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Protective effect of marine brown algal polyphenols against oxidative stressed zebrafish with high-glucose

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Abstract

The zebrafish (*Danio rerio*) is one of the most widely used vertebrate models in research studies in molecular genetics, development biology, drug discovery and human disease. This study has confirmed an increase in the production of reactive oxygen species (ROS) and induction of cell death by using high glucose treatment in zebrafish model.We found observed that the exposure to phlorotannins, which include 6,6-bieckol, phloroeckol, dieckol and phlorofucofuroeckol isolated from an edible brown alga, *Ecklonia cava*, significantly inhibited high glucose-induced ROS and cell death. Among the phlorotannins, DK (Dieckol) significantly reduced heart rates, ROS, nitric oxide (NO), lipid peroxidation generation and cell death in high glucose-induced oxidative stress. Further, high glucose levels induced the over expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), whereas DK treatment reduced its over expression. These findings indicate that the zebrafish is an efficient animal model that can be used to investigate hyperglycemia-stimulated oxidative stress. Therefore this model takes advantage of confirmation for functional antioxidant foods and nutraceuticals.

Key words: zebrafish; marine polyphenols; phlorotannins; oxidative stress; high glucose ;

1. Introduction

A model using zebrafish (*Danio rerio*) has several advantages for *in vivo* experiments its embryos are easily bred in large numbers, small size and optical transparency. Its larvae are approximately 1~4 mm in length and develop discrete organs and tissues, including the brain, heart, liver, pancreas, kidney, intestines, bone, muscles, nerve systems, and sensory organs within 120 hour post fertilization (hpf).[1] These tissues and organs are similar to mammals at the genetic, immunological, physiological, ethological, and anatomical levels.[2-4] Accordingly, adult zebrafish and zebrafish embryos have also been used in studies on various human diseases.

Several recent studies have verified that chronic hyperglycemia is considered to the increase of risk factors for chronic disease, especially diabetes, neuropathy, retinopathy, inflammation and vein endothelial dysfunction through the induction of reactive oxygen species (ROS) generation.[5-8] The high reactivity of ROS imparts cytotoxic effects on membrane phospholipids, thus causing a wide spectrum of cell damage, including lipid peroxidation and nitric oxide (NO) production, an increase in the levels of oxidants, and alterations in the activity of oxidant enzymes.[9,10] Multiple biochemical pathway of action have been implicated in the deleterious effects of chronic hyperglycemia and oxidative stress on the function of tissues.[11] Thus, prevent pathological damage associated with chronic disease attenuation of oxidative stress caused by a hyperglycemic condition is an important matter.[12]

Recently, seaweeds have been utilized as marine bioresources based on its potential health benefits that are related to substances such as alginates, polysaccharides, polyphenols (phlorotannins), minerals, and vitamins.[13] Especially, seaweeds contain appreciable amounts of polyphenols referred to as phlorotannins that have anti-oxidantive, anti-inflammatory,

anti-cancer, and anti-diabetes activities.[14-17] Brown seaweeds are particularly rich in polyphenols (phlorotannins); Polyphenols from *Ascophyllun nodosum* appeared to be responsible for the stimulatory activity on glucose uptake and improved blood antioxidant capacity in diabetic mice.[18] Previous reports have also shown that the edible brown algae, *Ecklonia cava* imparted regulatory effects on a type 1 diabetes mellitus model by controlling postprandial hyperglycemia and high glucose-induced oxidative stress in rat insulinoma and human umbilical vein endothelial cells.[8,19-21]. In this study, we confirm that zebrafish is an efficient *in vivo* model for hyperglycemia-stimulated oxidative stress related studies involving phlorotannins from *E. cava*.

2. Experimental

2.1. Chemicals and reagents

DCF-DA (2,7-dichlorodihydrofluorescin diacetate), DAF-FM-DA (diaminofluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate), DPPP (Diphenyl-l-pyrenylphosphine), PI (Propidium iodide), and 2-phenoxy ethanol were purchased from Sigma-Aldaich (St. Louis, MO, USA). Antibodies for iNOS and COX-2 were obtained from Cell Signaling Technology (Bedford, MA, USA) and GAPDH and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were of the purest grade available.

2.2. Materials

The marine brown alga *E. cava* was collected along the coast of Jeju Island, Korea, between March 2012 and June 2012. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached on its surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at −20°C. The frozen samples were then lyophilized and homogenized with a grinder prior to extraction.

2.3. Isolation of phlorotannins from *E. cava*

It exhibited the most efficient separation of four fractions from *E. cava* ethanol extracts under solvent the following conditions: n-hexane / ethyl acetate / methanol / water (2:8:3:7). Pure compounds of the fractions were identified as 6,6-bieckol (6,6-BK), phloroeckol (PK), dieckol (DK) and phlorofucofuroeckol-A (PFFK), respectively, by comparing the ^{1}H and ^{13}C-NMR data of each fraction with previous reports. The chemical structures of the phlorotannins are presented in Fig. 1.

2.4. Origin and maintenance of parental zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Seoul, Korea) and 15 fishes were kept in a 3.5 L acrylic tank under the following conditions; 28.5 ± 1 ^{o}C, and fed twice a day (Tetra GmgH D-49304 Melle Made in Germany) with a 14/10 h light/dark cycle. Zebrafish were mated and spawning was stimulated by the onset of light. Embryos were then obtained within 30 min of natural spawning and transferred Petri-dishes containing media.

2.5. Experimental design of high glucose-stimulated oxidative stress

The embryos (n = 15) were transferred to individual wells of 12-well plates containing 900 μL embryo media. At 7 to 9 hpf (hour post fertilization), a 50 μL of each sample was added to the wells. At 24 hpf, a 50 μL of the glucose solution was added to the embryo media, exposing the embryos for up to 2day post fertilization (dpf). The embryos were then rinsed with fresh embryo media.

2.6. Measurement of heart rate

The heart rates of both atrium and ventricle were measured at 2 dpf. Counting and recording of atrial and ventricular contractions were performed for 1 min under a microscope.

2.7. Estimation of oxidative stress-induced ROS generation and image analysis

ROS production in zebrafish was analyzed using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA was deacetylated intracellularly using nonspecific esterase, which was further oxidized to the highly florescent

compound dichlorofluorescein (DCF) in the presence of cellular peroxides [21]. At 4 dpf, the zebrafish larvae were transferred to one well of a 24-well plate, treated with a DCFH-DA solution (20 µg/mL) and incubated for 1 h in the dark at 28.5±1 ^{o}C. After the incubation, the zebrafish larvae were rinsed with fresh embryo media and anesthetized using 2-phenoxy ethanol (1:500 dilution) prior to observation and the photographed under a microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan). The fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.8. Estimation of oxidative stress-induced NO generation and image analysis

NO production in zebrafish was analyzed using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Transformation of DAF-FM DA by NO in the presence of dioxygen generates highly fluorescent triazole derivatives [22]. At 4 dpf, the zebrafish larvae were transferred to one well of a 24-well plate, treated with a DAF- FM DA solution (5 µM) and incubated for 2 h in the dark at 28.5±1 ^{o}C. The rest of the procedure was as described in Section 2.6.

2.9. Estimation of oxidative stress-induced lipid peroxidation generation and image analysis

Lipid peroxidation was measured to assess the extent of membrane damage in the zebrafish model. Diphenyl-l-pyrenylphosphine (DPPP) is a fluorescent probe commonly used for the detection of cell membrane lipid peroxidation. DPPP is naturally non-fluorescent, but it becomes fluorescent when oxidized. At 4 dpf, the zebrafish larvae were transferred to one well of a 24-well plate, treated with a DPPP solution (25 µg/mL), and incubated for 1 h in the dark at 28.5±1 ^{o}C. The rest of the procedure was as described in Section 2.6.

2.10. Estimation of oxidative stress-induced cell death and image analysis

Cell death was detected in the live embryos using Propidium iodide (PI) staining. PI is membrane impermeant and generally excluded from viable cells. PI is commonly used in identifying dead cells in a population. At 4 dpf, the zebrafish larvae were transferred to one well of a 24-well plate, treated with PI solution (80 µg/mL), and incubated for 30 min in the dark at 28.5±1 ^{o}C. The rest of the procedure was as described in Section 2.6.

2.11. Western blot analysis

The embryos (n = 50) were transferred to individual wells of 6-well plates containing 2,700 µL of embryo media. At 7 to 9 hpf, a 150 µL of DK was added to each of the wells. At 24 hpf, a 150 µL of 150 mM glucose solution was added to each well and incubated until 2 dpf. Then, embryos were rinsed with fresh embryo media. Embryos were transferred into Eppendorf tube, and then washed twice. The zebrafish were homogenized in lysis buffer using a homogenizer. The protein concentrations were determined using a BCA^{TM} protein assay kit (Bio-Rad, CA, USA). The lysate, which contained 50 µg of protein, was subjected to electrophoresis on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) using a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% methanol (v/v)]. The membranes were blocked in 5% blotting-grade blocker in TBST 2 h. The primary antibodies were used at a 1:1,000 dilution. Membranes were incubated with the primary antibodies at 4 ^{o}C overnight. The membranes were washed with TTBT, and then incubated with the secondary antibodies at a 1:3,000 dilution. The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit and exposed to X-ray films.

2.12. Statistical analysis

The data were expressed as the mean ± standard error (S.E.) and one-way ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences among the means of the parameters were determined using student's t-test (*$P< 0.05$, **$P<0.01$).

3. Results

3.1. Embryo toxicity of glucose

To determine the toxicity of glucose, we examined the survival rate, ROS production and cell death in zebrafish. The survival rates were 100%, 100%, 71% and 40% in 50, 100, 150 and 300 mM glucose-treated zebrafish, respectively (Fig. 2-A). The images were observed at 4 dpf. The levels of ROS were 105%, 132%, 147%, and 259% in the 50, 100, 150, and 300 mM of glucose-treated groups compared to that of the control group (Fig. 2-B). As shown in Fig. 2-A, cell death was recorded at 100%, 115%, 230% in the 283% in the 50, 100, 150 and 300 mM of glucose-treated groups, respectively. These results indicate that glucose imparts toxic effects when administered at the high concentrations (150 mM and 300 mM). The glucose concentrations of 150 mM was used in the subsequent experiments, owing to the lower survival rates, higher ROS generation and cell death levels observed using 300 mM of glucose.

3.2. Protective effect of phlorotannins isolated from *E. cava* against high glucose-stimulated oxidative stress in a zebrafish model

To confirm the reduction in oxidative stress by phlorotannins (6,6-BK, PK, DK and PFFK), we monitored survival rates as well as levels of ROS production and cell death. The survival rates of zebrafish treated with 150 mM glucose or co-treated with phlorotannins are presented in Fig. 3-A. The survival rate was 70% in the glucose-treated zebrafish. However, the survival rates increased to 95%, 83%, 95% and 90% (6,6-BK, PK, DK and PFFK), respectively in the groups treated with phlorotannins and 6,6-BK and DK significantly raised the survival rates. The level of ROS was 156% in the glucose-treated zebrafish compared to the control group (without glucose and samples). In contrast, the levels of ROS were 123%,

129%, 122% and 130% in the phlorotannin (6,6-BK, PK, DK and PFFK)-treated groups, respectively (Fig. 3-B). Phlorotannin treatment of the zebrafish significantly inhibited glucose-induced ROS production. And the glucose-induced cell death in zebrafish 109%, compared to the control group. However, the treatments of DK could significantly reduced cell death to 95% (Fig. 3-C). These results showed that DK contained the most effective compound for the reduction of ROS production and cell death among the phlorotannins. Therefore, DK possesses protective effects against high glucose oxidative stress.

3.3. Protective effect of DK against high glucose-induced oxidative stress in zebrafish model

On the basis of the previously presented results showing that DK had the highest protective effects against high glucose-induced oxidative stress among the phlorotannins. We compared its with that of resveratrol (Res) in terms of survival rate, heart beat rate, ROS generation, NO generation, lipid peroxidation, and cell death, as well as iNOS and COX-2 expressions. The survival rate was 76% in the glucose-treated zebrafish compared with the control group (Fig. 4-A). However, the survival rates in DK- and Res- treated groups at the concentrations of 10 μM and 20 μM significantly increased and the heart beat rate of the glucose-treated zebrafish increased to 113%, compared with that of the control group (Fig. 4-B). However, the treatments with DK and Res at a concentration of 20 μM significantly reduced and no difference between the both materials. These results showed that DK can protect the zebrafish from damage induced by high glucose. The level of ROS was 150% in the glucose-treated zebrafish, compared to the control group (Fig. 5-A). In contrast, the zebrafish exposed to DK and Res at the both tested concentrations (10 μM and 20 μM) with glucose showed a significant reduction in ROS generation, except for the Res treatment at a

10 μM concentration. The levels of glucose-induced NO generation are shown Fig. 5-B. The glucose-treated zebrafish showed a 117% expression of NO. Significant reductions in NO levels were observed with DK and Res treatment. Besides significant reductions in lipid peroxidation were also observed in the treatments of DK and Res at a concentration of 20 μM (Fig. 6-A), and the cell death in zebrafish was remarkably decreased when using 20 μM of DK group. These results prove that DK effectively reduced the elevated levels of ROS, NO, lipid peroxidation, and cell death that were earlier induced by high glucose. Lastly, the levels of iNOS and COX-2 expression were highly induced by the high glucose treatment, and DK and Res (20 μM) markedly reduced the expression of these proteins. The results of this experiment confirmed that DK and Res inhibited iNOS and COX-2 expression to protect against glucose-induced oxidative stress in zebrafish.

4. Discussion

The zebrafish has emerged as a popular model species in various fields of research. It has various advantages such as the rapid development of embryos and optical transparency. Further, zebrafish is similar to mammals in terms of its genetics, physiology, anatomical structure and immune functions. Accordingly, the zebrafish has been increasingly used for biomedical researches studies.[24,25]

Oxidative stress induced by hyperglycemia causes diabetes associated pathological damage. And hyperglycemia causes various complications, including cardiovascular and microvascular disease, periodontal disease, and increased susceptibility to other diseases.[8] Therefore, it is important to understand the mechanisms underlying high glucose-oxidative stress to prevent development of the complications of hyperglycemia. Previous *in vitro* experiments have shown that phlorotannins from the brown alga, *E. cava,* impart protective effects against high glucose-induced oxidative stress.[8,21] In this study, we confirmed that zebrafish could be used as an *in vivo* model for investigating the protective effects of *E. cava* phlorotannins against high glucose-stimulated oxidative stress.

Hyperglycemia initiates the production of free radicals, which can inhibit antioxidant system, increase lipid peroxidation, and instantly damage various biochemical, physiological lesions in diabetes. Such cellular damage often impairs metabolic functions, leading to cell death.[8,25] We determined the toxicity of glucose in zebrafish for in terms of survival rates, ROS production, and cell death (Fig 2). Glucose treatment reduced the survival rates in a dose-dependent manner, as well as increased the rates of ROS production and cell death. These results indicate that a high glucose induced oxidative stress in a zebrafish model.

Accordingly, we investigated the protective effects of phlorotannins (6,6-BK, PK, DK, and PFFK) isolated from *E. cava* on a high glucose-induced oxidative stress in zebrafish.

Exposure of zebrafish to the high level of glucose resulted in a significant increase in ROS production and cell death. However, DK showed the highest level of inhibition of ROS production and cell death among the phlorotannins, suggesting that DK can protect zebrafish from high glucose-induced toxicity (Fig.3).

Presently, we examined the protective effects of DK against high glucose-induced oxidative stress, compared to that of resveratrol which is well known for its anti-diabetes and anti-oxidants effects.[27,28] In the present study, zebrafish embryos exposed to high glucose levels showed a reduction in survival rates as well as an increase in heart rates. However, administration of DK increased the survival rates and decreased heart rates of glucose-treated embryos. The administration of high glucose levels resulted in ROS, NO production, and lipid peroxidation generation, as well as cell death. However, treatment of glucose-exposed embryos with DK inhibited these stress parameters (Figs. 5 and 6). These findings suggest that DK confers important protective effects against the oxidative stress induced by hyperglycemia.

Proinflammatory enzymes, including iNOS and COX-2, which influence many chronic diseases, are associated with oxidative stress. Especially, a large amount of NO was generated as a result of iNOS and COX-2 expression to confirm immune responses.[8,26,29] which was induced by high glucose treatment. However, DK treatment inhibited iNOS and COX-2 expression in zebrafish embryos (Fig. 7). These findings suggest that DK alleviates oxidative stress by down-regulating the expression of iNOS and COX-2.

5. Conclusion

This study has shown that DK imparts a protective effect against a high glucose-induced oxidative stressed in a zebrafish model. Thus, the zebrafish is effective model for investigation the mechanisms of high glucose-induced oxidative stress. Thus high glucose-induced zebrafish model can be used to identify of valuable functional antioxidant for foods and nutraceuticals.

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Figure legends

Fig. 1. Chemical structures of the phlorotannins isolated from *E. cava*.

Fig. 2. Measurement of toxicity level in glucose-stimulated survival rate (A), ROS generation (B), and cell death (C) in zebrafish. The zebrafish embryos were treated with various glucose concentrations at 24 hpf until 2 dpf. Control (a), 50 mM glucose(b), 100 mM glucose (c), 150 mM glucose (d) and 300 mM glucose (e). Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. *P<0.05, ** P<0.01.

Fig. 3. Inhibitory effects of phlorotannins on survival rate (A), ROS generation (B), and cell death (C) in zebrafish. The zebrafish embryos were exposed to phlorotannins at 7~9 hpf until 24 hpf. At 1 dpf, 150 mM glucose was administered to the zebrafish embryos until 2 dpf. Control (a), 150 mM glucose (b), 6,6-BK (c), PK (d), DK (e), and PFFK(f). Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. *P<0.05, ** P<0.01.

Fig. 4. Measurement of toxicity of DK and Res in glucose-stimulated survival rate and (A) heart rate (B) in zebrafish. The zebrafish embryos were exposed to DK and Res at 7~9 hpf until 24 hpf. At 1 dpf, 150 mM glucose was administered to the zebrafish embryos until 2 dpf. Control (a), 150 mM glucose (b), 10 μM DK (c), 20 μM DK (d), 10μM DK (e), and 10μM Res (f). Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and

glucose-treated zebrafish. *P<0.05, ** P<0.01.

Fig. 5. Inhibitory effect of DK and Res on glucose-stimulated ROS generation and (A) NO generation (B) in zebrafish. The zebrafish embryos were exposed to DK and Res at 7~9 hpf until 24 hpf. At 1 dpf, 150 mM glucose was administered to the zebrafish embryos until 2 dpf. Control (a), 150 mM glucose (b), 10 µM DK (c), 20 µM DK (d), 10µM DK (e), and 10µM Res (f). Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. *P<0.05, ** P<0.01.

Fig. 6. Inhibitory effect of DK and Res on glucose-stimulated lipid peroxidation generation and (A) cell death (B) in zebrafish. The zebrafish embryos were exposed to DK and Res at 7~9 hpf until 24 hpf. At 1 dpf, 150 mM glucose was administered to the zebrafish embryos until 2 dpf. Control (a), 150 mM glucose (b), 10 µM DK (c), 20 µM DK (d), 10µM DK (e), and 10µM Res (f). Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. *P<0.05, ** P<0.01.

Fig. 7. Effect of DK and Res on iNOS and COX-2 expression in zebrafish. Equal amounts of cell lysates (50 µg) were subjected to electrophoresis and analyzed for iNOS and COX-2 expressions by western blottng. GAPDH was used as an internal control. Experiments were performed in triplicate and the data are expressed as the mean ± SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. *P<0.05, ** P<0.01.

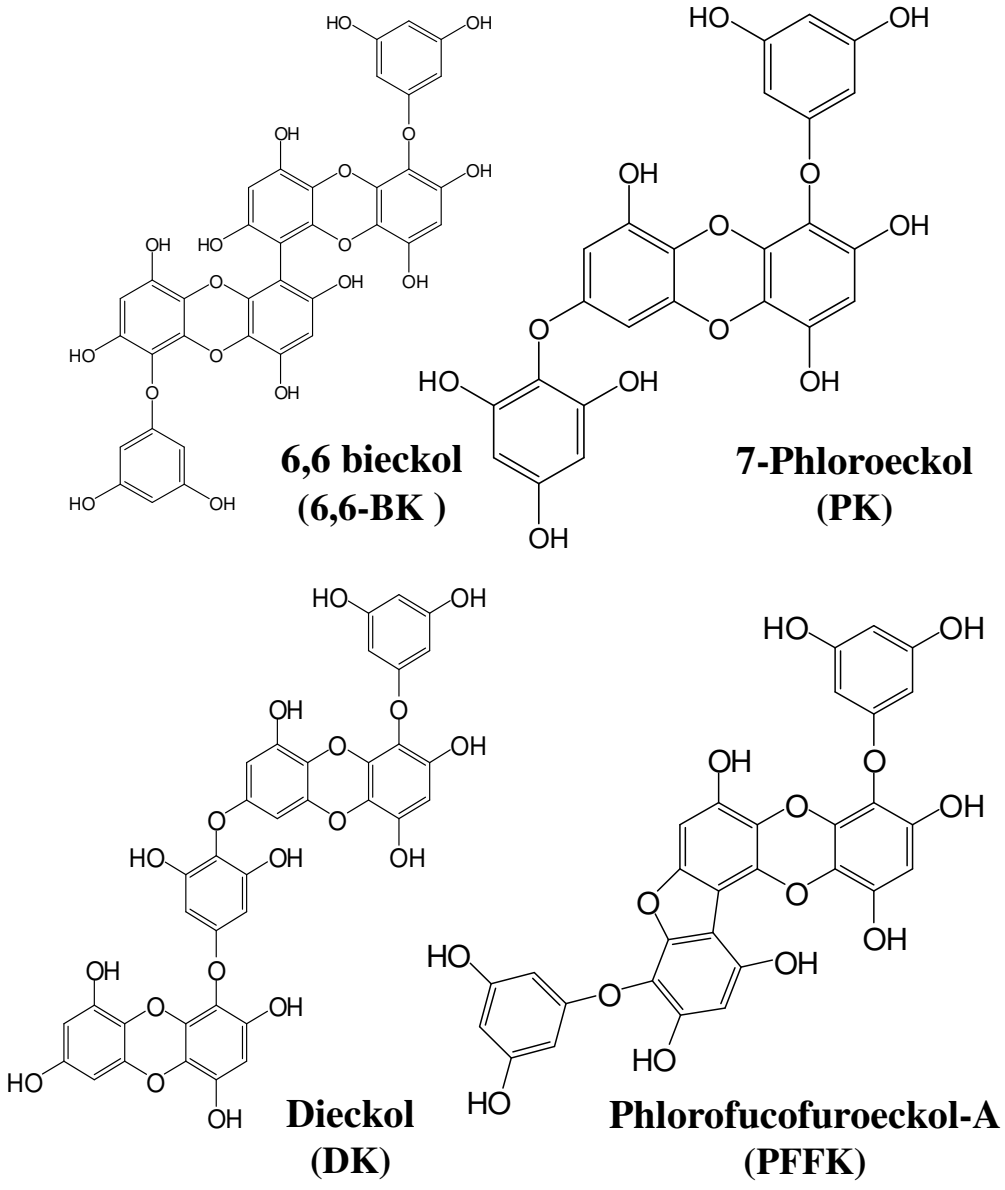


Figure 1

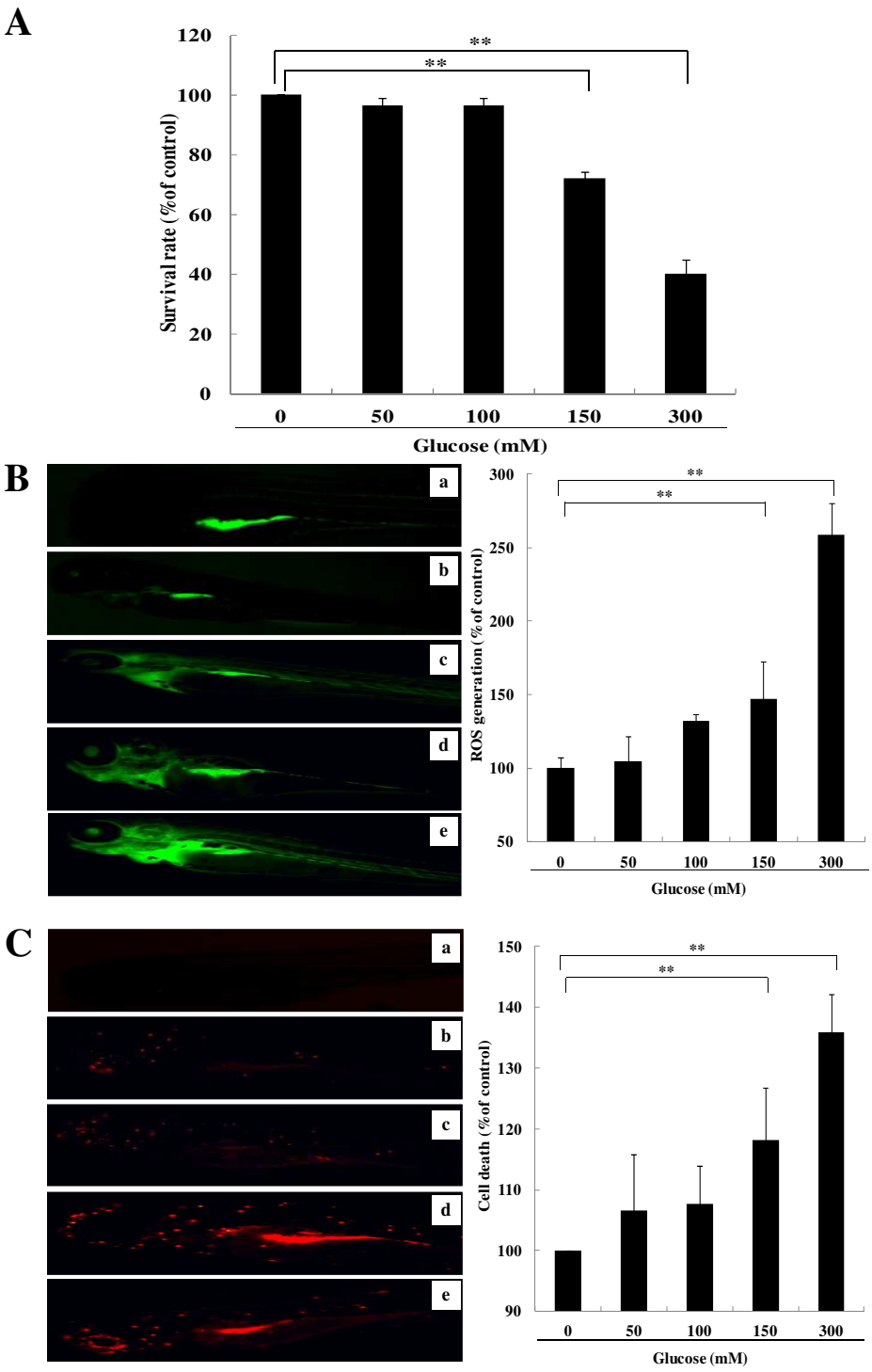
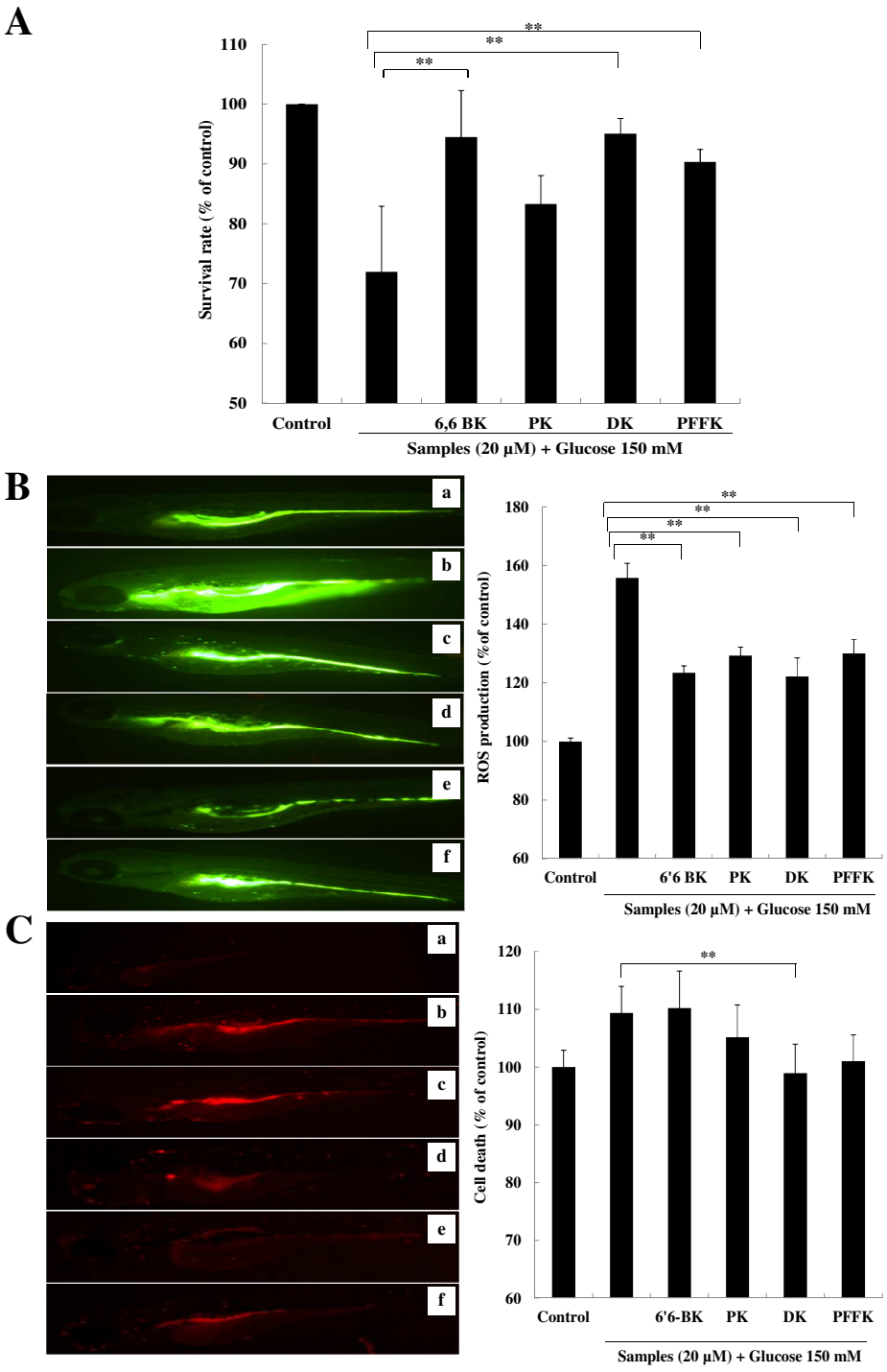


Figure 2



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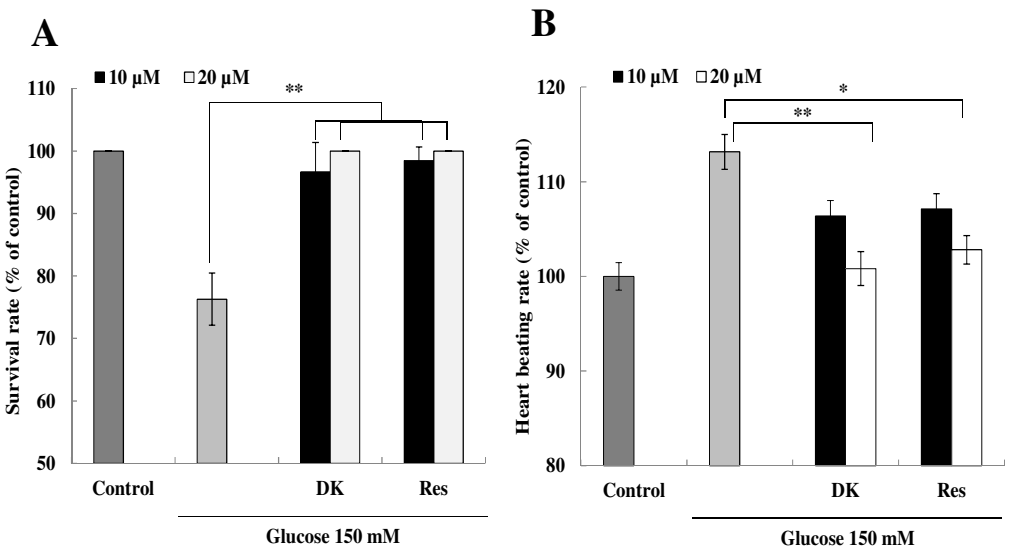

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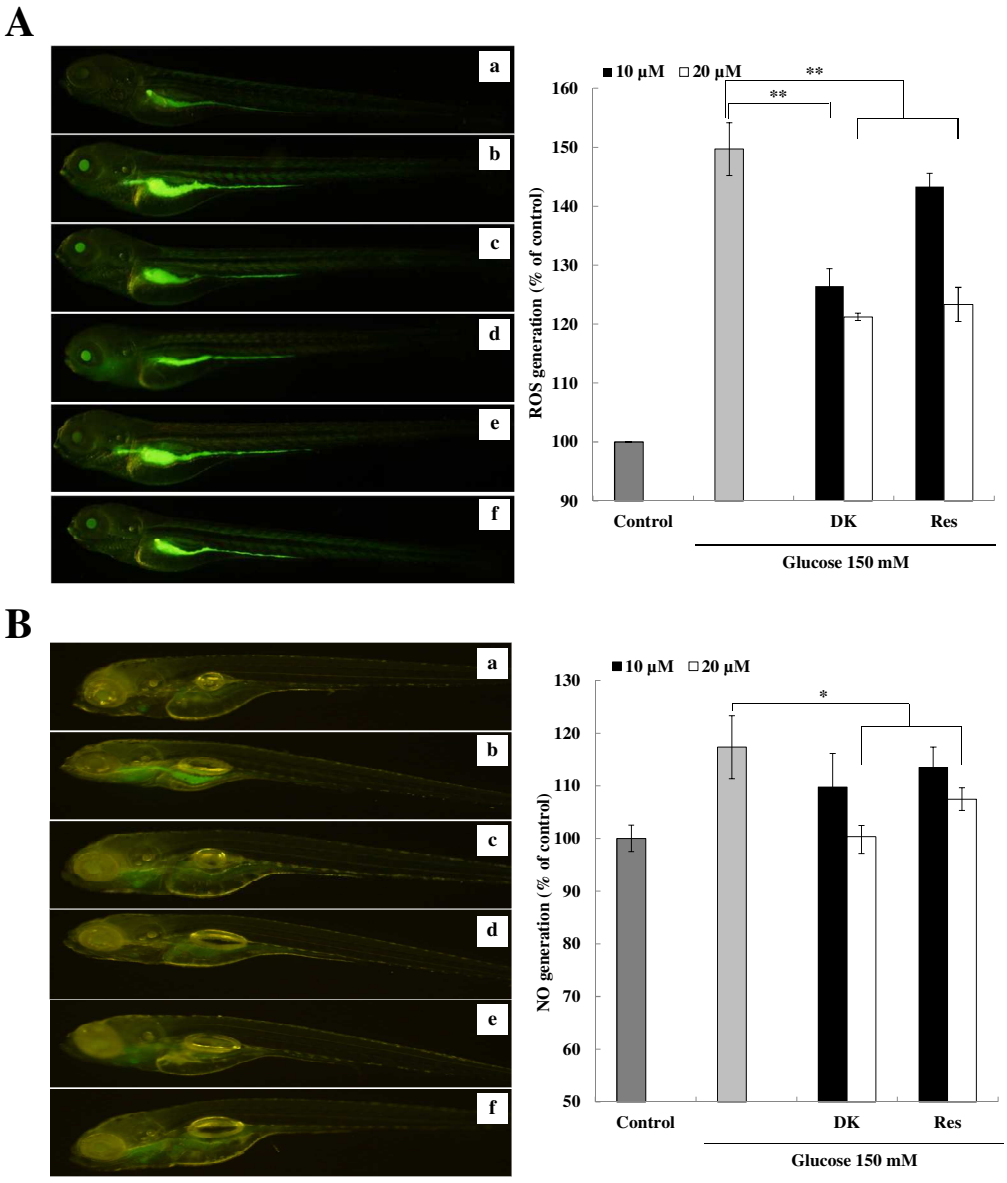

Figure 5

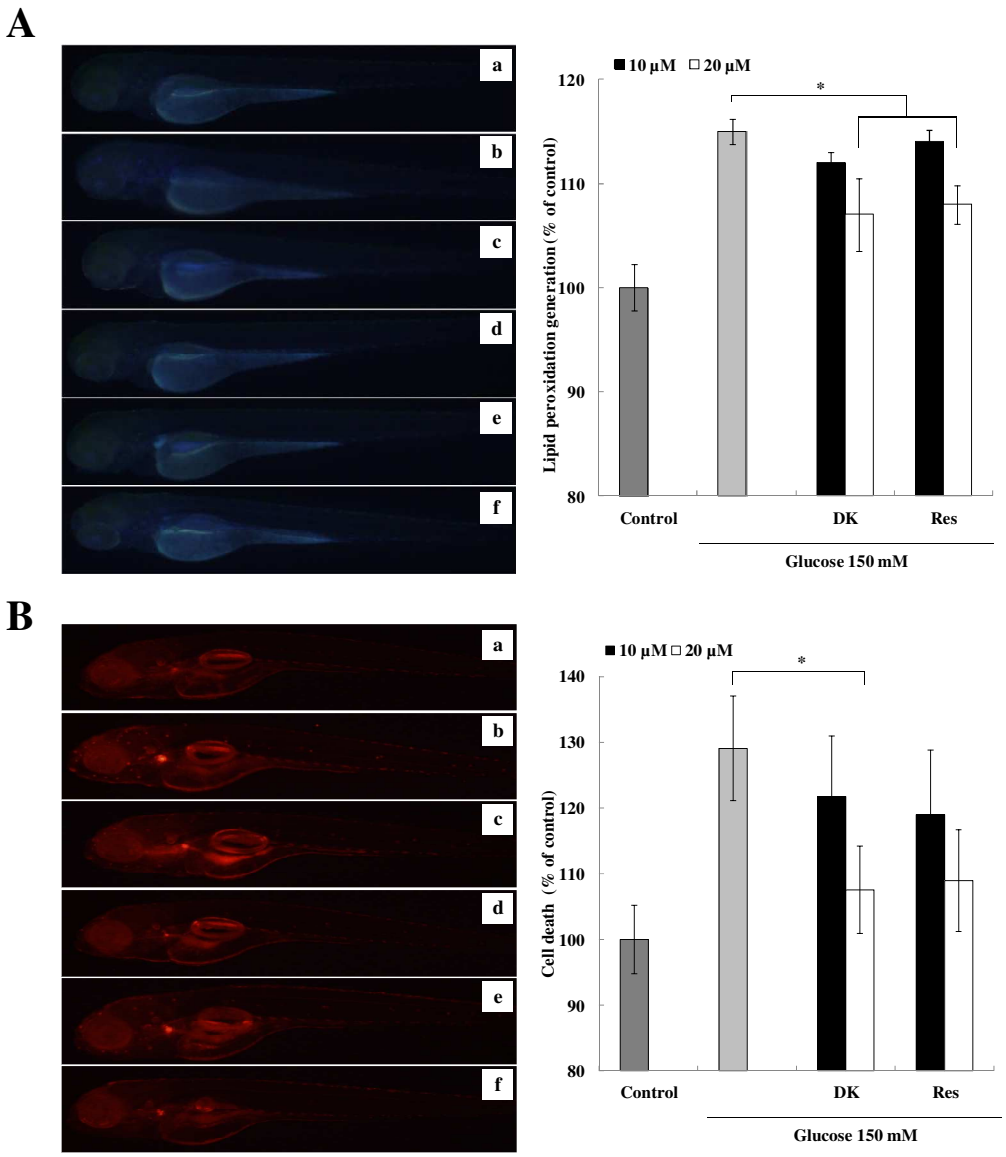

Figure 6

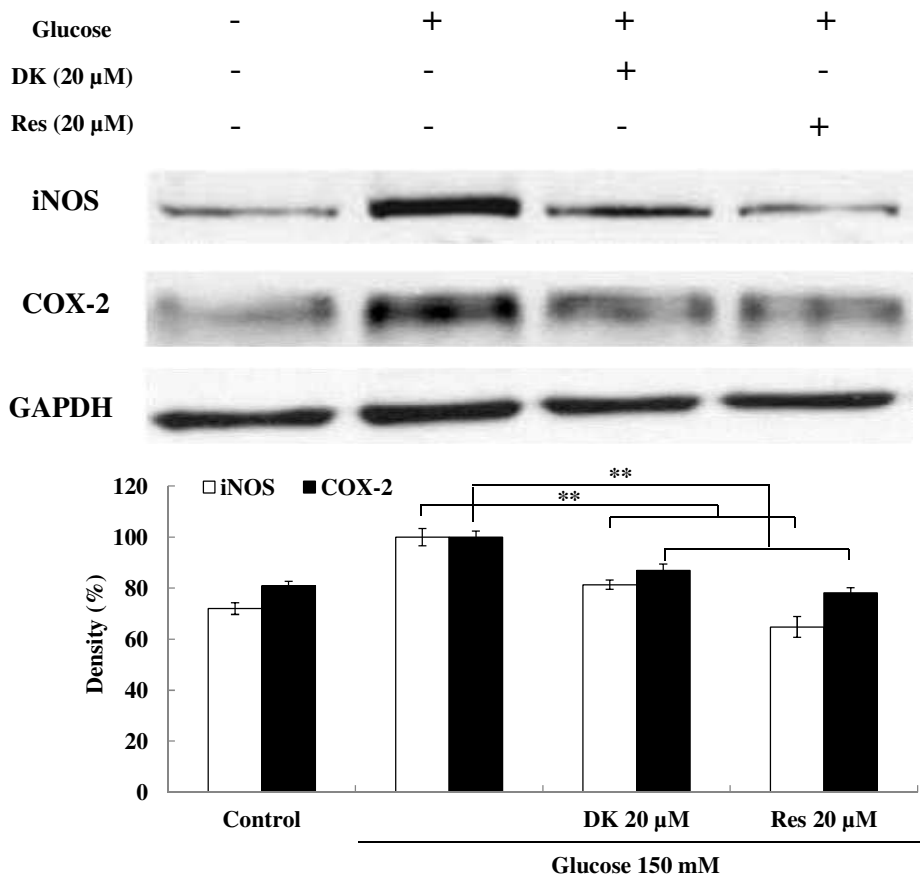

Figure 7