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The curcuminoid, EF-24, reduces cisplatin-mediated reactive oxygen species in zebrafish inner ear auditory and vestibular tissues

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Abstract

Cisplatin is a widely used chemotherapy drug that can damage auditory and vestibular tissue and cause hearing and balance loss through the intracellular release of reactive oxygen species (ROS). Curcumin has anticancer efficacy and can also counteract cisplatin's damaging effect against sensory tissue by scavenging intracellular ROS, but curcumin's applicability is limited due to its low bioavailability. EF-24 is a synthetic curcumin analog that is more bioavailable than curcumin and can target cancer, but its effects against cisplatin-mediated ROS in auditory and vestibular tissue is currently unknown. In this study, we employed a novel zebrafish inner ear tissue culture system to determine if EF-24 counteracted cisplatin-mediated ROS release in two sensory endorgans, the saccule and the utricle. The zebrafish saccule is associated with auditory function and the utricle with vestibular function. Trimmed endorgans were placed in tissue culture media with a fluorescent reactive oxygen species indicator dye, and intracellular ROS release was measured using a spectrophotometer. We found that cisplatin treatment significantly increased ROS compared to controls, but that EF-24 treatment did not alter or even decreased ROS. Importantly, when equimolar cisplatin and EF-24 treatments are combined, ROS did not increase compared to controls. This suggests that EF-24 may be able to prevent intracellular ROS caused by cisplatin treatment in inner ear tissue.

Keywords

tissue culture; zebrafish; reactive oxygen species; cisplatin; curcuminoid; inner ear

1. Introduction

The platinum-based chemotherapy compound, *cis*-diamminedichloridoplatinum(II) (cisplatin) (Fig. 1), is a widely-prescribed FDA-approved drug that can produce several negative side-effects including damage to auditory and vestibular tissue resulting in hearing

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and balance loss [1–9]. Curcumin, a naturally occurring plant compound, can act to prevent cisplatin-mediated damage to auditory tissue, although, its effects in vestibular tissue have not yet been characterized [4, 10]. However, curcumin exhibits poor solubility and bioavailability in mammals [11–12]. Therefore, there is considerable interest in discovering whether synthetic curcumin analogs (curcuminoids) with improved solubility and bioavailability can act like curcumin to prevent cisplatin-mediated damage to sensory tissue.

Cisplatin can induce ROS release in auditory and vestibular hair cells causing these sensory cells to undergo apoptosis [5–7, 13–16]. Unlike cisplatin, curcumin can scavenge ROS, but curcumin's efficacy is limited due to its low bioavailability [10–11, 17–20]. However, some synthetic curcuminoid compounds do not exhibit limited bioavailability [14, 21–24] and could potentially counteract intracellular ROS release. (3E,5E)-3,5-bis[(2-fluorophenyl) methylene]-4-piperidinone (EF-24) (Fig. 1) is a curcuminoid with anti-cancer efficacy that prevents reactive oxygen species damage in some tissues [25–26] and can counteract chemically-induced ROS in cancer cells [14]. These results suggested to us that EF-24 might be able to reduce ROS release in auditory and vestibular tissue treated with cisplatin.

Most auditory studies conducted in zebrafish focus on the lateral line system due to its ease of access and manipulation [27–31]. ROS release has been successfully studied in lateral line hair cells [32]. However, lateral line hair cells exhibit some differences from their mammalian counterparts including requiring mechanotransduction for cisplatin uptake [33], and functionally the lateral line is restricted to low frequency sound detection [30]. Although the zebrafish inner ear model can be used in drug-related studies [34], and it is sensitive to a broader spectrum of sound frequencies than the lateral line system [30], its use until now has been hampered by its inaccessibility.

Here, we have employed a novel zebrafish inner ear tissue culture system to test the effects of cisplatin and EF-24 treatment on intracellular ROS release. Using a spectrophotometric ROS indicator dye assay [35–36], we were able to determine the effects of cisplatin, EF-24 and combined cisplatin-EF-24 treatment on two zebrafish sensory endorgan tissues, the saccule and utricle (Fig. 2). The zebrafish saccule has primarily auditory function, and the utricle has primarily vestibular function [37–38]. We found that low (100 μM) and high (500 μM) cisplatin treatments caused significantly increased ROS release in both endorgan tissues relative to controls, but both low (100 μM) and high (500 μM) molarity EF-24 treatment either did not alter reactive oxygen species release or reduced it compared to controls. Interestingly, we found that administering equimolar concentrations of cisplatin and EF-24 did not change ROS release compared to controls, which indicates that EF-24 may counteract cisplatin-mediated ROS release in these zebrafish sensory tissues. Our results also suggest that the zebrafish inner ear tissue culture system may be a versatile new technique for assessing whether novel oto- and vestibuloprotective candidates, given in conjunction with known ototoxic therapies, modulate ROS release in sensory tissue.

2. Material and methods

2.1. Zebrafish maintenance

Zebrafish (*Danio rerio*) used in this study were obtained from a commercial supplier (Segrest Farms, Gibsonton, FL) and maintained in the Western Kentucky University animal facility following protocols approved by the Institutional Animal Care and Use Committee. All zebrafish were a mix of male and female adult animals at least 6 months of age and were maintained according to standard methods [39].

2.2. Tissue sample preparation

Tissue samples were prepared according to established procedures [34, 40]. First, zebrafish were euthanized using an overdose of tricaine methanesulfonate (MS-222) (Argent, Redmond, WA) per American Veterinary Medical Association (AVMA) protocol. Then, saccules and utricles were carefully dissected out and trimmed, and set aside in filter-sterilized phosphate buffer solution (LabChem, Pittsburgh, PA) for ROS analysis. A separate set of dissected and trimmed endorgans were photographed to provide illustrative samples of saccules and utricles used in the study (Fig. 2). These samples were first stained with Alexa Fluor 488-conjugated phalloidin (1:100; Life Technologies, Eugene, OR) for 30 minutes to label filamentous actin (F-actin). Tissue samples were then placed on glass slides and nuclei were labelled with Prolong Gold antifade reagent (4’,6-diamidino-2-phenylindole [DAPI], Life Technologies, Carlsbad, CA) and a cover-slip was placed over the samples. Saccules and utricles were subsequently viewed through the FITC and DAPI filters of a Zeiss Axioplan2 epifluorescence microscope (Carl Zeiss, Jena, Germany) at 5X and 10X magnification and photographed using an AxioCam MRm camera.

2.3. ROS measurement

Sets of 3–4 wells in black plastic 96-well plates (Nunclon, Roskilde, Denmark) were prepared for either experimental, control (tissue-media-dye; media-dye; DMSO-media-dye) or blank treatments. Experimentals consisted of either 100 μM cisplatin, 500 μM cisplatin, 100 μM EF-24 or 500 μM EF-24 in L-15 media supplemented with 10 mM HEPES, 0.5% w/v bovine serum albumin fraction V, 10 nM retinoic acid, 0.4 mg/L amphotericine B (Fungizone), 200 U/ml penicillin and 200 μg/ml streptomycin, as well as 5 μM H_2DCFDA indicator dye in phosphate-buffered saline (PBS). Tissue-media-dye and media-dye control treatments consisted of only L-15 media with supplementation and ROS indicator dye. DMSO-media-dye controls were prepared the same as the other controls except that DMSO concentrations equivalent to those introduced during the experiments were added. Blanks consisted of L-15 media prepared as before without dye. Stock solutions of EF-24 and H_2DCFDA were initially solubilized in DMSO and then diluted 1:100 in media (EF-24) or PBS (H_2DCFDA). Stock solutions of cisplatin were prepared in supplemented media. Trimmed saccule or utricle endorgans were then placed in experimental and tissue-media-dye control wells and incubated at 28 °C in a Quincy Lab (Chicago, IL) model 12–140E incubator for 45 minutes. Next, the 96-well plates were placed in a BioTek Synergy (Winooski, VT) microplate reader and read using the fluorescent mode set at 495 nm (excitation) and 527 nm (emission) wavelengths (t = 0 hours). Plates were then immediately placed back into the incubator and read again using the same spectrophotometer settings at t

= 18 hours. For experiments where cisplatin and EF-24 treatments were combined, either 100 or 500 μM cisplatin was initially placed in the wells and then tissue samples were introduced. This was followed by incubation for 45 minutes and then an initial (t = 0 hours) spectrophotometer reading as before. After 3 hours, equimolar to cisplatin concentrations of EF-24 (100 or 500 μM) were introduced. Plates were read a final time at t = 18 hours using the same excitation and emission wavelengths. ROS background measured in the media-dye control samples was subtracted from the experimental and tissue-media-control values to obtain final adjusted values which were then converted to a percent of control. Unless stated otherwise, reagents were purchased from Gibco (Gaithersburg, MD), Thermo Fisher (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).

2.4. Statistical analysis

GraphPad Prism v6 (La Jolla, CA) was used for all statistical analysis. Data sets were analyzed using unpaired two-tailed t tests ($p \leq 0.05$).

3. Results

A fluorescent spectrophotometric assay was used to measure ROS release within zebrafish saccules and utricles following cisplatin, EF-24 and cisplatin-EF-24 combination treatments. Our control results showed that DMSO and tissue only preparations did not exhibit altered ROS release compared to media-dye only controls (data not shown). However, samples treated with 100 μM of cisplatin exhibited a statistically significant higher ROS release in both saccules (169% of control; $p < 0.001$) and utricles (148% of control; $p < 0.05$) (Fig. 3A). Tissue samples treated with a higher (500 μM) concentration of cisplatin had significantly higher ROS release than the 100 μM samples for both saccules (298% of control; $p < 0.0001$) and utricles (263% of control; $p < 0.001$) (Fig. 3B).

We then investigated the effect of EF-24 treatment on ROS release in the dissected sensory endorgans. When tissue samples were treated with 100 μM of EF-24, ROS release was essentially unchanged in saccular tissue (95% of control) (Fig. 4A). In utricles, application of a 100 μM concentration caused a statistically significant decrease in ROS release (71% of control; $p < 0.01$) (Fig. 4A). Our 500 μM EF-24 treatments decreased ROS release by non-significant amounts in both saccules (71% of control) and utricles (87% of control) (Fig. 4B).

As a final experiment, we treated auditory endorgan samples initially with cisplatin and then an equimolar concentration of EF-24 after 3 hours had elapsed. When equimolar 100 μM treatments were introduced, ROS release in tissues increased slightly, but this was not statistically different from controls in both saccules (112% of control) and utricles (114% of control) (Fig. 5A). Similarly, ROS release in tissues exposed to combined 500 μM equimolar treatments of cisplatin and EF-24 were not significantly different from controls with saccules being equivalent (100% of control) and utricles exhibiting a slight decline (87% of control) (Fig. 5B). Overall, these results suggest that equimolar EF-24 treatment can counteract cisplatin-induced ROS release in these two zebrafish sensory endorgans.

4. Discussion

In this study, we used a novel zebrafish inner ear tissue culture method to determine if the curcuminoid, EF-24, prevents reactive oxygen species release in auditory and vestibular tissue treated with the chemotherapy compound and ototoxin, cisplatin. Once in cells, cisplatin enters the nucleus where it binds to DNA, causing apoptosis and increased ROS release that can damage auditory and vestibular cells [2, 4–9, 41]. As an initial step, we investigated whether cisplatin treatment caused ROS release in zebrafish saccular and utricular tissue samples and if release was concentration dependent. We found that ROS release in zebrafish saccular and utricular tissue is significantly higher than controls when treated with 100 μM cisplatin treatment (Fig. 3A). This concentration (100 μM) is approximately double that of reported cisplatin IC_{50} values in some cancer cell lines [42–43] and equivalent to or below IC_{50} values in some cisplatin resistant cell lines, e.g., A2780/C30, MDA/CH [42–44]. Therefore, our tissue culture assay found statistically significant ROS release at cisplatin concentrations encountered in cancer cell culture experiments. Further, we found that ROS release is sharply elevated from the 100 μM result at a cisplatin concentration of 500 μM (Fig. 3B). As the H_2DCFDA dye was prepared in DMSO, which can neutralize cisplatin [45], we diluted the dye 1:100 in PBS to prevent this effect. If the DMSO chemically affected cisplatin in these experiments, we would expect no or very minor changes in ROS release compared to control. However, our ROS yields with cisplatin treatment were extremely robust (148 to 169% of control at 100 μM and 263–298% of control at 500 μM), which strongly supports the interpretation that the cisplatin was intact throughout the experiments. Thus, our results suggest that the increased ROS release in the endorgan tissues was due solely from the cisplatin and follows a dosage-dependent profile.

EF-24 can act against cancer cells by increasing intracellular ROS release and enhance cisplatin's effect against cancer [46–50]. However, EF-24 can also protect non-malignant cells from cisplatin-mediated damage [46]. The curcuminoid, 4-[3,5-bis(2-chlorobenzylidene-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid (CLEFMA), causes lung cancer cell death via increased intracellular reactive oxygen species release but doesn't increase ROS release and cause damage in normal lung cells [51]. This suggested to us that EF-24 might not cause ROS release in non-cancerous auditory and vestibular tissue. We found that both lower (100 μM) and higher (500 μM) concentrations of EF-24 either did not change or even reduced ROS levels in either endorgan compared to controls (Fig. 4). This could mean that in cancer, EF-24 can signal through a mechanism that causes damaging ROS production, but that in normal sensory tissue, EF-24 does not activate a ROS-generating pathway. Additionally, like curcumin in non-cancerous tissue [52–53], our results could suggest that EF-24 functions in our auditory and vestibular tissue culture as a ROS scavenger.

Cisplatin-mediated reactive oxygen species release in auditory and vestibular tissue could be counteracted by EF-24 if the curcuminoid acts through a different pathway, pathway component or as a ROS scavenger. EF-24 and cisplatin can affect distinct antiapoptosis genes in malignant and normal mesothelioma cells [46]. Similarly, EF-24's ROS signaling in sensory tissue could be separate from cisplatin's and might function to counteract signals sent through cellular pathways induced by the platinum compound. This interpretation could

explain why the equimolar (100 μM and 500 μM) concentrations of EF-24 and cisplatin together did not alter ROS release (Fig. 5). This result could also be explained if EF-24 and cisplatin target different components of the same ROS pathway. EF-24 can form an adduct with the antioxidant glutathione in cancer cells; whereas, cisplatin instead targets glutathione S-transferase, an enzyme that catalyzes the binding of glutathione to ROS [54]. Therefore, in auditory and vestibular tissue, EF-24 could act on a different ROS pathway component than cisplatin, and this might explain how EF-24 can prevent increased ROS generation after cisplatin treatment. The restoration of normal reactive oxygen species levels when EF-24 and cisplatin are combined (Fig. 5) could also be explained by EF-24 neutralizing cisplatin-generated ROS.

In summary, we used a fluorescent spectrophotometric assay and zebrafish sensory tissue culture to show that the curcuminoid, EF-24, can counteract ROS generation caused by the platinum-based chemotherapy compound, cisplatin. Additional work using zebrafish sensory tissue cultures in combination with a variety of fluorescent probes could elucidate if EF-24 and other curcuminoids act as effective otoprotectants against platinum-based chemotherapy compounds that cause destructive reactive oxygen species release in auditory and vestibular hair cells.

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- Reactive oxygen species release increases in cisplatin-treated zebrafish endorgans
- Reactive oxygen species production in cisplatin only samples is dosage dependent
- Curcuminoid treatment does not increase endorgan reactive oxygen species
- Curcuminoid treatment counteracts cisplatin reactive oxygen species production

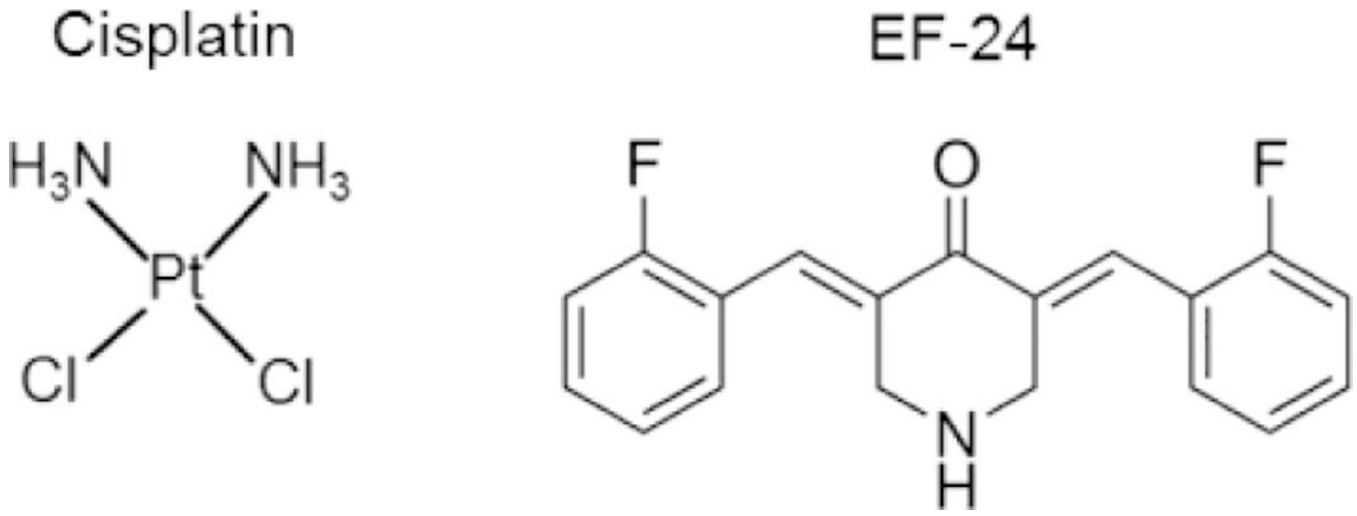

Figure 1.

The chemical structures of the platinum-based chemotherapy compound, cisplatin, and the curcuminoid, EF-24. Cisplatin treatment can cause intracellular ROS release that damages auditory and vestibular tissue. EF-24 can affect ROS release in cancer and non-cancer cells, but its effects on ROS in auditory and vestibular tissue is not known.

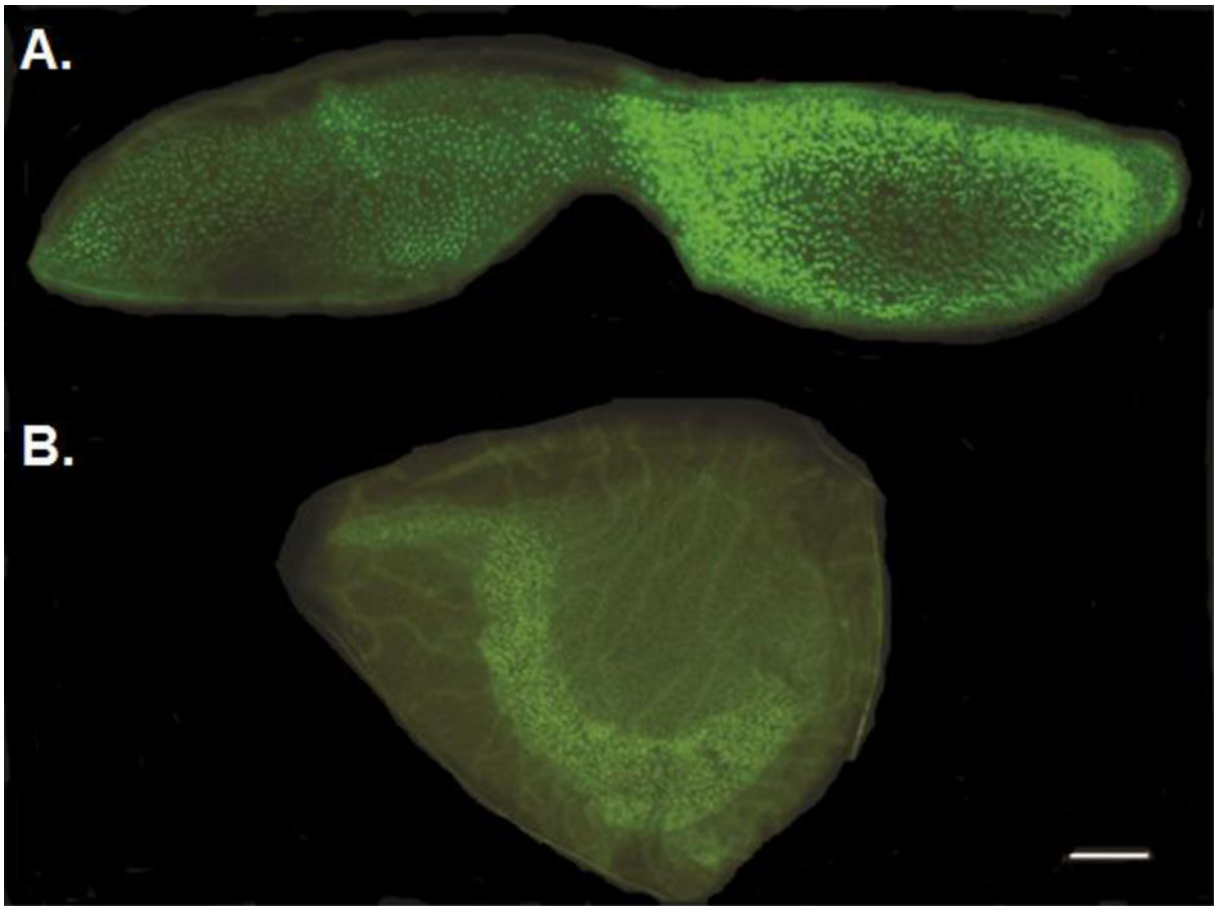

Figure 2.

Representative samples of dissected and trimmed zebrafish auditory and vestibular endorgans. A. Saccule (auditory endorgan) stained with the nuclear marker, DAPI, and the f-actin labelling marker, Alexa Fluor 488. B. Utricle (vestibular endorgan) stained with DAPI and Alexa Fluor 488. Bar = 10 μm.

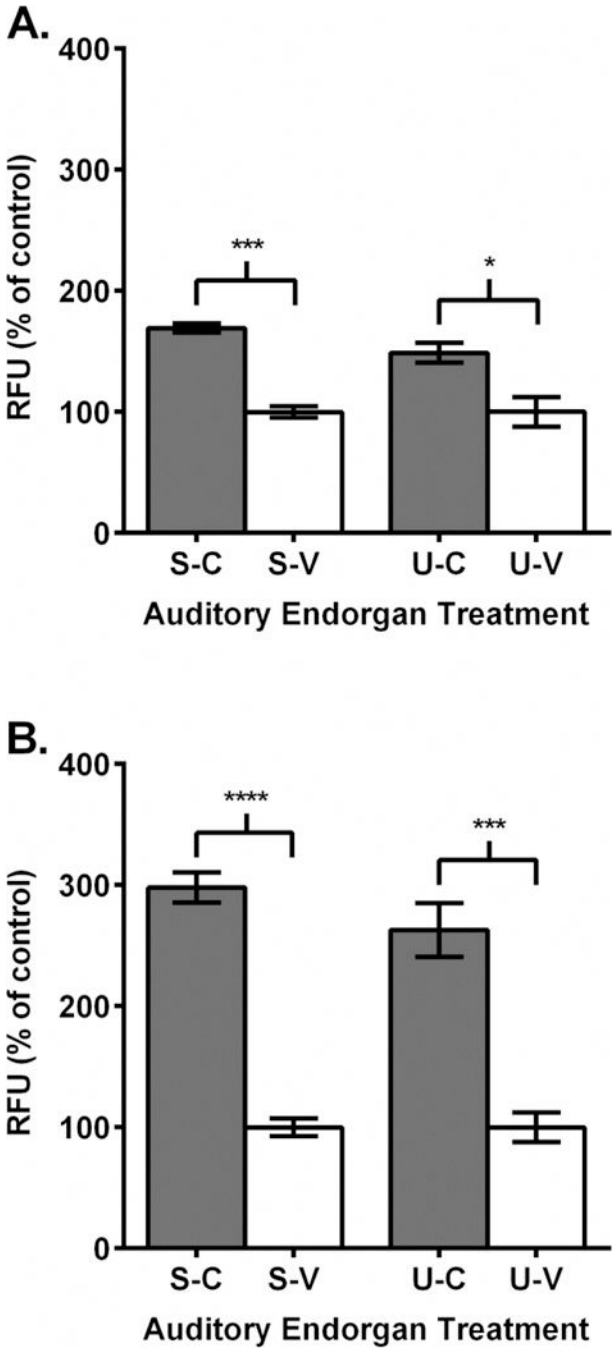

Figure 3.

Cisplatin treatment increased reactive oxygen species in zebrafish sensory endorgans. A.-B. Endorgan treatment labeling: saccule-cisplatin (S-C), saccule-vehicle (S-V), utricle-cisplatin (U-C), utricle-vehicle (U-V). A. 100 μM cisplatin (dark gray) treatment increased ROS in tissue samples compared to vehicle (white) treatment. B. 500 μM cisplatin (dark gray) treatment increased ROS in tissue samples compared to vehicle (white) treatment. RFU = relative fluorescence units. N = 3–6; "*", $p < 0.05$; "***", $p < .001$; "****", $p < 0.0001$.

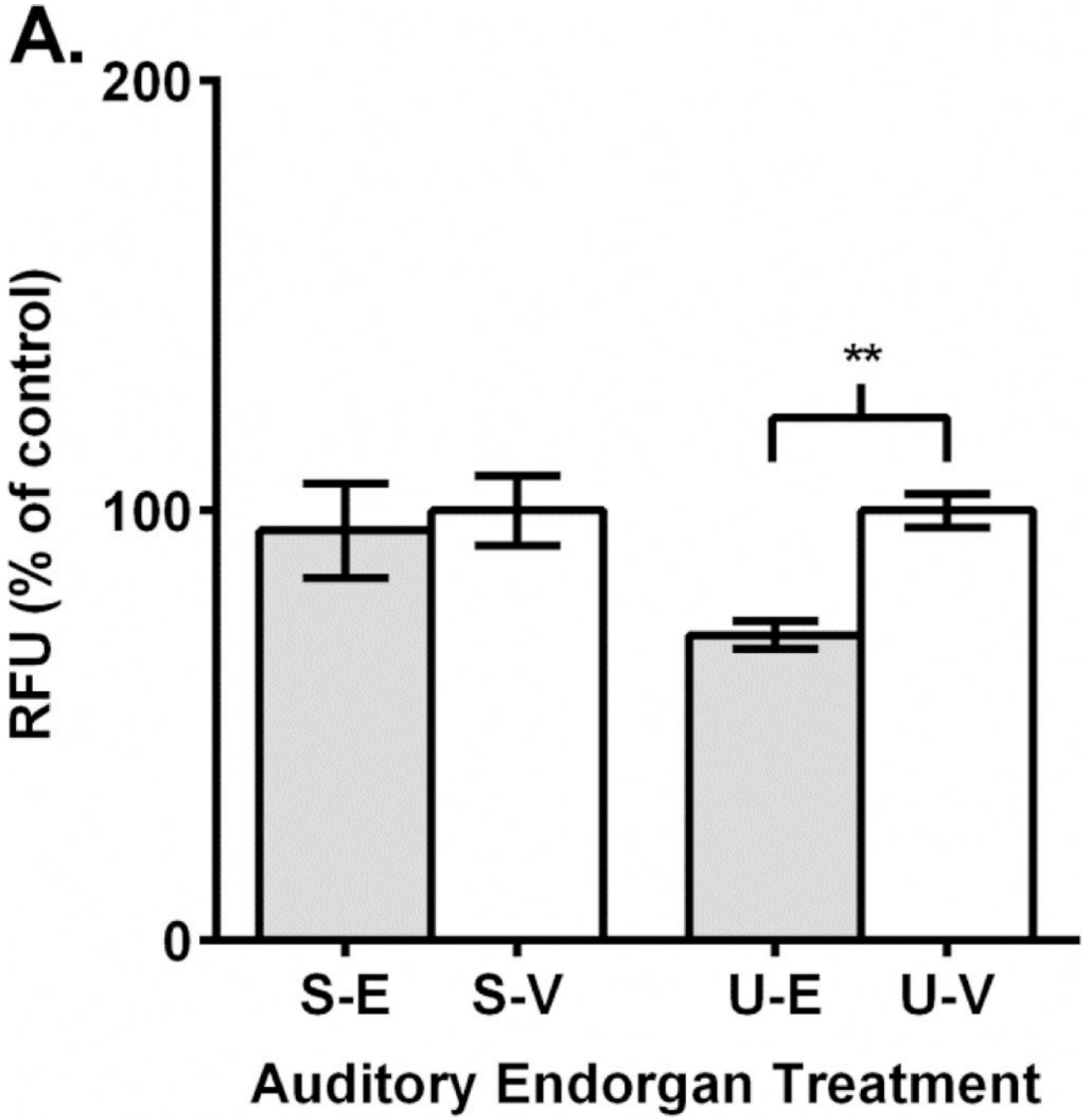

Figure 4.

Reactive oxygen species in zebrafish sensory endorgans is unchanged or reduced after EF-24 treatment. A.-B. Endorgan treatment labelling: saccule-EF-24 (S-E), saccule-vehicle (S-V), utricle-EF-24 (U-E), utricle-vehicle (U-V). A. 100 μM EF-24 (light gray) treatment does not alter ROS in saccular tissue but decreases ROS in utricular tissue compared to vehicle (white) treatment. B. 500 μM EF-24 (light gray) treatment does not alter ROS in tissue samples compared to vehicle (white) control. RFU = relative fluorescence units. N = 3–4; $p > 0.05$; "**", $p < 0.01$.

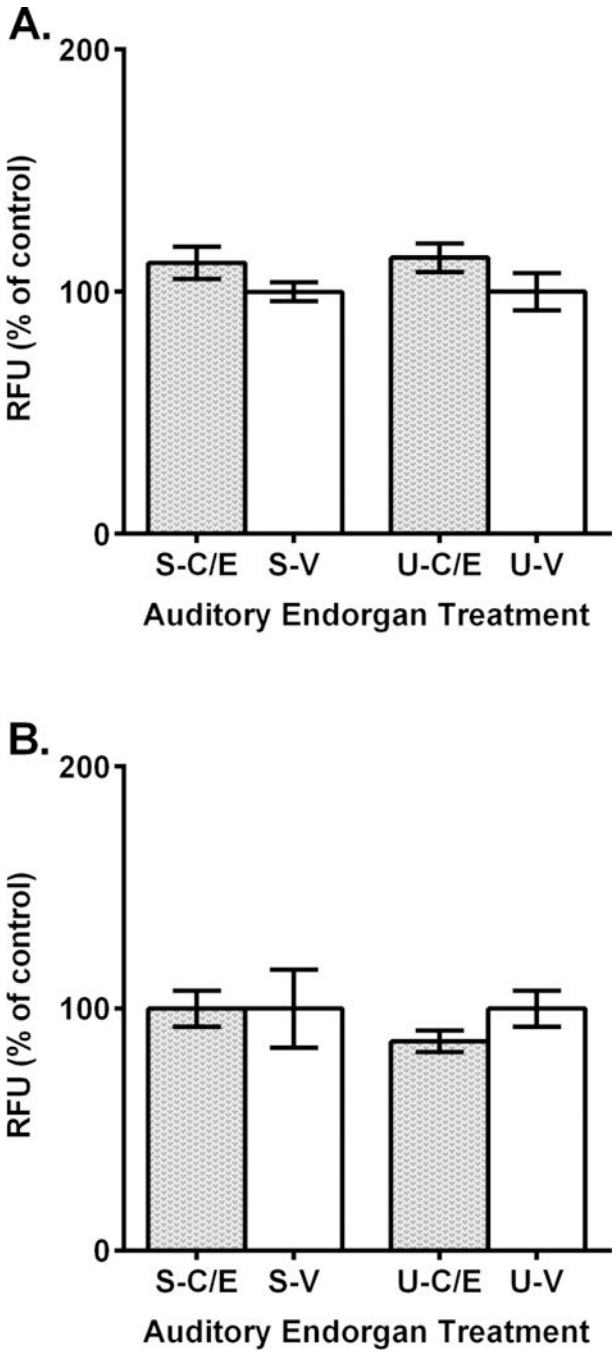

Figure 5.

EF-24 prevents reactive oxygen species in zebrafish sensory endorgan tissues treated with cisplatin. A.-B. Endorgan treatment labelling: saccule-cisplatin and EF-24 (S-C/E), saccule-vehicle (S-V), utricle-cisplatin and EF-24 (U-C/E), utricle-vehicle (U-V). A. ROS is not significantly different than control (white) treatment when 100 μM EF-24 treatment follows 100 μM cisplatin treatment (dotted gray). B. ROS is not significantly different than control

(white) treatment when 500 μM EF-24 treatment follows 500 μM cisplatin treatment (dotted gray). RFU = relative fluorescence units. N = 3; $p > 0.05$.