# Role of Endogenous Antioxidant System Regulation in the Acetaldehyde Resistance Enhanced by Quercetin and Its Intestinal Catabolites

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# PREFACE

The studies presented in this dissertation were conducted from October 2019 to September 2022 at Graduate School of Environment and Life Science (Doctor Course), Okayama University, Japan, under the supervision of Prof. Yoshimasa Nakamura. These studies are original work by the author, and any other assistance and collaboration from others are specially acknowledged.

This dissertation has never before been submitted in whole or in part to the council, a university, or any other professional organization for a degree, diploma, or other professional qualification.

**KEXIN LI**

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# ABBREVIATIONS

**AA,** acetaldehyde;

**AhR,** aryl hydrocarbon receptor;

**ALDH,** aldehyde dehydrogenase;

**ALDH1A1,** ALDH class-1A1;

**ALDH2,** ALDH class-2;

**ALDH3A1,** ALDH class-3A1;

**BSO,** buthionine sulfoximine;

**DCFH-DA,** dichlorofluorescin diacetate;

**DOPAC,** 3,4-dihydroxyphenylacetic acid;

**GCLC,** glutamate-cysteine ligase, catalytic subunit;

**GSH,** glutathione;

**HO-1,** heme oxygenase-1;

**Nrf2,** nuclear factor erythroid 2-related factor 2;

**OPAC,** 3-hydroxyphenylacetic acid;

**Que,** quercetin;

**Q4′G,** quercetin 4′-O-β-glucoside;

**ROS,** reactive oxygen species;

**SNPP,** tin protoporphyrin IX;

**xCT,** cystine/glutamate exchanger.

# ABSTRACT

Flavonoids are primary dietary polyphenols derived from various plant foods and beverages. Quercetin, the most ubiquitously distributed flavonoid, usually exists in its glycoside forms, such as quercetin 4′-O-β-glucoside (Q4′G), one of the major quercetin glycosides in onion. The digested quercetin glycosides are rarely detected in the plasma because most of them are metabolized before absorption or unabsorbed in the small intestine without metabolism. A previous study suggested that 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-hydroxyphenylacetic acid (OPAC) are the predominant catabolites of Q4′G, produced by its deglycosylation and ring fission reaction of the aglycone quercetin in intestinal microbiota. Even though current research on flavonoids has demonstrated their promising beneficial effects on health promotion, the role of the intestinal catabolites in the biological activities induced by the parent compounds remains to be clarified. Acetaldehyde, the product of ethanol oxidation, is highly toxic and is rapidly metabolized into acetic acid, mainly by the liver mitochondrial aldehyde dehydrogenase (ALDH), ALDH2. In contrast, the imbalance between the production and disposition of acetaldehyde causes severe toxicity. This study aimed to clarify the molecular mechanisms underlying the cytoprotective effects of quercetin and its microbiota catabolites on the acetaldehyde-induced cytotoxicity in the cellular hepatocyte model.

In chapter 2, I evaluated the protective effect of quercetin on the acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. Quercetin significantly inhibited the acetaldehyde-induced cytotoxicity and coincided with the enhancement of the total ALDH activity and the gene expression of ALDH1A1 and ALDH3A1. In addition to the electrophilic nature of acetaldehyde contributing to DNA and protein damage, reactive oxygen species (ROS) production via mitochondrial damage is also involved in its toxic mechanisms. Indeed, treating Hepa1c1c7 cells with acetaldehyde resulted in an enhancement of the intracellular ROS level. On the other hand, the pretreatment of quercetin significantly reduced the acetaldehyde-enhanced ROS level in a concentration-dependent manner. Simultaneously, the gene expressions of the representative phase 2 drug-metabolizing enzymes, such as heme oxygenase-1 (HO-1), glutamate-cysteine ligase, catalytic subunit (GCLC), and cystine/glutamate exchanger (xCT), were up-regulated by quercetin. The total intracellular glutathione (GSH) level was also significantly

increased by quercetin, suggesting that the up-regulation of the GSH level might be regulated by not only the enhanced biosynthesis but also the uptake of the substrate, cystine. An HO-1 inhibitor, tin protoporphyrin IX (SNPP), abolished the reduction of ROS production by quercetin, whereas a GSH biosynthesis inhibitor, buthionine sulphoximine (BSO), did not. These results suggested that the HO-1-dependent production of small molecular weight antioxidants, such as biliverdin/bilirubin and carbon monoxide, might play a pivotal role in the cytoprotective mechanism of quercetin.

In chapter 3, I showed the cytoprotective effects of the major microbiota catabolites of quercetin, DOPAC and OPAC, on the acetaldehyde-induced cytotoxicity. DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity, but OPAC did not. The DOPAC treatments significantly increased the gene expression of ALDH1A1 and ALDH3A1 in a concentration-dependent manner, whereas OPAC up-regulated only the ALDH3A1 gene expression. Both DOPAC and OPAC potentiated the total ALDH enzyme activity, even though neither DOPAC nor OPAC modulated the gene expression of ALDH2. These results implied that DOPAC and OPAC modulate the total ALDH activity through a transcription regulation of the isozymes other than ALDH2. Similar to quercetin, DOPAC significantly enhanced the mRNA levels of HO-1, GCLC, and xCT, while OPAC only up-regulated GCLC. The concentration dependency of DOPAC or OPAC for the enhanced intracellular GSH level was correlated with that for the gene expression of xCT, but not GCLC, suggesting that the up-regulation of GSH level might be due to the substrate uptake, but not by the enhanced biosynthesis.

In chapter 4, I demonstrated that the combination of DOPAC and OPAC protected mouse hepatoma Hepa1c1c7 cells from the acetaldehyde- and hydrogen peroxide-induced cytotoxicity at the concentration of which the single pretreatment of each compound showed no significant effect. This combination also inhibited the intracellular ROS level, suggesting the involvement of the antioxidative mechanism in its cytoprotection. The combinatory treatment enhanced the gene expression of not only ALDH1A1 and ALDH3A1, but also GCLC, the first rate-limiting enzyme of GSH synthesis. Accordingly, intracellular GSH level, as well as the total ALDH activity, was enhanced by DOPAC plus OPAC. Involvement of GSH in the cytoprotection as well as ALDH up-regulation by the combination, was confirmed by the experiments using BSO. Taken together, the present results suggested that the

quercetin intestinal catabolites concertedly protect the cells from acetaldehyde through the enhanced resistance against oxidative stress by the GSH-dependent up-regulation of the ALDH activity.

I have provided biological evidence through this study: (1) the intestinal catabolites, DOPAC and OPAC, as well as quercetin have a potential to protect the cells from the acetaldehyde-induced cytotoxicity, possibly through enhancement of the total ALDH activity by transcriptional regulation; (2) the acetaldehyde-enhanced ROS level could be reduced by quercetin in an HO-1-dependent mechanism; (3) the combination of DOPAC and OPAC concertedly protect the cells from acetaldehyde through the enhanced resistance against oxidative stress by the GSH-dependent up-regulation of ALDHs. In conclusion, this series of studies reveals the significant role of endogenous antioxidant system regulation in the acetaldehyde resistance enhanced by quercetin and its intestinal catabolites.

# Chapter 1

# General Introduction

## 1.1 Polyphenols

Plant-derived functional foods are gaining considerable attention due to their safety and therapeutic potential. In general, plant-based foods like kale, onions, and broccoli contain polyphenols (Dragovic-Uzelac et al., 2007). Polyphenols can be categorized into numerous groups, but the primary groups in polyphenols are phenolic acids, flavonoids, phenolic alcohols, and lignans. Bioactive compounds aid in the protection of chronic diseases, such as cardiovascular disease, osteoporosis, neurogenerative disease, cancer, and diabetes mellitus (D Archivio et al., 2007; Scalbert et al., 2005).

### 1.1.1 Flavonoids

Flavonoids are a significant group of natural ingredients; particularly, they belong to a group of plant secondary metabolites with a polyphenolic structure that is prevalent in fruits, vegetables, and certain beverages. They have a range of beneficial biochemical and antioxidant properties associated with various diseases including cancer, Alzheimer's disease (AD), atherosclerosis, etc (Banjarnahor & Artanti, 2014; Castañeda-Ovando et al., 2009; Lee et al., 2009). Flavonoids are essential in many nutraceuticals, pharmacological, medical, and cosmetic uses because they provide a wide range of health-promoting benefits (Panche et al., 2016). The subclasses of flavonoids include flavones, flavonols, flavanones, flavanonols, flavanols, catechins, anthocyanins, and chalcones. Almost every group of flavonoids has the capacity to serve as antioxidants. It has been reported that flavonoids have an ability to defend the body against reactive oxygen species. A number of studies have been indicated the properties of flavonoids as antioxidants and these studies emphasized that the flavonoids can be used as potential therapeutics to prevent the oxidative stress-related diseases (Hertog et al., 1997; Ishikawa et al., 1997; KITAGAWA et al., 1992; Lale et al., 1996).

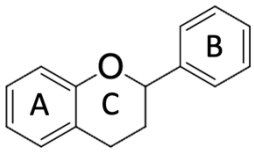
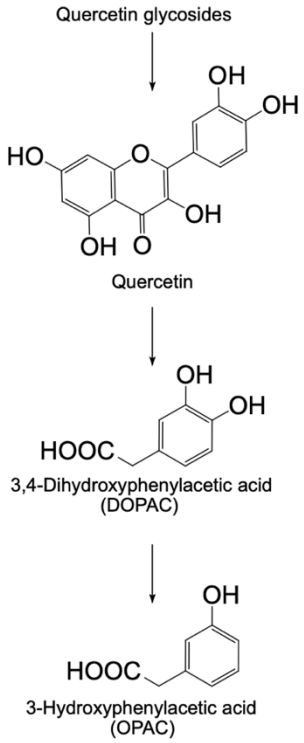



**Fig. 1.1** Flavan, a flavonoids' basic skeleton.

### 1.1.2 Quercetin

Quercetin, one of the most widely dispersed flavonoids, has received a lot of attention due to its favorable impact on human health (Boots et al., 2008). Generally, quercetin exists in foodstuff as glycoside forms with one or more sugar moieties. Q4′G is one of the major quercetin glycosides in onion, consumption of which accounts for 29% of the total flavonoid intake in human diets (Hervert-Hernández & Goñi, 2011). It is much superior to quercetin 3-glucoside, the most abundant quercetin glycoside in nature, in the inhibition of lipid peroxidation in the rat intestinal mucosa (Murota et al., 2004). A previous study revealed that Q4′G passes through the gastrointestinal tract of rats and that almost all of Q4′G is metabolized by




the gut microbiota (Mullen et al., 2008).

**Fig. 1.2** The root of the sequential formation of the colonic microbiota catabolites from quercetin glycosides in the large intestine.

There are abundant amounts of food compound-degrading enzymes in the gut microbiota. They can catalyze a number of processes, such as oxidation, reduction, decarboxylation, demethylation, and ring cleavage, which enable the formation of numerous dietary flavonoids catabolites, in addition to a number of hydroxylases, including glucosidases. The microbial enzymes first eliminate glycosides, glucuronides, and sulfates from the unabsorbed quercetin glycosides as well as metabolized conjugates and thus produce the aglycon. The gut microbiota utilizes these dissociated sugars as carbon sources (Hervert-Hernández & Goñi, 2011). The quercetin skeleton's C-ring can also be broken down by the microflora into a range of phenolic acid catabolites (Halliwell et al., 2005). The quercetin aglycone undergoes a ring fission reaction and is converted to 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAC is converted to 3-hydroxyphenylacetic acid (OPAC) by dehydroxylation.

**1.2 Ethanol Metabolism**

Alcohol consumption has a number of immediate and chronic impacts, some of which will affect the future course of drinking. Any of these effects of alcohol may result from ethanol or its metabolic products like acetaldehyde. The increment of acetaldehyde levels in humans have a number of typical effects, including euphoria, nausea, headaches, subjective feelings of heat and facial flushing, increased heart and respiration rates, lowered blood pressure, and the sensation of dry mouth or throat brought on by bronchoconstriction and allergic reactions. The term "alcohol sensitivity" is frequently used to characterize these symptoms (except euphoria) (Eriksson, 2001). Acetaldehyde, a very poisonous byproduct of ethanol oxidation, is quickly converted to acetate, mostly by a mitochondrial low $K_m$ aldehyde dehydrogenase (ALDH), which activity is markedly decreased by prolonged ethanol consumption (Hasumura et al., 1975). The imbalance between the production and disposition of acetaldehyde causes toxicity, such as forming protein adducts, resulting in antibody production, enzyme inactivation, and decreased DNA repair. It is also linked to a significant impairment in the liver's ability to utilize oxygen. Induction of mitochondria dysfunction and excessive generation of reactive oxygen species (ROS) have been linked to the toxic action of acetaldehyde (Farfán Labonne et al., 2009; Gomez-Quiroz et al., 2003).

### 1.3 Oxidative stress

The definition of oxidative stress is an imbalance between the production of reactive metabolites and an organism's ability to remove them via defense mechanisms known as the antioxidative system (Persson et al., 2014). It has been demonstrated that oxidative stress plays a role in the pathogenetic mechanisms of a number of diseases, including atherosclerosis, cancer, diabetes mellitus, inflammatory diseases, as well as psychiatric illnesses or the aging process (López-Alarcón & Denicola, 2013; Sies, 2020; Toda, 2011). Reactive oxygen species (ROS) are generated continuously during intracellular metabolism and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA (Freeman & Crapo, 1982). Additionally, ROS are regarded as host defense molecules that are released by neutrophils to destroy exogenous pathogens such as bacteria and to act as secondary messengers in signal transduction. The concept of biological antioxidants refers to any compounds that, when present at a lower concentration compared to that of an oxidizable substrate, are able to either delay or prevent the oxidation of the substrate (Gandhi & Abramov, 2012; Godic et al., 2014).

### 1.4 Antioxidant defense system

Antioxidant functions imply reducing oxidative stress, DNA mutations, and malignant transformations, as well as other cell damage characteristic. Both enzymatic and nonenzymatic antioxidant defense mechanisms can be found in the aqueous and membrane cell compartments. Another one is represented by repair processes, that remove the damaged biomolecules before their aggregation enables alteration of cell metabolism (Cheeseman & Slater, 1993).

#### 1.4.1 Glutathione biosynthesis

Glutathione (γ-glutamyl-L-cysteinylglycine) is the most prevalent non-protein thiol compound widely distributed in living organisms and, predominantly, in eukaryotic cells (Meister, 1983). While over 90% of the glutathione is normally present in the reduced form (GSH), several additional forms of glutathione are present in (microbial) cells, tissues, and plasmas. Glutathione disulfide GSSG (oxidized glutathione), formed upon oxidation of GSH, can be in turn be reduced to GSH by glutathione reductase at the expense of NADPH (Carmel-Harel & Storz, 2000).

GSH is synthesized in two steps, firstly, the enzyme $\gamma$-glutamylcysteine synthesis catalyzes dipeptide formation and then the product is converted to GSH by glutathione synthetase. In addition, glutamylcysteine synthetase can be blocked by buthionine sulfoximine (BSO), widely used in experiments to deplete cellular GSH levels.

### 1.4.2 HO-1

Heme oxygenase (HO), a member of the heat-shock protein family, plays a protective role in inflammation and oxidative stress. The overall objective of heme oxygenase is to eliminate a pro-oxidant (heme) whilst generating a putative antioxidant (bilirubin). Among three isoforms, HO-1 is an inducible isoform present in the brain, which is up-regulated by various stress stimuli including oxidative stress. The antioxidant response elements (ARE), found in the promoter regions of the genes of HO-1 and other antioxidant enzymes, serve as binding sites for the transcription factor Nrf2, whose activity is controlled by the redox state. HO-1 is regulated during oxidative stress and has been proposed to have a role in the regulation of inflammatory processes. The HO-1 absence in endothelial cells displays increased injury in the presence of oxidative challenge, demonstrating that the HO-1 pathway is a key cytoprotective mechanism against oxidative stress which contributes to cellular homeostasis (Hayashi et al., 2012; Wagener et al., 2003; Yao et al., 2007). HO can be inhibited and obviated protection by tin protoporphyrin (SNPP).

## 1.5 Study outline

In the current research, I have evaluated the intestinal catabolites, DOPAC and OPAC, as well as quercetin, which have potential cytoprotection against acetaldehyde-induced toxicity in mouse Hepatoma Hepa1c1c7 cells. Furthermore, the combination of DOPAC and OPAC possessed more significant protection than each compound alone at the same concentration. I also investigated the molecular mechanisms underlying the cytoprotective effects of quercetin and its catabolites on the acetaldehyde-induced cytotoxicity in the cells.

Quercetin enhanced the resistance against the acetaldehyde-induced cytotoxicity in Hepa1c1c7 cells, possibly via modulating the total ALDH activity and related gene expression. In addition, acetaldehyde-induced ROS generation can be decreased by quercetin in a concentration-dependent manner. The total intracellular GSH level

and the gene expressions of the representative phase 2 drug-metabolizing enzymes were up-regulated by quercetin. An HO-1 inhibitor, SNPP, abolished the reduction of ROS production by quercetin, whereas a GSH biosynthesis inhibitor, BSO, did not.

As the major microbiota catabolites of quercetin, DOPAC and OPAC, both of them inhibited the acetaldehyde-induced cytotoxicity in Hepa1c1c7 cells. DOPAC also showed a cytoprotective effect against hydrogen peroxide-induced cytotoxicity in the cells, but OPAC did not. The combination of DOPAC and OPAC possessed protected the cells from acetaldehyde- and hydrogen peroxide-induced cytotoxicity at the concentration of which the single pretreatment of each compound showed no significant effect. Furthermore, the results suggested that the quercetin intestinal catabolites concertedly protect the cells from acetaldehyde through the enhanced resistance against oxidative stress by the GSH-dependent up-regulation of the ALDH activity.

In conclusion, this series of studies reveals the significant role of endogenous antioxidant system regulation in the acetaldehyde resistance enhanced by quercetin and its intestinal catabolites.

# Chapter 2

# Quercetin attenuates acetaldehyde-induced cytotoxicity underlying the antioxidant mechanism in Hepa1c1c7 cells

## 2.1 Introduction

Quercetin, one of the most ubiquitously distributed flavonoids, has attracted much attention because of its beneficial effects on human health. It possessed free radical scavenging and metal-chelating properties as well as inhibition of some enzymes and protecting against DNA damage (Naderi et al., 2001). On the other hand, quercetin can also exhibit prooxidant effects (Wilms et al., 2008). Quercetin may be considered as an effective attenuating factor for preventing various disorders caused by environmental contaminants and free radical-mediated cytotoxicity and lipid peroxidation (Robaszkiewicz et al., 2007; Zhang, 2005). In general, quercetin exists in foodstuff as glycoside forms with one or more sugar moieties. Q4′G is one of the major quercetin glycosides in onion, consumption of which accounts for 29% of the total flavonoid intake in human diets. It is much superior to quercetin 3-glucoside, the most abundant quercetin glycoside in nature, regarding the inhibition of lipid peroxidation in the rat intestinal mucosa.

Excessive consumption of alcoholic beverages is a leading cause of liver disease, including cirrhosis, liver cancer, and acute and chronic liver failure, worldwide (Stickel et al., 2017). Ingested alcohol (ethanol) is absorbed mainly in the small intestine and, passing through the liver, is distributed throughout the entire organism. Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH), and acetaldehyde is further oxidized to acetic acid by ALDH (Lieber, 1991). The ALDH superfamily comprises 19 enzymes that catalyze the oxidation and detoxification of a wide spectrum of short and long aliphatic and aromatic aldehydes (Vasiliou & Pappa, 2000). The potential role of acetaldehyde in the pathogenesis of alcoholic liver diseases is recognized, in addition to adducts formation with proteins and DNA, and acetaldehyde can damage mitochondrial by a process that involves oxidative stress. Since mitochondrial damage induces both ROS formation and a decrease in the antioxidant defense system, it is possible that ROS has been associated with acetaldehyde-induced toxicity.

In the current study, I evaluated the protective effect of quercetin on the

acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. Quercetin significantly inhibited the acetaldehyde-induced cytotoxicity, possibly through enhancement of the gene expression of phase 2 drug-metabolizing enzymes, as well as the total ALDH activity through the transcriptional regulation. Furthermore, the acetaldehyde-enhanced ROS level could be reduced by quercetin in an HO-1-dependent and GSH-independent mechanism.

## 2.2 Materials and methods

### 2.2.1 Materials

Quercetin dihydrate and L-buthionine-sulfoximine (BSO) were obtained from Sigma Aldrich (St. Louis, MO, USA). α-Minimum essential medium (α-MEM) and Trizol reagent were purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Nichirei Corporation (Tokyo, Japan). β-Nicotinamide-adenine dinucleotide, oxidized form ($NAD^+$) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). ReverTra Ace was purchased from TOYOBO Co., Ltd. (Osaka, Japan). Taq polymerase was purchased from Takara Bio, Inc. (Kusatsu, Japan). The Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). PierceTM BCA Protein Assay Kit was purchased from Scientific (Meridian Rd., Rockford. The USA). Tin protoporphyrin (SNPP) was purchased from Funakoshi Co., Ltd (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemicals Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

### 2.2.2 Cell cultures

The mouse hepatoma cell line Hepa1c1c7, obtained from the American Type Culture Collection, were grown and maintained at 37 °C in α-MEM containing 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 95% air and 5% CO2. The cells were seeded in a complete medium for experiments and treated with each reagent or DMSO vehicle (final 0.1%, v/v).

### 2.2.3 Cell viability determination

Hepa1c1c7 cells were suspended at a density of $1\times10^4$ cells per well in a 96-well plate. After overnight pre-culture, the cells were incubated with quercetin for 24 h, followed by the additional treatment with acetaldehyde (10 mM) for 3 h. After stimulation with acetaldehyde, the MTT solution was added to each well, followed by the 2 h incubation. After aspiration of the medium, 150 μL DMSO was added to solute the formazan crystals. The absorbance was measured with a microplate reader (Benchmarkplus, Bio-Rad Laboratories, Hercules, CA, USA) at 530 nm. The obtained values were compared with each control incubated with a vehicle only.

### 2.2.4 Intracellular reactive oxygen species measurement and image analysis

The dichlorofluorescin diacetate (DCFH-DA) assay was used to detect the intracellular ROS level according to the method of Rosenkranz. For the inhibitory experiment of ROS production, SNPP or BSO was added 1 h before the catabolite treatment. The fluorescence levels in Hepa1c1c7 cells were analyzed by a Tali™ image-based cytometer (Life Technologies).

### 2.2.5 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Confluent Hepa1c1c7 cells were washed with ice-cold phosphate-buffered saline (PBS) (-) after treatment with quercetin at the indicated concentrations for 6 h. According to the manufacturer's recommendations, total cellular RNA was isolated using Trizol reagent and determined by measuring absorbance at 260 nm. Total RNA (5 μg) was reverse transcribed with Oligo dT to cDNA using Revertra Ace. PCR amplification was then performed with BIOTAQ DNA polymerase and gene-specific primers.

Primers used in PCR amplification are as follows: mβ-actin, (F) 5’-GTCACCCACACTGTGCCCATCTA-3’ and (R) 5’-GCAATGCCAGGGTACATGGTGGT-3’ (16 cycles, product size 455 bp); mHO-1, (F) 5’-ACATCGACAGCCCCACCAAGTTCAA-3’ and (R) 5’-CTGACGAAGTGACGCCATCTGTGAG-3’ (22 cycles, product size 66 bp); mGCLC, (F) 5’-GGCGATGTTCTTGAGACTCTGC-3’ and (R) 5’-TTCCTTCGATCATGTAACTCCCATA-3’ (26 cycles, product size 99 bp); mxCT, (F) 5’-CCTGGCATTTGGACGCTACAT-3’ and (R) 5’-TGAGAATTGCTGTGAGCTTGCA-3’ (25 cycles, product size 182 bp); mALDH1A1, (F) 5’-GACAGGCTTTCCAGATTGGCTC-3’ and (R) 5’-AAGACTTTCCCACCATTGAGTGC-3’ (26 cycles, product size 142 bp); mALDH2, (F) 5’-TGAAGACGGTTACTGTCAAAGTGC-3’ and (R) 5’-AGTGTGTGTGGCGGTTTTTCTC-3’ (26 cycles, product size 115 bp); mALDH3, (F) 5’-GATGCCCATTGTGTGTGTTCG-3’ and (R) 5’-CCACCGCTTGATGTCTCTGC-3’ (26 cycles, product size 138 bp).

### 2.2.6 Glutathione titration

Glutathione (GSH) contents were determined using 5,5’-dithiobis

(2-nitrobenzoic acid) (DTNB) and glutathione reductase according to the method of Baker.

### 2.2.7 ALDH activity assay

Hepa1c1c7 cells were treated with quercetin for 24 h. The ALDH activity was measured as previously described. Briefly, the cell pellets were prepared with 1 mL lysis buffer (25 mM EDTA, 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 0.1% sarcosyl). Then, 20 μL of 5 mM NAD+ and 20 μL of 5 mM propionaldehyde (substrate) were mixed with 200 μL of the total lysate. NADH was measured by the change in absorbance at 340 nm for 30 min. One unit was defined as the amount of enzyme activity that converts 1 μmol NAD+ to NADH per minute.

### 2.2.8 Statistical analysis

All the values are expressed as the mean of at least three independent experiments ± SD. Statistical significance was determined by the Student's paired two-tailed t-test or one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test using SPSS 26.0 software (IBM, Chicago, USA). A level of $p\text{-value} < 0.05$ was considered significant for all the comparisons.

## 2.3 Results

### 2.3.1 Quercetin protects the mouse hepatoma Hepa1c1c7 cells against the acetaldehyde-induced cytotoxicity

I initially examined whether quercetin has a protective effect against acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. When Hepa1c1c7 cells were pretreated with quercetin for 24 h, then treated with acetaldehyde (10 mM) for 3 h. Quercetin significantly inhibited the acetaldehyde-induced cytotoxicity in a concentration-manner (Fig. 2.1).

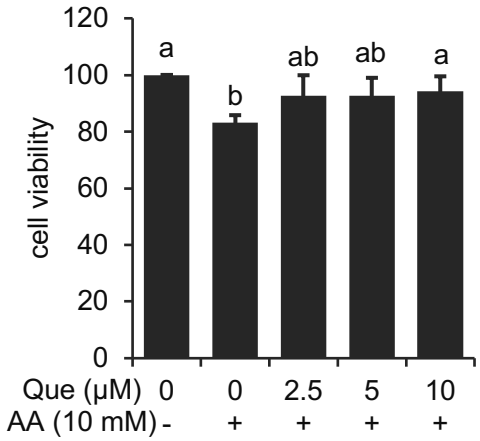



**Fig. 2.1** The cytoprotective effect of quercetin on cell viability. Hepa1c1c7 cells were treated or pre-treated with the indicated concentration of quercetin for 24 h, then were treated with acetaldehyde for 3 h. Cell viability was measured using an MTT assay. All values are expressed as means ± SD of three independent experiments and analyzed by a one-way ANOVA, followed by Tukey's HSD using SPSS software. The different letters above the bars indicate significant differences among the treatment for each condition ($\rho < 0.05$).

### 2.3.2 Quercetin enhances mRNA levels and activity of aldehyde dehydrogenase

Next, to investigate the possibility that quercetin affects the expression of the genes related to resistance against acetaldehyde, I checked the mRNA levels of the conventional ALDHs, such as ALDH1A1, ALDH2, and ALDH3A1, all of which are highly expressed in the liver and plays an important role in the acetaldehyde metabolism (Moreb et al., 2000). Quercetin enhanced the gene expression of ALDH1A1 and ALDH3A1, but did not that of ALDH2 (Fig. 2.2 (A)). According to

this result, the total ALDH activity was significantly increased by quercetin in a concentration-dependent manner (Fig. 2.2 (B)).

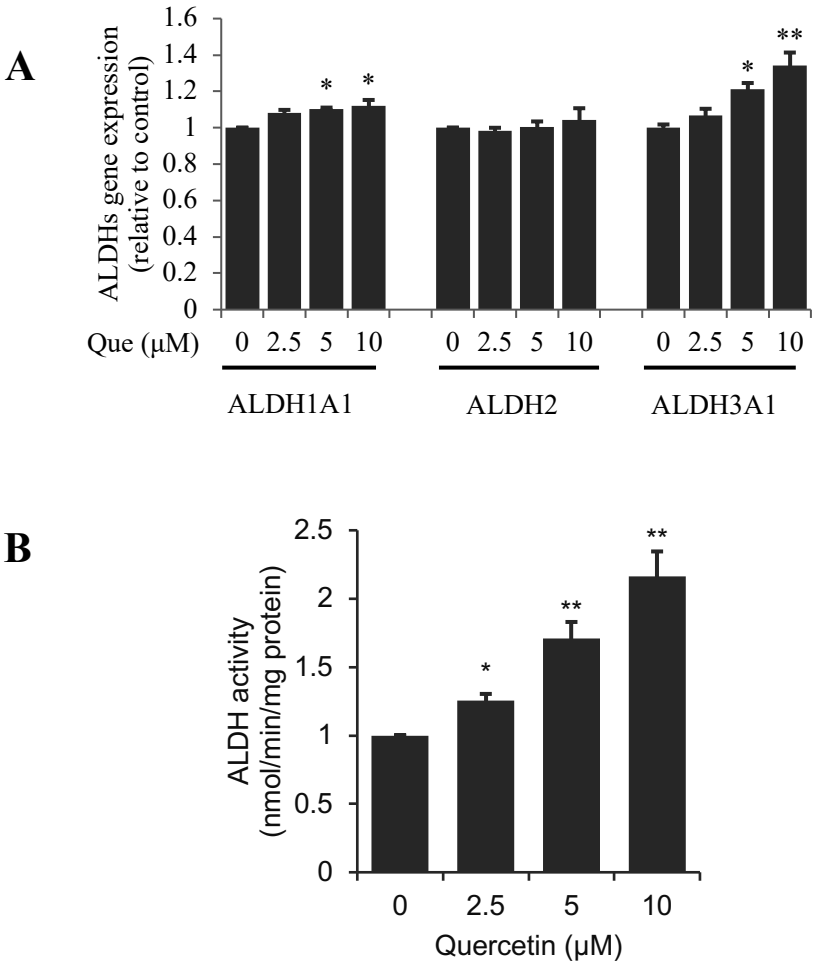



**Fig. 2.2** Modulating effect of quercetin on ALDHs in Hepa1c1c7 cells. (A) Modulating effects of quercetin on the gene expressions of the conventional family of ALDHs. The total RNA was extracted from the Hepa1c1c7 cells treated with quercetin for 6 h, then an RT-PCR analysis for each gene was carried out. (B) Modulating the effect of quercetin on the total ALDH activity. After Hepa1c1c7 cells were pretreated with quercetin for 24 h, then the total ALDH activity was determined. All values are expressed as means ± SD of three independent experiments and analyzed by a Student's t-test by using SPSS software (**, $\rho < 0.01$; *, $\rho < 0.05$ vs. control).

### 2.3.3 Quercetin decreases the acetaldehyde-enhanced reactive oxygen species (ROS) production

In fact, the electrophilic nature of acetaldehyde contributes not only to DNA and protein damage, but also to ROS production via mitochondrial damage is also involved in its toxic mechanisms. To further confirm this idea, the acetaldehyde-dependent change in the intracellular ROS level was evaluated by the experiments using a ROS-sensitive fluorescent dye, DCFH-DA. Indeed, the treatment of Hepa1c1c7 cells with acetaldehyde resulted in an enhancement of the intracellular ROS level. When the cells were pretreated with quercetin for 24 h, the acetaldehyde-enhanced ROS level was significantly reduced in a concentration-dependent manner (Fig. 2.3).

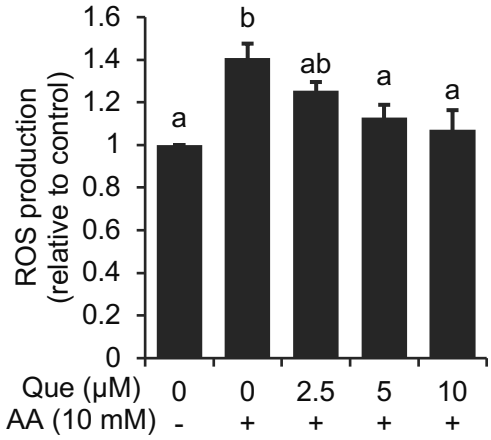



**Fig. 2.3** Inhibitory effect of quercetin on the acetaldehyde-induced enhancement of the intracellular ROS level. After Hepa1c1c7 cells were pretreated with quercetin for 24 h, the cells were treated with 10 mM acetaldehyde for 3 h, then a DCFH-DA assay using an image-based cytometer was carried out. All values are expressed as means ± SD of three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using SPSS software. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

### 2.3.4 Quercetin stimulated the gene expression of phase 2 drug-metabolizing enzymes, including HO-1, GCLC, and xCT, as well as GSH level

To examine the effect on the expression of the genes related with resistance against oxidative stress, I checked the mRNA levels of the phase 2 drug-metabolizing

enzymes, including HO-1, glutamate-cysteine ligase, catalytic subunit (GCLC), and cystine/glutamate exchanger (xCT), which are involved in the detoxification of prooxidative toxicants or production of antioxidative molecules (Nakamura & Miyoshi, 2010). The gene expressions of HO-1, GCLC, and xCT were up-regulated by quercetin (Fig. 2.4 (A)). The total intracellular glutathione (GSH) level was also significantly increased by quercetin (Fig. 2.4 (B)), suggesting that the up-regulation of the GSH level might be regulated by not only the enhanced biosynthesis but also the uptake of substrate, cystine.

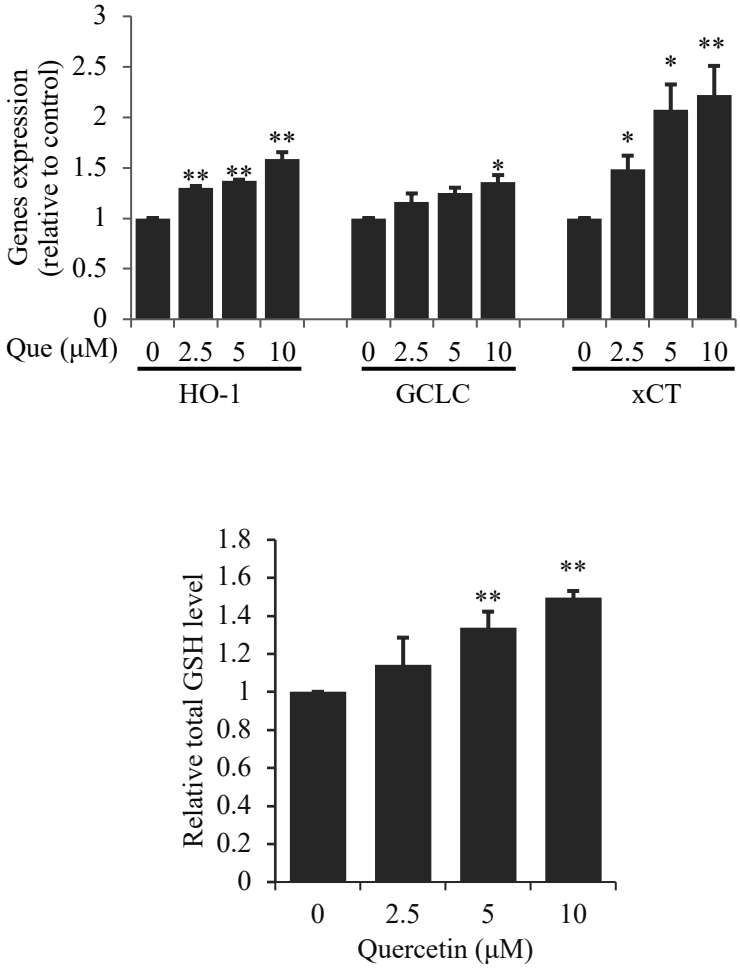
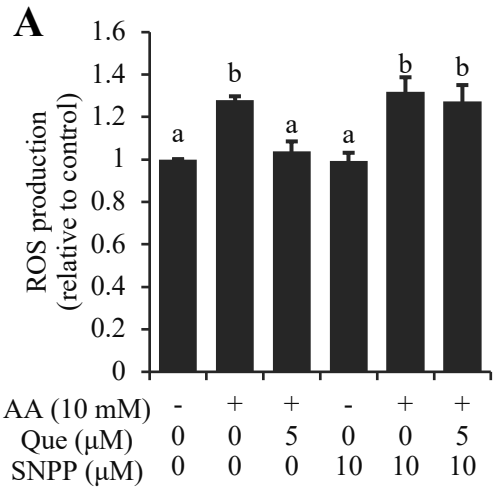



**Fig. 2.4** Modulating effects of quercetin on the gene expressions of phase 2 drug-metabolizing enzymes (A). The total RNA was extracted from the Hepa1c1c7 cells treated with quercetin for 6 h, then an RT-PCR analysis for each gene was carried out. Modulating the effect of quercetin on the total intracellular GSH level (B). After Hepa1c1c7 cells were pretreated with quercetin for 24 h, then the

intracellular GSH level was determined. All values are expressed as means ± SD of three independent experiments and analyzed by a Student's t-test by using SPSS software (**, $\rho < 0.01$; *, $\rho < 0.05$ vs. control).

### 2.3.5 Quercetin inhibits acetaldehyde-induced ROS level and cytotoxicity via an HO-1-dependent but GSH-independent mechanism

To clarify the role of HO-1 and GSH in the resistance against oxidative stress, experiments using an inhibitor of the heme oxygenase enzyme, SNPP, as well as an inhibitor of glutamylcysteine synthesizing enzyme (glutamate-cysteine ligase), BSO, was performed. The HO-1 inhibitor, SNPP, abolished the reduction of ROS production by quercetin (Fig. 2.5 (A)), whereas the GSH biosynthesis inhibitor, BSO, did not (Fig. 2.5 (B)). Furthermore, the cytoprotective effect of quercetin was significantly inhibited by the treatment of SNPP, whereas SNPP itself showed neither cytotoxicity nor cytoprotective effect in Hepa1c1c7 cells (Fig. 2.5 (C)). These results suggested that the HO-1-dependent production of small molecular weight antioxidants, such as biliverdin/bilirubin and carbon monoxide, might play a pivotal role in the cytoprotective mechanism of quercetin.



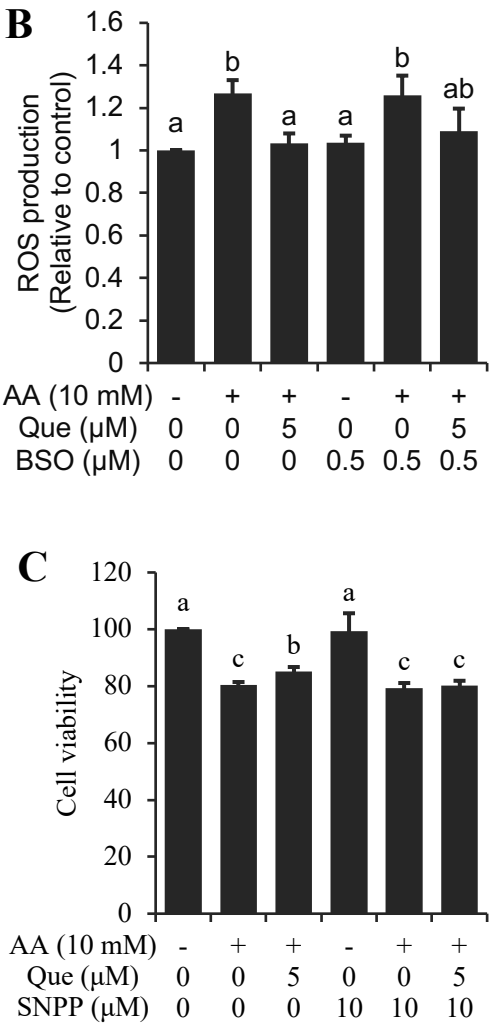



**Fig. 2.5.** Modulating effect of the inhibitor of HO-1, SNPP (A); the inhibitor of glutathione (GSH) biosynthesis, BSO (B), on the inhibition of quercetin on the acetaldehyde-induced enhancement of the intracellular reactive oxygen species (ROS) level. Hepa1c1c7 cells were pretreated with SNPP (10 μM) or BSO (0.5 μM) for 1 h, the cells were treated with quercetin for 24 h, next treated with 10 mM acetaldehyde for 3 h, then a DCFH-DA assay using an image-based cytometer was carried out. Modulating effect of SNPP on the acetaldehyde-induced cytotoxicity (C). After Hepa1c1c7 cells were pretreated with SNPP (10 μM) for 1 h, the cells were treated with quercetin for 24 h. the cells were treated with acetaldehyde (10 mM), then an MTT assay was carried out. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

## 2.4 Discussion

In the current study, I demonstrated that quercetin protected mouse hepatoma Hepa1c1c7 cells form the acetaldehyde-induced cytotoxicity in a concentration-dependent manner (Fig. 2.1). Acetaldehyde, the product of ethanol metabolism, is highly toxic and is rapidly metabolized to acetate. The imbalance between the production and disposition of acetaldehyde causes toxicity. Since the toxic effect of acetaldehyde is closely linked to the induction of mitochondrial dysfunction and thus overproduction of ROS, I supposed that the enhanced resistance against oxidative stress is a plausible mechanism underlying the cytoprotection against acetaldehyde. The intracellular ROS level also was decreased by quercetin in a concentration-dependent manner (Fig. 2.3), indicating that quercetin protected the cells from the acetaldehyde-induced cytotoxicity by enhancing the resistance against oxidative stress. There are numerous beneficial effects of quercetin in vivo and in vitro have been recognized (Legault et al., 2011). Quercetin could protect the sperm viability and structural integrity by reducing the effects of heat stress and maintain sperm quality via relieving the oxidative stress in mice and rabbits (Naseer et al., 2018; Shi et al., 2018).

Quercetin enhanced the total ALDH activity as well as the gene expression of ALDH1A1 and ALDH3A1, other than ALDH2 in a concentration-dependent manner (Fig. 2.2). In addition to ALDH2, ALDH1A1, a cytosolic enzyme expressed in the liver, and ALDH3A1, another cytosolic enzyme expressed in the liver, might assist ALDH2 in the ethanol metabolism. These results suggested that quercetin potentiate the total ALDH activity, possibly via the transcriptional regulation of these isozymes. Taken together, the enhancement total ALDH activity by the up-regulation of auxiliary ALDHs other than ALDH2 is likely to contribute to the enhanced tolerance toward acetaldehyde.

In addition to ALDHs, the gene expression of representative phase 2 drug-metabolizing enzymes including HO-1, GCLC, and xCT, were significantly enhanced by quercetin. Consistently, the intracellular GSH level was also enhanced by quercetin, suggesting that the GSH up-regulation might be not only regulated by the enhanced biosynthesis, but also by the substrate uptake. Recent reports indicated that the HO-1/CO system can stimulate mitochondrial biogenesis which may account in part for the cytoprotective roles of this system (MacGarvey et al., 2012; Suliman et

al., 2007). Recent research has elucidated the role of HO-1 and CO in cellular defense mechanisms against oxidative damage. Quercetin has gained much attention because of its ability to confer cytoprotective effects through induction of HO-1 in various cell lines and primary hepatocytes (Davis et al., 2009; Rayamajhi et al., 2013; Tang et al., 2016; Yao et al., 2007). Consistently, I demonstrated that SNPP, but not BSO, abolished the reduction of ROS production by quercetin (Fig. 2.5). These results strongly suggested that the HO-1-dependent production of small molecular weight antioxidants, such as biliverdin/bilirubin and carbon monoxide, but not GSH, might play a pivotal role in the cytoprotective mechanism of quercetin.

In conclusion, we demonstrated that quercetin underlying enhances cell viability against acetaldehyde-induced cytotoxicity, possibly through enhancement of the gene expression of phase 2 drug-metabolizing enzymes, as well as the total ALDH activity by transcriptional regulation. Furthermore, the acetaldehyde-enhanced ROS level could be reduced by quercetin in an HO-1-dependent and GSH-independent mechanism.

# Chapter 3

# 3,4-Dihydrooxyphenlacetic acid and 3-hydroxyphenylacetic acid as a potential agent in the mouse hepatoma Hepa1c17 cells

## 3.1 Introduction

Quercetin, one of the most ubiquitous flavonoids, shows beneficial effects for human health. In general, quercetin exists in foodstuff as glycoside forms. Since quercetin glycosides are mostly poorly absorbed in the small intestine, they can be degraded by the gut microbiota into DOPAC. DOPAC is converted to OPAC by dehydroxylation (LI et al., 2022).

DOPAC was identified as a major catabolite of other quercetin glycosides, such as rutin (Aura et al., 2002) and hyperoside (Yang et al., 2013), as well as procyanidins (Yang et al., 2013). DOPAC has also been confirmed as a predominant quercetin catabolite in humans (Peng et al., 2014). In the previous study, DOPAC was identified as the most potent antioxidant in vitro and in a cultured cell model among the phenolic acid catabolites (Tang et al., 2016). DOPAC has some advantages for application as a food chemical due to its lower cytotoxicity as well as colorless. This idea is also supported by the fact that humans have a metabolic pathway for DOPAC, also a metabolite of the neurotransmitter dopamine (Tang et al., 2016).

OPAC was identified as a major metabolite of Q4′G in the feces, whereas it was also detected in the urine and liver (Mullen et al., 2008). OPAC was also identified as a metabolite of different classes of flavonoids, such as flavonols, isoflavones, and flavanols (Appeldoorn et al., 2009; Feliciano et al., 2016; Guadamuro et al., 2016; Olthof et al., 2003; Serra et al., 2011). These findings strongly support the idea that OPAC is the most predominant and stable catabolite from the quercetin glycosides as well as several flavonoids in humans. OPAC has also the potential as a vasoactive and blood pressure-decreasing agent (Dias et al., 2022). The OPAC-induced vasodilation can be attained at its physiological concentrations dependent on the nitric oxide produced by the endothelium (Dias et al., 2022). OPAC was also utilized as a starting material for drug synthesis targeting receptors for γ-hydroxybutyric acid, a neurotransmitter (Chen et al., 2005).

Acetaldehyde, the most toxic metabolite of ethanol, is speculated to mediate the liver tissue damage and cognitive dysfunction induced by the chronic excessive consumption of alcohol. The principle enzyme responsible for the detoxification and metabolism of acetaldehyde into acetic acid is aldehyde dehydrogenase (ALDH) 2. The generation and accumulation of acetaldehyde by metabolism in the organisms may contribute to DNA, protein damage, and mitochondrial dysfunction. Hence, induction of mitochondrial dysfunction and overproduction of reactive oxygen species (ROS) have been linked to the action of acetaldehyde (Farfán Labonne et al., 2009; Gomez-Quiroz et al., 2003).

In this study, to explore the possibility of DOPAC and OPAC, as mainly quercetin intestinal catabolites, as potential protective agents, we examined the cytoprotection of DOPAC and OPAC alone on the acetaldehyde- or hydrogen peroxide-induced cytotoxicity in mouse hepatoma Hepa1c1c7 cells. Our results showed that DOPAC and OPAC individually showed protective effects against acetaldehyde-induced toxicity in the cells. Furthermore, DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity, while OPAC did not.

## 3.2 Materials and methods

### 3.2.1 Materials

DOPAC and OPAC were obtained from Sigma Aldrich (St. Louis, MO, USA).

### 3.2.2 Cell cultures

See chapter 2.

### 3.2.3 Cell viability determination

See chapter 2.

### 3.2.4 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

See chapter 2.

### 3.2.5 Glutathione titration

See chapter 2.

### 3.2.6 ALDH activity assay

See chapter 2.

### 3.2.7 Statistical analysis

See chapter 2.

### 3.3 Results

#### 3.3.1 DOPAC and OPAC protects the mouse hepatoma Hepa1c1c7 cells against the acetaldehyde-induced cytotoxicity

I initially examined whether DOPAC and OPAC have a protective effect against the acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. Hepa1c1c7 cells were pretreated with DOPAC or OPAC for 6 h, then treated with acetaldehyde (10 mM) for 3 h. Both DOPAC and OPAC significantly inhibited the acetaldehyde-induced cytotoxicity in a concentration-manner (Fig. 3.1 (A)(B)). In addition, DOPAC also inhibited hydrogen peroxide-induced cytotoxicity, but OPAC did not (Fig. 3.1 (C)(D)).

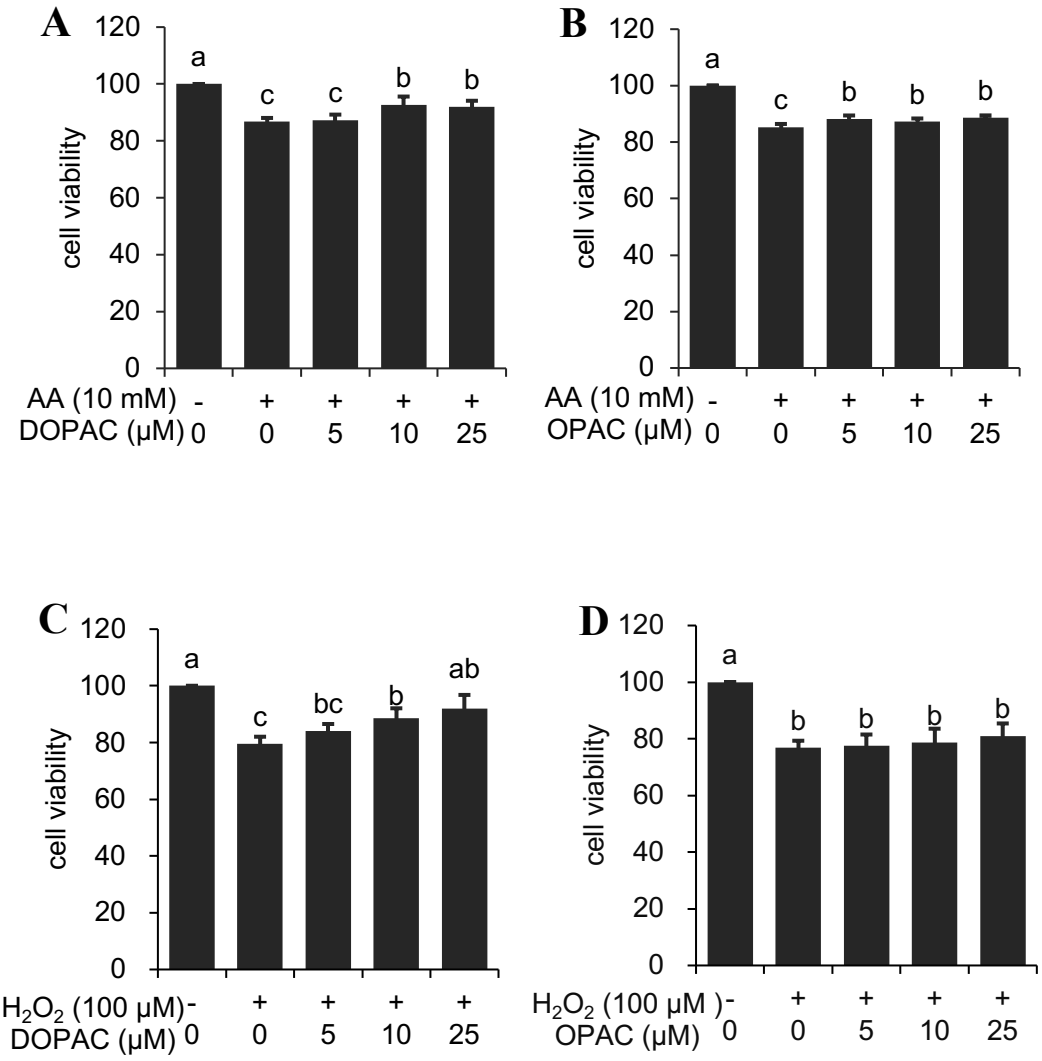
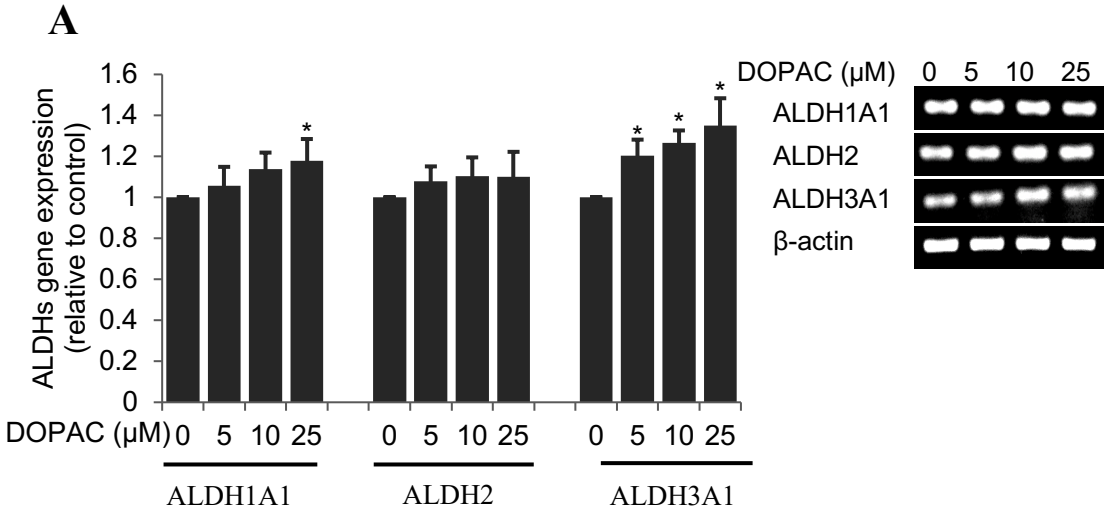



**Fig. 3.1** Protective effects of DOPAC and OPAC on the acetaldehyde- and hydrogen peroxide-induced cytotoxicity. Inhibitory effect of DOPAC or OPAC on the

acetaldehyde-induced cytotoxicity. After Hepa1c1c7 cells were pretreated with DOPAC(A) or OPAC (B) for 6 h, the cells were treated with 10 mM acetaldehyde for 3 h, then an MTT assay was carried out. Inhibitory effect of DOPAC (C) or OPAC (D) on the hydrogen peroxide-induced cytotoxicity. After Hepa1c1c7 cells were pretreated with DOPAC or OPAC for 6 h, the cells were treated with 100 μM hydrogen peroxide for 6 h; then an MTT assay was carried out. All values are expressed as means ± SD of three separate experiments and analyzed by a Student's t-test or a one-way ANOVA followed by Tukey's HSD using SPSS software. The different letters above the bars indicate significant differences among the treatments for each condition ($P < 0.05$).

### 3.3.2 DOPAC and OPAC enhance mRNA levels and activity of aldehyde dehydrogenase

Next, to investigate the possibility that quercetin affects the expression of the genes related to resistance against acetaldehyde, I checked the mRNA levels of the conventional ALDHs, such as ALDH1A1, ALDH2, and ALDH3A1, all of which are highly expressed in the liver and play an important role in the acetaldehyde metabolism (Moreb et al., 2000). The DOPAC treatments significantly increased the gene expression of ALDH1A1 and ALDH3A1 in a concentration-dependent manner, whereas OPAC up-regulated only the ALDH3A1 gene expression (Fig. 3.2 (A) (B)). Both DOPAC and OPAC potentiated the total ALDH enzyme activity, even though neither DOPAC nor OPAC modulated the gene expression of ALDH2 (Fig. 3.2 (C) (D)).



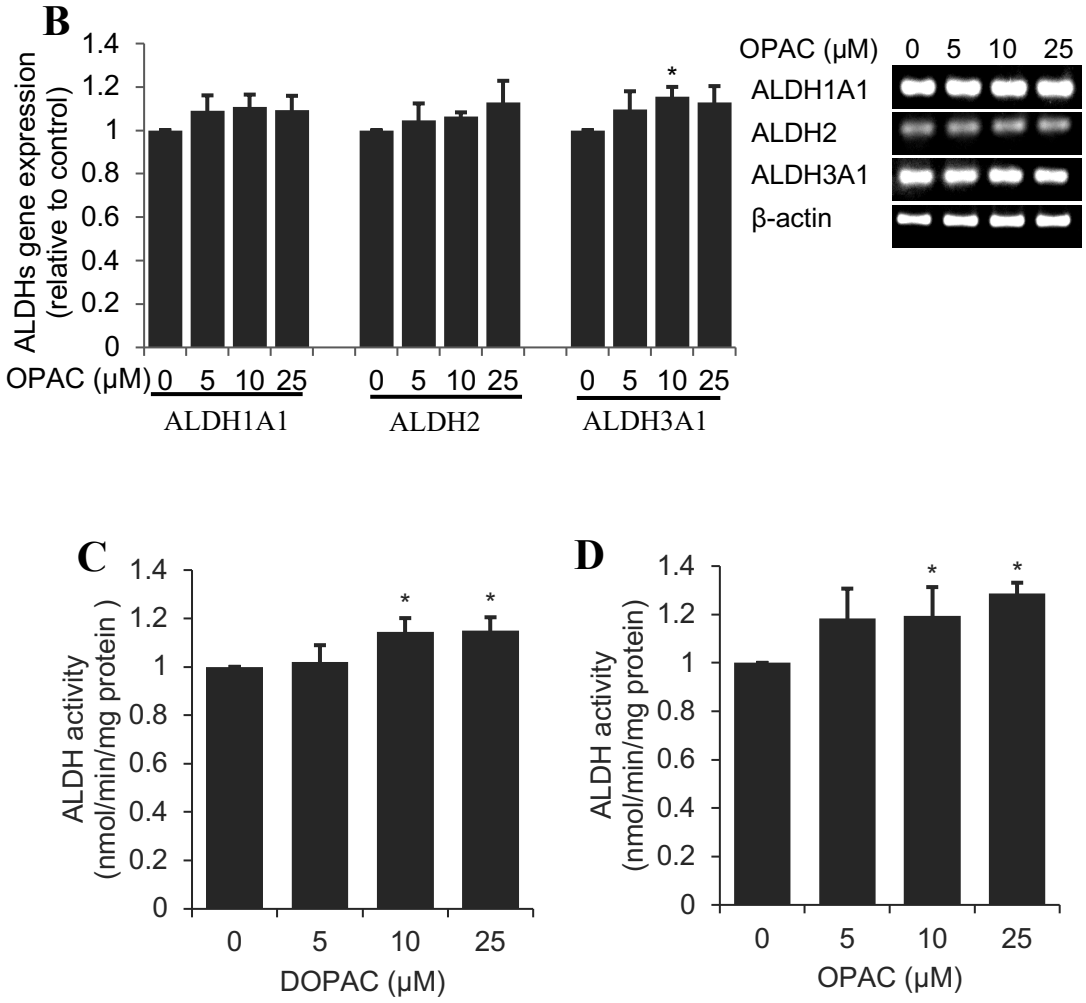



**Fig. 3.2** Modulating effects of DOPAC and OPAC on the gene expressions of the classical family of ALDHs (A) (B). The total RNA was extracted from the Hepa1c1c7 cells treated with DOPAC or OPAC for 6 h, then an RT-PCR analysis for each gene was carried out. Modulating the effect of DOPAC and OPAC on the total ALDH activity (C) (D). After Hepa1c1c7 cells were pretreated with DOPAC or OPAC for 6 h, then the total ALDH activity was determined. Representative blots and quantitative data for ADLH1A1, ADLH2, and ADLH3A1 are shown. All values are expressed as means ± SD of three independent experiments and analyzed by a Student's t-test by using SPSS software (*, $\rho < 0.05$ vs. control).

### 3.3.3 DOPAC and OPAC stimulated the gene expression of phase 2 drug-metabolizing enzyme, as well as GSH level

Similar to quercetin, DOPAC significantly enhanced the mRNA levels of HO-1, GCLC, and xCT (Fig. 3.3 (A)), while OPAC only up-regulated GCLC (Fig. 3.3 (B)).

The concentration dependency of DOPAC or OPAC for the enhanced intracellular GSH level was correlated with that for the gene expression of xCT, but not GCLC, suggesting that the up-regulation of GSH level might be due to the substrate uptake, but not by the enhanced biosynthesis (Fig. 3.3 (C) (D)).

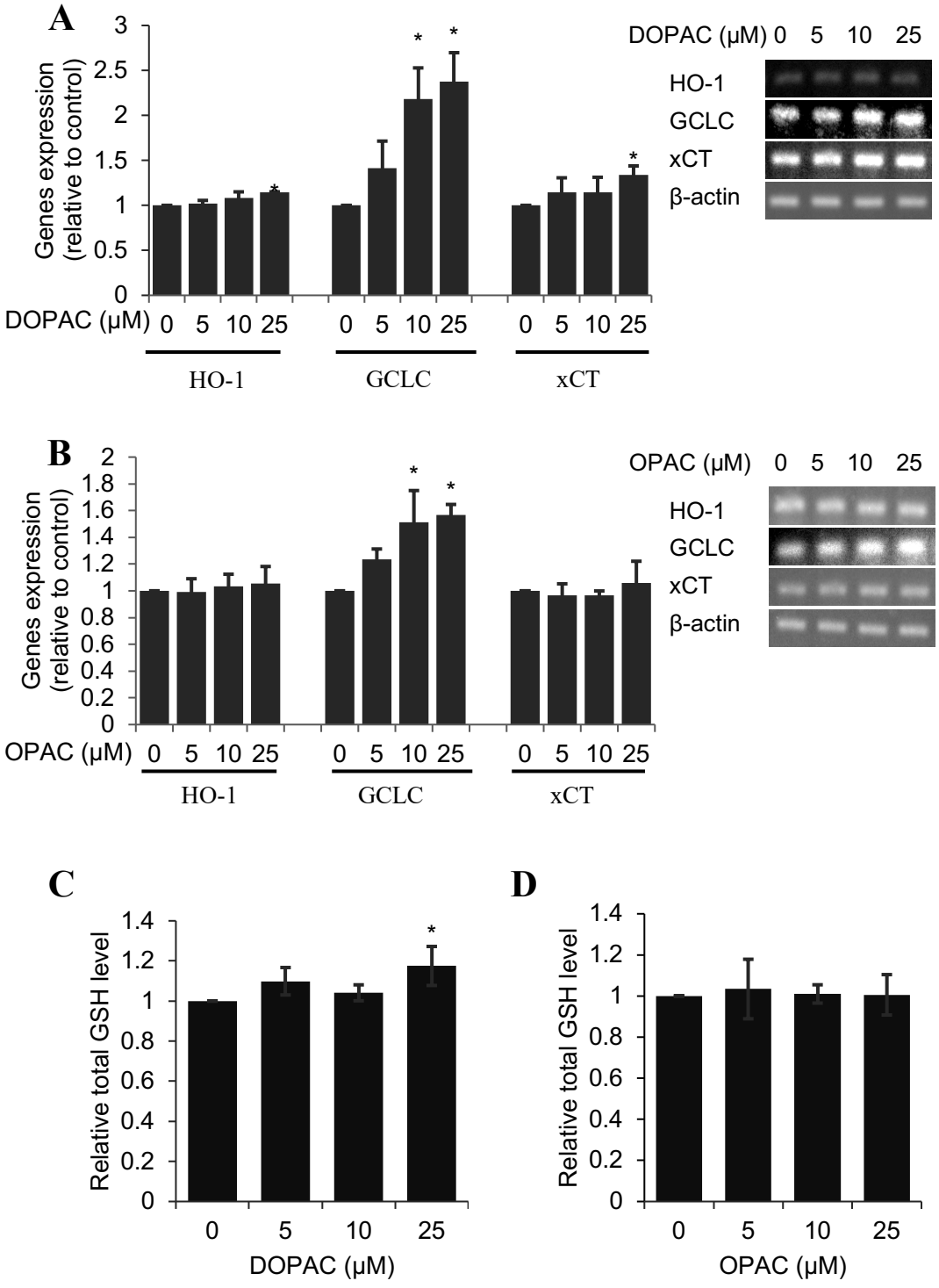



**Fig. 3.3** Modulating effects of DOPAC and OPAC on the gene expressions of phase 2

drug-metabolizing enzymes (A) (B). The total RNA was extracted from the Hepa1c1c7 cells treated with DOPAC or OPAC for 6 h, then an RT-PCR analysis for each gene was carried out. Modulating the effect of DOPAC and OPAC on the total intracellular GSH level (C) (D). After Hepa1c1c7 cells were pretreated with DOPAC or OPAC for 6 h, then the intracellular GSH level was determined. All values are expressed as means ± SD of three independent experiments and analyzed by a Student's t-test by using SPSS software (*, $\rho < 0.05$ vs. control).

### 3.4 Discussion

Each the quercetin intestinal catabolite, DOPAC and OPAC, significantly inhibited the acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. The minimal concentration of each compound for a significant enhancement of the total ALDH activity was 10 μM, which is consistent with the previous studies (Liu et al., 2017; Liu et al., 2022). The DOPAC treatments significantly increased the gene expression of ALDH1A1 and ALDH3A1 in a concentration-dependent manner, whereas OPAC up-regulated only the ALDH3A1 gene expression. Both DOPAC and OPAC potentiated the total ALDH enzyme activity, even though neither DOPAC nor OPAC modulated the gene expression of ALDH2. These results implied that DOPAC and OPAC modulate the total ALDH activity through a transcription regulation of the isozymes other than ALDH2.

Furthermore, DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity, while OPAC did not. Similar to quercetin, DOPAC significantly enhanced the mRNA levels of HO-1, GCLC, and xCT, while OPAC up-regulated only GCLC. The concentration dependency of DOPAC for the enhanced intracellular GSH level was correlated with that for the gene expression of xCT, but not GCLC, suggesting that the up-regulation of GSH level might be due to the substrate uptake, but not by the enhanced biosynthesis. However, the findings of the current study were not consistent with the previous study, indicating that OPAC is neither a radical scavenger (chemical antioxidant) nor an inducer of antioxidant enzymes (biologically active antioxidant) after 24 h treatment (Tang et al., 2016). A possible explanation for these results may be due to the difference in incubation time of OPAC among these studies, which is also future effort to be investigated.

Taken together, we identified DOPAC and OPAC, major intestinal catabolites of quercetin glycosides, as potential protectors against the alcohol-induced liver diseases and might be active metabolites after the ingestion of quercetin-rich diets. DOPAC is also a biologically active antioxidant with the inducible potency of an antioxidative defense system, including the gene expression and the total intracellular GSH level.

# Chapter 4

# The microbiota catabolites of quercetin glycosides concertedly enhance the resistance against the acetaldehyde-induced oxidative stress

## 4.1 Introduction

As shown in Chapter 3, I demonstrated that DOPAC and OPAC each showed the cytoprotective effect on the acetaldehyde-induced cytotoxicity. Each of them also significantly enhanced the total ALDH activity as well as the gene expression of certain ALDH isozymes (Liu et al., 2017; Liu et al., 2022). Contrarily, there is much difference of biological activities between DOPAC and OPAC, even though their structures quite similar to each other except for the number(s) of phenolic hydroxyl group (Fig. 1.2). For example, DOPAC enhanced the gene expression of the phase 2 drug-metabolizing enzymes, such as HO-1 and GCLC as well as acted as a radical scavenger, whereas OPAC showed no significant effects (Tang et al., 2016). DOPAC also showed significant cytoprotection against the ROS-induced toxicity coincided with an enhancement of the intracellular glutathione level (Tang et al., 2016). Therefore, the up-regulation of antioxidant molecules might, at least partly, be involved in the mechanism underlying the cytoprotection against acetaldehyde by DOPAC, but not by OPAC. Even though we have evaluated the solo effects of each intestinal catabolites of quercetin glycosides on the acetaldehyde-induced cytotoxicity and clarified their mechanisms, the effect of their combination remains to be examined.

In the present study, I examined the modulating effect of the combination of DOPAC and OPAC, two predominant intestinal catabolites of quercetin glycosides, on the hydrogen peroxide-toxicity as well as the acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells. I demonstrated that their combination significantly inhibited both acetaldehyde- and hydrogen peroxide-induced cytotoxicity at the concentration of which the single treatment of each catabolite showed no significant effect. I also checked whether the combination of DOPAC and OPAC affected the acetaldehyde-enhanced intracellular ROS level as well as the gene expression of ALDHs and phase 2 drug-metabolizing enzymes. The present

data indicated that GSH plays an important role in the DOPAC and OPAC combination-induced resistance against the acetaldehyde-induced oxidative stress.

## 4.2 Materials and methods

### 4.2.1 Materials

See chapter 3.

### 4.2.2 Cell cultures

See chapter 2.

### 4.2.3 Cell viability determination

See chapter 2.

### 4.2.4 Intracellular reactive oxygen species measurement and image analysis

See chapter 2.

### 4.2.5 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

See chapter 2.

### 4.2.6 Glutathione titration

See chapter 2.

### 4.2.7 ALDH activity assay

See chapter 2.

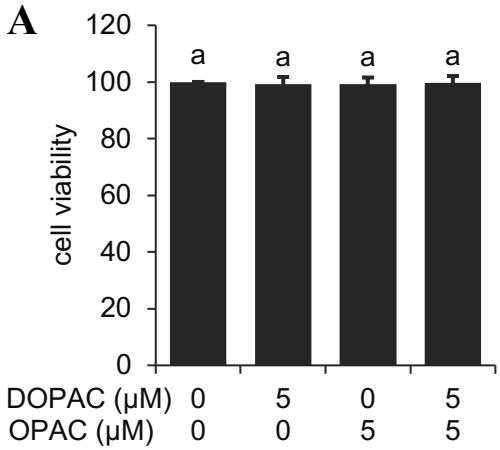
### 4.2.8 Statistical analysis

See chapter 2.

## 4.3 Results

### 4.3.1 Protective effect of the combination of intestinal catabolites of quercetin glycosides (DOPAC and OPAC) on the acetaldehyde-induced cytotoxicity

In this study, the mouse hepatoma cell line Hepa1c1c7 cells were used for the cultured hepatocyte model since its response to DOPAC or OPAC was quite similar to that of the human hepatocyte model (Liu et al., 2017; Liu et al., 2022). In the previous study, the concentration of DOPAC or OPAC required for the cytoprotection against acetaldehyde was 10 μM or higher (Liu et al., 2017; Liu et al., 2022). Therefore, 5 μM was chosen as the tested concentration for use in the following experiments to evaluate the effect of the combination of these compounds. In addition, a metabolic study using radiolabeled Q4′G revealed that substantial amounts of both DOPAC and OPAC were detected in the colon, and their ratio in the colon is approximately 1:1 (Mullen et al., 2008). Therefore, the ratio of two compounds for the combinatory experiments was decided to be 1:1. When Hepa1c1c7 cells were treated with DOPAC, OPAC, or their combination for 6 h, no cytotoxic effect was observed (Fig. 4.1 (A)). Next, to examine the combinatory effect on the acetaldehyde-induced cytotoxicity, acetaldehyde (10 mM) was treated for 3 h after the 6 h preculture with the catabolite treatment. Although the single treatment of DOPAC or OPAC did not show a significant effect, the combination of DOPAC and OPAC significantly alleviated the decreased cell viability induced by acetaldehyde (Fig. 4.1 (B)).



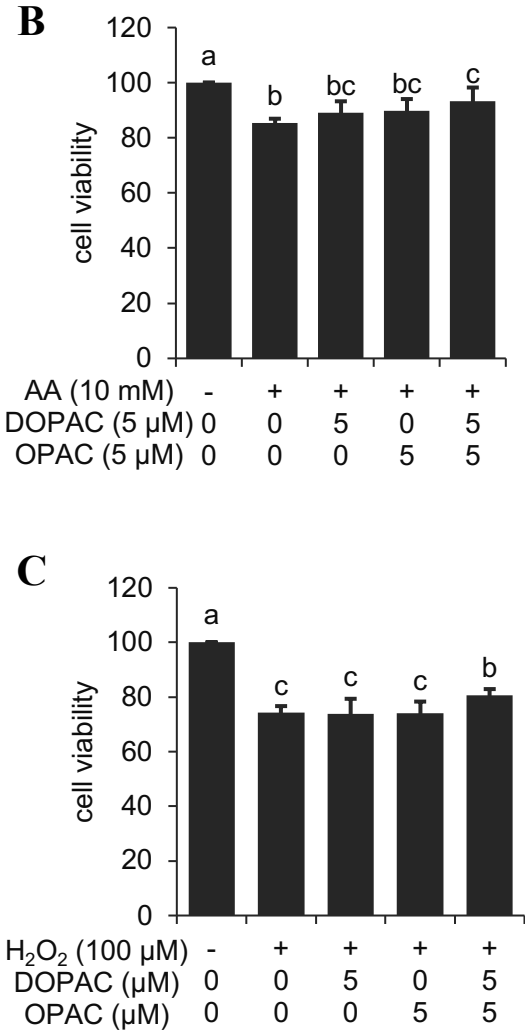



**Fig. 4.1.** Protective effects of the combination of DOPAC and OPAC on the acetaldehyde- and hydrogen peroxide-induced cytotoxicity. (A) Effect of DOPAC, OPAC, or their combination on cell viability. Hepa1c1c7 cells were treated with the indicated concentrations of the test compounds for 6 h, then an MTT assay was carried out. (B) Inhibitory effect of DOPAC, OPAC, or their combination on the acetaldehyde-induced cytotoxicity. After Hepa1c1c7 cells were pretreated with DOPAC, OPAC, or their combination for 6 h, the cells were treated with 10 mM acetaldehyde for 3 h, then an MTT assay was carried out. (C) Inhibitory effect of DOPAC, OPAC, or their combination on the hydrogen peroxide-induced cytotoxicity. After Hepa1c1c7 cells were pretreated with DOPAC, OPAC, or their combination for 6 h, the cells were treated with 100 μM hydrogen peroxide for 6 h; then an MTT assay was carried out. All values are expressed as means ± SD of three separate experiments and analyzed by a Student's t-test or a one-way ANOVA followed by Tukey's HSD using SPSS software. The different letters above the bars indicate

significant differences among the treatments for each condition ($P < 0.05$).

### 4.3.2 Inhibitory effect of the combination of DOPAC and OPAC on the oxidative stress-induced cytotoxicity.

Next, to check the effect on the oxidative stress-related cytotoxicity, hydrogen peroxide (100 μM) was treated for 6 h after the 6 h pretreatment of the catabolites. As shown in Fig. 4.1 (C), the DOPAC and OPAC combination also protected the cells from the hydrogen peroxide-induced toxicity, whereas the single treatment of each compound showed no protective effect. These results suggested that the combinatory treatment of DOPAC and OPAC potentiates the cytoprotective effect, possibly through the enhanced resistance against oxidative stress.

To further confirm this idea, the acetaldehyde-dependent change in the intracellular ROS level was evaluated by the experiments using a ROS-sensitive fluorescent dye, DCFH-DA. The treatment of Hepa1c1c7 cells with acetaldehyde for 3 h resulted in a significant enhancement of the ROS level by approximately 1.2-fold (Fig. 4.2). When the cells were pretreated with DOPAC (5 μM) or OPAC (5 μM) alone slightly but significantly decreased. Furthermore, the combination of these compounds showed a more potent inhibitory effect than that of each compound (Fig. 4.2).

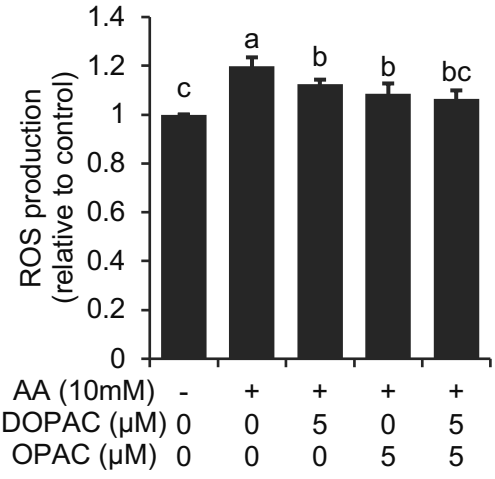



**Fig. 4.2** Inhibitory effect of DOPAC, OPAC, or their combination on the acetaldehyde-induced enhancement of the intracellular ROS level. After Hepa1c1c7 cells were pretreated with DOPAC, OPAC, or their combination for 6 h, the cells were treated with 10 mM acetaldehyde for 3 h, then a DCFH-DA assay using an image-based cytometer was carried out. All values are expressed as means ± SD of

three separate experiments and analyzed by a one-way ANOVA followed by Tukey's HSD using SPSS software. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

### 4.3.3 Modulating effect of the combination of DOPAC and OPAC on the resistance against acetaldehyde and oxidative stress

To examine the possibility that the combination of DOPAC and OPAC affects the expression of the genes related with the resistance against acetaldehyde, I checked the mRNA levels of the conventional ALDHs, such as ALDH1A1, ALDH2, and ALDH3A1, all of which are highly expressed in the liver and playing an important role in the acetaldehyde metabolism (Chen et al., 2015; Lind et al., 2008). The combination of DOPAC (5 μM) and OPAC (5 μM) significantly up-regulated the ALDH1A1 and ALDH3A1 gene expression, but not that of ALDH2 (Fig. 4.3 (A)). Next, to examine the effect on the expression of the genes related with the resistance against oxidative stress, I checked the mRNA levels of the phase 2 drug-metabolizing enzymes, including HO-1, NAD(P)H: quinone oxidoreductase 1 (NQO1), GCLC, and xCT, which are involved in the detoxification of prooxidative toxicants or production of antioxidative molecules (Nakamura & Miyoshi, 2010). The combination of DOPAC (5 μM) and OPAC (5 μM) significantly enhanced the gene expression only of GCLC (Fig. 4.3 (B)).

To further confirm whether the combination of DOPAC and OPAC modulates the resistance against acetaldehyde or oxidative stress, the effects on the total ALDH enzyme activity and intracellular GSH level were examined. The combination of DOPAC (5 μM) and OPAC (5 μM) significantly increased not only the total ALDH activity but also the intracellular GSH level, whereas the single treatment of each compound showed no significant effect (Figs. 4.4 (A) and (B)).

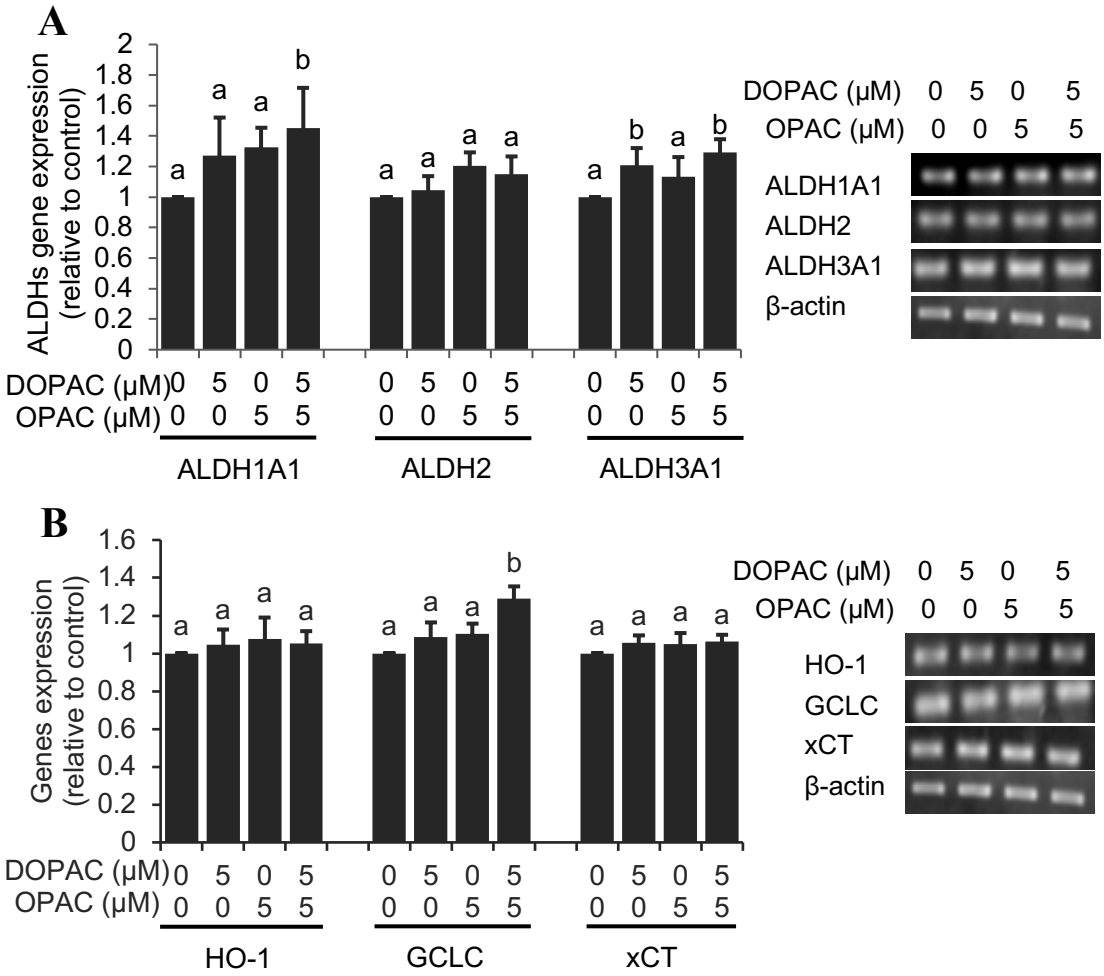



**Fig. 4.3** Modulating effects of DOPAC, OPAC, or their combination on the gene expressions of the classical family of ALDHs (A) and phase 2 drug-metabolizing enzymes (B). The total RNA was extracted from the Hepa1c1c7 cells treated with DOPAC, OPAC, or their combination for 6 h, then an RT-PCR analysis for each gene was carried out. Representative blots and quantitative data for ADLH1A1, ADLH2, ADLH3A1, HO-1, GCLC, and xCT are shown. All values are expressed as means ± SD of three separate experiments. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

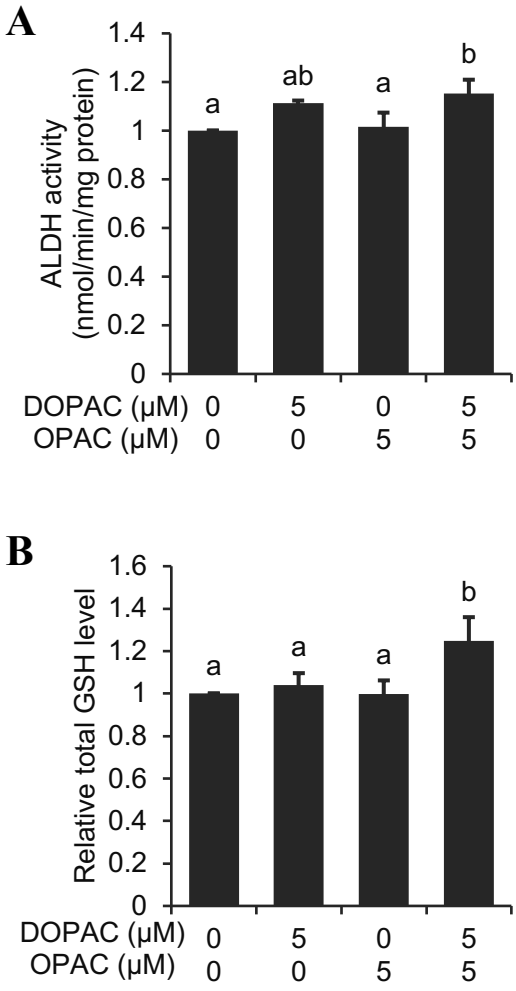
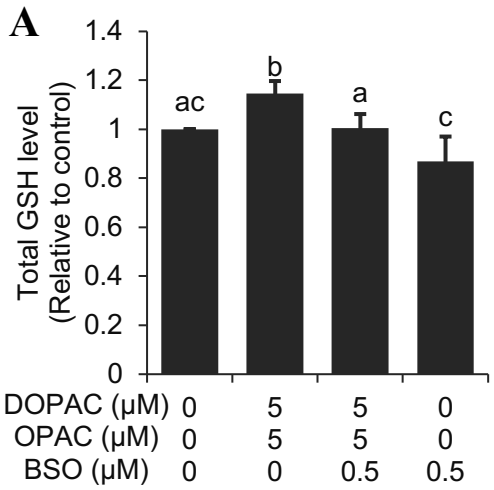


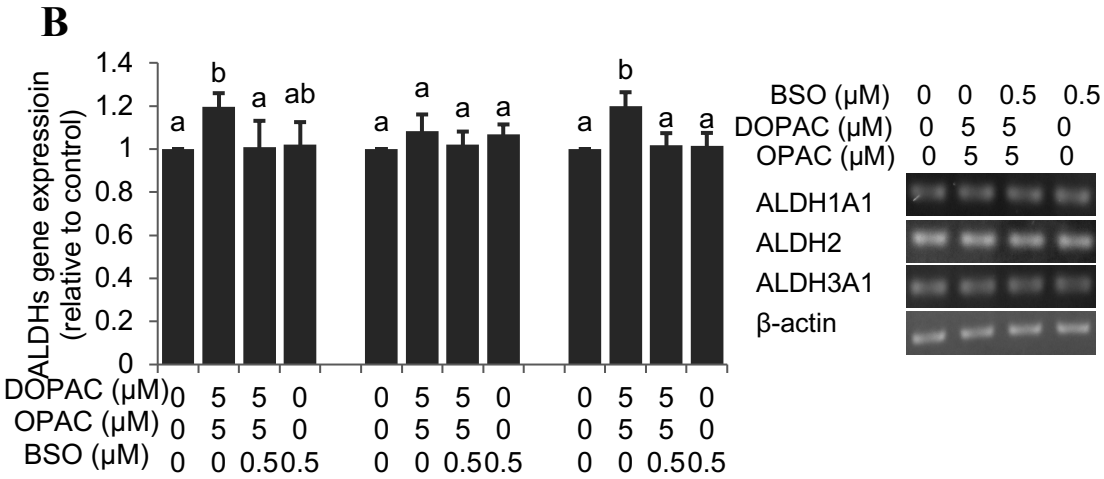

**Fig. 4.4.** Modulating effects of DOPAC, OPAC, or their combination on the total ALDH activity (A) or the intracellular GSH levels (B) in mouse hepatoma Hepa1c1c7 cells. After Hepa1c1c7 cells were pretreated with DOPAC, OPAC, or their combination for 6 h, then the total ALDH activity and intracellular GSH level were determined. All values are expressed as means ± SD of three separate experiments. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

### 4.3.4 Repelled effect of a GSH synthesis inhibitor on the combination-induced cytoprotection

To clarify the role of GSH in the resistance against oxidative stress, experiments using an inhibitor of the glutamylcysteine synthesizing enzyme (glutamate-cysteine ligase), BSO, was performed. As shown in Fig. 4.5 (A), the intracellular level enhanced by the combination of DOPAC (5 μM) and OPAC (5 μM) was decreased by

BSO (0.5 μM) to the control level, whereas BSO itself slightly decrease the basal GSH level. Unexpectedly, the combination-induced enhancement of the total ALDH activity, as well as the gene expression of ALDH1A1 and ALDH3A1, was also cancelled by BSO (Figs. 4.5 (B) and (C)). Finally, the cytoprotective effect of the combination of DOPAC (5 μM) and OPAC (5 μM) was significantly inhibited by the treatment of BSO, whereas BSO itself showed neither cytotoxicity nor cytoprotective effect in Hepa1c1c7 cells (Fig. 4.5 (D)). These results strongly suggested that the enhanced GSH biosynthesis is, at least partly, attributable to not only the cytoprotection against acetaldehyde but also the up-regulation of ALDHs by the combination of DOPAC and OPAC.



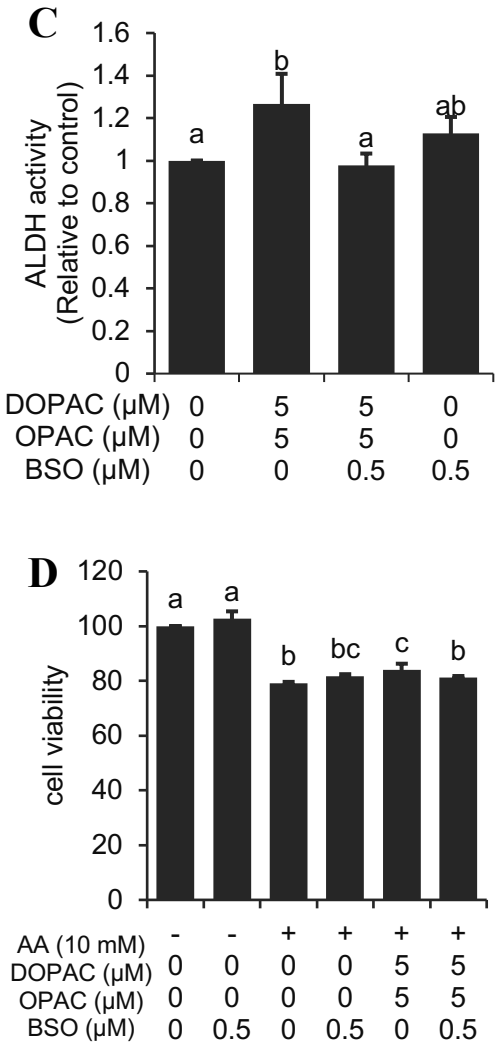



**Fig. 4.5**. Modulating effect of the inhibitor of GSH biosynthesis, BSO, on the cytoprotection against acetaldehyde by the combination of DOPAC and OPAC. (A) Modulating effect of BSO on the intracellular GSH level. After Hepa1c1c7 cells were pretreated with BSO (0.5 μM) for 1 h, the cells were treated with DOPAC, OPAC, or their combination for 6 h, then the intracellular GSH level was determined. (B) Modulating the effect of BSO on the total ALDH activity. After Hepa1c1c7 cells were pretreated with BSO (0.5 μM) for 1 h, the cells were treated with DOPAC, OPAC, or their combination for 6 h, then the ALDH activity was determined. (C) Modulating effect of BSO on the gene expressions of the classical family of ALDHs. (D) Modulating effect of BSO on the acetaldehyde-induced cytotoxicity. After Hepa1c1c7 cells were pretreated with BSO (0.5 μM) for 1 h, the cells were treated with DOPAC, OPAC, or their combination for 6 h. The cells were treated with 10 mM acetaldehyde for 3 h, then an MTT assay was carried out. All values are

expressed as means ± SD of three separate experiments. The different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

## 4.4 Discussion

In the present study, I demonstrated the combination of DOPAC and OPAC protected mouse hepatoma Hepa1c1c7 cells from the acetaldehyde-induced cytotoxicity at the concentration of which the single pretreatment of each compound showed no significant effect (Fig. 4.1 (B)). Among the intestinal phenolic acid catabolites derived from quercetin glycosides, DOPAC and OPAC were identified as potential cytoprotectants against acetaldehyde (Liu et al., 2017; Liu et al., 2022). The minimum concentration of DOPAC or OPAC alone required for protection against acetaldehyde was reported to be 10 μM (Liu et al., 2017; Liu et al., 2022), which is the same as the total concentration of DOPAC and OPAC in combination for this effect (Fig. 4.1 (B)). This suggested that the combination of DOPAC and OPAC concertedly induces the cytoprotective effect to the equivalent extent to DOPAC or OPAC alone. Furthermore, this combination also significantly inhibited the hydrogen peroxide-induced cytotoxicity (Fig. 4.1 (C)), even though OPAC has a much lower capability of inducing the gene expression of the phase 2 drug-metabolizing enzymes as well as scavenging free radicals (Tang et al., 2016). The combination of OPAC and DOPAC also decreased the intracellular ROS level (Fig. 4.2). Since the toxic effect of acetaldehyde is closely linked to the induction of mitochondrial dysfunction and thus overproduction of ROS (Farfán Labonne et al., 2009; Gomez-Quiroz et al., 2003), these results led me to the speculations that the enhanced resistance against oxidative stress is a plausible mechanism underlying the cytoprotection against acetaldehyde, and the antioxidative effect of this combination is not merely the additive of each compound, but mechanistically interactive.

The combination of DOPAC and OPAC also enhanced the gene expression of ALDH1A1 and ALDH3A1 (Fig. 4.3 (A)). ALDHs play a crucial role in the metabolism of aldehydes and, among the 19 ALDH family enzymes, ALDH2 plays a major role in the acetaldehyde metabolism (Dias et al., 2022). In addition to ALDH2, ALDH1A1, a cytosolic enzyme highly expressed in the liver (Li et al., 2011), and ALDH3A1, another cytosolic enzyme expressed in the liver, might assist ALDH2 in the ethanol metabolism (Chen et al., 2015). Consistent with this result, the combination of DOPAC and OPAC significantly enhanced the total ALDH enzyme activity (Fig. 4.4 (A)). Previous studies indicated that the ALDH2-knockout animals as well as ALDH2-deficient cells have the reduced ability to detoxify acetaldehyde

and thus show the lower resistance against ethanol- or acetaldehyde-induced toxicity (Jamal et al., 2016; Yu et al., 2009). Therefore, the increased total ALDH activity by the transcriptional regulation of auxiliary ALDHs other than ALDH2 is likely to contribute to the enhanced tolerance toward acetaldehyde.

In addition to ALDHs, the gene expression of GCLC, but not other phase 2 drug-metabolizing enzymes, such as HO-1 and NQO-1, was significantly enhanced by the combination of DOPAC and OPAC (Fig. 4.3 (B)). Consistently, the intracellular GSH level was also enhanced by this combination (Fig. 4.4 (B)), suggesting that the GSH up-regulation might be regulated by the enhanced biosynthesis and not by the substrate uptake. The previous study showed that neither OPAC nor DOPAC alone affects the xCT gene expression (Fig. 4.3 (B)). Even though the gene expression of GCLC is mainly regulated by Nrf2 as well as AP-1 and NFκB (Lu, 2013), OPAC has little ability to activate the Nrf2-dependent transcriptional regulation (Liu et al., 2022). Therefore, the combined effects of DOPAC and OPAC may be neither additive to the common mechanism exhibited by these compounds nor due to one compound potentiating the effect of the other. Since the GCLC-dependent regulation of GSH biosynthesis by DOPAC and OPAC combination is so unique that each compound alone does not have, a future study will be concerned with further understanding the signaling pathways involved in the GSH up-regulation.

The GSH up-regulation might be one of the plausible mechanisms for the resistance against the acetaldehyde-induced oxidative stress, because GSH not only reacts directly with ROS but also acts as a substrate for peroxidases (Aldini et al., 2018). Furthermore, BSO, the inhibitor of glutamate-cysteine ligase, significantly abolished not only the enhancement of intracellular GSH level (Fig. 4.5 (A)), but also the cytoprotective effects induced by the combination of DOPAC and OPAC (Fig. 4.5 (D)), implying that the enhanced GSH biosynthesis is also, at least partly, attributable to the protection against the acetaldehyde-induced cytotoxicity by the combination of DOPAC and OPAC. In addition to the antioxidant action by GSH itself, the DOPAC /OPAC combination-enhanced total ALDH activity as well as the up-regulation of ALDH1A1 and ALDH3A1 genes was canceled by BSO (Figs. 4.5 (B) (C)). This result suggested that the intracellular GSH level might be a prerequisite for the regulation of the gene expression of some ALDHs. A previous study demonstrated that the aryl hydrocarbon receptor (AhR)-dependent CYP1A1 gene expression was impaired by the GSH depletion, but enhanced by the GSH up-regulation (Omidi et al.,

2018). Furthermore, the depletion of intracellular GSH also inhibited the enhanced ALDH3c expression by a polycyclic hydrocarbon through the AhR-dependent mechanism (Pappas et al., 1995). The AhR-dependent pathway might be regulated by both DOPAC and OPAC, whereas the Nrf2-dependent one was enhanced by DOPAC and not by OPAC (Liu et al., 2022). Taken together, the GSH-dependent regulation of ALDH1A1 and ALDH3A1 gene expression by the combination might involve the AhR pathway activation. This idea was preliminarily supported by a luciferase assay using the reporter gene having a xenobiotic response element showing that the combination of DOPAC and OPAC significantly increased the transcriptional activity of AhR, which was abolished by BSO, even though not only its effective concentration but also the cell line used was different (data not shown).

In conclusion, we demonstrated that the major quercetin intestinal catabolites, DOPAC and OPAC, in combination concertedly, but not additively, protected the cells from the acetaldehyde-induced toxicity. Not only the enhanced ALDH activity but also the increased intracellular GSH level play a key role in this effect of the combination. Our findings indicated that these combinations of quercetin intestinal catabolites have more advantages for application as food chemicals than each compound alone because of their equivalent efficacy and lower cytotoxicity. On the other hand, the cultured mouse hepatocyte model has some limitations, such as not reflecting the characteristics of hepatocytes in vivo, and being the acute and transient model, not the chronic one. In addition, the concentrations of DOPAC and OPAC required for its cytoprotection (~5 μM) might be beyond physiological, since a previous human preclinical study uncovered that the plasma OPAC level in the individuals after the consumption of polyphenols was approximately 100~400 nM (Koli et al., 2010). Future studies will be concerned with the significance of the combination of DOPAC and OPAC in several in vivo models as well as human hepatocyte models.

# CONCLUSIONS

In the current study, the molecular mechanisms underlying the cytoprotective effects of quercetin and its microbiota catabolites on acetaldehyde-induced cytotoxicity in the mouse hepatoma Hepa1c1c7 cells were examined.

In chapter 2, the protective effect of quercetin on acetaldehyde-induced cytotoxicity was evaluated.

(1) Quercetin significantly inhibited the acetaldehyde-induced cytotoxicity coincided with the enhancement of the total ALDH activity as well as the gene expression of ALDH1A1 and ALDH3A1.
(2) The pretreatment of quercetin significantly reduced the acetaldehyde-enhanced ROS level in a concentration-dependent manner.
(3) the gene expressions of the representative phase 2 drug-metabolizing enzymes, such as HO-1, GCLC, and xCT were up-regulated by quercetin.
(4) The intracellular GSH level was also significantly increased by quercetin.
(5) An HO-1 inhibitor, SNPP, abolished the reduction of ROS production and cytoprotection by quercetin, whereas a GSH biosynthesis inhibitor, BSO did not.

In chapter 3, the cytoprotective effects of the major microbiota catabolites of quercetin, DOPAC and OPAC, on the acetaldehyde-induced cytotoxicity were investigated.

(1) DOPAC or OPAC alone inhibited acetaldehyde-induced cytotoxicity. Furthermore, DOPAC also inhibited the hydrogen peroxide-induced cytotoxicity, while OPAC did not.
(2) The DOPAC treatments significantly increased the gene expression of ALDH1A1 and ALDH3A1 in a concentration-dependent manner, whereas OPAC up-regulated only the ALDH3A1 gene expression.
(3) DOPAC or OPAC alone potentiated the total ALDH enzyme activity.

In chapter 4, the combination of DOPAC and OPAC concertedly protected the cells from the acetaldehyde- and hydrogen peroxide-induced cytotoxicity.

(1) the combination of DOPAC and OPAC protected mouse hepatoma Hepa1c1c7 cells from the acetaldehyde- and hydrogen peroxide-induced cytotoxicity at the concentration of which the single pretreatment of each compound showed no

significant effect.

(2) This combination also inhibited the intracellular ROS level.

(3) The combinatory treatment enhanced the gene expression of not only ALDH1A1 and ALDH3A1, but also GCLC, the first rate-limiting enzyme of GSH synthesis. Accordingly, intracellular GSH level, as well as the total ALDH activity, was enhanced by DOPAC plus OPAC.

(4) BSO abolished the combinations of cytoprotective effects against acetaldehyde.

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Kexin Li
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# REFERENCES


Aldini, G., Altomare, A., Baron, G., Vistoli, G., Carini, M., Borsani, L., & Sergio, F. (2018). N-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free radical research*, *52*(7), 751-762.

Appeldoorn, M. M., Vincken, J. P., Aura, A. M., Hollman, P. C., & Gruppen, H. (2009). Procyanidin dimers are metabolized by human microbiota with 2-(3, 4-dihydroxyphenyl) acetic acid and 5-(3, 4-dihydroxyphenyl)-γ-valerolactone as the major metabolites. *Journal of agricultural and food chemistry*, *57*(3), 1084-1092.

Aura, A. M., O'leary, K., Williamson, G., Ojala, M., Bailey, M., Puupponen-Pimiä, R., Nuutila, A. M., Oksman-Caldentey, K. M., & Poutanen, K. (2002). Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. *Journal of agricultural and food chemistry*, *50*(6), 1725-1730.

Banjarnahor, S. D., & Artanti, N. (2014). Antioxidant properties of flavonoids. *Medical Journal of Indonesia*, *23*(4), 239-244.

Boots, A. W., Haenen, G. R., & Bast, A. (2008). Health effects of quercetin: from antioxidant to nutraceutical. *European journal of pharmacology*, *585*(2-3), 325-337.

Carmel-Harel, O., & Storz, G. (2000). Roles of the glutathione-and thioredoxin-dependent reduction systems in the Escherichia coli and Saccharomyces cerevisiae responses to oxidative stress. *Annual Reviews in Microbiology*, *54*(1), 439-461.

Castañeda-Ovando, A., de Lourdes Pacheco-Hernández, M., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, *113*(4), 859-871.

Cheeseman, K. H., & Slater, T. F. (1993). An introduction to free radical biochemistry. *British medical bulletin*, *49*(3), 481-493.

Chen, C. H., Cruz, L. A., & Mochly-Rosen, D. (2015). Pharmacological recruitment of aldehyde dehydrogenase 3A1 (ALDH3A1) to assist ALDH2 in acetaldehyde and ethanol metabolism in vivo. *Proceedings of the National Academy of Sciences*, *112*(10), 3074-3079.

Chen, W., Wu, H., Hernandez, R. J., Mehta, A. K., Ticku, M. K., France, C. P., & Coop, A. (2005). Ethers of 3-hydroxyphenylacetic acid as selective gamma-hydroxybutyric acid receptor ligands. *Bioorganic & medicinal chemistry letters*, *15*(13), 3201-3202.

D Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., & Masella, R. (2007). Polyphenols, dietary sources and bioavailability. *Annali-Istituto Superiore di Sanita*, *43*(4), 348.

Davis, J. M., Murphy, E. A., Carmichael, M. D., & Davis, B. (2009). Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *296*(4), R1071-R1077.

Dias, P., Pourová, J., Vopršalová, M., Nejmanová, I., & Mladěnka, P. (2022). 3-Hydroxyphenylacetic Acid: A Blood Pressure-Reducing Flavonoid Metabolite. *Nutrients*, *14*(2), 328.

Dragovic-Uzelac, V., Levaj, B., Mrkic, V., Bursac, D., & Boras, M. (2007). The content of polyphenols and carotenoids in three apricot cultivars depending on stage of maturity and geographical region. *Food Chemistry*, *102*(3), 966-975.

Eriksson, C. P. (2001). The role of acetaldehyde in the actions of alcohol (update 2000). *Alcoholism: Clinical and Experimental Research*, *25*, 15S-32S.

Farfán Labonne, B. E., Gutiérrez, M., Gómez-Quiroz, L. E., Konigsberg Fainstein, M., Bucio, L., Souza, V., Flores, O., Ortíz, V., Hernández, E., & Kershenobich, D. (2009). Acetaldehyde-induced mitochondrial dysfunction sensitizes hepatocytes to oxidative damage. *Cell biology and toxicology*, *25*(6), 599-609.

Feliciano, R. P., Boeres, A., Massacessi, L., Istas, G., Ventura, M. R., Dos Santos, C. N., Heiss, C., & Rodriguez-Mateos, A. (2016). Identification and quantification of novel cranberry-derived plasma and urinary (poly) phenols. *Archives of biochemistry and biophysics*, *599*, 31-41.

Freeman, B. A., & Crapo, J. D. (1982). Biology of disease: free radicals and tissue injury. *Laboratory investigation; a journal of technical methods and pathology*, *47*(5), 412-426.

Gandhi, S., & Abramov, A. Y. (2012). Mechanism of oxidative stress in neurodegeneration. *Oxidative medicine and cellular longevity*, *2012*.

Godic, A., Poljšak, B., Adamic, M., & Dahmane, R. (2014). The role of antioxidants in skin cancer prevention and treatment. *Oxidative medicine and cellular longevity*, *2014*.

Gomez-Quiroz, L., Bucio, L., Souza, V., Escobar, C., Farfán, B., Hernández, E., Konigsberg, M., Vargas-Vorackova, F., Kershenobich, D., & Gutiérrez-Ruiz, M. C. (2003). Interleukin 8 response and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde or lipopolysaccharide. *Hepatology Research*, *26*(2), 134-141.

Guadamuro, L., Jimenez-Giron, A. M., Delgado, S., Florez, A. B., Suarez, A., Martin-Alvarez, P. J., Bartolome, B., Moreno-Arribas, M. V., & Mayo, B. (2016). Profiling of phenolic metabolites in feces from menopausal women after long-term isoflavone supplementation. *Journal of agricultural and food chemistry*, *64*(1), 210-216.

Halliwell, B., Rafter, J., & Jenner, A. (2005). Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *The American journal of clinical nutrition*, *81*(1), 268S-276S.

Hasumura, Y., Teschke, R., & Lieber, C. (1975). Hepatic microsomal ethanol-oxidizing system (MEOS): dissociation from reduced nicotinamide adenine dinucleotide phosphate oxidase and possible role of form I of cytochrome P-450. *Journal of pharmacology and experimental therapeutics*, *194*(2), 469-474.

Hayashi, Y., Matsushima, M., Nakamura, T., Shibasaki, M., Hashimoto, N., Imaizumi, K., Shimokata, K., Hasegawa, Y., & Kawabe, T. (2012). Quercetin protects against pulmonary oxidant stress via heme oxygenase-1 induction in lung epithelial cells. *Biochemical and biophysical research communications*, *417*(1), 169-174.

Hertog, M., Sweetnam, P. M., Fehily, A. M., Elwood, P. C., & Kromhout, D. (1997). Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. *The American journal of clinical nutrition*, *65*(5), 1489-1494.

Hervert-Hernández, D., & Goñi, I. (2011). Dietary polyphenols and human gut microbiota: a review. *Food reviews international*, *27*(2), 154-169.

Ishikawa, T., Suzukawa, M., Ito, T., Yoshida, H., Ayaori, M., Nishiwaki, M., Yonemura, A., Hara, Y., & Nakamura, H. (1997). Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. *The American journal of clinical nutrition*, *66*(2), 261-266.

Jamal, M., Ameno, K., Tanaka, N., Ito, A., Takakura, A., Kumihashi, M., & Kinoshita, H. (2016). Ethanol and acetaldehyde after intraperitoneal administration to Aldh2-knockout mice-reflection in blood and brain levels. *Neurochemical research*, *41*(5), 1029-1034.

KITAGAWA, S., FUJISAWA, H., & SAKURAI, H. (1992). Scavenging effects of dihydric and polyhydric phenols on superoxide anion radicals, studied by electron spin resonance spectrometry. *Chemical and pharmaceutical bulletin*, *40*(2), 304-307.

Koli, R., Erlund, I., Jula, A., Marniemi, J., Mattila, P., & Alfthan, G. (2010). Bioavailability of various polyphenols from a diet containing moderate amounts of berries. *Journal of agricultural and food chemistry*, *58*(7), 3927-3932.

Lale, A., Herbert, J., Augereau, J., Billon, M., Leconte, M., & Gleye, J. (1996). Ability of different flavonoids to inhibit the procoagulant activity of adherent human monocytes. *Journal of natural products*, *59*(3), 273-276.

Lee, Y. K., Yuk, D. Y., Lee, J. W., Lee, S. Y., Ha, T. Y., Oh, K. W., Yun, Y. P., & Hong, J. T. (2009). (−)-Epigallocatechin-3-gallate prevents lipopolysaccharide-induced elevation of beta-amyloid generation and memory deficiency. *Brain research*, *1250*, 164-174.

Legault, J., Perron, T., Mshvildadze, V., Girard-Lalancette, K., Perron, S., Laprise, C., Sirois, P., & Pichette, A. (2011). Antioxidant and anti-inflammatory activities of quercetin 7-O-β-D-glucopyranoside from the leaves of Brasenia schreberi. *Journal of Medicinal Food*, *14*(10), 1127-1134.

LI, K., NAKAMURA, T., & NAKAMURA, Y. (2022). RING FISSION CATABOLITES OF QUERCETIN GLYCOSIDES. *Journal of Environmental Science for Sustainable Society*, *11*(Supplement), MR02_p05-MR02_p08.

Li, X., Wang, X., Chen, D., & Chen, S. (2011). Antioxidant activity and mechanism of protocatechuic acid in vitro. *Functional Foods in Health and Disease*, *1*(7), 232-244.

Lieber, C. S. (1991). Hepatic, metabolic and toxic effects of ethanol: 1991 update. *Alcoholism: Clinical and Experimental Research*, *15*(4), 573-592.

Lind, P. A., Eriksson, C., & Wilhelmsen, K. C. (2008). The role of aldehyde dehydrogenase-1 (ALDH1A1) polymorphisms in harmful alcohol consumption in a Finnish population. *Human genomics*, *3*(1), 1-12.

Liu, Y., Kurita, A., Nakashima, S., Zhu, B., Munemasa, S., Nakamura, T., Murata, Y., & Nakamura, Y. (2017). 3, 4-Dihydroxyphenylacetic acid is a potential aldehyde dehydrogenase inducer in murine hepatoma Hepa1c1c7 cells. *Bioscience, biotechnology, and biochemistry*, *81*(10), 1978-1983.

Liu, Y., Myojin, T., Li, K., Kurita, A., Seto, M., Motoyama, A., Liu, X., Satoh, A., Munemasa, S., Murata, Y., Nakamura, T., & Nakamura, Y. (2022). A major intestinal catabolite of quercetin glycosides, 3-hydroxyphenylacetic acid, protects the hepatocytes from the acetaldehyde-induced cytotoxicity through the enhancement of the total aldehyde dehydrogenase activity. *International Journal of Molecular Sciences*, *23*(3), 1762.

López-Alarcón, C., & Denicola, A. (2013). Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. *Analytica chimica acta*, *763*, 1-10.

Lu, S. C. (2013). Glutathione synthesis. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1830*(5), 3143-3153.

MacGarvey, N. C., Suliman, H. B., Bartz, R. R., Fu, P., Withers, C. M., Welty-Wolf, K. E., & Piantadosi, C. A. (2012). Activation of mitochondrial biogenesis by heme oxygenase-1–mediated NF-E2–related factor-2 induction rescues mice from lethal Staphylococcus aureus sepsis. *American journal of respiratory and critical care medicine*, *185*(8), 851-861.

Meister, A. (1983). Glutathione. *Annu. Rev. Biochem.*, *52*, 711-760.

Moreb, J. S., Maccow, C., Schweder, M., & Hecomovich, J. (2000). Expression of antisense RNA to aldehyde dehydrogenase class-1 sensitizes tumor cells to 4-hydroperoxycyclophosphamide in vitro. *Journal of pharmacology and experimental therapeutics*, *293*(2), 390-396.

Mullen, W., Rouanet, J.-M., Auger, C., Teissedre, P.-L., Caldwell, S. T., Hartley, R. C., Lean, M. E., Edwards, C. A., & Crozier, A. (2008). Bioavailability of [2-14C] quercetin-4′-glucoside in rats. *Journal of agricultural and food chemistry*, *56*(24), 12127-12137.

Murota, K., Mitsukuni, Y., Ichikawa, M., Tsushida, T., Miyamoto, S., & Terao, J. (2004). Quercetin-4 ‘-glucoside is more potent than quercetin-3-glucoside in protection of rat intestinal mucosa homogenates against iron ion-induced lipid peroxidation. *Journal of agricultural and food chemistry*, *52*(7), 1907-1912.

Naderi, G. A., Asgary, S., Sarraf-Zadegan, N., Shirvany, H., & Gharipour, M. (2001). Anti-oxidant effect of flavonoids on the susceptibility of LDL oxidation. *Journal of Molecular and Cellular Cardiology*, *6*(33), A82.

Nakamura, Y., & Miyoshi, N. (2010). Electrophiles in foods: the current status of isothiocyanates and their chemical biology. *Bioscience, biotechnology, and biochemistry*, *74*(2), 242-255.

Naseer, Z., Ahmad, E., Şahiner, H. S., Epikmen, E. T., Fiaz, M., Yousuf, M. R., Khan, S. A., Serin, İ., Ceylan, A., & Aksoy, M. (2018). Dietary quercetin maintains the semen quality in rabbits under summer heat stress. *Theriogenology*, *122*, 88-93.

Olthof, M. R., Hollman, P. C., Buijsman, M. N., Van Amelsvoort, J. M., & Katan, M. B. (2003). Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *The Journal of nutrition*, *133*(6), 1806-1814.

Omidi, M., Ghafarian-Bahraman, A., & Mohammadi-Bardbori, A. (2018). GSH/GSSG redox couple plays central role in aryl hydrocarbon receptor-dependent modulation of cytochrome P450 1A1. *Journal of Biochemical and Molecular Toxicology*, *32*(7), e22164.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: an overview. *Journal of nutritional science*, *5*.

Pappas, P., Vasiliou, V., Karageorgou, M., Stefanou, P., & Marselos, M. (1995). Studies on the induction of rat class 3 aldehyde dehydrogenase. *Enzymology and Molecular Biology of Carbonyl Metabolism 5*, 143-149.

Peng, X. C., Zhang, Z. C., Zhang, N., Liu, L., Li, S. T., & Wei, H. (2014). In vitro catabolism of quercetin by human fecal bacteria and the antioxidant capacity of its catabolites. *Food & nutrition research*, *58*(1), 23406.

Persson, T., Popescu, B. O., & Cedazo-Minguez, A. (2014). Oxidative stress in Alzheimer’s disease: why did antioxidant therapy fail? *Oxidative medicine and cellular longevity*, *2014*.

Rayamajhi, N., Kim, S.-K., Go, H., Joe, Y., Callaway, Z., Kang, J.-G., Ryter, S. W., & Chung, H. T. (2013). Quercetin induces mitochondrial biogenesis through activation of HO-1 in HepG2 cells. *Oxidative medicine and cellular longevity*, *2013*.

Robaszkiewicz, A., Balcerczyk, A., & Bartosz, G. (2007). Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell biology international*, *31*(10), 1245-1250.

Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: antioxidants and beyond. *The American journal of clinical nutrition*, *81*(1), 215S-217S.

Serra, A., Macià, A., Romero, M.-P., Anglés, N., Morelló, J.-R., & Motilva, M.-J. (2011). Metabolic pathways of the colonic metabolism of procyanidins (monomers and dimers) and alkaloids. *Food Chemistry*, *126*(3), 1127-1137.

Shi, X., Hu, H., Ji, G., Zhang, J., Liu, R., Zhang, H., & Li, M. (2018). Protective effect of sucrose and antioxidants on cryopreservation of sperm motility and DNA integrity in C57BL/6 mice. *Biopreservation and biobanking*, *16*(6), 444-450.

Sies, H. (2020). Oxidative eustress and oxidative distress: Introductory remarks. In *Oxidative Stress* (pp. 3-12). Elsevier.

Stickel, F., Datz, C., Hampe, J., & Bataller, R. (2017). Pathophysiology and management of alcoholic liver disease: update 2016. *Gut and liver*, *11*(2), 173.

Suliman, H. B., Carraway, M. S., Ali, A. S., Reynolds, C. M., Welty-Wolf, K. E., & Piantadosi, C. A. (2007). The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. *The Journal of clinical investigation*, *117*(12), 3730-3741.

Tang, Y., Nakashima, S., Saiki, S., Myoi, Y., Abe, N., Kuwazuru, S., Zhu, B., Ashida, H., Murata, Y., & Nakamura, Y. (2016). 3, 4-Dihydroxyphenylacetic acid is a predominant biologically-active catabolite of quercetin glycosides. *Food Research International*, *89*, 716-723.

Toda, S. (2011). Polyphenol content and antioxidant effects in herb teas. *Chinese Medicine*, *2*(1), 29.

Vasiliou, V., & Pappa, A. (2000). Polymorphisms of human aldehyde dehydrogenases. *Pharmacology*, *61*(3), 192-198.

Wagener, F. A., Volk, H.-D., Willis, D., Abraham, N. G., Soares, M. P., Adema, G. J., & Figdor, C. G. (2003). Different faces of the heme-heme oxygenase system in inflammation. *Pharmacological reviews*, *55*(3), 551-571.

Wilms, L. C., Kleinjans, J. C., Moonen, E. J., & Briedé, J. J. (2008). Discriminative protection against hydroxyl and superoxide anion radicals by quercetin in human leucocytes in vitro. *Toxicology in vitro*, *22*(2), 301-307.

Yang, J., Qian, D., Guo, J., Jiang, S., Shang, E.-x., Duan, J.-a., & Xu, J. (2013). Identification of the major metabolites of hyperoside produced by the human intestinal bacteria using the ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Journal of ethnopharmacology*, *147*(1), 174-179.

Yao, P., Nussler, A., Liu, L., Hao, L., Song, F., Schirmeier, A., & Nussler, N. (2007). Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways. *Journal of hepatology*, *47*(2), 253-261.

Yu, H. S., Oyama, T., Isse, T., Kitakawa, K., Ogawa, M., Pham, T. T. P., & Kawamoto, T. (2009). Characteristics of aldehyde dehydrogenase 2 (Aldh2) knockout mice. *Toxicology mechanisms and methods*, *19*(9), 535-540.

Zhang, Y. M. C. (2005). Protective effect of quercetin on Aroclor 1254–induced oxidative damage in cultured chicken spermatogonial cells. *Toxicological Sciences*, *88*(2), 545-550.