppresses neomycin-induced oxidative stress by reducing the accumulation of ROS in hair cells.

Semi-quantitative image analysis using ImageJ showed that fluorescence intensity was markedly increased in the neomycin-treated group compared with the control group, whereas this increase was significantly suppressed in the neomycin + Shimotsuto group (p<0.01) (Figure 2J). These findings support that Shimotsuto treatment attenuates neomycin-induced ROS generation in lateral line hair cells.

Hair cells of the CBA/N mice utricles

Mouse utricles were cultivated with neomycin (2 mM) alone or in the presence of Shimotsuto for 24 hr in order to determine the effects of Shimotsuto on the survival of mammalian hair cells in response to neomycin treatment. The utricles were fixed, and calmodulin immunolabelling was done to detect

remaining hair cells (Figure 3A-C). Neomycin caused a decrease in the density of hair cells, but the density was higher in the presence of Shimotsuto than it was in the absence of Shimotsuto (Figure 3D).

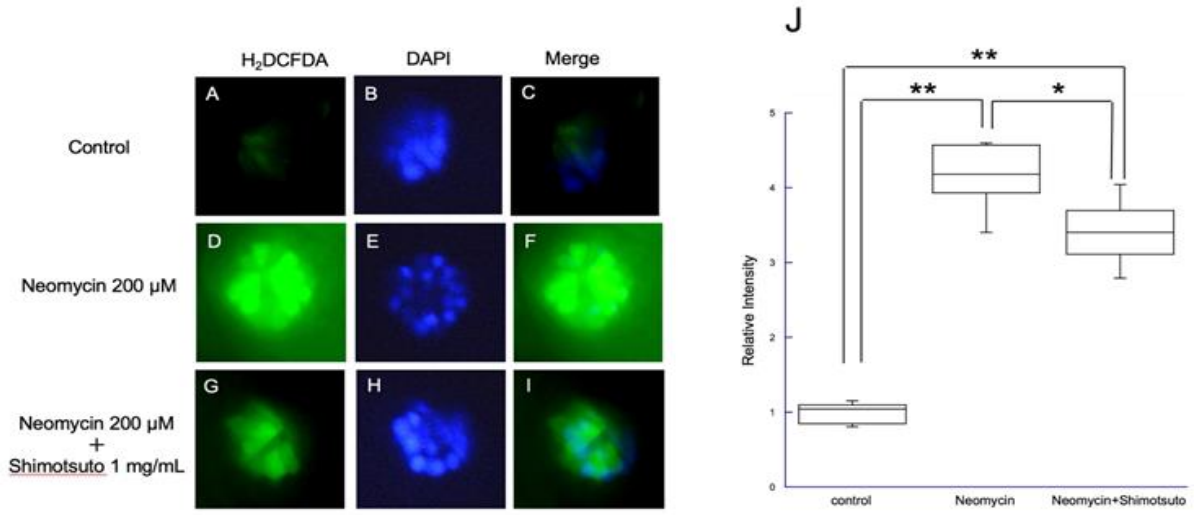

Figure 2. Suppression of neomycin-induced ROS generation. Hair cells in the neomycin-treated group showed increased staining for H_2DCFDA (D-F) compared to those in the control group (A-C). The signals were reduced in the Shimotsuto-treated group (G-I). Semi-quantitative analysis of fluorescence intensity using ImageJ showed that the neomycin-treated group exhibited significantly higher fluorescence compared with the control group, whereas this increase was significantly suppressed in the neomycin + Shimotsuto group ($p<0.01$). Data are presented as mean ± SE (n = 6 per group) (J).

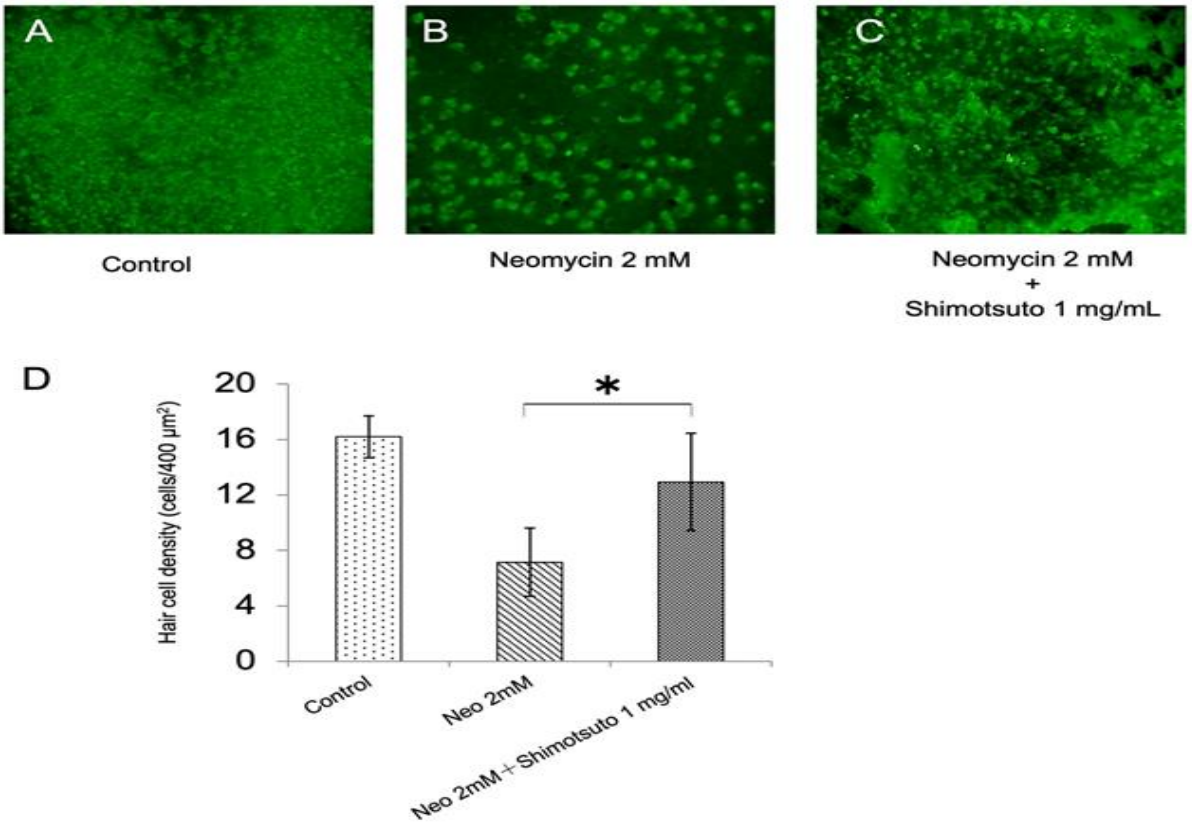

Figure 3. Protective effect of Shimotsuto on the mammalian hair cells. Mouse utricles were cultured for 24 hr without neomycin (A) and with 2 mM neomycin (B), neomycin, and 1 mg/ml Shimotsuto (C). Hair cells were labeled with calmodulin immunoreactivity. The number of surviving hair cells was significantly higher in the Neomycin + Shimotsuto group than in the neomycin group (D). Data are presented as mean ± SE (n = 5 utricles per group). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test ($p<0.05$).

Discussion

As lateral line hair cells evolve into inner ear hair cells, they are structurally similar to those of the inner ear and are damaged by ototoxic drugs, such as aminoglycosides and cisplatin (Ou et al. 2007). As they are located on the body surface, it is easy to visualise hair cells without dissection. Therefore, zebrafish have been used to screen for medicines that affect hair cells (Owens et al. 2007). They have been used to screen the ototoxic effects of a library of anti-cancer drugs and the protective effects of FDA-approved drugs and supplements (Hirose et al. 2011; Ou et al. 2009). Furthermore, since the hair cells are on the body surface, it is easy to evaluate oxidative stress in cells (Hirose et al. 2016).

In the present study, the effects of eight herbal medicines on hair cells were evaluated using a zebrafish model. Many studies have shown that herbal medicines provide neuronal protection *in vivo* and *in vitro*. Gua Lou Gui Zhi decoction exerts neuroprotective effects against ischaemic stroke and glutamate-induced apoptosis by inhibiting cell apoptosis (Zhang et al. 2014). Danggui-Shaoyao-San, also called Tokishakuyakusan, exerts a protective effect against neurological diseases through anti-inflammatory and antioxidant activities (Qian et al. 2008).

In contrast, inner ear hair cell damage is caused by the generation of hydroxyl radicals in aminoglycoside-induced, noise-induced, and age-related hearing loss in rodents (Yamasoba et al. 1999). Therefore, one method to prevent hearing loss is to suppress the generation of hydroxyl radicals in the inner ear. Shimotsuto, which showed a particularly strong protective effect in our study, scavenged the hydroxyl radicals generated by exposure to neomycin. Although Shimotsuto showed reduced antioxidant marker activity in our assay, its protective effect might involve mechanisms other than direct radical scavenging. Several constituents of Shimotsuto, such as ligustilide, have been reported to suppress inflammation by inhibiting NF-κB and MAPK signaling and reducing the expression of inflammatory mediators including iNOS (Inducible Nitric Oxide Synthase) and COX-2 (Cyclooxygenase-2) (Chung et al. 2012; Wang et al. 2010; Wang et al. 2019). These findings suggest that the protective effect of Shimotsuto may involve modulation of inflammatory signaling or regulation of cell survival pathways, in addition to antioxidative processes. The chemical composition of Shimotsuto has been characterized previously by Takiyama et al. using HPLC and LC–MS/MS, with a 3D HPLC profile provided in Supplementary Figure 1 and key constituents (catalpol, paeoniflorin, albiflorin, ferulic acid, ligustilide, senkyunolide A, and butylphthalide) identified by authentic standards (Takiyama et al. 2021). Building on these data, future studies will investigate whether neomycin exposure alters the relative abundance or activity of these components. Although the present study qualitatively demonstrated the suppression of oxidative stress using H_2DCFDA staining, all exposure conditions for drug treatment and imaging parameters were kept identical among experimental groups to ensure comparability. Quantitative analysis of fluorescence intensity using a plate reader or image analysis software would provide more objective data and will be performed in future studies. In addition to Shimotsuto, Unseiin exhibited a marked protective effect in the zebrafish screening assay. Although additional experiments could not be performed in the current study, Unseiin is considered another promising candidate, and its mechanism of action will be investigated in future work.

Hangeshashinto has been reported to inhibit hydroxyl radicals and protects hair cells against gentamicin-induced loss in rat cochlea (Niwa et al. 2016). Similarly, the other herbal medicines examined in this study are known to possess antioxidant and anti-inflammatory properties (Borchers et al. 2000; Kaneko et al. 2004; Kato et al.

2014; Li et al. 2014; Makino et al. 2006; Wang et al. 1997), which are likely responsible for their protective effects against aminoglycoside-induced hair cell damage. Although the effect of Shimotsuto on hair cells has not been directly reported, its antioxidant activity (Nishimura et al. 2011) may contribute to the observed protection. These findings support the potential of herbal medicines as tool for preventing progressive sensorineural hearing loss.

This study has some limitations. Our results suppressed acute-phase damage to hair cells, but chronic-phase damage such as progressive deafness could not be examined. In addition, the present study evaluated the protective effect of Shimotsuto using vestibular hair cells, but cochlear hair cells and auditory function were not assessed. Future studies using cochlear explant cultures and Auditory Brainstem Response (ABR) testing will be necessary to confirm whether Shimotsuto also protects auditory sensory cells and preserves hearing function. Further research is needed for clinical efficacy.

This study demonstrated that zebrafish models are useful for screening herbal medicines with hair cell-protective effects. Among the eight tested medicines, all showed protective effects, with Shimotsuto also exhibiting efficacy in mammalian hair cells. These findings support the potential utility of zebrafish for identifying candidate drugs to treat inner ear disorders.

Acknowledgment

We thank Ms. Kazuko Muto for assistance with animal husbandry and microscopy. We would like to thank Honyaku Center Inc. for English language editing.

Conflicts of interest

The authors report there are no competing interests to declare.

Funding

The Japanese Ministry of Education, Culture, Sports, Science, and Technology provided grants to support this work (18K09321, 20K09733, 24K19752).

Ethical Considerations

The Yamaguchi University Institute for Life Science authorized the experimental protocols (Approval No. 43-051). The Animal Welfare Law and the management guidelines for animals were followed during the performance of this investigation.

Code of Ethics

This study involved no human participants. All animal experiments were conducted in accordance with institutional and national ethical guidelines.

Authors' Contributions

Yosuke Takemoto and Yoshinobu Hirose contributed to the experimental design and optimization of the protocols. Yosuke Takemoto, Junko Tsuda, Yoshinobu Hirose, and Mei Sakamoto conducted the experiments and collected the data. Shogo Nishimura, Youhei Yamamoto, and Makoto Hashimoto analyzed the data and interpreted the results. Yosuke Takemoto and Kazuma Sugahara drafted the manuscript. All authors critically reviewed the manuscript and approved the final version.

References

Ajibade TO, Esan OO, Osawere IM, et al. (2024) Cardiovascular and renal oxidative stress-mediated toxicities associated with bisphenol-A exposures are mitigated by Curcuma longa in rats. Avicenna J Phytomed 14(2):202-214 doi:10.22038/ajp.2023.23367

Andoh T, Al-Akeel A, Tsujii K, Nojima H, Kuraishi Y (2004) Repeated treatment with the traditional medicine Unsei-in inhibits substance P-induced itch-associated responses through downregulation of the expression of nitric oxide synthase 1 in mice. J Pharmacol Sci 94(2):207-10

doi:10.1254/jphs.94.207

Borchers AT, Sakai S, Henderson GL, et al. (2000) Shosaiko-to and other Kampo (Japanese herbal) medicines: a review of their immunomodulatory activities. J Ethnopharmacol 73(1-2):1-13 doi:10.1016/s0378-8741(00)00334-2

Chung JW, Choi RJ, Seo EK, et al. (2012) Anti-inflammatory effects of (Z)-ligustilide through suppression of mitogen-activated protein kinases and nuclear factor-κB activation pathways. Arch Pharm Res 35(4):723-32 doi:10.1007/s12272-012-0417-z

Cunningham LL (2006) The adult mouse utricle as an in vitro preparation for studies of ototoxic-drug-induced sensory hair cell death. Brain Res 1091(1):277-81 doi:10.1016/j.brainres.2006.01.128

Fetoni AR, Ferraresi A, Greca CL, et al. (2008) Antioxidant protection against acoustic trauma by coadministration of idebenone and vitamin E. Neuroreport 19(3):277-81 doi:10.1097/WNR.0b013e3282f50c66

Ghysen A, Dambly-Chaudiere C (2004) Development of the zebrafish lateral line. Curr Opin Neurobiol 14(1):67-73 doi:10.1016/j.conb.2004.01.012

Hirose Y, Simon JA, Ou HC (2011) Hair cell toxicity in anti-cancer drugs: evaluating an anti-cancer drug library for independent and synergistic toxic effects on hair cells using the zebrafish lateral line. J Assoc Res Otolaryngol 12(6):719-28 doi:10.1007/s10162-011-0278-z

Hirose Y, Sugahara K, Kanagawa E, Takemoto Y, Hashimoto M, Yamashita H (2016) Quercetin protects against hair cell loss in the zebrafish lateral line and guinea pig cochlea. Hear Res doi:10.1016/j.heares.2016.10.001

Irahara M, Yasui T, Tezuka M, et al. (2000) Evidence that Toki-shakuyaku-san and its ingredients enhance the secretion of a cytokine-induced neutrophil chemoattractant (CINC/gro) in the ovulatory process. Methods Find Exp Clin Pharmacol 22(10):725-30 doi:10.1358/mf.2000.22.10.802288

Kaneko T, Chiba H, Horie N, et al. (2004) Effect of two different groups of Chinese medicines on nitric oxide production by mouse macrophage-like cells. In Vivo 18(6):771-8

Kato T, Hino S, Horie N, et al. (2014) Anti-UV activity of Kampo medicines and constituent plant extracts: re-evaluation with skin keratinocyte system. In Vivo 28(4):571-8

Khordad E, Akbaribazm M, Hosseini SM (2024) Heracleum persicum L. extract protects gentamicin-induced testicular toxicity. Avicenna J Phytomed 14(5):585-599 doi:10.22038/ajp.2024.24347

Li Z, Hu H, Lin R, et al. (2014) Neuroprotective effects of Gua Lou Gui Zhi decoction against glutamate-induced apoptosis in BV-2 cells. Int J Mol Med 33(3):597-604 doi:10.3892/ijmm.2013.1612

Makino T, Tsubouchi R, Murakami K, Haneda M, Yoshino M (2006) Generation of reactive oxygen species and induction of apoptosis of HL60 cells by ingredients of traditional herbal medicine, Sho-saiko-to. Basic Clin Pharmacol Toxicol 98(4):401-5 doi:10.1111/j.1742-7843.2006.pto_328.x

Minami M, Konishi T, Makino T (2018) Effect of Hochuekkito (Buzhongyiqitang) on Nasal Cavity Colonization of Methicillin-Resistant Staphylococcus aureus in Murine Model. Medicines (Basel) 5(3) doi:10.3390/medicines5030083

Miura N, Fukutake M, Yamamoto M, et al. (2007) An herbal medicine orengedokuto prevents indomethacin-induced enteropathy. Biol Pharm Bull 30(3):495-501 doi:10.1248/bpb.30.495

Nishimura K, Osawa T, Watanabe K (2011) Evaluation of oxygen radical absorbance capacity in kampo medicine. Evid Based Complement Alternat Med 2011:812163 doi:10.1093/ecam/nen082

Niwa K, Matsunobu T, Kurioka T, et al. (2016) The beneficial effect of Hangesha-shin-to (TJ-014) in gentamicin-induced hair cell loss in the rat cochlea. Auris Nasus Larynx 43(5):507-13 doi:10.1016/j.anl.2015.12.012

Ou HC, Cunningham LL, Francis SP, et al. (2009) Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (Danio rerio) lateral line and mouse (Mus musculus) utricle. J Assoc Res Otolaryngol 10(2):191-203 doi:10.1007/s10162-009-0158-y

Ou HC, Raible DW, Rubel EW (2007) Cisplatin-induced hair cell loss in zebrafish (Danio rerio) lateral line. Hear Res 233(1-2):46-53 doi:10.1016/j.heares.2007.07.003

Owens KN, Cunningham DE, MacDonald G,

Rubel EW, Raible DW, Pujol R (2007) Ultrastructural analysis of aminoglycoside-induced hair cell death in the zebrafish lateral line reveals an early mitochondrial response. J Comp Neurol 502(4):522-43 doi:10.1002/cne.21345

Qian YF, Wang H, Yao WB, Gao XD (2008) Aqueous extract of the Chinese medicine, Danggui-Shaoyao-San, inhibits apoptosis in hydrogen peroxide-induced PC12 cells by preventing cytochrome c release and inactivating of caspase cascade. Cell Biol Int 32(2):304-11 doi:10.1016/j.cellbi.2007.10.004

Raible DW, Kruse GJ (2000) Organization of the lateral line system in embryonic zebrafish. J Comp Neurol 421(2):189-98

Shimizu I (2000) Sho-saiko-to: Japanese herbal medicine for protection against hepatic fibrosis and carcinoma. J Gastroenterol Hepatol 15 Suppl:D84-90 doi:10.1046/j.1440-1746.2000.02138.x

Takeno N, Inujima A, Shinohara K, et al. (2015) Immune adjuvant effect of Juzentaihoto, a Japanese traditional herbal medicine, on tumor vaccine therapy in a mouse model. Int J Oncol 47(6):2115-22 doi:10.3892/ijo.2015.3208

Takiyama M, Matsumoto T, Sanechika S, Watanabe J (2021) Pharmacokinetic study of traditional Japanese Kampo medicine shimotsuto used to treat gynecological diseases in rats. J Nat Med 75(2):361-371 doi:10.1007/s11418-020-01474-x

Tanaka M, Satou T, Koike K (2013) Anxiolytic-like effect of Shigyakusan extract with low side effects in mice. J Nat Med 67(4):862-6 doi:10.1007/s11418-013-0746-3

Wang J, Du JR, Wang Y, Kuang X, Wang CY (2010) Z-ligustilide attenuates lipopolysaccharide-induced proinflammatory response via inhibiting NF-kappaB pathway in primary rat microglia. Acta Pharmacol Sin 31(7):791-7 doi:10.1038/aps.2010.71

Wang K, Chen T, Ying X, et al. (2019) Ligustilide alleviated IL-1β induced apoptosis and extracellular matrix degradation of nucleus pulposus cells and attenuates intervertebral disc degeneration in vivo. Int Immunopharmacol 69:398-407 doi:10.1016/j.intimp.2019.01.004

Wang LM, Yamamoto T, Wang XX, et al. (1997) Effects of oren-gedoku-to and unsei-in, Chinese traditional medicines, on interleukin-8 and superoxide dismutase in rats. J Pharm Pharmacol 49(1):102-4 doi:10.1111/j.2042-7158.1997.tb06760.x

Yamasoba T, Schacht J, Shoji F, Miller JM (1999) Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res 815(2):317-25

Zhang Y, Li H, Huang M, et al. (2014) Neuroprotective effects of Gualou Guizhi decoction in vivo and in vitro. J Ethnopharmacol 158 Pt A:76-84 doi:10.1016/j.jep.2014.10.020