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Oryzadine, a new alkaloid of *Oryza sativa* cv. *Heugjinjubyeo*, attenuates oxidative stress-induced cell damage *via* a radical scavenging effect

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ABSTRACT

The antioxidant properties of oryzadine, a new alkaloid, obtained from *Oryza sativa* cv. *Heugjinjubyeo* was investigated by applying various methods based on cell-free and cell experiments. Oryzadine showed scavenging effects on the hydroxyl radical, superoxide radical, and intracellular reactive oxygen species (ROS). Oryzadine inhibited H_2O_2-induced DNA damage, which was demonstrated by DNA tail formation, lipid peroxidation which was demonstrated by the formation of thiobarbituric acid reactive substance (TBARS), and protein oxidation which was demonstrated by protein carbonyl formation. Therefore, oryzadine protected H_2O_2-induced cell damage. Our results show that the cytoprotective effects of oryzadine stem from its ability to inhibit H_2O_2-induced apoptosis, as demonstrated by a decrease in apoptotic body formation and the inhibition of mitochondrial membrane potential ($\Delta\Psi$) loss. Taken together, these findings suggest that oryzadine protected cells against H_2O_2-induced cell damage *via* ROS scavenging effect. Therefore, oryzadine could be considered a significant natural source of antioxidant.

1. Introduction

For aerobic organisms, the oxidative process is an essential mechanism, providing energy for biological processes. However, oxygen-related free radicals and other reactive oxygen species (ROS), which are produced continuously *in vivo*, result in cell death and tissue damage. Oxygen radicals have been implicated in several diseases, including cancer, diabetes, cardiovascular diseases, and ageing (Ozsoy, Can, Yanardag, & Akev, 2008). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical-induced oxidative stress. There is an increasing interest in natural antioxidants such as polyphenols present in medicinal and dietary plants, which might help prevent oxidative damage (Silva, Ferreres, Malva, & Dias, 2005). In the oxidation process, antioxidants are preferentially oxidized through the donation of an electron or hydrogen ion to the potentially damaging radicals. This occurs due to their high redox potentials, allowing them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelating agents, illustrating their antioxidant properties (Tsao & Deng, 2004). Considerable research into phenolic antioxidants has been done in an attempt to treat or eliminate ROS-mediated clinical disorders. These classes of antioxidants are considered beneficial for human health, decreasing the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001; Pulido, Bravo, & Saura-Calixto, 2004; Silva et al., 2004). Given these results, researchers are now searching for natural antioxidants exhibiting strong pharmacological activity without the cytotoxic properties. Bioactive compounds can represent extra-nutritional constituents that are naturally present in small quantities within a food matrix. The selection of phytochemicals, originating natural sources and exhibiting biological activity, are considered critical with regard to the promotion of human health. A host of crops have proven to be important sources of a number of phytochemicals and secondary metabolites. Anthocyanin-pigmented rice was first produced by genetic engineering techniques in the 1970s. This rice has a characteristic dark purple colour, which is attributable to a genomic modification affecting the rice grains. It is used as enriched rice, and is favoured in Korea for both its taste and health benefits. As a component of our ongoing research on the identification of functional activities of anthocyanin-pigmented rice, we focused on the antioxidant activities of oryzadine isolated from rice bran of *Oryza sativa* cv. *Heugjinjubyeo* to give value-added grains (Kim, Shin, Hyun, & Chung, 2009). The aim of this study is to evaluate the antioxidant effect of oryzadine and to elucidate the cytoprotective effect and its mechanism

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against oxidative stress induced cellular component damage and apoptosis.

2. Materials and methods

2.1. Reagents

Oryzadine, (methyl 3-(6-(2,4-dihydroxy-6-oxo-1,6-dihydropyridin-3-yl) benzo[d][1,3]dioxole-5-carbonyl)-2-hydroxy-2,3-dihydrobenzofuran-5-carboxylate), (Fig. 1) was obtained from professor Ha Sook Chung of DukSung Women's University (Seoul, Korea). The 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), Hoechst 33342, and propidium iodide were purchased from the Sigma Chemical Company (St. Louis, MO), thiobarbituric acid from BDH Laboratories (Poole, Dorset, UK), and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine chloride) from Invitrogen Corporation (Carlsbad, CA). All other chemicals and reagents used were of analytical grade.

2.2. Cell Culture

Chinese hamster lung fibroblast cells (V79-4) were obtained from the American Type Culture Collection, and the cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 in air, and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated foetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

2.3. Detection of hydroxyl radical

The hydroxyl radicals were generated by the Fenton reaction (H_2O_2 + $FeSO_4$), and then reacted with a nitrone spin trap, DMPO. The resultant DMPO-OH adducts was detected using a JES-FA electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan). The ESR spectrum was recorded at 2.5 min after mixing with phosphate buffer solution (pH 7.4) with 0.2 ml of 0.3 M DMPO, 0.2 ml of 10 mM $FeSO_4$, 0.2 ml of 10 mM H_2O_2, and oryzadine at 30 μM. The parameters of the ESR spectrometer were set at a magnetic field of 336 mT, power of 1.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 200, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 s, and a temperature of 25 °C (Rimbach et al., 2004).

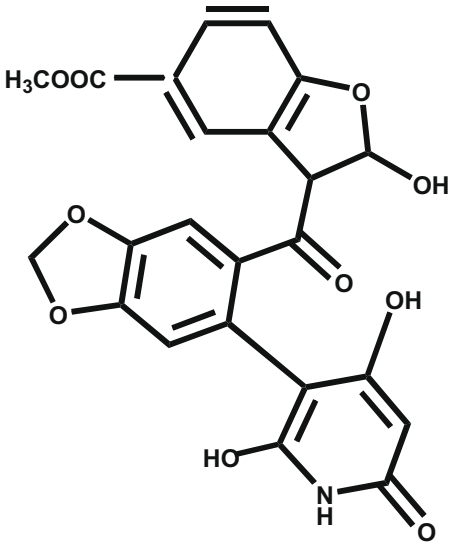

Fig. 1. Chemical structure of oryzadine.

2.4. Detection of superoxide radical

The superoxide radical was produced by reaction of the xanthine/xanthine oxidase system and reacted with spin trap DMPO. The DMPO-·OOH adducts were detected using an ESR spectrometer. ESR signalling was recorded 5 min after 20 μl of xanthine oxidase (0.25 U/ml) was mixed with 20 μl of xanthine (5 mM), 20 μl of DMPO (1.5 M), and 20 μl of oryzadine at 30 μM (Zhao, Joseph, Zhang, Karoui, & Kalyanaraman, 2001).

2.5. Intracellular reactive oxygen species (ROS) measurement

The DCF-DA method was used to detect the levels of intracellular ROS (Rosenkranz et al., 1992). V79-4 cells were seeded in a 96-well plate at 2×10^4 cells/well. Sixteen h after plating, the cells were treated with oryzadine at 0.3, 3, and 30 μM. After 30 min, 1 mM of H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37 °C. After the addition of 25 μM of DCF-DA solution for 10 min, the fluorescence of 2',7'-dichlorofluorescein was detected using a Perkin Elmer LS-5B spectrofluorometer. The intracellular ROS scavenging activity (%) was calculated as [(optical density of H_2O_2 treatment) − (optical density of oryzadine + H_2O_2 treatment)]/(optical density of H_2O_2 treatment) × 100. The level of intracellular ROS was also detected by flow cytometry. Cells were loaded for 30 min at 37 °C with 10 μM DCF-DA at indicated time and supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. Fluorescence of DCF-DA loaded cells was measured using a flow cytometer.

2.6. Comet assay

A comet assay was performed to assess oxidative DNA damage (Rajagopalan, Ranjan, & Nair, 2003). The cell pellet (1.5×10^5 cells) was mixed with 100 μl of 0.5% low melting agarose (LMA) at 39 °C and spread on a fully frosted microscopic slide that had been precoated with 200 μl of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 μl of 0.5% LMA and immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100 and 10% DMSO, pH 10) for 1 h at 4 °C. The slides were placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow DNA unwinding and the expression of the alkali labile damage. An electrical field was applied (300 mA, 25 V) for 20 min at 4 °C to draw negatively charged DNA toward an anode. After electrophoresis, the slides were washed 3 times for 5 min at 4 °C in a neutralising buffer (0.4 M Tris pH 7.5) before being stained with 75 μl ethidium bromide (20 μg/ml). The slides were observed using a fluorescence microscope and image analysis (Komet, Andor Technology, Belfast, UK). Both the percentage of total fluorescence in the tail and the tail length of the 50 cells per slide were recorded.

2.7. Lipid peroxidation assay

Lipid peroxidation was assayed by the thiobarbituric acid reaction (Ohkawa, Ohishi, & Yagi, 1979). The cells were washed with cold phosphate buffered saline (PBS), scraped and homogenised in ice-cold 1.15% KCl. One hundred microlitre of the cell lysates was mixed with 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (adjusted to pH 3.5) and 1.5 ml of 0.8% thiobar-

bituric acid (TBA). The mixture was made up to a final volume of 4 ml with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 5 ml of *n*-BuOH and pyridine mixture (15:1, v/v) was added to each sample and shaken. After centrifugation at 1000*g* for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm. The amount of TBARS was determined using a standard curve with 1,1,3,3-tetrahydroxypropane.

2.8. Protein carbonyl formation

The cells were treated with 30 μM of oryzadine, and after 1 h, 1 mM of H_2O_2 was added to the plate, and the mixture was incubated for 24 h. The amount of carbonyl formation in protein was determined using an Oxiselect™ protein carbonyl ELISA kit purchased from Cell Biolabs (San Diego, CA) according to the manufacturer's instructions. Cellular protein was isolated using protein lysis buffer (50 mM Tris (pH 7.5), 10 mM EDTA (pH 8), 1 mM PMSF) and quantified using a spectrophotometer.

2.9. Cell viability

The effect of oryzadine at 30 μM on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay. Fifty microlitre of the MTT stock solution (2 mg/ml) was then added into each well to attain a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800*g* for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethyl sulfoxide and read at A_{540} on a scanning multi-well spectrophotometer.

2.10. Nuclear staining with Hoechst 33342

One point five microlitre of Hoechst 33342 (stock 10 mg/ml), a DNA specific fluorescent dye, was added to each well and incubated for 10 min at 37 °C. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro colour digital camera, in order to examine the degree of nuclear condensation.

2.11. Mitochondrial membrane potential ($\Delta\psi$) analysis

The cells were harvested, washed and suspended in PBS containing JC-1 (10 μg/ml). After 15 min of incubation at 37 °C, the cells were washed, suspended in PBS and analysed by flow cytometer (Troiano et al., 2007). For image analysis, the cells were harvested, and after changing the media, JC-1 was added to each well and was incubated for an additional 30 min at 37 °C. After washing with PBS, the stained cells were mounted onto microscope slide in mounting medium (DAKO, Carpinteria, CA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on confocal microscope with red and green filters. JC-1 at low concentration (low $\Delta\psi$) exists mainly in a monomeric from, which emits green fluorescence. JC-1 at a high concentration (high $\Delta\psi$) forms aggregates called "J" complex, which emit red fluorescence (Simeonova, Garstka, Kozioł-Lipińska, & Mostowska, 2004).

2.12. Statistical analysis

All values were represented as means ± standard error of the mean (SEM). The results were subjected to an analysis of ANOVA

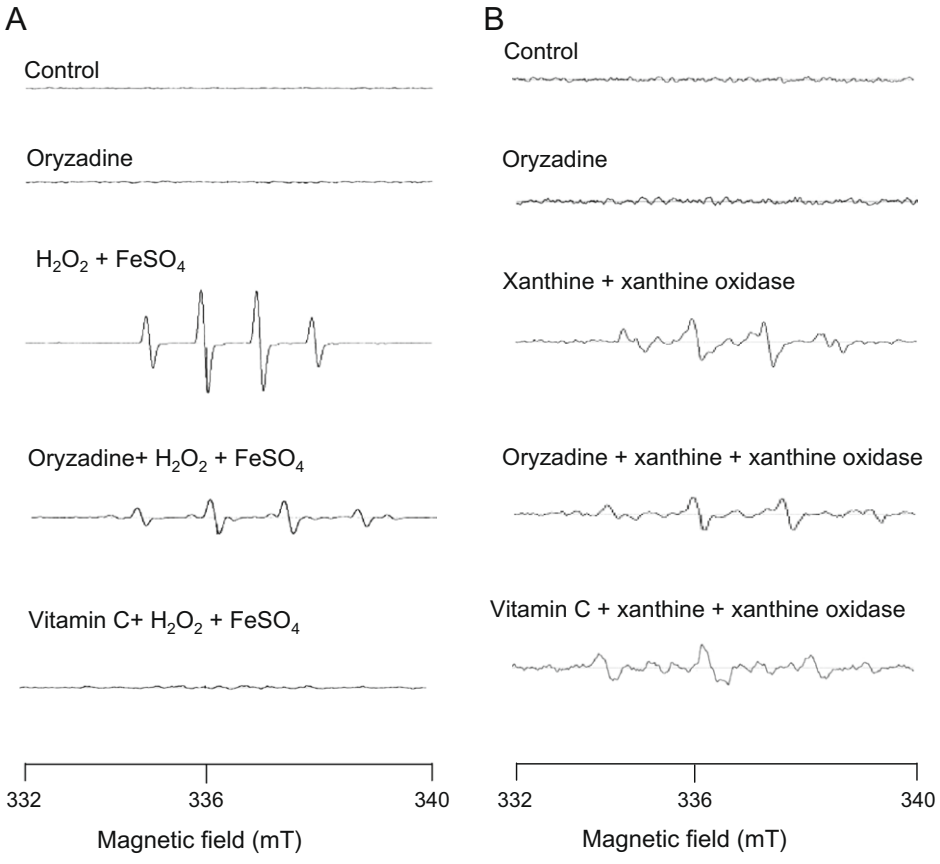

Fig. 2. The efficacy of oryzadine as a scavenger of hydroxyl radical and superoxide radicals. (A) The hydroxyl radicals generated by the Fenton reaction (H_2O_2 + $FeSO_4$) were reacted with DMPO, and the resultant DMPO-OH adducts were detected by ESR spectrometry. (B) The superoxide radicals generated by the xanthine and xanthine oxidase were reacted with DMPO, and the resultant DMPO-·OOH adducts were detected by ESR spectrometry.

using the Tukey test to analyse the differences. $P < 0.05$ was considered significant.

3. Results

3.1. Radical scavenging activity of oryzadine in a cell-free system

The ESR results revealed that the specific signal of the hydroxyl radical was not clearly detected in the control and the 30 μM of oryzadine, however the hydroxyl radical signal increased up to 3467 in the $FeSO_4 + H_2O_2$ system. Oryzadine at 30 μM and vitamin C at 60 μM, which is a well known antioxidant, treatment was found to decrease the hydroxyl radical signal to 956 and 110, respectively (Fig. 2A). Consistent with its hydroxyl radical scavenging activity, oryzadine was also able to reduce the generation of superoxide radicals (Fig. 2B). Oryzadine and vitamin C treatment reduced the superoxide radical level to 811, and 850, respectively, as compared to the value of 1246 for the superoxide radical in the xanthine and xanthine oxidase. From the above results, oryzadine is a better scavenger of hydroxyl radicals than superoxide radicals.

3.2. The effect of oryzadine on intracellular ROS scavenging activity

The intracellular ROS scavenging activity of oryzadine was 26% at 0.3 μM, 56% at 3 μM, 72% at 30 μM, respectively, compared to 75% of vitamin C at 60 μM (Fig. 3A). The level of ROS detected using flow cytometry after DCF-DA staining (Fig. 3B) showed a value of 133 in H_2O_2-treated cells with oryzadine at 30 μM, compared to a value of 1196 fluorescence intensity in only H_2O_2-treated cells. Taken together, these results suggest that oryzadine has an antioxidant effect.

3.3. The effect of oryzadine against H_2O_2-induced cellular components damage

H_2O_2-induced damage of cellular components represents the primary cause of cell viability loss. The effect of oryzadine on the damage of cellular DNA, membrane lipid, and protein in H_2O_2-treated cells was investigated. Cellular DNA breakage induced by H_2O_2 treatment was detected using an alkaline comet assay. Exposure of cells to H_2O_2 increased the tail length and the percentage of DNA in the tails of the cells. Upon exposure to H_2O_2, the percentage of DNA in the tail increased to 46%, and treatment with oryzadine resulted in a decrease to 32% (Fig. 4A and B). The cells exposed to H_2O_2 showed a 30% increase in lipid peroxidation compared to the control, as measured by the generation of TBARS. However, oryzadine treatment was found to decrease the H_2O_2-induced peroxidation of lipids by 10% (Fig. 4C). The formation of protein carbonyl serves as a biomarker for cellular oxidative protein damage. Moreover, the accumulation of oxidatively modified protein carbonyls may abolish cellular function either by the loss of catalytic and structural integrity or by the interruption of regulatory pathways (Stadtman & Levine, 2000). Moreover, the protein carbonyl content in cells after H_2O_2 treatment increased to 44% of protein carbonylation compared to the control, whereas oryzadine decreased the H_2O_2-induced protein carbonyl formation by 25% (Fig. 4D). These results suggest that oryzadine inhibits the H_2O_2-induced damage of cellular components including DNA, membrane lipid, and protein.

3.4. Effect of oryzadine on H_2O_2-induced apoptosis

The protective effect of oryzadine on cell survival in H_2O_2-treated cells was measured by using a MTT test. The cell viability was

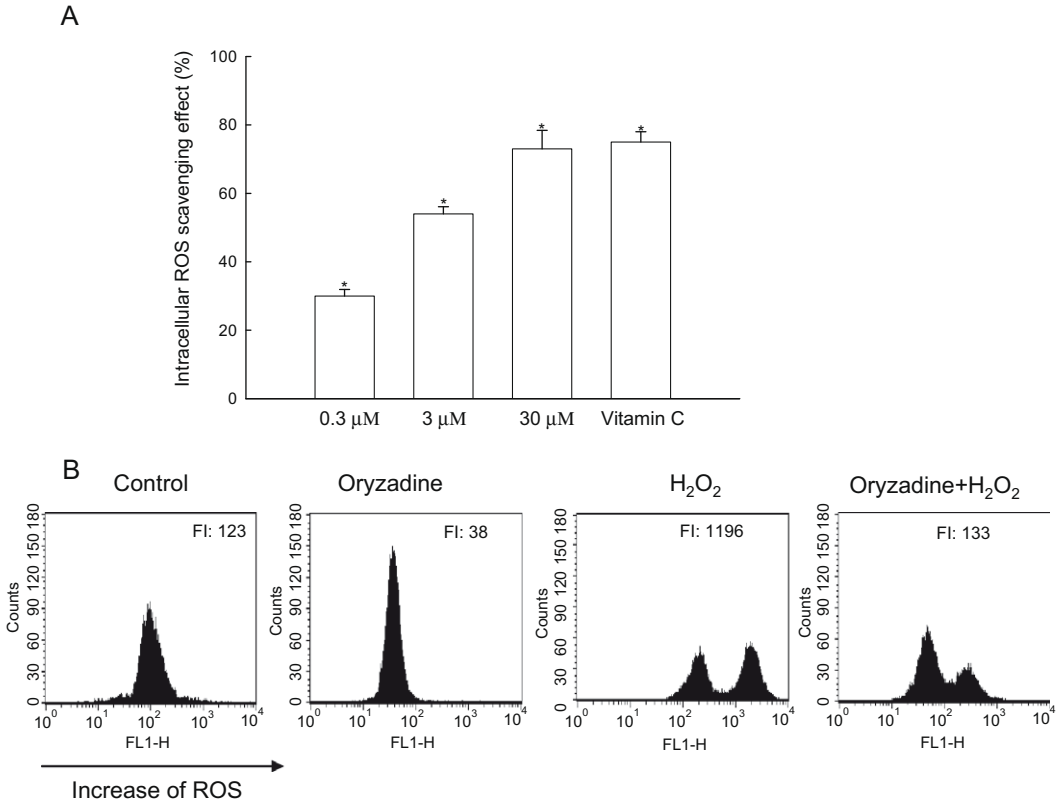

Fig. 3. The efficacy of oryzadine as a scavenger of intracellular ROS. Cells were treated with oryzadine and after 30 min, 1 mM of H_2O_2 was added to the plate. After an additional 30 min, the intracellular ROS generated were detected by spectrofluorometry (A) and flow cytometry (B) after the DCF-DA treatment. FI indicates the fluorescence intensity of DCF-DA. *Significantly different from control cells ($p < 0.05$).

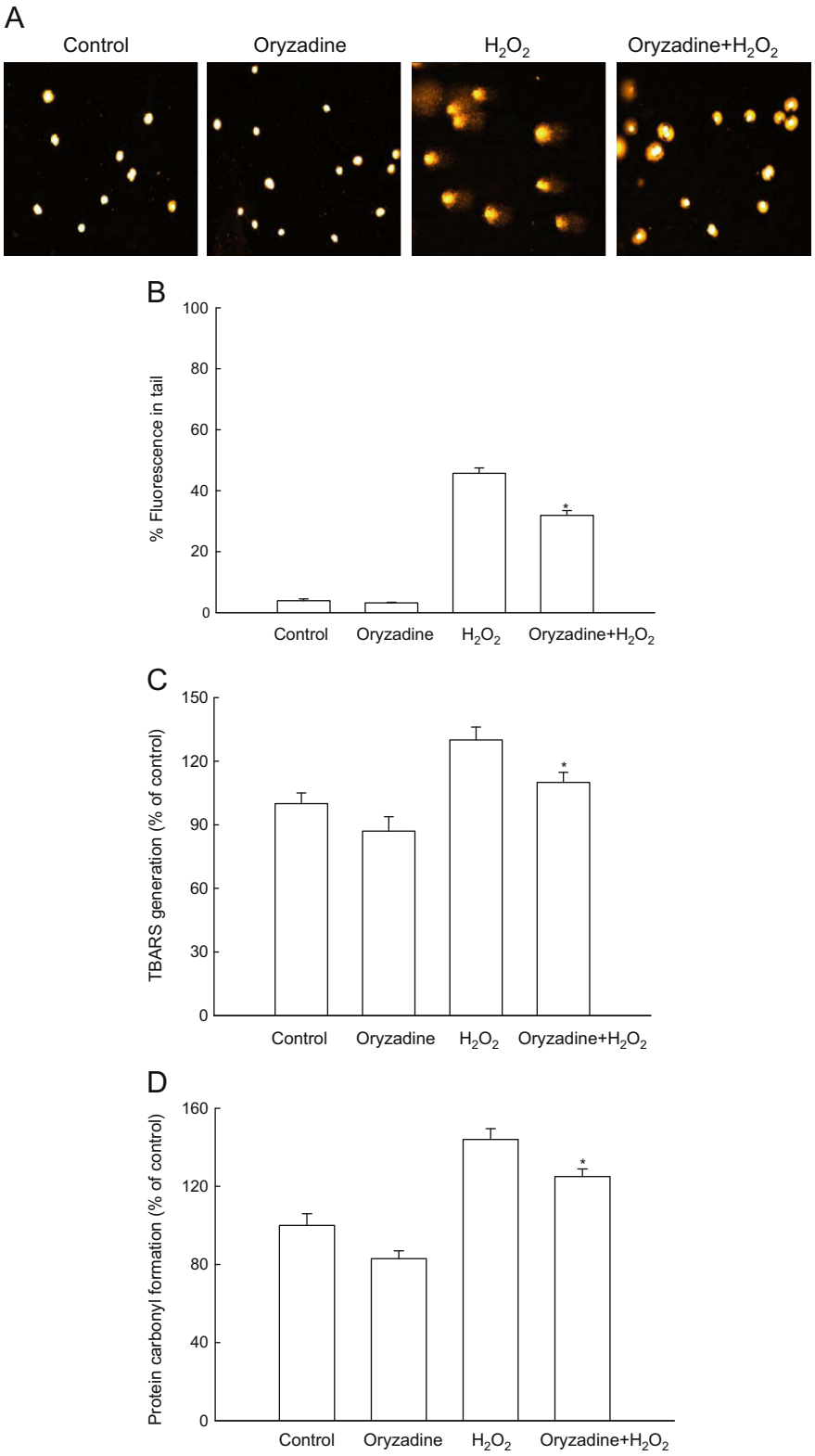

Fig. 4. The protective effect of oryzadine on H_2O_2-inducd DNA damage, lipid peroxidation, and protein modification. (A) Representative images and (B) percentage of cellular DNA damage were detected by an alkaline comet assay. The values are expressed as means ± SEM. *Significantly different from H_2O_2-treated cells ($P < 0.05$). (C) Lipid peroxidation was assayed by measuring the amount of TBARS formation. *Significantly different from H_2O_2-treated cells ($P < 0.05$). (D) The protein oxidation was assayed by measuring the amount of carbonyl formation. *Significantly different from H_2O_2-treated cells ($P < 0.05$).

determined 24 h later by the MTT assay. Treatment with oryzadine increased the cell survival by 70% compared to 52% with the H_2O_2 treatment (Fig. 5A), but oryzadine itself did not show cytotoxicity at 30 μM considering cell survival was 107% as compared to the control. To evaluate the cytoprotective effect of oryzadine on H_2O_2-induced apoptosis, the nuclei of V79-4 cells were stained

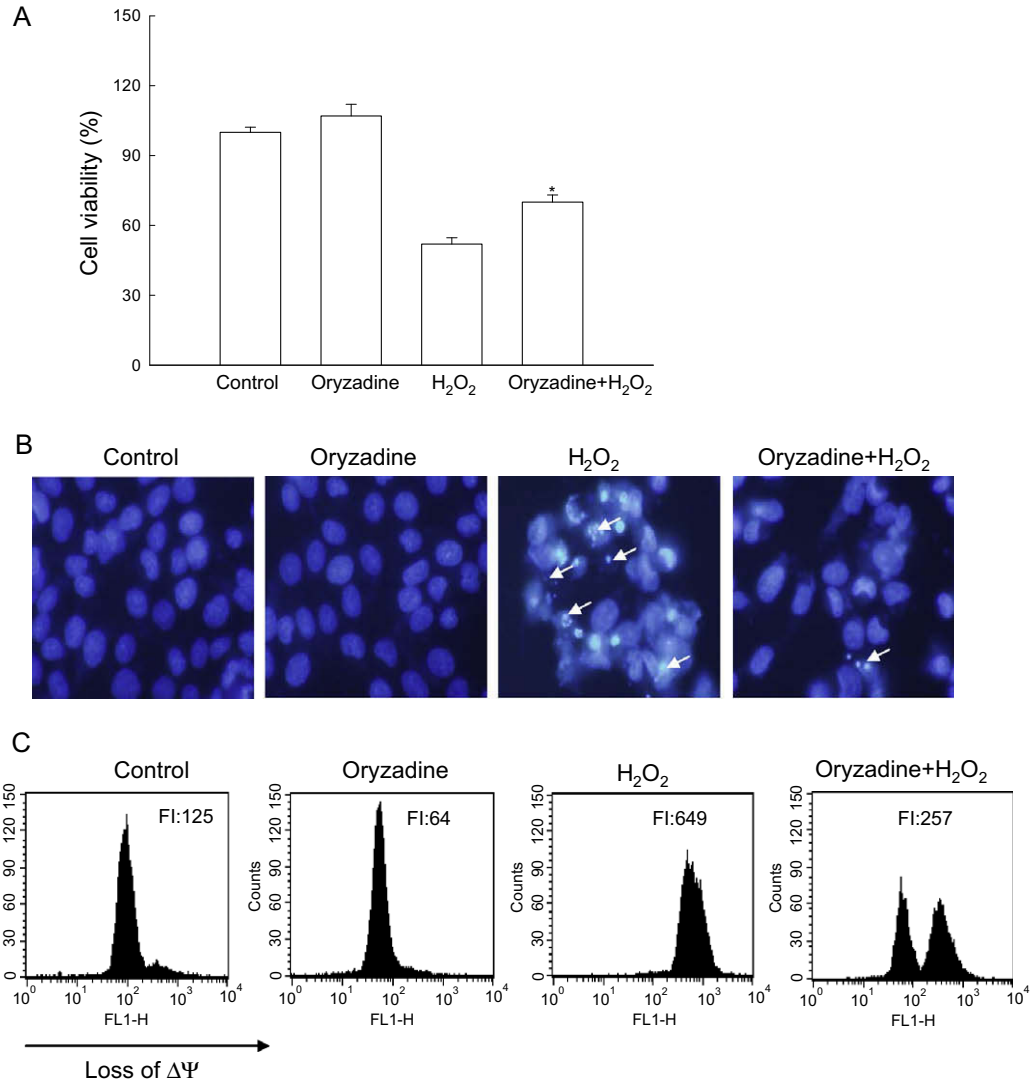

Fig. 5. The protective effect of oryzadine on H_2O_2-induced apoptosis. (A) The viability of V79-4 cells was determined by MTT assay. *Significantly different from H_2O_2-treated cells ($P < 0.05$). (B) Apoptotic body formation was observed under fluorescent microscope after Hoechst 33342 staining. Apoptotic bodies are indicated by arrows. The mitochondrial membrane potential ($\Delta\psi$) was analysed using (C) flow cytometer and (D) confocal microscope after staining cells with JC-1.

with Hoechst 33342 and assessed using microscopy. The images in Fig. 5B show that the control cells had intact nuclei, while the H_2O_2-treated cells showed significant nuclear fragmentation, a distinctive characteristic of apoptosis. However, oryzadine decreased nuclear fragmentation. In addition to the morphological evaluation, the anti-apoptotic effect of oryzadine was also confirmed by a mitochondrial membrane potential ($\Delta\psi$) analysis. The H_2O_2-treated cells resulted in the loss of $\Delta\psi$, as substantiated by an increase of 649 fluorescence intensity in fluorescence (FL-1) with the JC-1 dye (Fig. 5C). However, oryzadine blocked the loss of $\Delta\psi$ in H_2O_2-treated cells to 257 fluorescence intensity. In non-apoptotic cells, the monomer form of JC-1 accumulates as aggregates in the mitochondria, which emits a red fluorescence whereas in apoptotic cells, JC-1 does not accumulate and remains a monomer, which emits a green fluorescence. The control cells and only the oryzadine-treated cells exhibited the strong red fluorescence in the mitochondria (Fig. 5D). However, H_2O_2 treatment resulted in a decrease in red fluorescence in the mitochondria and increase in green fluorescence, suggesting that H_2O_2 treatment disrupts the mitochondrial $\Delta\psi$. Oryzadine at 30 μM decreased the green fluorescence in H_2O_2 treatment, suggesting that oryzadine inhibited the loss of $\Delta\psi$ in response to H_2O_2 treatment.

4. Discussion

In aerobic organisms, the process of maintaining cellular function results in the reduction of oxygen and the corresponding production of the superoxide radicals. Superoxide radicals have unpaired electrons and to return to a stable state, they require an additional electron. They take an electron from an adjacent source, such as DNA, lipid, protein as well as non enzymatic antioxidants such as glutathione, thioredoxin and vitamins C or E (Carmody & Cotter, 2001; Jang & Surh, 2003; Kim et al., 2001). In doing so, superoxide radicals have been shown to damage cells, enzymes, DNA, and cell membranes (Fridovich, 1978). Superoxide radicals may also play an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Nice & Robinson, 1992). Furthermore, superoxide radicals can be more cytotoxic by generating other reactive species, such as hydrogen peroxide, by the addition of one or more electron. Hydrogen peroxide itself is not a radical and is not very reactive; however, it can sometimes be toxic to cells, since it can give rise to destructive species such as the hydroxyl radical and peroxynitrite (Fubini & Hubbard, 2003; Ozsoy et al., 2008). The primary mechanism of hydrogen peroxide toxicity is the formation of highly reactive spe-

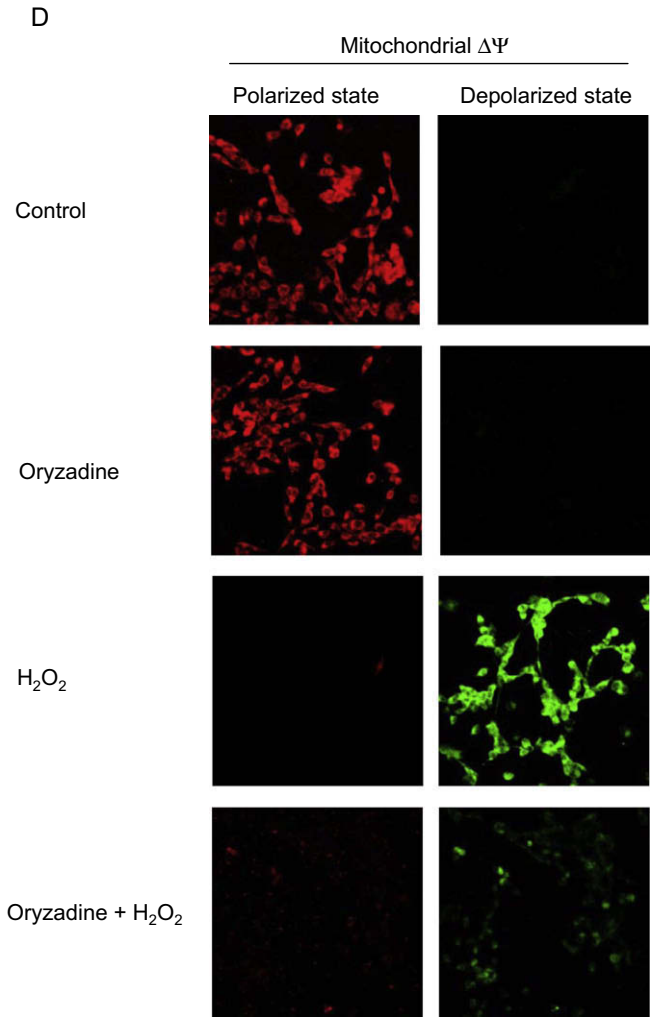

Fig. 5 *(continued)*

cies (hydroxyl radicals) in the presence of transition metal ions or other various mechanisms (Halliwell, Gutteridge, & Cross, 1992). The hydroxyl radical is the most reactive among ROS, and bears the shortest half-life, and induces severe damage to adjacent biomolecules (Sakanaka, Tachibana, & Okada, 2005). Therefore, the evaluation of the scavenging effects of oryzadine on superoxide and hydroxyl radical is one of the most important ways of explaining the mechanism of antioxidant activity. In our data of ESR to the detection of radicals, oryzadine showed an antioxidant effect by directly scavenging the superoxide radical and hydroxyl radical. It is known that the antioxidant mechanism of flavonoids is based on the ability to scavenge radicals and to chelate transition metal like iron, thereby suppressing the hydrogen peroxide-driven Fenton reaction. Chelating properties of flavonoids depend on the structural features like catechol group on the B-ring, 4-carbonyl and 5-hydroxy group, and Fenton-induced oxidation is strongly inhibited by flavonoids with 3′,4′-catechol, 4-carbonyl, and 5-hydroxy groups (Cheng & Breen, 2000; Heim, Tagliaferro, & Bobilya, 2002; Leopoldini, Russo, Chiodo, & Toscano, 2006). Thus iron chelation properties and radical scavenging activity of flavonoids are closely related. However, in the case of oryzadine it might be difficult to find a possible site for iron chelation in its chemical structure. Thus, the antioxidant activity of oryzadine might be related to a scavenging effect rather than chelation. Therefore, further investigation regarding the contribution of iron chelation to the antioxidant activity of oryzadine is required. Oryzadine attenuated H_2O_2-induced damage to the lipid membrane, protein, and DNA, suggesting that oryzadine has cytoprotective properties. The cells exposed to H_2O_2 exhibited the distinct features of apoptosis, including nuclear fragmentation and disruption of mitochondrial membrane potential ($\Delta\psi$). However, cells pretreated with oryzadine had a significantly reduced percentage of apoptotic cells. In conclusion, antioxidant oryzadine prevented the cellular DNA damage, lipid peroxidation, and protein modification against oxidative stress induced by H_2O_2 and protected cells against apoptosis *via* inhibition of mitochondrial disruption.

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