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The Plant-derived Natural Compound Flavin 7® Attenuates Oxidative Stress in Cultured Renal Proximal Tubule Cells

AGOSTON EMBER[1], JEB S. CLARK[2], TIMEA VARJAS[3], ISTVAN KISS[3], ISTVAN EMBER[3], RADHAKRISHNA BALIGA[2] and ISTVAN ARANY[2]

[1]Department of Surgery, and [3]Department of Public Health and Preventive Medicine, University of Pecs, Hungary;
[2]Department of Pediatrics, Division of Pediatric Nephrology, University of Mississippi Medical Center, Jackson, MS, U.S.A.

Abstract. *Background: Cancer therapies and cancer progression can increase oxidative stress that might account for renal toxicity in cancer patients. Flavin 7® (F7) is a natural polyphenol-containing dietary supplement with potential antioxidant activity. Therefore, it might help to attenuate renal toxicity of chemotherapeutics. Materials and Methods: Cultured mouse renal proximal tubule cells were subjected to H_2O_2-mediated oxidative stress. Potential antioxidant effects of F7 were assessed by measuring the production of reactive oxygen species (ROS), mitochondrial depolarization and injury (lactate dehydrogenase release as well as trypan blue exclusion) in cells that were pretreated with F7 prior to treatment with H_2O_2. Results: F7 pretreatment significantly attenuated H_2O_2-induced ROS production, mitochondrial depolarization and consequent injury in renal proximal tubule cells. Conclusion: F7 supplementation might be beneficial for cancer patients in order to prevent renal toxicity of anticancer drug- or cancer progression-related oxidative stress.*

It has been shown that various antitumor therapeutics (1) and tumor progression (2, 3) can increase generation of reactive oxygen species (ROS) that might impose an increased risk for injury of the proximal tubules in the kidney. Indeed, higher incidence of renal failure has been demonstrated in cancer patients (4, 5) and antioxidants have been shown to ameliorate renal toxicity of chemotherapeutics (6-8). Thus, cancer patients might benefit from various antioxidant therapies (9). Plant-derived natural products such as polyphenols have shown renoprotective effects from oxidative stress in humans and animal models (10, 11). Flavin7® (F7) is a fruit extract that contains natural polyphenols in high concentration (220 mg/10 ml). F7 has been advised for use in enhancing the health of those coping with cancer and chemotherapy as it may attenuate side-effects of chemotherapeutics. Due to its high polyphenol content, it might exert these beneficiary effects in cancer patients through antioxidant activities.

Thus, the aim of this study was to determine whether or not F7 demonstrates antioxidant properties in immortalized renal proximal tubule cells that were exposed to oxidative stress.

Materials and Methods

Cell culture and treatment. Immortalized mouse proximal tubule cells (TKPTS-gift from Dr. Reuss) (12) were grown in 5% CO_2 atmosphere at 37˚C as described elsewhere (13). Oxidative stress was induced by treatment of semi-confluent cells with 400 μM H_2O_2 for 24 hours similar to the model described elsewhere (14). In certain experiments cell cultures were pretreated with 50 μl/ml F7 (Crystal Institute Kft, Eger, Hungary) for 30 minutes prior to treatment with H_2O_2. This dose of F7 was found to be non-toxic by us and others in previous experiments (15, 16).

Assessment of cell injury. Cell morphology was determined by a phase-contrast inverted microscope (Nikon Eclipse TS-100F; magnification: ×100). The number of surviving cells was determined by trypan blue exclusion and expressed as a percentage of that of untreated cells. Cell injury was assessed by the fluorescent CytoTox-One Homogenous Membrane Integrity assay kit (Promega, Madison, WI, USA). Accordingly, 24 hours after the appropriate treatment lactate dehydrogenase (LDH) content of the cell growth medium and the monolayer was determined according to the manufacturer's recommendation. LDH release was calculated as a percentage of LDH content in the medium compared to the total LDH content.

ROS production assay. The intracellular generation of ROS was determined by a microplate assay using the oxidant-sensitive 2',7'-dichlorofluorescein-diacetate (DCFDA; Invitrogen, Grand Island, NY, USA). Accordingly, cells were loaded with 100 μM DCFDA for 30 minutes at 37˚C. After washing with Hank's balanced salt solution

Correspondence to: Istvan Arany, 2500 N. State St. Research Wing, Room R129B, University of Mississippi Medical Center, Jackson, MS 39216, U.S.A. Tel: +1 6018159464, Fax: +1 601984598, e-mail: iarany@ped.umsmed.edu

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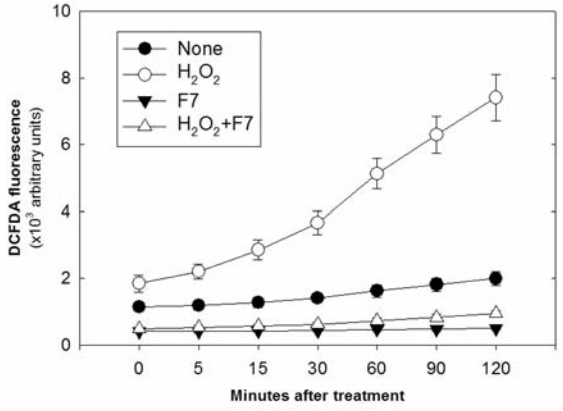

Figure 1. *ROS production in mouse renal proximal tubule cells after treatment with H_2O_2 in the presence or absence of F7. TKPTS cells were loaded with the oxidant-sensitive fluorescent dye DCFDA as described in Materials and Methods and pretreated with 50 μl/ml F7 for 30 minutes prior to treatment with 400 μM H_2O_2. Untreated, F7- and 400 μM H_2O_2-treated controls were also included. ROS production was determined as an increase in DCFDA fluorescence as measured in a fluorescence plate reader at 485nm$_{exc}$/530nm$_{em}$. All experiments were performed in triplicate and results expressed as mean±S.D.*

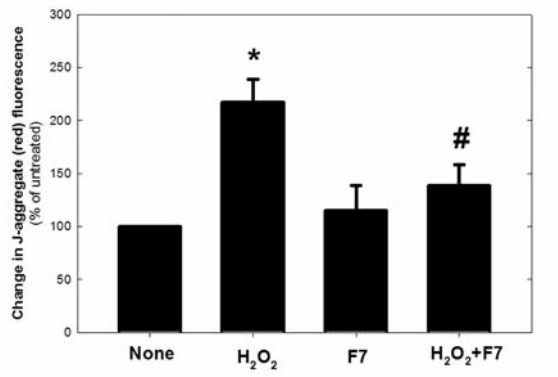

Figure 2. *Mitochondrial depolarization in renal proximal tubule cells after treatment with H_2O_2 in the presence or absence of F7. TKPTS cells were loaded with the mitochondrial polarity-sensing dye JC-1 (see Materials and Methods) and treated with 50 μg/ml F7 for 30 minutes then 400 μM H_2O_2 was added. Untreated, F7- and H_2O_2-treated cells were also processed. Mitochondrial depolarization was calculated as decrease in J aggregate fluorescence/5×10^5 cells/10 minutes and expressed as a percentage of that of untreated cells. N=3, mean±S.D., *p<0.05 compared to untreated control; #p<0.05 compared to H_2O_2-treated group.*

(HBSS) cells were isolated by trypsinization, counted and 5×10^5 cells were placed in wells of a 96-well plate. The following groups were set up: i) untreated cells; ii) cells treated with 400 μM H_2O_2; iii) cells incubated with 50 μg/ml F7; and iv) cells incubated with 50 μg/ml F7 for 30 minutes then 400 μM H_2O_2. DCFDA fluorescence was detected at various time points (between 0 and 120 minutes) in a fluorescence plate reader (Packard) at 485nm$_{exc}$/530nm$_{em}$.

Assessment of mitochondrial depolarization. JC-1 (Invitrogen) is a fluorescent dye that accumulates potential-dependently in mitochondria. In polarized mitochondria, J aggregates are formed that exhibit red fluorescence. Conversely, depolarization of mitochondria is indicated by a decrease in red fluorescence. Cell suspensions in HBSS were loaded with 2 μM JC-1 for 30 minutes at 37˚C. After washing with HBSS buffer cells were seeded in 96-well plates (5×10^5 cells/well) and treated with 50 μg/ml F7 for 30 minutes followed by 400 μM H_2O_2. Untreated cells, F7- and H_2O_2 -treated cells were also included as different controls. The J aggregate fluorescence was monitored in a fluorescence plate reader at 530nm$_{exc}$/590nm$_{em}$ according to the manufacturer's recommendation. Mitochondrial depolarization was calculated as the decrease in J aggregate fluorescence/5×10^5 cells/10 minutes and expressed as a percentage of that of untreated cells.

Statistical evaluation. Statistical differences between the treated and control groups were determined by Student's *t*-test. Differences between means were considered significant if $p<0.05$. All analyses were performed using a SigmaStat 3.5 software package (Systat, San Jose, CA, USA).

Results

F7 Attenuates H_2O_2-induced ROS production. As shown in Figure 1, H_2O_2 treatment significantly increased ROS production (DCFDA fluorescence) compared to the control (untreated) cells. While F7 treatment alone did not significantly affect ROS production, pretreatment with F7 prior to treatment with H_2O_2 virtually quenched ROS release.

F7 Attenuates H_2O_2-mediated mitochondrial depolarization. The source of the H_2O_2-mediated ROS production in renal proximal tubule cells is the mitochondria (manuscript in preparation). It has been well established that increased mitochondrial ROS release results in mitochondrial depolarization and consequent cell injury (17, 18). Thus, we determined whether the H_2O_2-mediated ROS production was associated with depolarization of the mitochondria using the mitochondrial polarity-sensing fluorescent dye (JC-1) as described in Materials and Methods. As seen in Figure 2, H_2O_2 treatment significantly ($p<0.05$) depolarized the mitochondria as assessed by decrease in J aggregate fluorescence. Importantly, while F7 itself did not change mitochondrial polarity, pretreatment with F7 significantly ($p<0.05$) attenuated H_2O_2-induced mitochondrial depolarization.

F7 attenuates H_2O_2-induced cytotoxicity and consequent cell death. In this set of studies, we evaluated whether attenuation of H_2O_2-mediated ROS production and mitochondrial depolarization by F7 would affect survival of cells that undergo necrotic death in the oxidative injury model (14). First, the effects of F7 on H_2O_2-induced cytotoxicity were assessed by LDH release 24 after treatment. As shown in Figure 3, H_2O_2 treatment greatly increased LDH release, which was significantly reduced ($p<0.05$) in cells that were pretreated with F7 prior to

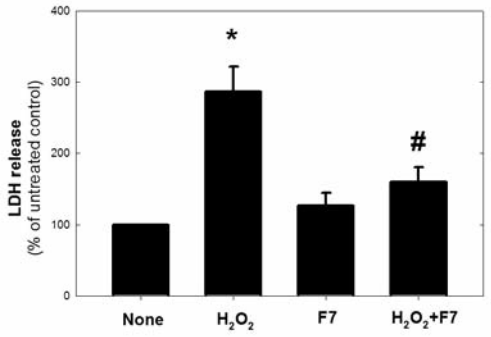

Figure 3. *LDH release in renal proximal tubule cells after treatment with H_2O_2 in the presence or absence of F7. TKPTS cells grown in 12-well-plates were pretreated with 50 μg/ml F7 for 30 minutes then 400 μM H_2O_2 was added: some wells received F7 or H_2O_2 treatment only, or were left untreated. After 24 hours' incubation at 37˚C, LDH release was determined as described in Materials and Methods. Each experiment was carried out in triplicate: values are expressed as percentage of untreated control (N=3, mean±S.D.) *p<0.05 compared to the untreated control; #p<0.05 compared to H_2O_2-treated group.*

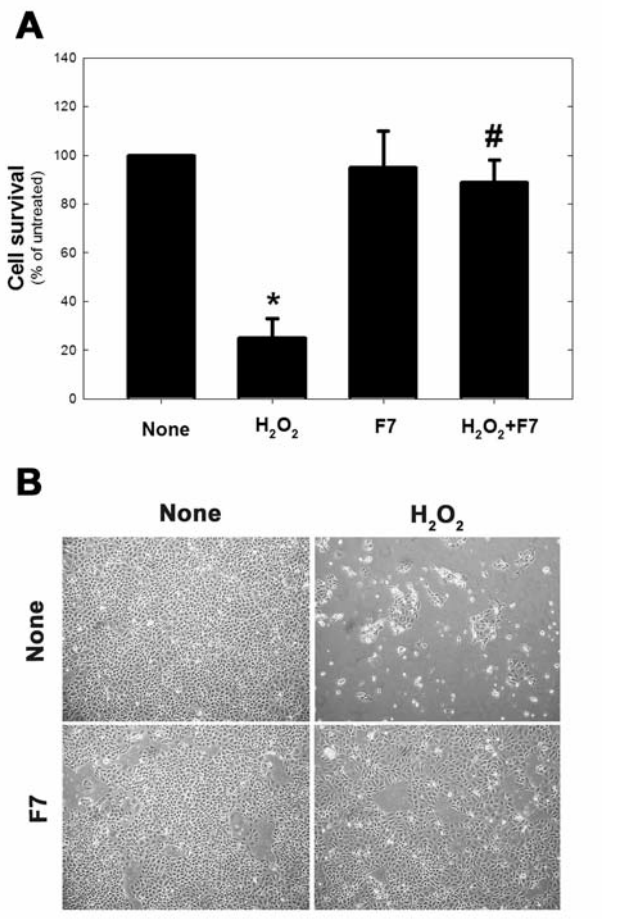

Figure 4. *Morphology (A) and surviving cell counts (B) of TKPTS cells after treatment with H_2O_2 in the presence or absence of F7. A, TKPTS cells were pretreated or not with 50 μl/ml F7 for 30 minutes prior to treatment with 400 μM H_2O_2. After 24 hours' incubation at 37˚C, images were taken (magnification: ×100). Images shown are representative of three independent experiments. B, Cell numbers were determined by trypan blue exclusion in cells that were treated in (A). Cell survival was expressed as cell counts of untreated values. *p<0.05 compared to the untreated control; #p<0.05 compared to H_2O_2-treated group.*

treatment with H_2O_2. F7 itself did not change LDH release significantly. We also showed that cell morphology and the number of surviving cells significantly ($p<0.05$) changed after treatment with H_2O_2, consistent with a necrotic cell death as described earlier (14). While F7 treatment itself did not change either cell count or morphology, pretreatment with F7 virtually protected TKPTS cells from H_2O_2-mediated cell death (Figure 4).

Discussion

Generation of ROS plays an important role in the development of kidney diseases (19). Cancer therapies and cancer progression have shown to be associated with increased ROS production (2, 3), with potential involvement of renal damage. Therefore, antioxidants and ROS scavengers should prevent oxidative stress and consequent renal damage (20-22). Indeed, some plant-derived flavonoids showed protection against oxidative injury of the kidney [reviewed in (21)]. F7 is a natural bioflavonoid-containing dietary supplement with potential antioxidant activity. Our results showed that F7, indeed, significantly reduced ROS production triggered by H_2O_2 treatment in cultured renal proximal tubule cells (Figure 1).

Mitochondrial dysfunction is identified in either the etiology or underlying pathology of a variety of renal diseases that are associated with oxidative stress (23, 24). The elevated ROS production contributes to the opening of the mitochondrial permeability transition pore (25) that rapidly depolarizes the mitochondrial membrane: a process termed mitochondrial permeability transition. Mitochondrial permeability transition is a major contributor to apoptotic and necrotic cell death in a variety of cell types, including renal cells (26). Here, we showed that increased ROS production initiated by H_2O_2 treatment *in vitro* increased mitochondrial depolarization (Figure 2) and consequent injury as demonstrated by increased LDH release (Figure 3), changes in cell morphology (Figure 4A) and decrease in cell counts (Figure 4B). Importantly, pretreatment of cells with F7 attenuated depolarization of mitochondria (Figure 2) and LDH release (Figure 3). As a result of F7 pretreatment cells were virtually protected from H_2O_2-induced cell death (Figure 4).

These experiments suggest that F7 protects renal proximal tubule cells from oxidative injury from ROS, preventing the consequent mitochondrial depolarization. Thus, F7

supplementation might be beneficial for cancer patients in order to prevent renal toxicity of chemotherapy. Whether this effect is due to activation of antioxidant defenses or direct inhibition of ROS production needs further evaluation.

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