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RESEARCH ARTICLE

Flavone is efficient to protect zebrafish fins from UV-induced damage

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Abstract
We used ultraviolet (UV)-induced fin damage in zebrafish as a system for evaluating the chemopreventive potential of flavonoids. Chemopreventive effects of each compound, including flavone, flavanone, and chalcone, on fin development were evaluated using Kaplan-Meier analysis and Cox proportional hazards regression. Results showed that 1) flavone has the highest capacity to protect zebrafish fins from UV-induced damages among other groups; 2) zebrafish fins in the UV+1 ppm flavone group are 1.02~9.60 times more likely to return to normal fins than ones in the UV-only group, but fins in the UV+20 ppm flavone group are only 0.45~5.66 times more likely to recover; and 3) flavone significantly reduced ROS production in UV-exposed zebrafish embryos, which may attenuate UV-mediated apoptosis. In conclusion, zebrafish can be used as a system for comparing the UV-protection efficacy of flavonoids.

Keywords: Chalcone, fin, flavone, UV, zebrafish

Introduction

Ultraviolet (UV) radiation is a well-known risk factor responsible for most skin cancers (Young, 1990; Naylor, 1997). After exposure to UV radiation, a series of biological effects, such as inflammation, oxidative stress, and DNA damage, are initiated (Girard et al., 2008; Liao and Chen, 2010). Among them, generation of reactive oxygen species (ROS) is considering as a common consequence after UV exposure. One effective method for UV protection is by enhancing the cellular defense response by adding ROS scavengers from natural dietary products. Many natural dietary compounds, such as ascorbic acid, β-carotene, (−)-epigallocatechin gallate, resveratrol, sulforaphane, and flavonoids, have been proven to have UV-protection activities (Aziz et al., 2005; Kim et al., 2005; Dinkova-Kostova et al., 2006; Cimino et al., 2007; Stahl and Sies, 2007; Talalay et al., 2007; Wang et al., 2009).

Flavonoids are a class of plant phenolics. They are widely distributed in the leaves, seeds, bark, and flowers of plants (Harborne and Williams, 2000; Heim et al., 2002). In plants, flavonoids are the most important pigments for flower coloration and can afford protection against UV radiation, pathogens, and herbivores (Reifenrath and Müller, 2007; Galeotti et al., 2008). In medical applications, flavonoids have been reported to possess many pharmacological effects, such as antioxidant, -viral, -tumor, -inflammatory, neuroprotective, and cardioprotective activities (Mazur et al., 1999; Ono et al., 2005; Pan et al., 2005; Lee et al., 2007; Wang et al., 2009; Chirumbolo, 2010; Huang et al., 2010). These observations suggest that flavonoids have great potential as a possible dietary nutrition supplement.

To date, approximately 3,000–5,000 flavonoids have been identified, and many of them have been shown to possess UV-protection activity (Kootstra, 1994; Stahl and Sies, 2007). We previously described an emerging platform for screening UV-protection compounds using zebrafish larvae (Wang et al., 2009). In this study, we have used that model to generate a series of time- and

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dose-dependent flavonoid (e.g., flavone, flavanone, and chalcone) exposure experiments to compare their chemoprotection effects against UV-induced cytotoxicity. This strategy is effective to estimate or compare the UV-protective activities for different flavonoids and serves as a platform for further studying their structure-activity relationship (SAR).

Methods

Fish embryos culture, UV treatment, and chemopreventive experiments

The procedures for zebrafish culture and embryo collection have been described previously (Chen et al., 2001). Embryos developed at 72 hours postfertilization (hpf) were collected, randomly divided into 30 embryos per experimental group, and exposed 6 times separated by 30-minute intervals, receiving 100 mJ/cm^2 of energy each time (Wang et al., 2009). For UV-protection experiments, embryos at 72 hpf were collected, randomly divided into seven groups (30 embryos each), and exposed to either water (UV only) or water containing flavone (1 and 20 ppm), flavonone (1 ppm), and chalcone (1 ppm) in parallel to receive 100 mJ/cm^2 of UV for 6 times. The chemical structures of flavone, flavonone, and chalcone are shown in Figure 1; all of them were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

Fin morphology recording and microscopy

To get a quantitative view of fin morphology, fins were compared to fins of healthy nonexperimental fish and subjectively classified as normal (at least 90% of the fin was intact), reduced (20–90% intact), or absent (<20% intact) (Chen et al., 2011). All embryos were observed at specific stages under a microscope (DM 2500; Leica Microsystems, Buffalo Grove, Illinois, USA) equipped with Nomarski differential interference contrast optics. Photographs of embryos at specific stages were taken with a DFC490 CCD (Leica).

Detection of apoptotic cells

We performed terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate (dUTP) nick-end labeling (TUNEL) experiments to detect apoptotic cells. By 8 dpf (i.e., 5 days after exposure with UV), embryos from the mock control (fish from the same population of embryos, but which were not treated with UV), UV (no flavone), UV+1 ppm flavone, and UV+20 ppm

flavone groups were fixed overnight at 4°C in 4% paraformaldehyde and TUNEL was performed using a protocol previously reported (Chen et al., 2009; Pai and Chen, 2010; Peng et al., 2010).

Detection of ROS

For detecting the accumulation of ROS in zebrafish embryos, embryos from the mock control, UV (no flavone), UV+1 ppm flavone, and UV+20 ppm flavone groups were incubated with 500 ng/mL of dihydrodichlorofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, Oregon, USA). Intracellular H2DCFDA was deesterified to dichlorodihydrofluorescein, which is oxidized by ROS to produce the fluorescent compound, dichlorofluorescein (DCF). After a 150-minute incubation at 28°C, fluorescence intensity was measured at excitation/emission = 485/530 nm.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (RT-PCR)

One hundred embryos derived from the UV-only, UV+1 ppm flavone, and UV+20 ppm flavone groups were collected and their total RNA were isolated by using the standard procedure, as described previously (Chen et al., 2007). Approximately 25 μg of total RNA from each group were used for complementary DNA (cDNA) synthesis; 1% of the cDNA was used for each quantitative PCR reaction. Quantitative PCR was performed under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C, then 1 minute at 60°C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) and 200 nM of forward and reverse primers. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicate, and expression fold-changes were derived using the comparative C_T method (https://products.appliedbiosystems.com). An antiapoptotic gene, *bcl2*, and two *p53*-induced apoptosis pathway-related genes, such as *p53* and *p21*, were selected as targets. β-actin was used as an endogenous control for relative quantification.

Statistical analysis

All analyses in this study were carried out by JMP statistical software (version 4.02; SAS Institute Inc., Cary, North Carolina, USA). We treated "return to normal fin development" as the event of interest and regarded embryos that did not achieve "return" before death or at the end of the experiment as censored data. The Kaplan-Meier method was used to describe time-to-return phenomena and estimate the average time of "return to normal" for each group. The log-rank test was applied to examine the difference in malformed (i.e., nonreturn) rate between groups, and the Cox proportional hazards fit was employed to quantify the relative probability of return for each treatment group, compared to the control group. A *P*-value less than 0.05 was considered statistically significant in all analyses.
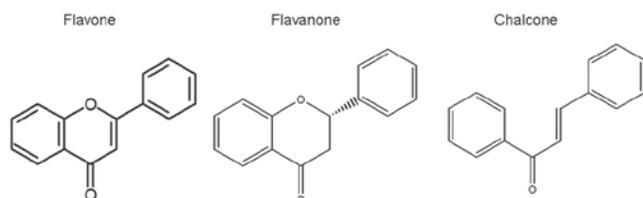

Figure 1. Structure of flavonoids used in this study. Left: flavone; middle: flavanone; right: chalcone.

Results

Flavone increased the rate of fin repair

Our previous studies have shown that embryonic zebrafish fins are very sensitive to UV exposure (Wang et al., 2009; Chen et al., 2011). Thus, fin morphology became an efficient index for evaluating UV-induced damage. In this study, we examined the preventive effect of three flavonoids (e.g., flavone, flavanone, and chalcone) on the pelvic fin after UV exposure. As shown in Figure 2, all of the mock control embryos (i.e., not treated with UV) displayed normal fins (Figure 2A), but embryos exposed to UV showed a higher incidence of malformed-fin phenotypes, including fin absence or reduction (Figure 2B and C). We first applied the Kaplan-Meier method to describe time-to-return phenomena for each experimental group. In addition to the malformed (or nonreturn) rate curve (Kaplan-Meier estimate) for each group is presented in Figure 2D, the mean time of return to normal and its corresponding standard error are listed in Table 2. Results reveal that the UV+flavone 1 ppm experimental group had the shortest average time of return to normal (4.11; Table 1), and the pelvic fin malformed rates were 79.17% for the UV-only, 52.86% for UV+flavone 1 ppm, 84.62% for UV+flavanone 1 ppm, and 91.48% for UV+chalcone

1 ppm groups estimated by 5 days after exposure to UV (Figure 2D).

We next used the log-rank test to examine the homogeneity of the malformed rate curves across the groups. The result shows a significant difference in time to return among these groups ($P = 0.0054$), confirming the UV+1 ppm flavone experimental group had a significantly optimal repair effect. These results suggest that flavone has the greatest efficacy, in comparison with flavanone and chalcone.

To investigate the concentration effect of flavone extract, we further treated zebrafish embryos with different dosage levels of flavone (0, 1, and 20 ppm). Figure 2E depicts malformed rates over time for three dosage-level groups by Kaplan-Meier analysis. The mean time of return to normal (standard error) for control, flavone 1 ppm, and flavone 20 ppm were 4.92 (0.09), 3.90 (0.21), and 4.59 (0.18) days, respectively. We further performed the Cox proportional hazards regression to evaluate the chemoprotective effects of flavone. As shown in Table 2, the relative probability of return (with corresponding confidence limits) for the 1- and 20-ppm flavone groups, compared to the UV-only group, was 2.76 (1.02~9.60) and 1.48 (0.45~5.66), respectively. This indicates that, in terms of "cure" for the dorsal fin, a zebrafish in the 1-ppm flavone group was 2.76 times more
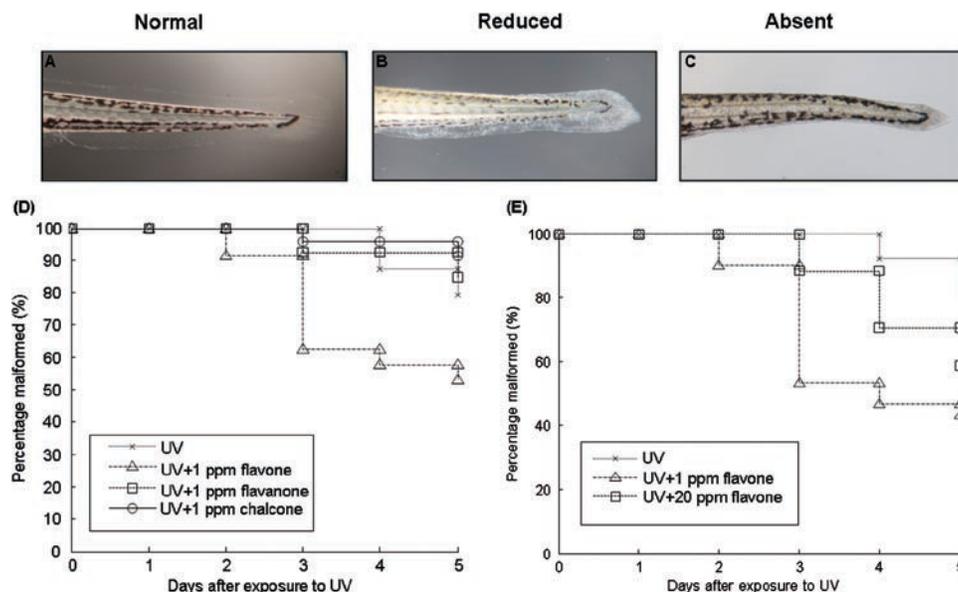

Figure 2. UV-induced malformed fin phenotypes. (**A**) Embryos display normal fins before UV exposure, but exhibit reduced- (**B**) or absent-fin phenotypes (**C**) after exposure to UV. Kaplan-Meier analysis to determine the number of days for pelvic fin in which zebrafish embryos returned to normal after exposure to (**D**) 1 ppm of flavone, flavanone, and chalcone or to (**E**) different concentrations of flavone.

Table 1. Summarized results based on the Kaplan-Meier method for each experimental group: control, flavone (1 ppm), flavanone (1 ppm), and chalcone (1 ppm).

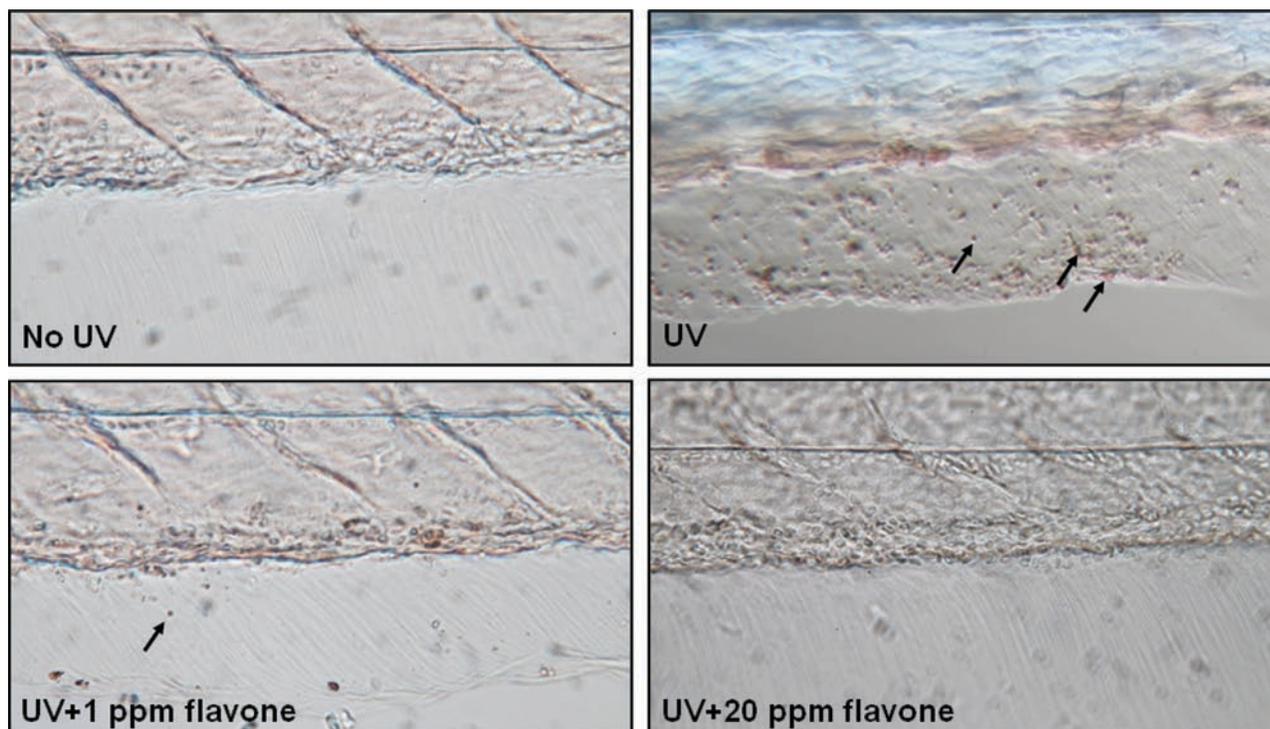| Experiment group	Mean time of return to normal (day)	Standard error of mean time
UV	4.88	0.08
UV+1 ppm flavone	4.11	0.25
UV+1 ppm flavanone	4.85	0.21
UV+1 ppm chalcone	4.92	0.12

Figure 3. UV exposure results for cell apoptosis in the fin region. Lateral views of mock control embryos without (**A**) and with UV exposure (**B**) after TUNEL assay staining. (**C**) Lateral views of embryos derived from the UV+1 ppm flavone group or (**D**) UV+20 ppm flavone after TUNEL assay staining. Arrows indicate apoptotic cells.

Table 2. Cox proportional hazards regression for assessing the effect of flavone concentration on time to return.

Experimental group	L-R Chi-square	*P*-value	Relative probability	Lower CL	Upper CL
UV+1 ppm flavone	3.98	0.046	2.76	1.02	9.60
UV+20 ppm flavone	0.40	0.526	1.48	0.45	5.66

CL, confidence limit.

likely to be cured than one in the UV-only group (Table 2). Thus, we suggest that the 1-ppm flavone group had better UV protection ability than the 20-ppm flavone group.

Flavone protect zebrafish larvae from UV-mediated fin damages by preventing cells from apoptosis

Previous studies have shown that zebrafish embryos undergo apoptosis after exposure to UV, which, consequently, causes fin damage (Nowak et al., 2005; Wang et al., 2009). Our data suggested that the UV-induced, malformed-fin phenotypes can be attenuated by coexposure to flavone (Figure 2). Here, we carried out a TUNEL assay to further confirm whether flavone could protect cells from UV-induced apoptosis. Results showed that no apoptotic signals were observed in the mock control embryos (Figure 3A), but many apoptotic signals accompanying malformed-fin phenotypes were found in the embryos after exposure to UV (indicated by arrow in Fig. 3B). However, few or no signals were found when those embryos were coexposed to UV with flavone (Figures 3C and D). Thus, we propose that flavone has the chemoprevention ability to protect UV-damaged fin cells from apoptosis.

The ROS-scavenging ability of flavone may contribute to its UV-protection efficiency

Because UV exposure is associated with the generation of ROS (Sinha and Häder, 2002), we detected the level of ROS in zebrafish embryos treated with UV and various concentrations of flavone with the cellular oxidation of H2DCFDA, a probe that is oxidized to green fluorescent DCF by various peroxide-like ROS intermediates. As shown in Figure 4, the level of ROS in zebrafish embryos treated with flavone was decreased in a concentration-dependent manner, with 24.4 and 80.9% decrease for 1- and 20-ppm flavone treatment, compared with that of UV group (i.e., no flavone). These data demonstrated that flavone significantly reduced ROS production in UV-exposed zebrafish embryos, which may attenuate UV-mediated apoptosis.

Possible mechanisms of chemoprevention of UV-induced fin damages by flavone

From the molecular aspect, UV-exposed mammalian cells have been shown to accumulate the expression of the P53 protein and to regulate the balance of Bax/Bcl-2 (Slee et al., 2004; Brown and Attardi, 2005). In this

Table 3. Relative quantification of *bcl2, p53*, and *p21* expression levels using the comparative C_T method.

Sample	Target average C_T	β-actin average C_T	ΔC_T, target-(β-actin)	$\Delta\Delta C_T$, ΔC_T-ΔC_T(UV)	Relative fold to UV group
*bcl*2					
UV	24.00 ± 0.44	19.79 ± 0.44	4.22	0	1.00
UV+1 ppm flavone	24.44 ± 0.68	20.26 ± 1.49	4.19	−0.03	1.02
UV+20 ppm flavone	25.27 ± 0.83	20.23 ± 1.32	5.04	0.82	0.57
*p*21					
UV	22.06 ± 0.48	19.79 ± 0.44	2.27	0	1.00
UV+1 ppm flavone	24.08 ± 0.43	20.26 ± 1.49	3.83	1.55	0.34
UV+20 ppm flavone	24.52 ± 0.78	20.23 ± 1.32	4.29	2.02	0.25
*p*53					
UV	22.90 ± 0.58	19.79 ± 0.44	3.12	0	1.00
UV+1 ppm flavone	24.01 ± 0.46	20.26 ± 1.49	3.76	0.64	0.64
UV+20 ppm flavone	23.89 ± 0.50	20.23 ± 1.32	3.66	0.54	0.69

study, we carried out quantitative RT-PCR experiments to further investigate the molecular mechanisms for the chemoprevention of UV-induced fin damage by flavone. As shown in Table 3, the expression levels of *bcl2*, *p21*, and *p53* in the embryos derived from UV+1 and 20 ppm flavone groups decreased to 0.57-, 0.25-, and 0.69-fold, respectively, in comparison with those of embryos derived from the UV group. This suggests that flavone might regulate the expression of *bcl2*, *p21*, and *p53* to protect fin cells from UV-induced damage.

Discussion

In this study, we demonstrated that fin damages in zebrafish embryos caused by UV can be attenuated by treatment with flavonoids, such as flavone. In human skin cells, flavonoids have been shown to have UV-protection activity. For example, it has been demonstrated that three flavone derivatives, 3,4′-dihydroxy flavone, 5,7,3′,4′-tetrahydroxyflavanone, and 5,6,7,8,3′,4′-hexamethoxy flavone, can significantly suppress the UV-induced cell death of human HaCaT and normal epidermal kerationcyte cells (Tanaka et al., 2004; Lee et al., 2007). Stahl and Sies (2007) applied flavonoids on human volunteers, and found that flavonoids may contribute to nutritional protection against damage from sunlight. In another animal model, topical treatment of 4′,5,7-trihydroxyflavone on SK-1 mouse skin inhibits the formation of squamous cell carcinoma induced by UV (Birt et al., 1997). This evidence indicates that the chemoprotective activity of flavone and flavone derivatives are effective in a wide range of hosts, such as fish, mice, and humans.

For toxicological studies, the exposure dosage of a toxin is most important. In this study, we found that 1–20 ppm of flavone is efficient to protect zebrafish fin from UV-induced damaged. The average weight of each zebrafish embryo is ~1.2 mg. After appropriate calculation, 1–20 ppm of flavone is estimated to be 16.7–333 mg/kg (Chen et al., 2008). This range is very close to the acceptable daily intakes of flavone in adult people (20–50 mg per day) (Cermak and Wolffram, 2006). We
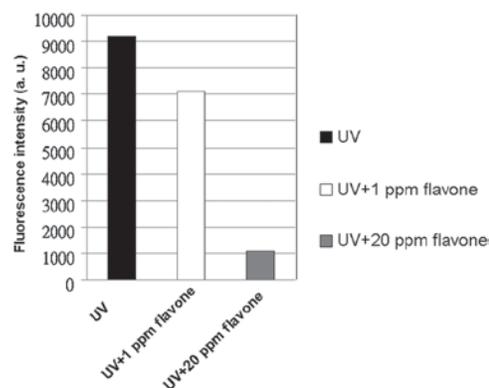

Figure 4. Repression of UV-induced ROS production by flavone. ROS levels were measured using the oxidant-sensitive probe, H2DCFDA (Molecular Probes, Eugene, Oregon, USA). The *x*- and *y*-axes represent concentrations of flavone and fluorescence intensity, respectively. a.u., arbitrary unit.

suggest that daily intake of flavone might be beneficial for UV protection.

This study suggests that flavone (but not flavonone or chalcone) can protect zebrafish fin from UV-induced damage, implying that it may have a complex SAR in the differential UV-protection activities of various flavonoids.

Declaration of interest

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