Abstract: In zebrafish, UV exposure leads to fin malformation phenotypes including fin reduction or absence. The present study evaluated UV-protective activities of comfrey leaves extracts in a zebrafish model by recording fin morphological changes. Chemopreventive effects of comfrey leave extracts were evaluated using Kaplan-Meier analysis and Cox proportional hazards regression. The results showed that (1) the mean times of return to normal fin in the UV+comfrey (50 and 100 ppm) groups were 3.43 and 2.86 days and were quicker compared with that in the UV only group (4.21 days); (2) zebrafish fins in the UV+comfrey (50 and 100 ppm) groups were 2.05 and 3.25 times more likely to return to normal than those in the UV only group; and (3) comfrey leave extracts had UV-absorbance abilities and significantly reduced ROS production in UV-exposed zebrafish embryos, which may attenuate UV-mediated apoptosis. In conclusion, comfrey leaves extracts may have the potential to be developed as UV-protective agents to protect zebrafish embryos from UV-induced damage. (DOI: 10.1293/tox.2013-0053; J Toxicol Pathol 2014; 27: 115–121)

Key words: comfrey, fin, UV, zebrafish

Introduction

Comfrey (Symphytum officinale L.) is a plant of the borage family, which is native to Europe and distributed throughout Ireland, Britain and Russia. It is a fast growing plant, producing huge numbers of leaves. It is commonly used in herbal medicine and cosmetic products. For example, comfrey root extract has been used for the topical treatment of painful muscle and joint complaints. Topical comfrey creams (especially leave extracts) have been used to treat minor wounds, bruises, sprains, and varicose veins. These observations suggest that comfrey has many applications, especially in terms of medical uses.

Ultraviolet (UV) radiation is a well-known environmental risk factor. Inflammation, oxidative stress and DNA damage are caused by exposure to UV radiation. Importantly, generation of reactive oxygen species (ROS) is considered the most important adverse effect after UV exposure. In aquaculture, short-term exposure to UV radiation is used to protect juvenile fish from parasite infection. However, fish exposed to excessive UV will experience some pathogenic effects, such as “solar dermatitis” and “summer lesion syndrome”. These observations suggest that over-exposure to UV radiation is harmful to aquatic animals; in this regard, it is important to develop a low-cost and highly efficient UV-protective substance for aquaculture application.

One effective method of UV protection is enhancement of the cellular defense response by addition of ROS scavengers from natural products. Many active compounds have been proven to have UV protection activities, including (−)–epigallocatechin gallate, resveratrol, sulforaphane and flavones. However, these active compounds are too expensive to be applied to aquaculture. Searching for a low-cost alternative is an important issue that should be addressed. Since comfrey is a fast growing plant with plenty of leaves, in this study, we used the zebrafish as a model and generated a series of time- and dose-dependent leave extracts in comfrey exposure experiments in order to evaluate their chemoprotection effects on UV-induced cytotoxicity. These results should be applicable to aquaculture.
Materials and methods

Preparation of comfrey samples

Comfrey was kindly supplied by Yan Ten Biotech Corp., Taiwan. After eight weeks of the nutrition period, comfrey leaves were collected. The leaves were washed, air dried and ground into small particles in the presence of methanol (55 g/400 mL). The mixture was filtered to obtain a green solution. The solution was further processed through a small C18 cartridge to remove the chlorophyll. Comfrey may contain a certain amount of pyrrolizidine alkaloids, which are capable of being removed by extraction with dichloromethane. Finally, a powder sample was obtained by evaporation of methanol and water to dryness at room temperature in the dark. The fine particles were put into a glass bottle for further drying in the presence of phosphorus pentoxide (P2O5) under high vacuum for 18 h. Finally, around 1.4 g of powder were obtained and were ready for further examination.

Fish embryos culture, UV treatment and chemopreventive experiments

The procedures for zebrafish culture and embryo collection have been described previously20. For survival rate analysis, embryos developed at 72 hours post fertilization (hpf) were collected, randomly divided into 30 embryos per experimental group and soaked in different concentrations of comfrey leave extracts (50, 100 and 1000 ppm) without UV exposure (comfrey only) or with exposure to 302 nm UV (UVB, generated by a UV Crosslinker; Spectronics, Westbury, NY, USA) 6 times at 30-min intervals. To get a quantitative view of fin morphology, fins were observed at specific stages under a microscope (DM 2500, Leica) 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 deoxyuridine triphosphate [dUTP] nick end labeling (TUNEL) experiments to detect apoptotic cells. By 8 dpf (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; no UV), UV (no comfrey; UV only), UV+50 ppm comfrey, UV+100 ppm comfrey and UV+1000 ppm comfrey groups were fixed overnight at 4 °C in 4% paraformaldehyde, and TUNEL was performed using a protocol previously reported20-22.

Detection of ROS

To detect the accumulation of ROS in zebrafish embryos, embryos from the UV only (no comfrey) and UV + comfrey groups (50, 100 and 1000 ppm) were incubated with 500 ng/ml dihydrodichlorofluorescein diacetate (H2DCFDA, Molecular Probes). Intracellular H2DCFDA was de-esterified to dichlorohydrofluorescein, which is oxidized by ROS to produce the fluorescent compound dichlorofluorescein (DCF). After a 150-min incubation at 28 °C, the fluorescence intensity of embryos (FI) was measured with excitation/emission wavelengths of 485/530 nm. All data were presented as “ROS-scavenging rates”, which were calculated with the following equation: ROS-scavenging rates (%) = (FIUV+comfrey – FIUV only/FIUV only)*100%. FIUV+comfrey and FIUV only represent the fluorescence intensity (FI) of the UV+comfrey group and the UV only group, respectively. A positive ROS-scavenging rate indicates that treatment with the comfrey led to the generation of ROS. A negative ROS-scavenging rate indicates that the tested comfrey group has ROS-scavenging activities20,23.

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

One hundred embryos derived from the UV only, UV+50 ppm comfrey and UV+100 ppm comfrey groups were collected, and their total RNAs were isolated by using the standard protocol as described previously24-26. Around 25 μg of total RNA from each group were used for cDNA synthesis; 1% of cDNA was used for each quantitative PCR reaction. Quantitative PCR was performed under the following conditions: 2 min at 50, 10 min at 95, and 40 cycles of 15 sec at 95 and 1 min at 60 using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, 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 fold-changes in expression were derived using the comparative C target method (https://products.appliedbiosystems.com). An anti-apoptotic gene, bcl2 (F, 5’-CCTTTCAATAAAAGCAGTG-GAGGAA-3'; R, 5’-CGGGCTATCATGGGATTCCA-3'), and several p53-induced apoptosis pathway-related genes, such as p53 (F, 5’-GGCTCTTTTGGCTGGACATCAT-3'; R, 5’-TGGATGGCGATGCGGTCT-3'), p21 (F, 5’-CAGCTCGGTTCTCTGAC-3'; R, 5’-CTCGAGTTGAGTGGGC-3') and mdm2 (F, 5’-GTGACAC- CAGATCCGAGACC-3'; R, 5’-TGAATCTTGAAGTGG-3') were selected as targets. The β-actin (F, 5’-CAGAAGCAGGAGTGATGGT-3'; R, 5’-TGAATCTTGAAGTGG-3') was used as an endogenous control for relative quantification.

Statistical analysis

All analyses in this study were carried with the JMP statistical software (version 4.02; SAS Institute Inc., Cary, NC, USA). We treated “return to normal fin development”
as the event of interest and regarded embryos that did not achieve “return” prior to death or at the end of the experiment as censored data. The Kaplan-Meier method was used to describe the malformation (non-return) rate over time and estimate the average time of return to normal for each experiment group. The log-rank test was applied to examine the difference in malformation rate between groups, and the Cox proportional hazards fit was employed to quantify the relative probability of return for each treatment group compared with the control group. The Tukey-Kramer HSD (honestly significant difference) test was used to compare the population marginal mean number of apoptotic cells for each treatment group. A significance level 0.05 was used in ANOVA analysis, and a familywise error rate of 0.05 was applied for the Tukey-Kramer HSD test.

Results

**Comfrey extracts increased the rate of fin repair**

Our previous studies have shown that embryonic zebrafish fins are very sensitive to UV exposure\(^1\), \(^2\), \(^3\). Thus, fin morphology has become an efficient index for evaluating UV-induced damage. In this study, we examined the preventive effect of comfrey leave extracts at different dosages on pelvic fins after UV exposure. First, we treated zebrafish embryos with different dosages of comfrey extracts (50, 100 and 1000 ppm) with or without UV exposure and calculated their survival rates. As shown in Fig. 1, there were no significant differences in survival rates between comfrey-only (100.0 ± 0%; mean ± standard error; SE) and (UV+comfrey) groups (95.7 ± 2.6% to 100.0 ± 0%; n = 30 (numbers of tested embryos in each group), N = 3 (in triplicate experiments)], suggesting that treatment with 50–1000 ppm of comfrey is not toxic to zebrafish embryos. Then, we recorded the fin morphology among all groups. As shown in Fig. 2, all of the mock control embryos (not treated with UV) displayed normal fins, but embryos exposed to UV showed a higher incidence of malformed-fin phenotypes, including fin absence or reduction. To get a better statistical point of view, we first applied the Kaplan-Meier method to describe time-to-return phenomena for each experimental group. In addition to the malformation (or non-return) rate curve (Kaplan-Meier estimate) for each group presented in Fig. 2, the mean time of return to normal and its corresponding standard error are listed in Table 1. The results revealed that UV+100 ppm comfrey experimental group had the shortest average time of return to normal (Table 1) and that the pelvic fin malformation rates, estimated 5 days after exposure to UVB, were 61.90%, 37.08% and 18.24% for the UV only, UV+50 ppm comfrey and UV+100 ppm comfrey groups, respectively (Fig. 2). We next used the log-rank test to examine the homogeneity of the malformation rate curves across the
groups. The result showed a significant difference in time-to-return among these groups (p-value < 0.0001), confirming that UV+100 ppm comfrey experimental group had a significantly optimal repair effect.

The Cox proportional hazards regression analysis (Table 2) demonstrates that the relative probabilities of return to normal fin (with corresponding confidence limits) for the UV+50 ppm comfrey and UV+100 ppm comfrey groups compared with control (UV only) group were 2.05 (1.11–3.90) and 3.25 (1.83–6.04). The former indicates with statistical significance (p-value=0.022) that a zebrafish in the UV+50 ppm comfrey group was 2.05 times more likely to achieve return than one in the UV only group. The latter significantly suggests that a zebrafish in the UV+100 ppm comfrey group was 3.25 times more likely to achieve return than one in the control group (p-value=0.000). This indicates that the comfrey extracts increased the rate of fin repair in a dose-dependent manner.

### Comfrey protects zebrafish larvae from UV-mediated fin damage by preventing apoptosis of cells

It has been demonstrated that UV-induced zebrafish fin damage is due to apoptosis. Our data demonstrated that the UV-induced malformed fin phenotypes can be attenuated by co-exposure to comfrey leaf extracts (Fig. 2). Here, we carried out a TUNEL assay to further confirm whether comfrey leaf extracts can protect cells from UV-induced apoptosis. The results showed that no apoptotic signals were observed in the embryos derived from the no UV group (Fig. 3A), but many apoptotic signals accompanying malformed fin phenotypes were found in the embryos after exposure to UV (UV only group; indicated by an arrow in Fig. 3B). However, few or no signals were found when these embryos were co-exposed to UV with 50–100 ppm of comfrey extracts (Figs. 3C–3E). To pinpoint which treatment means were significantly different from each other, the Tukey-Kramer HSD test was further used for pairwise comparisons. Figure 3F presents the mean numbers and their 95% confidence intervals for the five treatment groups. The test revealed that the mean numbers for the no UV, UV only, UV+50 ppm comfrey, UV+100 ppm comfrey and UV+1000 ppm comfrey groups were 13.67, 162.50, 93.33, 56.03 and 7.57, with the common standard error being 2.82, and also identified that the mean numbers for the five treatment groups were significantly different from each other, except those for the no UV and UV+1000 ppm comfrey groups (Fig. 3F). This indicates that the UV+1000 ppm comfrey group had the potential to let the UV-treated zebrafish fins return to normal. Thus, we propose that comfrey extract has a chemoprevention ability that protects UV-damaged fin cells from apoptosis.

### The ROS-scavenging and UV-absorbance abilities of comfrey leaf extract may contribute to its UV-protection efficiency

Previous studies have shown that UV exposure is associated with the generation of ROS. In this study, we detected the level of ROS in zebrafish embryos treated with UV and 50–100 ppm of comfrey leaf extracts. As shown in Fig. 4, the level of ROS in zebrafish embryos treated with comfrey extract was decreased in a concentration-dependent manner, with 51.7%, 82.8% and 93.1% decreases for the 50, 100 and 1000 ppm comfrey extract treatments compared with the UV only group (no comfrey). For the UV absorbance experiment, 0, 50 and 100 ppm of comfrey leaf extracts were used to measure the absorbance between 280–410 nm. As shown in Fig. 5, comfrey extracts indeed had photochemical properties, especially in the wavelength range of 290–340 nm. These data demonstrated that the ROS-scavenging and UV-absorbance abilities of comfrey leaf extract may contribute to its UV-protection efficiency.

### Possible mechanisms of chemoprevention of UV-induced fin damage by comfrey

From the molecular point of view, UV-induced cell apoptosis has been shown to accumulate the expression of p53 and its downstream target, p21. mdm2 is a negative regulator of p53, whereas bcl2 is a cell cycle regulator proteins that is thought to have anti-apoptotic activity. In this study, we carried out quantitative RT-PCR experiments to further investigate the molecular mechanisms for chemoprevention of UV-induced fin damage by comfrey extract. As shown in Table 3, the expression levels of p53 and p21 in the embryos derived from UV+comfrey (50 and 100 ppm) groups increased by 1.4- to 2.7-fold, in comparison with those of embryos derived from the UV only group; the expression levels of mdm2 were downregulated by 0.6-fold. This suggests that comfrey treatment might induce the p53-related pathway. However, the expression levels of bcl2 were increased by 1.2- to 1.5-fold. Taken together, we propose that comfrey may increase the expressions of bcl2 to protect fin cell UV-induced apoptosis.

---

**Table 1.** Summarized Results Based on the Kaplan-Meier Method for Each Experimental Group: Control (only UV), UV+50 ppm comfrey and UV+100 ppm comfrey

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>Mean time of return to normal (day)</th>
<th>Standard error of mean time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only UV</td>
<td>4.21</td>
<td>0.19</td>
</tr>
<tr>
<td>UV+50 ppm comfrey</td>
<td>3.43</td>
<td>0.23</td>
</tr>
<tr>
<td>UV+100 ppm comfrey</td>
<td>2.86</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Table 2.** Cox Proportional Hazards Regression for Assessing the Effect of Comfrey Concentration on Time to Return

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>L-R chi-Square</th>
<th>P-value</th>
<th>Relative probability</th>
<th>Lower CL</th>
<th>Upper CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV+50 ppm comfrey</td>
<td>5.28</td>
<td>0.022</td>
<td>2.05</td>
<td>1.11</td>
<td>3.90</td>
</tr>
<tr>
<td>UV+100 ppm comfrey</td>
<td>16.84</td>
<td>0.000</td>
<td>3.25</td>
<td>1.83</td>
<td>6.04</td>
</tr>
</tbody>
</table>

CL: confidence limit.
Fig. 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+50 ppm comfrey group, (D) UV+100 ppm comfrey group or UV+1000 ppm comfrey group (E) after TUNEL assay staining. Arrows indicate the apoptotic cells. (F) The Tukey-Kramer HSD (honestly significant difference) test reported the marginal mean cell counts and corresponding 95% confidence intervals for all groups. The means of two groups are significantly different if their intervals are disjoint and are not significantly different if their intervals overlap.

Fig. 4. Repression of UV-induced ROS production by comfrey. UV-induced ROS levels are regulated by comfrey. The ROS levels were measured using the oxidant-sensitive probe H2DCFDA. The X- and Y-axes represent the different concentrations of comfrey and ROS-scavenging rates, respectively. ROS-scavenging rates were calculated using the following equation: ROS-scavenging rates (%) = (FI_{UV+comfrey} − FI_{UV only})/FI_{UV only} × 100%.

Fig. 5. Absorbance spectrum of comfrey leave extract between 280-410 nm. Comfrey leave extracts of 0 (circle), 50 (triangle) and 100 (square) ppm were used to measure the absorbance between 280–410 nm, respectively. The instrument used was a JASCO V-550 UV/VIS spectrophotometer, and a quartz cuvette was used. The path length was 1 cm.
Discussion

In this study, we demonstrated that fin damage in zebrafish embryos caused by UV can be attenuated by treatment with comfrey leave extracts. In order to apply comfrey extracts to aquaculture and fish physiology, the toxicants of the comfrey extracts should be removed. It was reported that comfrey contains dangerous levels of toxic pyrrolizidine alkaloids and that its use led to severe liver injury and death. Because of its toxicity, comfrey (leaves and roots) crude extracts have often been processed as topical cream, and it has been recommended that they never be taken by mouth or even applied comfrey to broken skin.

In general, the root of the plant contains more pyrrolizidine alkaloids than the leaves. To avoid the poison effect of pyrrolizidine alkaloids, we selected comfrey leave extracts as materials and used a pyrrolizidine alkaloid-free purification protocol. Our study indicated that treatments with 50–100 ppm of purified comfrey leave extract are not toxic to zebrafish fins from UV-induced damage, implying that it may be applied to aquaculture to enhance the survival of juvenile fish.

Acknowledgments: Comfrey was kindly supplied by Yan Ten Biotech Corp, Taiwan. This project was supported by the National Science Council, Republic of China, under grant number NSC 96-2313-B-032-001-MY3.

References

6. Araújo LU, Reis PG, Barbosa LC, Saúde-Guimarães DA, Grabe-Guimarães A, Mosqueira VC, Carneiro CM, and Silva-Barcellos NM. In vivo wound healing effects of Symphytum officinale L. leaves extract in different topical for-
8. Girard PM, Pozzebon M, Delacôte F, Douki T, Smirnova V, and Sage E. Inhibition of S-phase progression triggered by UVA-induced ROS does not require a functional DNA damage checkpoint response in mammalian cells. DNA Repair (Amst). 7: 1500–1516. 2008. [Medline] [CrossRef]
9. Liao YF, and Chen YH. Zebrafish is emerging to be a model to find sun-protective compounds. Household Personal Care Today. 4: 6–8. 2010.
21. Tsai IT, Yang ZS, Lin ZY, Wen CC, Cheng CC, and Chen YH. Flavone is efficient to protect zebrafish fins from UV-induced damage. Drug Chem Toxicol. 35: 341–346. 2012. [Medline] [CrossRef]
25. Shih TL, Hsiao CA, Lin ZY, and Chen YH. An alternative synthesis of 3′,4′-diaminoflavones to evaluate their antioxidant ability and cell apoptosis of zebrafish larvae. Molecules. 17: 8206–8216. 2012. [Medline] [CrossRef]