Oxidative Medicine and Cellular Longevity

Xenobiotics, Oxidative Stress, and Antioxidants

Lead Guest Editor: Fatma M. El-Demerdash
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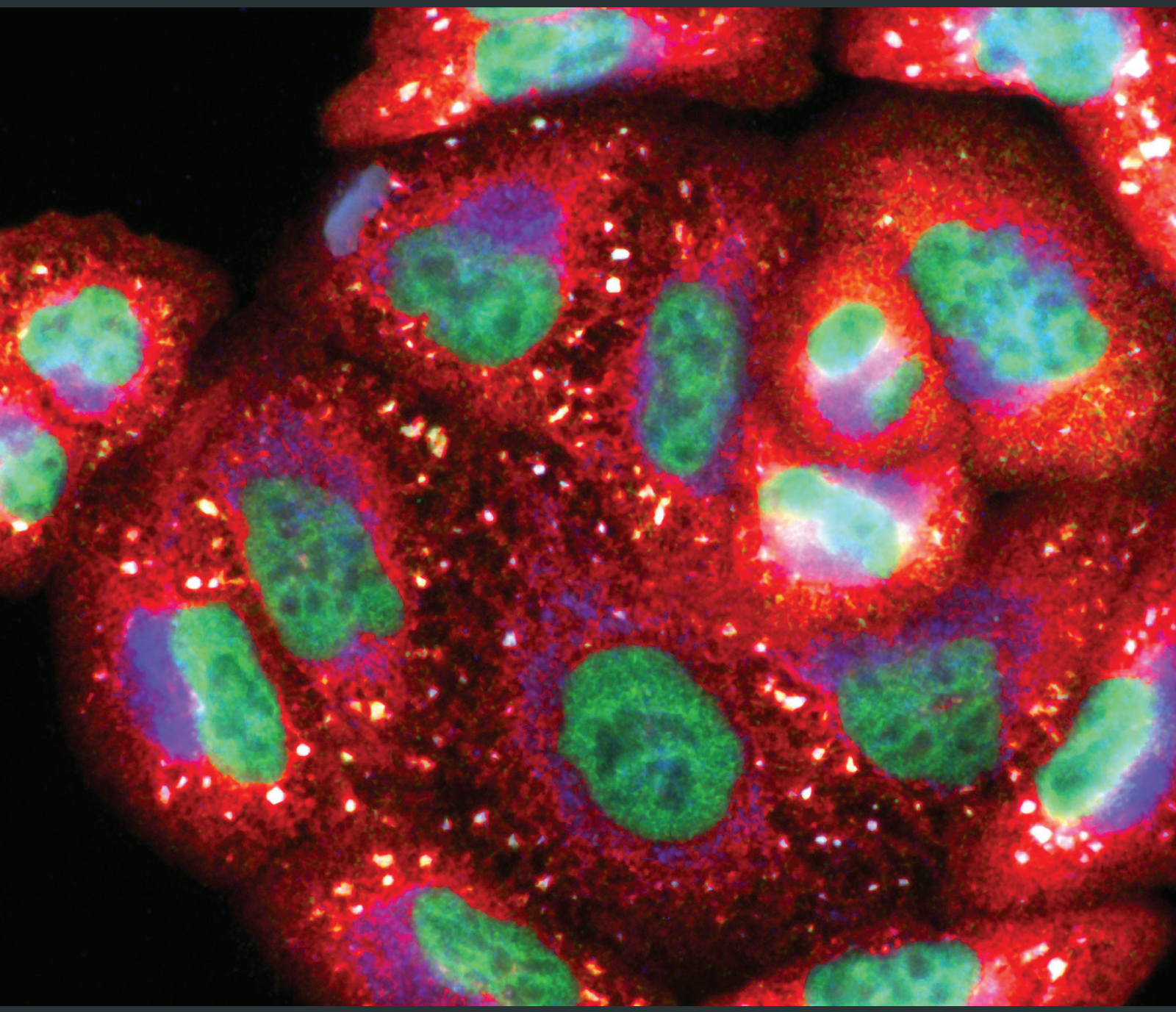

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Editorial Board

Contents

Hindawi
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Editorial

Xenobiotics, Oxidative Stress, and Antioxidants

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Human and animals are exposed to large number of biological and environmental hazards. Free radicals and oxidant species may act as deleterious and toxic products, involved in cellular and organ dysfunction. Overproduction of these species may result in DNA, lipid, and protein damage. Nevertheless, low or moderate concentrations of reactive oxygen species (ROS) or reactive nitrogen species (RNS) are also involved in physiological responses as part of signaling processes and defense mechanisms. Oxidative stress is arguably the most common mechanism in the toxicology of environmental agents. Cells are equipped with multiple complementary energy-dependent systems for maintaining redox homeostasis in the face of environmental oxidative stress. The cell has several means available to tackle free radical generation including antioxidants and antioxidant enzymes. There is a growing interest of natural products in human diet. Increased consumption of plant-based, antioxidant-rich foods including, fruits, vegetables, whole grains, and nuts is associated with the reduced risk of several chronic diseases. In addition, a great number of spices and aromatic herbs contain chemical compounds exhibiting antioxidant properties since they have a variety of active phytochemicals including vitamins, carotenoids, terpenoids, alkaloids, flavonoids, lignans, simple phenols, and phenolic acids. In this special issue on Xenobiotics, Oxidative Stress, and Antioxidants, five papers are included.

In this issue, K. Michalak et al. addressed that the main directions of possible effective treatment of fluoroquinolone-associated disability (FQAD) to reduce oxidative stress, restoring reduced mitochondrion potential, supplementation of uni- and bivalent cations that are chelated by FQs. FQAD may also ineffectively transported to the cell, stimulating the mitochondrial proliferation, removing fluoroquinolones permanently accumulated in the cells, and regulating the disturbed gene expression as well as enzyme activity. G. Murtaza et al. reported that neurodegeneration and cancer diseases are associated with aging, which is affected by many genetic and environmental factors. Healthy aging conceives human longevity, possibly due to carrying the defensive genes. FOXO (forkhead box O) genes are involved in the multiple cellular pathways, which regulate growth, stress resistance, metabolism, cellular differentiation, and apoptosis in mammals. D. Yuan et al. evaluated the antioxidant and anti-inflammatory effects of *Eucommia ulmoides flavones* (EUF) using diquat-challenged piglet models. They showed that EUF supplementation improved the growth performance of diquat-treated piglets from day 14 to 21. Diquat also induced oxidative stress and inflammatory responses and impaired intestinal morphology. EUF alleviated these negative effects induced by diquat to decease serum concentrations of pro-inflammatory cytokines and increase antioxidant indexes as well as anti-inflammatory cytokines on day 14. They indicated that EUF attenuated the inflammation and oxidative stress of piglets caused by diquat injection. T. Zhang et al. demonstrated that avenanthramides, a group of

diphenolic acids found only in oats are bioavailable to human, have anti-inflammatory and antioxidant effects as a useful natural phytochemical in sports science and chronic disease prevention. L. Lin et al. showed that *N*-hydroxycinnamoylphenalkylamides (36H) is an effective skin-whitening agent that has the potential for cosmetic applications due to the biofunctions of antioxidation and melanin suppression.

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Research Article

36H: A Novel Potent Inhibitor for Antimelanogenesis

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N-Hydroxycinnamoylphenalkylamides (36H) exhibited both antioxidation and antityrosinase abilities. The compound was studied for its antioxidative properties, using a 1,1-diphenyl-2-picrylhydrazul- (DPPH-) scavenging test, a ferric ion-reducing antioxidant power assay (FRAP) assessment, and a metal-chelating power assay. The results showed that 36H had antioxidative capabilities in the DPPH-scavenging and ferric-reducing power examinations but the chelating power assay did not demonstrate antioxidative capability. 36H was also measured for tyrosinase inhibitory activity applying various species platforms, including in vitro mushroom, B16F10 mouse melanoma, and human melanocyte cells. In terms of in vitro mushroom tyrosinase suppression, 36H restrained the melanogenesis processes. It is assumed that 36H blocked the tyrosinase active site as a competitive inhibitor for mushroom tyrosinase, hence not decreasing the human normal melanocyte cellular viability. A quantitative real-time polymerase chain reaction (qRT-PCR) and western blot discovered that 36H downregulated melanogenesis-related RNA and proteins, including pigment production (MITF, tyrosinase, TRP-1, and TRP-2), melanosome maturation (Rab27a), and melanosome transportation (Myo5a, MLPH and Mreg). Overall, 36H displayed the biofunctions of antioxidation and melanin suppression, so there was a possibility for its application as a food additive or a skin-whitening agent.

1. Introduction

The human skin is the largest organ in the human body; it maintains bodily functions and prevents the loss of water, electrolytes, and biomolecules. The skin is composed of the epidermis, the dermis, and the hypodermis. The epidermis is further divided into five separated layers: the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum, and the stratum basale [1]. The base layer of the epidermis, which is connected to the dermis, is the stratum basale that contains melanocytes. Melanocytes have dendrites to transfer melanin to keratinocytes, which

determine the surface of the skin color due to the melanin content within these keratinocytes [2].

Free radicals are composed of an atom or a group of atoms that have one or more unpaired electrons. They are involved in physiological, metabolic, and immune reactions and signal transfer functions. Although they are a part of the normal healthy biochemical processes in the body, they also are responsible for damage. Reactive oxygen species (ROS) and various free radicals are stimulated by the generation of superoxide anions or hydrogen peroxides. This can cause confused messaging, damage to cell membranes, and damage to ion cell communications due to lipid peroxidation. This affects intracellular molecules that feature SH groups and acting proteins and DNA [3]. The amount of ROS is controlled by the self-defense systems that are antioxidant-mediated in their normal state, such as antioxidants. These include vitamin C, vitamin E, or glutathione [4], which scavenge free radicals to prevent cellular damage. However, they disrupt the balance of the cellular oxidation state which results in too many ROS. Previous studies have shown that many degenerative diseases, such as aging and cancer, are caused by active oxygen species or free radicals. The antioxidant properties suggest that the peroxidation of lipid within melanocyte membranes increases the content of intracellular glutathione, which may account for the depigmentation [5, 6].

Darkening of the skin, eyes, and hair is caused by the synthesis of melanin, which is a pigmented biopolymer that is synthesized in the melanosome of melanocytes. Melanin is a protective mechanism against damage by UV light [7]. Congenital genes can affect the skin color, but UV radiation induction, inflammation, and hormonal changes cause melanocytes to synthesize more melanin. Nevertheless, the overproduction of melanin can induce pigment disorders, such as melasma, senile lentigo, freckles, and hyperpigmentation [8]. These are usually treated using cosmetics or pharmaceutical ingredients that contain skin-whitening components. Even though these elements are from natural sources, only some agents are used in cosmetics or medicines because of safety concerns or the concerns of whitening effectiveness. Melanogenesis involves the binding of the α-melanocyte-stimulating hormone to the melanocortin-1, which increases cyclic adenosine monophosphate (cAMP) and activates microphthalmia-associated transcription factors (MITF) [9]. MITF upregulates the expression of a melanogenic enzyme, tyrosinase, which serves an important function in the hydroxylation of L-tyrosine to dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to dopaquinone [10]. Medical and cosmetic treatments are increasingly using tyrosinase activity inhibitors. Melanogenesis production and the transfer of melanosomes from melanocytes to keratinocytes are necessary for the coloration of skin [11]. Melanosomes are tethered to the actin cytoskeleton at the periphery of melanocytes via the tripartite complex formed by Ras-related protein Rab-27 (Rab27a), melanophilin (MLPH), and myosin Va (Myo5a). Rab27a is a membrane-bound protein involved in protein transportation and small GTPase-mediated signal transduction. Melanophilin forms a ternary complex with Rab27a in the GTP-bound site with Myo5a which is the motor protein. The visible mammal pigmentation in the hair and skin is according to this tri-protein complex to tether the pigment-producing organelles. If there is a deficiency in the complex protein, the connection between the F-actin and melanosome is broken down [12].

Within the positive therapeutic potential compounds, *N*-hydroxycinnamoylphenalkylamides (36H) are reported to have an inhibitory effect on the expression of matrix metalloproteinases- (MMP-) 9 in THP-1, a human leukemia monocytic cell line, which is stimulated by tumor necrosis factor-α (TNF-α) [7]. A previous study showed that the induced TNF-α expression of MMP-9 for both protein and mRNA levels was fully inhibited in a concentration-dependent manner (1–20 μM). It has been found that 36H markedly suppressed nuclear factor-κ light polypeptide gene enhancer in B cell (NF-κB) signaling as detected by the NF-κB reporter gene assay but had no effect on the degradation of (nuclear factor of κ light polypeptide gene enhancer in B cell inhibitor α (IκBα) or the translocation of NF-κB. Chromatin immunoprecipitation data illustrated that the affiliation between MMP-9 and NF-κB p65 subunit (p65) promoter gene was entirely negated by 36H and the phosphorylation of p65 was not influenced. In general, a previous report demonstrated that 36H suppressed MMP-9 secretion by the nuclear-targeted downregulation of NF-κB pathway mechanisms in THP-1. 36H is an analogue of caffeic acid phenethyl ester (CAPE), which is a known suppressor of cancer metastasis and invasion [14]. The antioxidant activities and free radical-scavenging of some CAPE analogues have been studied, and the results of those studies prompted this study to determine whether 36H reduced tyrosinase activity and downregulated melanogenesis-related RNA and proteins. This study finds that 36H is a potential and positive antioxidant and a skin-whitening agent.

2. Materials and Methods

2.1. Reagents and Materials. Dimethyl sulfoxide (DMSO) and L-tyrosine were obtained from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). All reagents and alternative buffers were acquired at the highest commercial purity.

2.2. Chemical Synthesis of 36H. 36H was provided by Professor Yueh-Hsiung Kuo, who used the subsequent methods from amide-binding coupling to obtain the compounds. Benzotriazol-1-yloxytris (dimethylamino)-phosphonium hexafluorophosphate (BOP) was combined with a mixture of R2-NH2, R1-COOH, and triethylamine (Et3N) in dimethylformamide (DMF). The reaction solution was mixed for 30 min at 0°C and then blended at 25°C for 2 hrs. When the chemical liquid had been evaporated, the residue was divided between H_2O and ethyl acetate (AcOEt). The residue was then filtered and purified using column chromatography with an eluting solution (AcOEt–CH_2Cl_2, 1 : 1, *v*/*v*) on silica gel (70–230 and 230–400 mesh, Merck 7734). The products were recrystallized from AcOEt to obtain the best

and most ideal crystals. A Finnigan TSQ-46C mass spectrometer was used for the electron impact mass spectrometry (EIMS). 1H and 13C NMR spectra were obtained using a Bruker Avance 500 spectrometer. A Nicolet Magna-IR 550 spectrophotometer recorded the IR spectra. 36H was initially dissolved in DMSO solution before the following studies. For each assay, DMSO with a constant and final concentration of 0.2% (*v*/*v*) was used. This compound has not been known as an antioxidant or skin whitener [7, 13], and the structure is shown in Figure 1. Samples were dissolved in DMSO, and various concentrations were prepared with a final DMSO concentration less than 0.5%.

2.3. Assay of DPPH Radical-Scavenging Capacity. DPPH is 2,2-diphenyl-1-picrylhydrazyl, which becomes dark purple when it has a free radical. When the antioxidant scavenges the radical from DPPH˙, it reduces DPPH˙ to a stable DPPH, which has a light-yellow color [14]. Various dosages of the 36H (2 μL in total volume) were combined in 98 μL of DPPH (60 μM) mixture, and the plate was examined using a 517 nm enzyme-linked immunosorbent assay (ELISA) spectroscopic reader. L-ascorbic acid was used as the positive control. The ratios of residual DPPH were mapped beside the sample to determine the amount of antioxidant that decreases the former DPPH concentration. The scavenging property (%) was determined as

$$\text{Scavenging activity}\,(\%) = \frac{\left(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}\right)}{\text{OD}_{\text{control}}} \times 100\%. \quad (1)$$

2.4. Assay of Reducing Power. The ferric ion-reducing antioxidant power assay (FRAP) is often applied to evaluate the antioxidative capability of beverages, foods, fruits, and nutritional supplements, including flavonoids or polyphenols. Various dosages of 36H were introduced into a 67 mM phosphate buffer mixture (0.085 mL, pH 6.8) and 20% potassium ferricyanide [$K_3Fe(CN)_6$, 2.5 μL). The solution reacted for 20 min at 50°C and then trichloroacetic acid (10%, 0.16 mL) was added into the solution, before it was centrifuged at 3000*g* for 10 min [14]. The solution supernatant (75 μL) was combined with 2% $FeCl_3$ (25 μL), and then an ELISA spectroscopic reader measured the resulting absorbance at 700 nm, for a 96-well plate. Butylated hydroxyanisole (BHA) was employed as the positive control. The greater the reductive qualification, the stronger is the absorbance.

2.5. Assay of Metal-Chelating Activity. Chlorophyll's ion-chelating ferrous potential was measured using the technique of Chen et al. [14]. In brief, specific dosages of the samples were dissolved into DMSO and were introduced into a mixture of $FeCl_2{\cdot}4H_2O$ (2.0 mM, 0.05 mL). The addition of ferrozine (5 mM, 0.2 mL) initiated the reaction when the solution was forcefully shaken and then left for 10 min at 25°C. When the reaction had reached equilibrium, the absorbance values for the solution were determined at 560 nm using a blank vehicle. EDTA was used as the positive control. The calculation formula for the chelating activity was identical to (1).

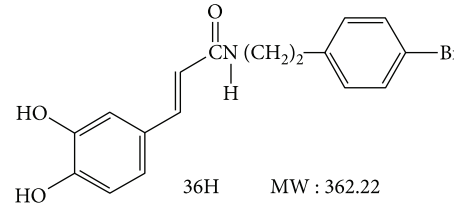

Figure 1: The chemical structure of *N*-hydroxycinnamoyl phenalkylamides (36H).

2.6. Assay of the Mushroom Tyrosinase Activity. Both the mushroom tyrosinase activity suppression and the cellular tyrosinase inhibition were spectrophotometrically determined using the previous method, with minor modifications [2]. Kojic acid was used as the positive control for the tyrosinase activity assay. The materials were first dissolved in aqueous DMSO and cultured in L-tyrosine (2.5 mg/mL) with phosphate buffer (50 mm, pH 6.8). All models used DMSO, which is irrelevant to tyrosinase activity when DMSO constitutes <0.5% of the total volume. Subsequently, 25 U/mL of mushroom tyrosinase with an identical buffer was then added, and the solution was incubated at 37°C for 30 min. An ELISA spectroscopic reader was used to conduct the absorbance measurements for the assays at 475 nm, using 96-well microplates (Molecular Devices, CA, USA).

2.7. Human Melanocyte Cell (HMC) Cultures. Primary human epidermal melanocytes from neonatal foreskin were acquired from Cascade Biologics. This was also applied by Wang et al. [2]. It was cultured in Medium 254 (M-254-500; Cascade Biologics, Portland, OR, USA) and enhanced with human melanocyte growth supplement (HMGS, cat. number S-002-5). Medium 254 is a basal medium with some nonessential and essential vitamins, organic compounds, amino acids, inorganic salts, and trace minerals. The human melanocyte growth supplement included fetal bovine serum, bovine insulin, bovine pituitary extract, bovine transferrin, hydrocortisone, basic fibroblast growth factor, phorbol 12-myristate 13-acetate, and heparin. All cells were incubated at 37°C in a humidified incubator with 5% CO_2 atmosphere.

2.8. Determination of Cell Viability of HMC. Cell viability was determined using a MTT assay [3]. When a yellow tetrazole is combined with the mitochondrial dehydrogenase (ubiquinone) in living cells, the tetrazolium rings of MTT cleave and reduce to purple formazan. The cells were seeded at a density of 9×10^3 cells/well in 96-well plates. When the cells had been cultured for a day, the medium was changed, and different concentrations of 36H were added into a final medium volume of 100 μL. After these had incubated for 48 hrs, the medium was substituted with a fresh medium (100 μL), including MTT (0.5 mg/mL). The plate was then cultured in an incubator at 37°C for 2 hrs with 5% CO_2. DMSO (100 μL) was then added to each well to dissolve the purple formazan crystals. To ensure that the dishes achieved maximum dissolution, the dishes were gently shaken and mixed

in the dark for 10 min and measured at 595 nm. Cell growth was calculated as

$$\text{Cell growth}(\%) = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100\%. \quad (2)$$

2.9. Measurement of Tyrosinase Activity from HMC. To determine the tyrosinase activity in human melanocyte cells (HMC) (5×10^4 cells per well), they were positioned in 12-well plates in 1000 μL of medium with different dosages of 36H and cultured for 48 hrs [2]. PBS buffer was used to wash the sample-treated cells, and these were then lysed with 1% Triton X-100/PBS. The enzyme extract was then collected from the resulting cellular lysate and combined with 50 μL of 2 mM L-tyrosine. The extracts were then incubated for 3 hrs at 37°C in darkness. A spectrophotometer was then used to determine their absorbance at 490 nm.

2.10. Determination of the Melanin Content in HMC. With some minor modifications, the previous technique was also used for this experiment [2]. Cell pellets were dissolved in 2.0 N NaOH with 10% DMSO and heated to 90°C for 1 hr. The suspensions were separated by centrifuging for 10 min at 10,000*g*. Using a spectrophotometer, the melanin content was determined via the data produced at an absorbance level of 475 nm.

2.11. Quantitative Real-Time Polymerase Chain Reaction. For the qRT-PCR, a 20 μL reaction contained a 0.4 mL mixture of two reverse transcriptases: 10 μL of 2 × QuantiTect SYBR Green Master Mix (QIAGEN, Valencia, CA, USA) with the hot start Taq polymerase, 0.8 mL of primers, and 0.5 mL (10 ng/mL) of template. The primer sequences are listed in Table 1. The StepOnePlus™ System was used for all real-time PCR assays. The reaction was completed by performing the RT reaction at 42°C for 20 min and then activating the FastStart Taq DNA polymerase at 95°C for 5 min. This was then amplified for 40 or 50 cycles at 95°C for 5 sec for denaturation, annealing, and acquisition at 60°C for 5 sec. It was finally elongated at 72°C for 15 sec. Fluorescence can also be measured after the elongation phase, but for this experiment, it was measured after the annealing phase. With a 96-well plate, 9 μL of the lysate was added to 11 μL of the reaction mix, which consisted of 10.6 μL MasterMix from the Eurogentec qPCR core kit for SYBR Green I (1.4 μL of 50 mM $MgCl_2$, 2 μL 10x reaction buffer, 0.8 μL of 5 mM dNTP mix, 0.6 μL SYBR Green I, 5.7 μL of nuclease-free water, and 0.1 μL of hot GoldStar Taq polymerase), 0.1 μL MS2 RNA, 0.1 μL 10x diluted RNAse inhibitor, and 0.1 μL of the REV primer (100 μM), as well as the MS2 FWD for SG-PERT assays on the ABI 7300 qPCR system. The qRT-PCR reaction, activation of the hot GoldStar Taq enzyme, 40-cycle amplification, annealing and acquisition, denaturation at 95°C, and the elongation at 72°C used the ABI 7300 instrument. To prepare the assay, all of the reagents were kept either on a cooling block or on ice. Duplicate SG-PERT reactions were performed on each lysate sample. Using qPCR software and instruments, an ABI 7300 with its threshold determined manually and a LightCycler® 480 with its maximum second-derivative method, generated the cycle of quantification (Cq) values. Using the same software for both instruments, the melting peaks were also automatically calculated.

Table 1: The nucleotide sequences of primers used in this study.

Tyrosinase	
	Forward: 5′-CTGCCAACGATCCTATCTTCCT-3′
	Reverse: 5′-GGTTATGTCCAATGGGTGCATT-3′
MITF	
	Forward: 5′-TTGGTGCCACCTAAAACATTGT-3′
	Reverse: 5′-CCGTTGGGCTTGCTGTATG-3′
TRP-1	
	Forward: 5′-GGTTTATTTGACACGCCTCCTT-3′
	Reverse: 5′-AGACTTCGAACAGCAGGGTCAT-3′
DCT	
	Forward: 5′-GTTCCTTTCTTCCCTCCAGTGACTA-3′
	Reverse: 5′-GTGGGCCAACCTGGAGTTT-3′
Pmel17	
	Forward: 5′-GGATGGTACAGCCACCTTAAGG-3′
	Reverse: 5′-CAGGATCTCGGCACTTTCAATAC-3′
Rab27a	
	Forward: 5′-CAGGGAAAAAAGAGTGGTGTACAGA-3′
	Reverse: 5′-ACGCTGTCGTTAAGCTACGAAAC-3′
Myo5a	
	Forward: 5′-GCCCAGATTGTGAAAGTGTTGA-3′
	Reverse: 5′-CCTGTCTCGTAAACGCATCTGT-3′
MLPH	
	Forward: 5′-AAGAGACCAGAGGACCCAAATG-3′
	Reverse: 5′-TTTCCGATCAAAAGAATCATCATC-3′
Mreg	
	Forward: 5′-TGGTGAGGGATGATGAGAAGAAT-3′
	Reverse: 5′-TCTGCCACTCCTCTGAGTCTTTG-3′

2.12. Western Blotting. A total of 1×10^5 cells were treated with sample groups or the blank vehicle control for two days. The cells were harvested and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 137 mM sodium chloride, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 2 μM leupeptin, 0.1 mM sodium orthovanadate, and 2 μg/mL aprotinin) [14]. Lysates were centrifuged at 20,000 ×g for 30 min, and the protein concentrations within the supernatant solution were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to a nitrocellulose membrane (PALL Life

Science, Ann Arbor, MI, USA). The membrane was blocked for 1 hr with 5% nonfat milk in PBS-T buffer (PBS containing 0.1% Tween 20). The transfer film was carefully removed from the wet transfer tank and semidry transfer slot and placed in a box that had 5% skimmed milk in 1x TBST for 1 hr at room temperature. It was then gently washed with 1x TBST to remove traces of the skimmed milk. The membrane was then incubated with respective primary antibodies. In each case, the membranes were incubated with horseradish peroxidase-conjugated antirabbit or mouse antibody and then treated with ECL detection reagents (PerkinElmer, ECL1 : ECL2 = 1 : 1). A mini-sized chemiluminescent imaging system from Life Science was used for measurement to detect the bands [14].

2.13. Statistical Analysis. Three of each concentration for the standard and the samples were used. Using Student's *t*-test, the results were statistically compared and were expressed using the average of the mean values ± standard deviation (SD).

3. Results

3.1. DPPH Free Radical-Scavenging Activity. ROS can generate lipid peroxides, DNA damage, protein expression, tissue aging, and cancer genesis when the balance between free radicals and inherent antioxidants is disturbed. Free radical chain reactions are blocked by neutralizing the electron donations or acceptances and chelating free lone pairs. Reducing oxidative stress from intrinsic and extrinsic facets is one way to increase health. This study examined antioxidative properties, by determining the ferric-reducing power, the DPPH free radical-scavenging capacity, and the metal-chelating power. The increased generation and accumulation of ROS produces lipid oxidation in food and cosmetics and natural antioxidative activity. In particular, free radical-scavenging properties are vital in functional nutrition additives and skin care products.

The first oxidation inhibitory assay was the DPPH free radical-scavenging test. This is a simple and economical experimental platform, in which antioxidants act to prevent oxidation products. Antioxidants change the color of the stable radical DPPH reagent from purple to the light yellow of diphenyl-picrylhydrazine. To determine the antioxidative properties of 36H, a 100 μM dose was applied to determine the scavenging ability. Table 2 shows that 36H exhibited excellent free radical scavenging ability and scavenges 60% of the DPPH free radical and vitamin C at the same concentration (100 μM) scavenges 85.3%.

3.2. Ferric Reducing Antioxidant Power. The second oxidation inhibitory assay is the ferric-reducing power test, which is a common and reliable way to measure the synthesis of Fe(III)–ferricyanide complex. A functional agent reduces Fe^{3+}/ferricyanide complexes to the ferrous form. This combination had a blue color at 700 nm, because $K_4Fe(CN)_6$ reacted with Fe^{3+} to form $Fe[Fe(CN)_6]_3$. In the experiment, the color of the solution changed from light yellow to different shades of blue and green, depending on the reducing power of the target compound. Table 2 presents that BHA at 100 μM had a reducing power value of 0.95 and 36H at 100 μM had a reducing power value of 0.85 compared to BHA. Therefore, 36H was shown to have good ferric-reducing antioxidant power.

Table 2: DPPH free radical-scavenging property, reducing power, and chelating ability of 36H at different concentrations (10, 50, and 100 μM) are presented. The concentration of three specific positive controls, vitamin C, BHA, and EDTA, is 100 μM. The data is representative of 3 experiments as mean value ± SD.

Samples	DPPH inhibition (%)	Ferric-reducing power (OD value)	Chelating capacity (%)
Vitamin C (100 μM)	85.3 ± 6.2	—	—
BHA (100 μM)	—	0.95 ± 0.07	—
EDTA (100 μM)	—	—	80.2 ± 0.4
36H (10 μM)	<10.0	0.19 ± 0.01	<10.0
36H (50 μM)	25.8 ± 5.1	0.42 ± 0.04	13.1 ± 2.3
36H (100 μM)	60.4 ± 2.8	0.85 ± 0.07	43.2 ± 3.6

3.3. Ferrous Ion-Chelating Capacity. The chelating activity for ferrous ion of 36H is shown in Table 2. EDTA (100 μM) was used as a positive control. Fe^{2+} and ferrozine form complexes quantitatively. The formation of reagent complexes is often disrupted, because of chelating agents, which results in a reduction in the red color of the complex. 36H at a dosage of 10 μM had no significant effect on Fe^{2+}-scavenging capacity, and at the concentration of 100 μM, it presented 43.2% inhibition. The positive control, EDTA, had approximately 80% ion-chelating capacity at 100 μM.

3.4. Effect of 36H on Mushroom Tyrosinase Activity. The inhibition of tyrosinase activity was described in numerous reports, most of which used mushroom tyrosinase as the model. The advantages are being well-developed by scientists, easy procedures, low cost, rapid pigmentation process, similar structural and functional characteristics as mammals, and convenience in observing melanin development. The inhibition of mushroom tyrosinase activity by 36H was studied, and the inhibitory ability had a positive correlation with the concentration of 36H (Figure 2(a)). In the fifteenth minute, it was seen that 36H at different concentrations (1, 5, and 10 mM) compared to the control reduces the OD values. The enzyme activity decreases by about 10% at 1 mM, 30% at 5 mM, and 40% at 10 mM, compared to the vehicle control (Figure 2(b)). The relationship between tyrosinase activity and its concentration in 36H was studied. Different concentrations of the inhibitor give a group of lines that all pass through the origin. The inhibition of tyrosinase activity by 36H had no effect on the amount of enzyme, which illustrated that 36H was a reversible inhibitor (Figure 2(c)). The type of inhibition on tyrosinase by 36H was confirmed using a Lineweaver-Burk double-reciprocal plot. The plots of $1/v$ versus $1/[s]$ gave a family of straight lines through the origin, which presented that 36H was a competitive complex. The kinetics of the enzyme are presented in Figure 2(d).

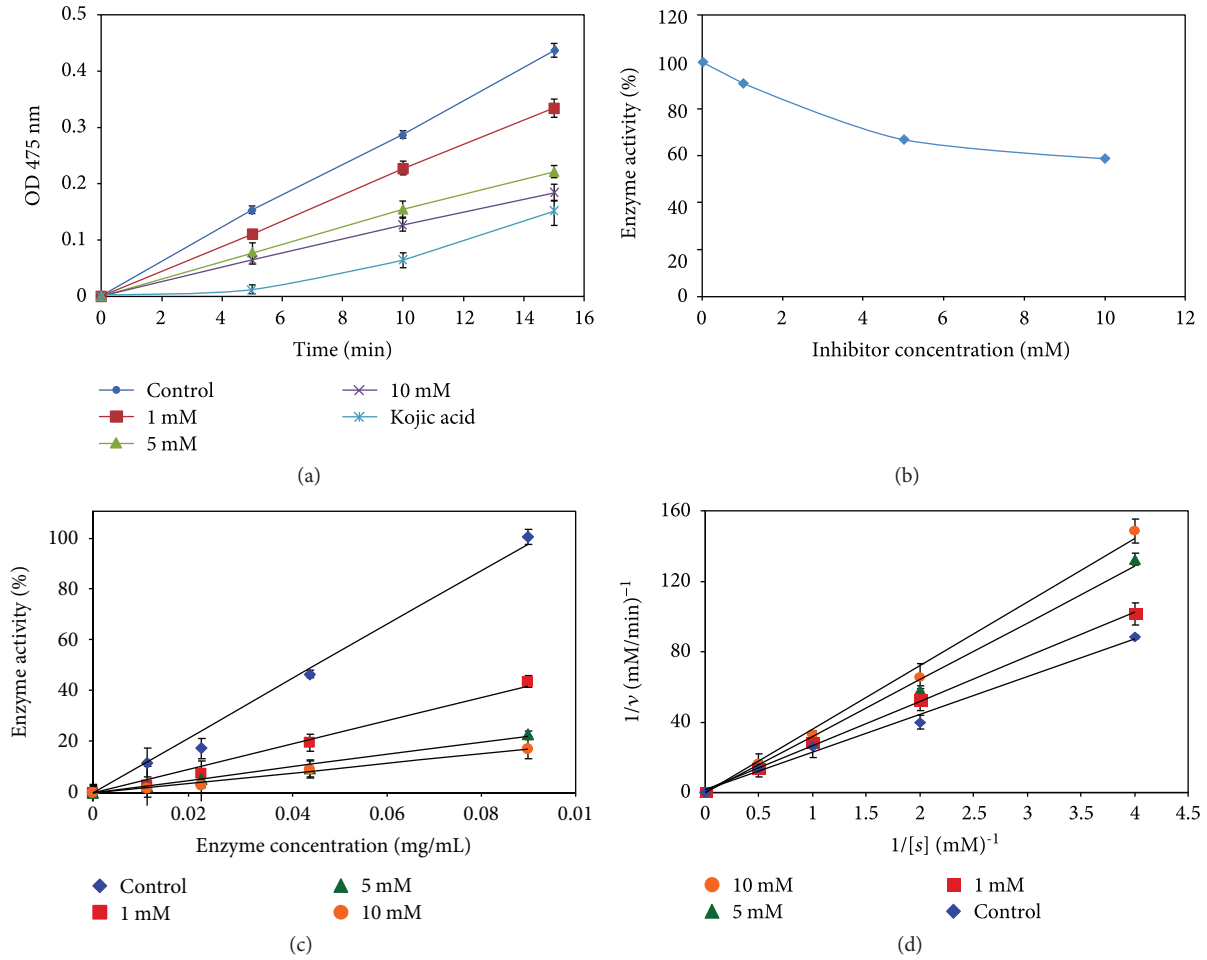

Figure 2: Inhibitory enzyme kinetic effects of 36H on mushroom tyrosinase. (a) 36H at different concentrations (1, 5, and 10 mM) affects mushroom tyrosinase activity. The concentration of positive control, Kojic acid, is 10 mM. Data are representative of 3 experiments. (b) The inhibition of mushroom tyrosinase enzyme activity of 10 mM. (c) Inhibition effects of 36H at different concentrations (1, 5, and 10 mM) on the activity of mushroom tyrosinase for the oxidation of L-tyrosine. (d) Inhibitory effect of 36H at different concentrations (10, 50, and 100 mM) on mushroom tyrosinase. The data for Lineweaver-Burk plots were obtained as mean values of three independent assays with various concentrations of L-tyrosine (0.125, 0.25, 0.5, 1, and 2 mM) as the substrate.

3.5. The Effect of Cell Viability, Cellular Tyrosinase, and Melanin Content on 36H in HMC. As a potent skin-lightening compound, the component should be harmless, without undesirable cytotoxic side effects. There are some well-known melanin synthesis inhibitors, including kojic acid, arbutin, PTU, or hydroquinone, that are being utilized globally as cosmetic ingredients at present. We also discovered that these melanogenic inhibitors might induce human skin tumorigenicity at high concentration doses or frequent use [2]. HMC was cultured in 36H for 48 hrs at various concentrations of 1, 5, 10, and 25 μM, and the cell viability was determined to show low cellular toxicities in Figure 3(a). When considering the agent for therapeutic or cosmetic usage in human beings, we found that the cytotoxic consequences on human dermal cellular viabilities were insignificant. To understand the inhibitory effect of 36H on melanogenesis, we assessed intracellular tyrosinase activity in HMC. The results for tyrosinase activity in HMC showed that there was an obvious variation as the concentration increased, and the tyrosinase activity decreased for low cell toxicity. After treatment, the tyrosinase activity was reduced to 39 ± 2.2% at 25 μM in a dose-dependent manner (Figure 3(b)). These results showed that 36H inhibited intracellular tyrosinase activity. The melanin assay results clearly showed that 36H reduces the melanin content in HMC in a dose-dependent manner (Figure 3(c)). The percentage of control represents the melanin content. After treatment, the melanin contents were 77% at 25 μM, 84% at 10 μM, 88% at 5 μM, and 90% at 1 μM. These results showed that 36H had a significant inhibitory effect on the synthesis of melanin in HMC at 25 μM.

3.6. The Melanin Biosynthesis-Related mRNA and Proteins Were Influenced by 36H in HMC. Melanin biosynthesis occurs in melanosome, and the initial amino acid substrate is tyrosine. Tyrosine is firstly catalyzed to L-DOPA via tyrosinase, and using the same enzyme, tyrosinase, DOPA is catalyzed to dopaquinone. Dopaquinone is catalyzed by dopachrome tautomerase (tyrosinase-related protein-2, TRP-2), TRP-1, and tyrosinase to form eumelanin.

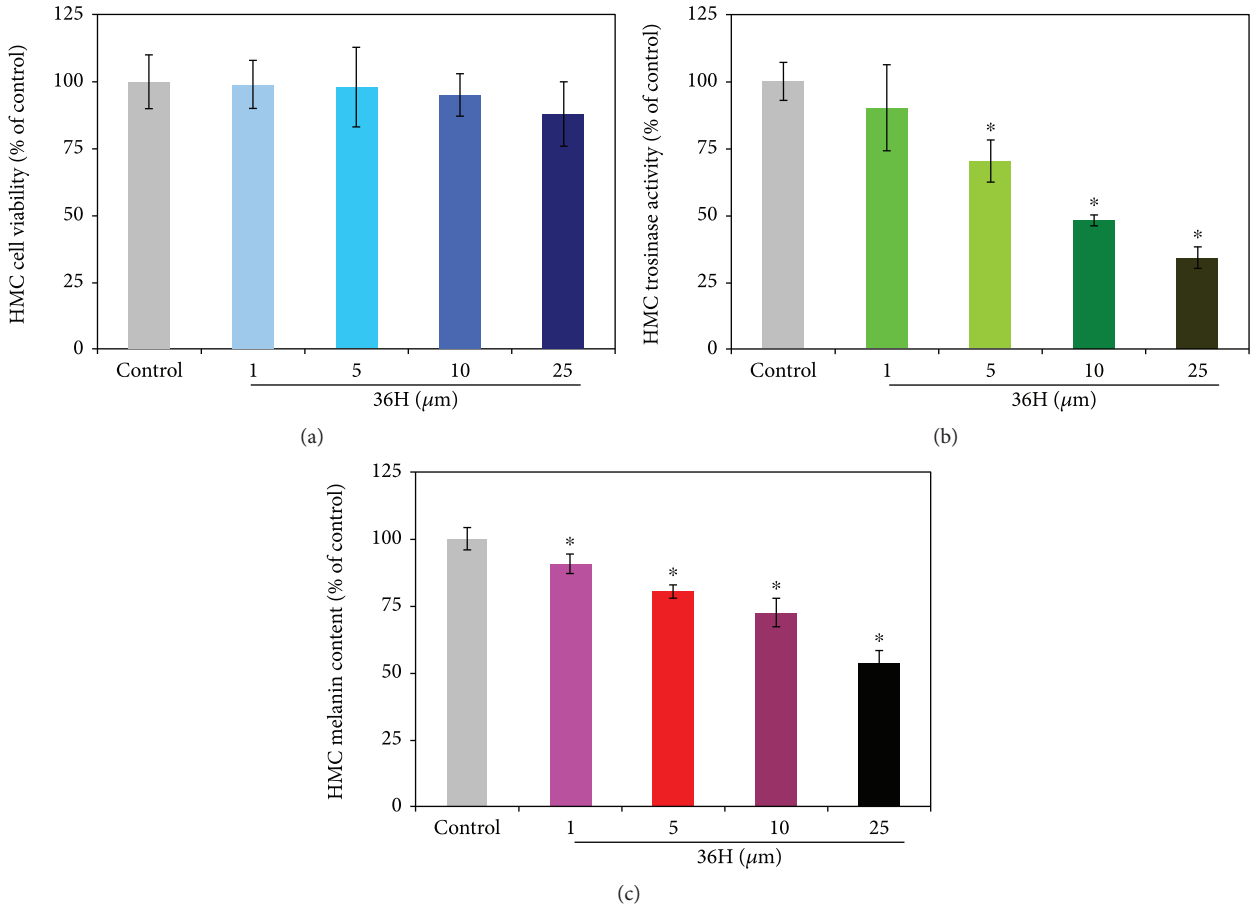

FIGURE 3: The inhibitory effect of 36H in HMC. (a) The impact of 36H at different concentrations (1, 5, 10, and 25 μM) to human melanocyte cell viabilities. (b) The tyrosinase activity of the human melanocyte cell treated with various concentrations of 36H. (c) The melanin content of the human melanocyte cell treated with various concentrations of 36H. Data are shown as mean ± SD; $n = 3$; $^*P < 0.005$, compared with the control groups.

In HMC, 36H downregulated the cellular melanogenesis-related RNAs and proteins as demonstrated in Figure 4.

3.7. Effect of 36H on the Melanosome Maturation. The melanosome is an organelle that relies on melanin synthesis within melanocytes. The more melanosomes mature, the more melanin is formed. Therefore, melanosome maturation is important in the mechanism of skin whitening. Premelanosome protein 17 (Pmel17) is targeted to precursors of the pigment organelle, the melanosome, where it is proteolytically processed to several small fragments. Some of these fragments form nonpathological amyloids that assemble into sheets and form the striated pattern that underlies the melanosomal ultrastructure. This study demonstrated that 36H downregulated Pmel17 RNA and protein expression in HMC (Figure 5).

3.8. Effect of 36H on the Melanosome Transport. Rab27a, MLPH, and Myo5a form a tri-protein complex to bind melanosomes at the melanocyte peripheries. In the process of melanosome transport, the ternary complex is the connection between actin cytoskeleton and melanosome. A lack of these proteins affects the transport, and melanoregulin (Mreg) drives melanosome transfer from melanocytes to keratinocytes via a regulated shedding mechanism. This study illustrated that 36H decreased the melanosome transport by affecting these related RNA and proteins (Figure 6).

4. Discussion

This study shows that antioxidants inhibit melanogenesis in two ways. In the melanin biosynthesis process [15], tyrosinase first transforms hydroxide tyrosine to DOPA, then oxidizes DOPA to dopaquinone [2]. Melanin scavenges free radicals to inhibit lipid peroxidation and protects the skin from UV damage, but melanin can also be deoxidized by an antioxidant. Therefore, melanin is called the radical sink [2, 16, 17]. A lack of melanin reduces the protection for the skin, so ROS stimulates melanocyte to produce more melanin [18]. Consequently, a good antioxidant can reduce tyrosinase activity and inhibit parts of the melanin synthesis. 36H had antioxidant properties in the DPPH free radical-scavenging ability and ferric-reducing power.

Before injecting the protein samples into SDS-PAGE, we normalized all protein levels. The protein normalization is a significant process applied to remove both experimental biological errors and artificial unexpected variabilities [2]. Genes encoded in DNA are transcribed into pre-messenger RNA

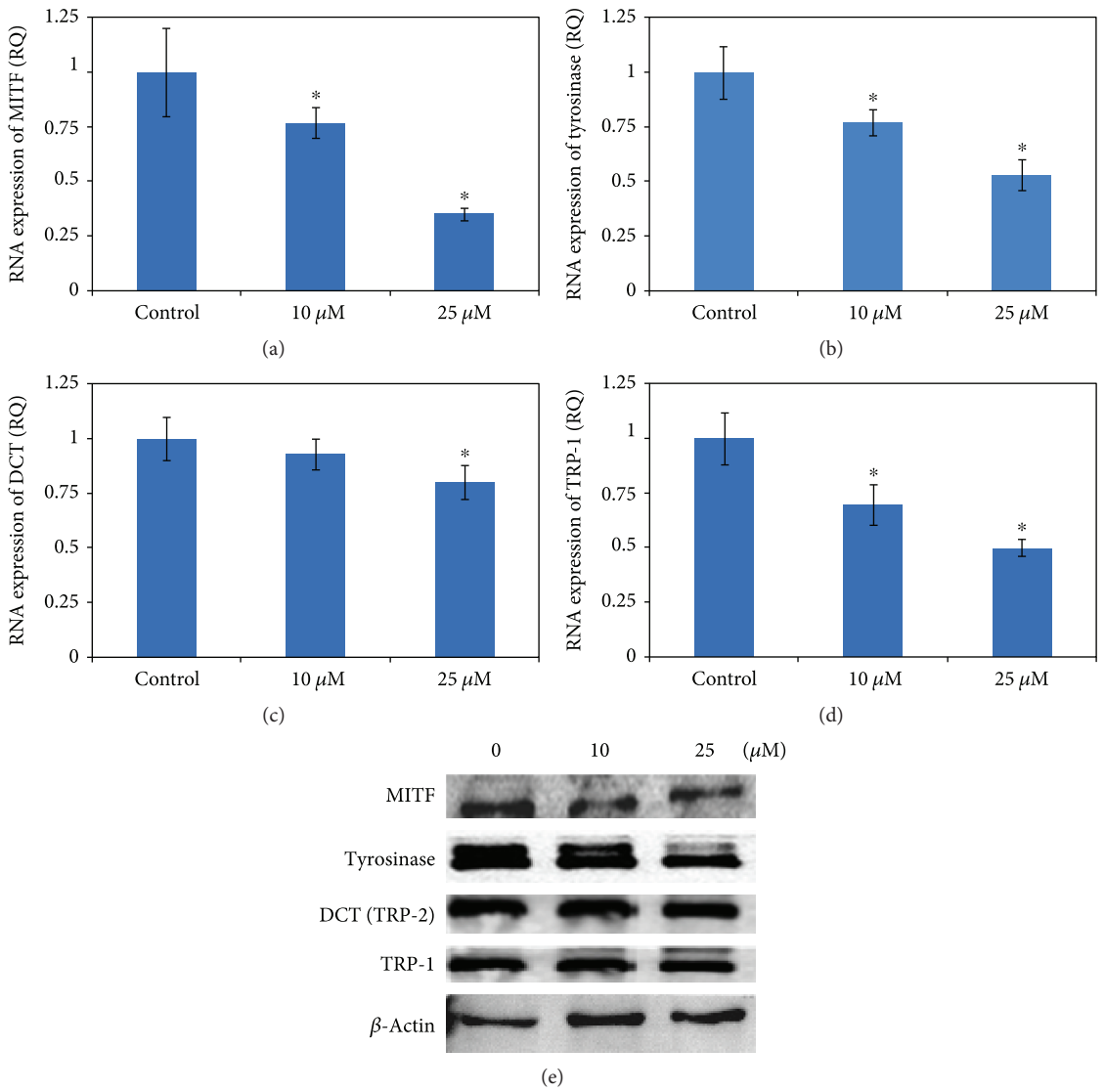

Figure 4: The RNA and protein expressions associated with melanin biosynthesis of human melanocyte cell treated with various concentrations (0, 10, and 25 μM) of 36H. Data are representative of 3 experiments. $^*P < 0.05$ as compared with the control.

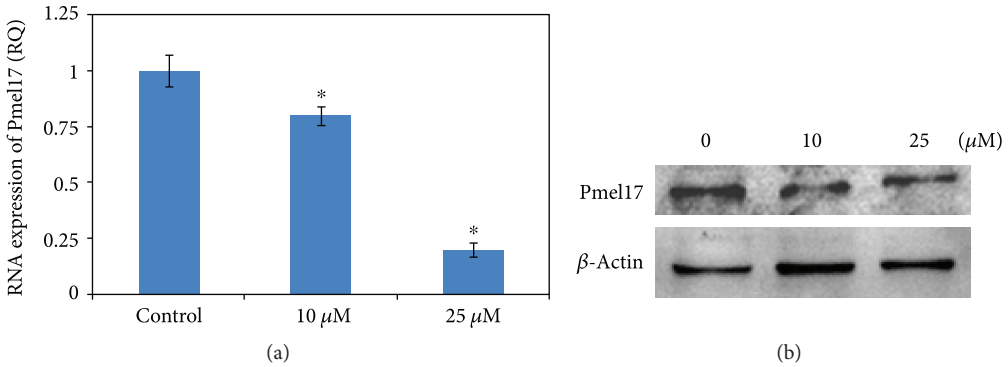

Figure 5: The RNA and protein expressions associated with melanosome maturation of human melanocyte cell treated with various concentrations (0, 10, and 25 μM) of 36H. Data are representative of 3 experiments. $^*P < 0.05$ as compared with the control.

(mRNA) by RNA polymerase, and then most organisms develop it using various posttranscriptional modification forms to generate the matured-mRNA, which is applied as a template for protein syntheses via ribosomes. The transcription unit is a stretched DNA to transcribe into RNA and transcripts mRNA which is provided as a template on

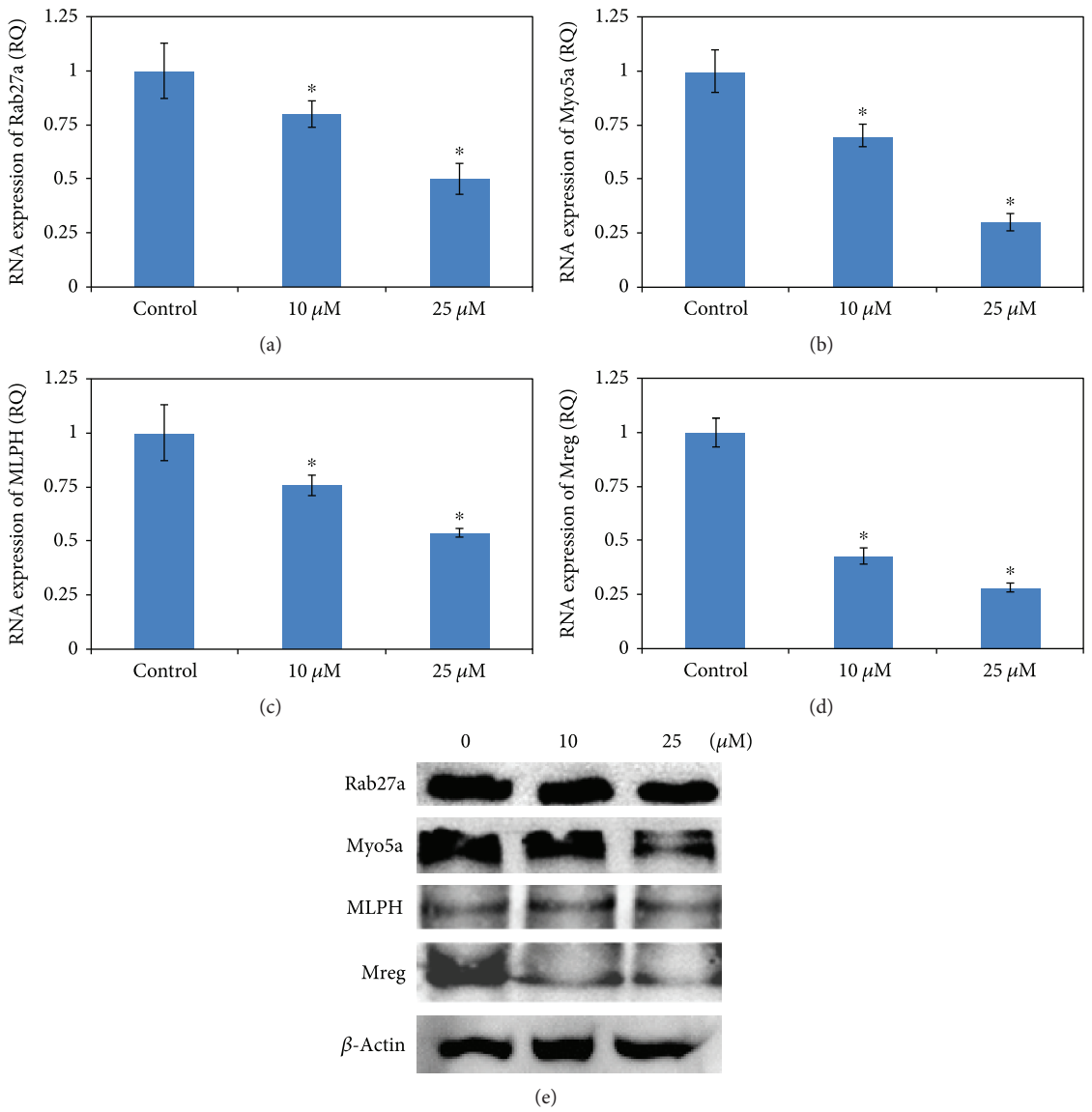

Figure 6: The RNA and protein expressions associated with the melanosome transport of human melanocyte cell treated with various concentrations (0, 10, and 25 μM) of 36H. Data are representative of 3 experiments. $^*P < 0.05$ as compared with the control.

the protein translation for the syntheses [3, 6]. In human beings, mRNA is in the cellular nucleus to be translocated across the nuclear membrane into the cytoplasm, which is the location where protein syntheses take place. The relationship between mRNA and protein is a complex network. The regulation of NDA transcriptions and translations could be differently changed. In cells, proteases degrade the functions of proteins into small amino acids or polypeptides. Due to intracellular breakdown, amino acids can be recycled for protein syntheses again. This mechanism cleans abnormal or damaged proteins and ones that are no longer needed to prevent unnecessary protein accumulations. Although we would consider that the amount of proteins decreased when the transcription of the encoding genes was reduced, there were other mechanisms regulating the protein abundances. For example, the protein's half-life might be increased due to a decreased rate of biodegradation. Another possibility was that the mRNA was more preferentially translated during the process. The human skin color is also affected by the melanosome regulative degradation autophagy in keratinocytes [19].

When keratinocytes are exposed to UV [20], they release α-melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), and prostaglandins E_2 (PGE_2) [21]. These signaling molecules activate the downstream signaling pathway of adenylate cyclase through the melanocortin 1 receptor (Mc1R) on the membrane of the melanocyte to induce melanogenesis by enhancing MITF, tyrosinase, TRP-1, and TRP-2 [22] and through the IP3/DAG mechanism to activate the inactive-form tyrosinase to the active form. Our work demonstrated that 36H altered *MITF* RNA expression, but there was insignificant change in the amount of protein production. Tyrosinase affects melanin biosynthesis and TRP-2 and TRP-1 [23]. Dopachrome is

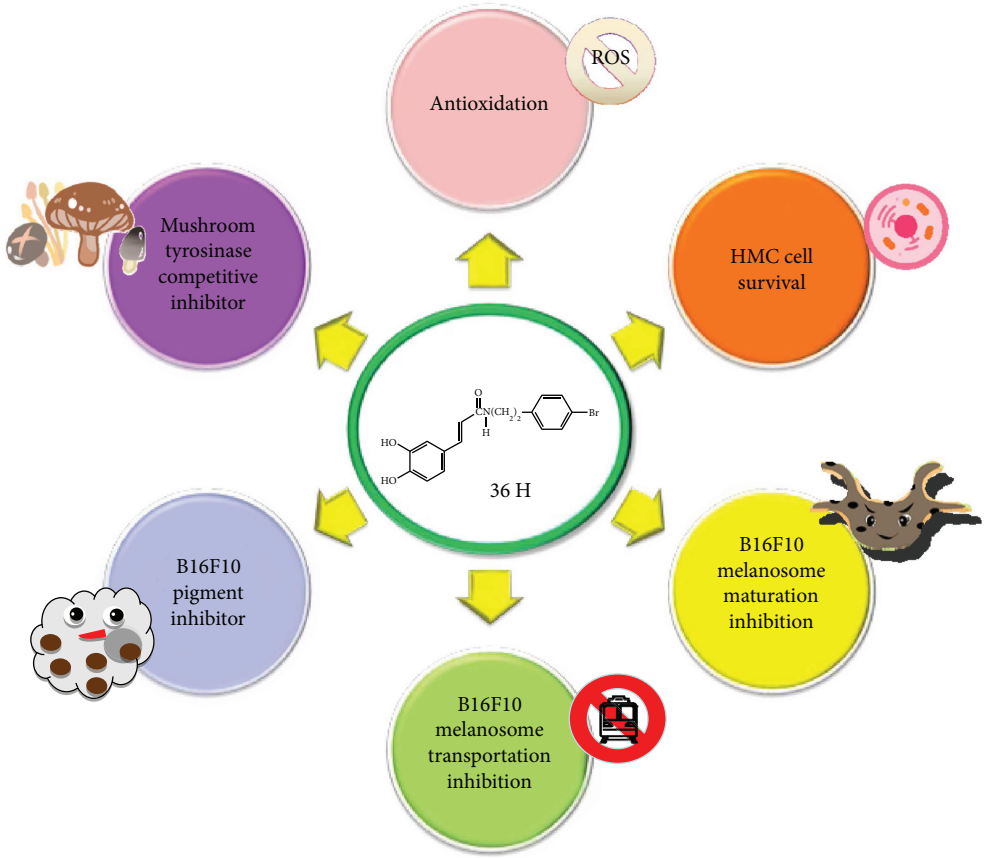

Figure 7: Proposed schematic diagram of compound 36H biofunctions.

catalyzed to 5,6-dihydroxyindole-2carboxylic acid by TRP-2, and 5,6-dihydroxyindole-2carboxylic acid is transferred to indole-5,6-quinone carboxylic acid via TRP-1 [24], which is then synthesized into eumelanin [16]. In mushroom tyrosinase and cellular tyrosinase assays, 36H downregulated tyrosinase activity. TRP-2 and TRP-1 were diminished at the RNA level, but there were insignificant differences in protein levels, compared to the control group. In melanosome maturation, Pmel17 is the precursor of melanosome. It is proteolyzed to fragments to form the striated pattern that underlies melanosomal ultrastructure [25]. Using a western blot, Pmel17 was shown to decrease in both RNA and protein expressions, which interrupted the maturation of melanosome.

Human skin melanin is driven by the intercellular movement of melanin-containing melanosomes from the extremities of HMC dendrites to neighboring keratinocytes. When it is carried by the actin filament, melanosome moves to the dendritic tail section, through exocytosis, and is transported into keratinocytes [26]. The greater the amount of melanin that is transferred into keratinocytes, the darker is the color of the skin [27]. The movement on the microtubule depends on the dynein-dynactin motor complex. Mreg forms a complex with Rab-interacting lysosomal protein and p150(Glued) which is a subunit of dynactin [28]. Mreg adjusts a shedding system which transports melanosome from HMC to keratinocytes. The shedding process from HMC of melanosome-rich packages undergoes the phagocytosis of keratinocytes. The shedding not only takes place principally at dendritic extremities but also around the center areas, having adhesion to keratinocytes, tightening behind the forming packages, and apparent self-abscissions [29]. The movement on the actin filament requires Myo5a, Rab27a, and MLPH as the connecting bridge [30]. 36H downregulated the protein expression for Myo5a and might prevent a darkening of skin color. Collectively, the data shows that 36H is an effective skin-whitening agent that has the potential for cosmetic applications (Figure 7).

Conflicts of Interest

The authors have no competing interests regarding the publication of this study.

Authors' Contributions

Li-Ching Lin, Byeong Hee Hwang, Yueh-Hsiung Kuo, and Hui-Min David Wang conceived and designed the experiments; Li-Ching Lin, Chung-Yi Chen, Chia-Hung Kuo, and Yun-Sheng Lin performed the experiments and analyzed the data; Yueh-Hsiung Kuo contributed the reagents, materials, and analysis tools; Li-Ching Lin, Chung-Yi Chen,

Tina Kaiting Wang, and Hui-Min David Wang wrote the paper. Li-Ching Lin and Chung-Yi Chen contributed equally to this work.

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Clinical Study

Absorption and Elimination of Oat Avenanthramides in Humans after Acute Consumption of Oat Cookies

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Background. Avenanthramides (AVA) are a group of diphenolic acids found only in oats that have anti-inflammatory and antioxidant effects. Absorption of AVAs in humans after oral consumption of natural oat flour is unknown. *Objective*. To examine the appearance of AVAs in plasma after oral ingestion of oat cookies and estimate key pharmacokinetic parameters. *Methods*. Male and female nonobese participants ($n = 16$) consumed three cookies made with oat flour containing high (229.6 mg/kg, H-AVA) or low (32.7 mg/kg, L-AVA) amounts of AVAs, including AVA-A, AVA-B, and AVA-C. Blood samples were collected at 0, 0.5, 1, 2, 3, 5, and 10 h after ingestion. Plasma total (conjugated and free) AVA concentrations were quantified using UPLC-MS, and pharmacokinetic parameters for each AVA were estimated. *Results*. AVAs reached peak concentrations in plasma between 2 and 3 h for the H-AVA group and between 1 and 2 h for the L-AVA group. Maximal plasma concentrations for AVAs were higher in the H-AVA than in the L-AVA group. AVA-B demonstrated a longer half-life and slower elimination rate than AVA-A and AVA-C. *Conclusions*. AVAs found naturally in oats are absorbed in the plasma after oral administration in humans. AVA-B has the slowest elimination rate and the longest half-life compared to AVA-A and AVA-C, while AVA-C demonstrated the lowest plasma concentrations. This study is registered with ClinicalTrials.gov identifier NCT02415374.

1. Introduction

Avenanthramides (AVAs) are a group of diphenolic acids found only in oats (*Avena sativa*) [1]. Of all the AVAs identified, AVA-A (2p), AVA-B (2f), and AVA-C (2c) are the most abundant and differ only by a single moiety on the hydroxycinnamic acid ring. Early research indicates that oat bran contains a high concentration of antioxidants [2], and increased oat consumption is associated with decreased serum lipid and cholesterol levels [3] and low-density lipoprotein oxidation [4]. Additional in vitro studies show that AVAs have the anti-inflammatory and antiatherogenic effects of decreasing monocyte adhesion to aortic endothelial cells and decreased expression of proinflammatory (e.g., tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1α, IL-6) markers and chemotactins (e.g., intercellular adhesion molecule-1, vascular cell adhesion molecule-1, monocyte chemotactic protein-1, and c-elastin) [5]. These effects are derived from decreased nuclear factor- (NF-) κB activity [6, 7]. Ji et al. [8] first showed that rats fed with an AVA 2c-supplemented diet for 50 days display lower reactive oxygen species (ROS) generation and lipid peroxidation in skeletal muscle and higher levels of superoxide dismutase (SOD) and glutathione peroxidase activity

in the muscle, heart, liver, and kidney. Also, Koenig et al. report that 8 weeks of AVA supplementation among young and postmenopausal women abolishes plasma TNF-α and C-reactive protein responses to downhill running-induced inflammation. Compared with women in the low AVA group, women in the high AVA group showed a suppressed neutrophil respiratory burst 0 and 24 h after downhill running and lower IL-6 and monocyte NF-κB activation [9, 10]. These studies show that AVAs may have potential effect to prevent chronic diseases and sports injuries.

AVA bioavailability has been demonstrated in animals and humans. Chen et al. [11] reported that serum levels of AVA 2p, 2f, and 2c reach a peak 2 h after humans consume an AVA-enriched mixture (AEM) and then gradually return to resting levels after 10 h. We previously found that rats orally gavaged with a mixture of synthetic AVAs show significant quantities of all three AVA fractions in the plasma, heart, liver, and skeletal muscle during a 48-hour period [12]. Using glucuronidase-sulfatase to cleave AVA conjugate bonds, we confirmed that most AVA was in a conjugated form. Although previous studies report that AVAs are bioavailable and detectable in humans [11, 13], these studies utilized an AEM or specially treated oats, which might contain much higher amounts of AVAs than regular oats.

Here, we examined the metabolic fate of orally ingested oat AVAs by measuring plasma concentrations of three major fractions of AVAs, AVA-A, AVA-B, and AVA-C. Our results provide the first evidence of AVA absorption in humans after acute consumption of oat cookies made with natural oat flour.

2. Methods

2.1. Participants and Study Design. Male and female nonobese participants (body mass index (BMI) greater than 18 kg/m^2 and less than 28 kg/m^2, age: 20–45 years, $n = 16$) were recruited from the Minneapolis-St. Paul community. All participants signed informed consent forms and were willing to avoid oat consumption at least 24 hours prior to and throughout the testing period and to consume a low-flavonoid diet 1 week prior to the test. This study was approved by the Institutional Review Board of the University of Minnesota and was registered at ClinicalTrials.gov (NCT02415374). Foods rich in flavonoids were defined as having berries, apples, pears, citrus fruits, fruit juices, onions, chocolate, wine, coffee, tea, beans, nuts, soy products, and most spices. Exclusion criteria included the presence of gastrointestinal conditions that interfere with absorption; clinically significant endocrine, cardiovascular, pulmonary, renal, hepatic, pancreatic, biliary, or neurologic disorders; major trauma or surgery within 3 months of the test; cancer within 2 years of the test; allergy to oat products; pregnancy or currently lactating; smoking; drinking more than five alcoholic drinks per week; using nutraceuticals, blood pressure medication, nonsteroidal anti-inflammatory drugs (>800 mg ibuprofen per week), vitamin supplements, anticoagulants, or hypoglycemic drugs; oat product consumption at least 24 hours before the test; and consuming high-flavonoid foods 1 week prior to the test.

2.2. Diet. Participants received three cookies made with oat flour containing a high amount of AVA (H-AVA; 7.2 mg/cookie; 21.6 mg total, PepsiCo Inc., USA) or a low amount of AVA (L-AVA; 1.03 mg/cookie; 3.09 mg total, PepsiCo Inc., USA). H-AVA and L-AVA oat flour are compared and selected from 26 natural oat groats incorporating different AVA contents. The concentrations of AVA in the two types of cookies were verified by our laboratory and International Chemistry Testing (Milford, MA). Each cookie was made of 30 g flour, 1 tsp unsweetened applesauce (8 g), 1 tsp noncaloric sweetener (3 g), and 2 tsp water (14 g) and was baked at 212°F (100°C) for 15 min to avoid excessive AVA disintegration.

2.3. Study Visits and Blood Collection. After an overnight fast with water allowed, participants reported to the Laboratory of Physiological Hygiene and Exercise Science in the morning. Participants completed a food history questionnaire to ensure that they met the selection criteria; those who consumed vegetables, fruits, or other food containing over 200 mg of polyphenols during the previous week were excluded. Over-the-counter and prescription medication consumed within the past 4 weeks was recorded.

Before cookie consumption, mixed venous blood was drawn from an antecubital vein into an EDTA-coated Vacutainer tube (7 ml) and immediately centrifuged at 500 ×g for 30 min at 20°C to obtain plasma. Participants then consumed three cookies containing either H-AVA or L-AVA in random order with 150 ml water within 10 min. After 0.5, 1, 2, 3, and 5 h, additional blood samples were drawn as described above. The final blood sample was drawn when participants returned to the lab after 10 h. Samples were stored at −80°C until analysis. Participants were retested after a 2-week washout period after consuming three cookies containing the opposite concentration of AVA.

2.4. Plasma AVA Extraction. AVA was extracted from plasma according to the procedures described by Chen et al. [4] with modification. Briefly, 50 μl vitamin C-NaH_2PO_4 and 50 μl α-glucuronidase/sulfatase were added to 500 μl plasma, and the mixture was incubated at 37°C for 45 min. Next, 2000 μl acetonitrile was added to a 5 ml Eppendorf tube and vortexed. After 5 min, samples were centrifuged at 5000 rpm for 30 min. The supernatant was removed, dried by nitrogen, and reconstituted in 100 μl MeOH:H_2O (1 : 1). Following centrifugation at 14800 rpm for 15 min, 50 μl of the supernatant was transferred into a clean vial for analysis.

2.5. UPLC-MS Detection. Concentrations of total AVA in plasma were measured by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS; Waters XEVO G2-S Q-TOF) using a C18 column (Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm). Gradient elution composing solvent A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile) was performed starting with 0.5% solvent B for 0.5 min, gradually increased

to 20% solvent B for 3.5 min, and to 95% solvent B for 4 min, and to 100% solvent B for 1 min. Solvent B was decreased from 100% to 0.5% in the last 1 min. The injection volume was 5 μl for each sample. The mass range was measured from 50 to 1000 m/z. Instrument control and data acquisition were performed using Waters MassLynx MS software (version 4.1). The concentrations of AVAs in samples were calculated using an AVA standard curve generated with synthetic standards provided by Dr. Mitchell Wise (USDA Cereal Research Laboratory, Madison, WI). The sensitivity of detection was in the ng/ml range.

2.6. Pharmacokinetic Parameters. Maximum plasma concentration (C_{max}) and time to reach maximal concentration (T_{max}) of AVAs were calculated and presented as mean ± standard deviation (SD). Half-life (i.e., time taken for concentration to decrease to half the initial value; $T_{1/2}$) was calculated from log-transformed plasma concentration-time data and presented as mean ± SD. Area under the curve (AUC) for each participant was calculated using the trapezoidal rule, and mean AUC_{0-t} was presented as mean ± SD. Elimination rate constant (K_{el}) was calculated using the following equation: $K = 0.693/T_{1/2}$. C_{max}, T_{max}, $T_{1/2}$, and AUC_{0-t} were calculated with PKSolver, an add-in program for Microsoft Excel [14].

2.7. Statistical Analysis. The mean and SD of plasma total (conjugated and free) AVA was calculated for each time point and plotted against time. AUC, C_{max}, T_{max}, and $T_{1/2}$ for each AVA were compared between H-AVA and L-AVA groups using *t*-tests and among AVA-A, AVA-B, and AVA-C compounds using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. AVA Content in H-AVA and L-AVA Cookies. Table 1 shows the total AVA content in H-AVA and L-AVA cookies. Participants in the H-AVA group consumed higher amounts of AVA-A, AVA-B, and AVA-C than participants in the L-AVA group.

3.2. Participant Characteristics. Table 2 shows the average age (range: 24.9 to 26.3 years) and BMI (range: 21.4 to 23.8 kg/m^2) of participants, which did not differ between males and females.

3.3. TOF-MS Chromatographs of AVAs. Representative retention times for AVA-A, AVA-B, and AVA-C in standards as measured by Q-TOF-MS were 4.68 min (Figure 1(a)), 4.80 min (Figure 1(b)), and 4.26 min (Figure 1(c)).

3.4. Plasma AVA Concentrations. Participants in the H-AVA group showed higher plasma concentrations of AVA-A, AVA-B, and AVA-C than participants in the L-AVA group. AVA-A concentration reached maximum at 2.50 ± 1.2 h in the H-AVA group and 1.80 ± 1.3 h in the L-AVA group and returned to baseline after approximately 10 h (Figure 2(a)). AVA-B concentrations reached maximum values at 2.04 ± 0.9 h in the H-AVA group and 1.50 ± 1.5 h in the L-AVA group but did not return to baseline levels after approximately 10 h (Figure 2(b)). AVA-C concentrations reached maximum values at 2.29 ± 1.0 h in the H-AVA group and 1.32 ± 1.0 h in the L-AVA group and returned to baseline levels after approximately 10 h (Figure 2(c)).

Table 1: AVA content in H-AVA and L-AVA cookies.

	AVAs	One cookie (55 g)	Per 100 g	Per serving (3 cookies, 165 g)
H-AVA	Total	7.20	13.10	21.61
	A (mg)	1.70	3.09	5.09
	B (mg)	2.89	5.25	8.67
	C (mg)	2.62	4.76	7.85
L-AVA	Total	1.03	1.86	3.08
	A (mg)	0.19	0.35	0.57
	B (mg)	0.32	0.58	0.95
	C (mg)	0.52	0.94	1.55

Table 2: Participant characteristics.

	Male ($n = 8$)	Female ($n = 8$)
Age (years)	26.3 ± 3.0	24.9 ± 3.4
BMI (kg/m^2)	23.8 ± 3.0	21.4 ± 2.2

3.5. AVA Pharmacokinetics. Table 3 lists kinetic parameter determined for the three AVA fractions. Maximum plasma concentrations (C_{max}) for AVA-A, AVA-B, and AVA-C were higher in the H-AVA than in the L-AVA group. Also, AVA-A and AVA-B had higher C_{max} than AVA-C in the H-AVA group. AVA-A, AVA-B, and AVA-C were present at peak concentrations in plasma (T_{max}) between 2 and 3 h for the H-AVA group and between 1 and 2 h for the L-AVA group. AVA-B had longer $T_{1/2}$ than AVA-A and AVA-C in the H-AVA group.

Participants in the H-AVA group demonstrated larger mean AUC_{0-t} for AVA-A, AVA-B, and AVA-C compared with participants in the L-AVA group (Table 4). AVA-C had the lowest AUC_{0-t} among the three AVAs no matter in H-AVA or L-AVA groups.

4. Discussion

Oats (*Avena sativa*) are rich in dietary fibers and known for its cardiac protection attributed to the abundant soluble fiber α-glucan [15]. Recently, other components in oat with additional health benefits have been further investigated, including vitamin E, phenolic compounds (e.g., AVA), phytic acids, sterols, and flavonoids [16]. AVAs are a group of diphenolic acids unique to oats that exert anti-inflammatory and anti-atherogenic effects by decreasing expression of proinflammatory markers (e.g., TNF-α and IL-6) [5], possibly due to inhibition of NF-κB pathway [6, 7]. To elucidate absorption and elimination profiles of oat AVAs, we measured plasma concentrations of AVAs in humans after acute ingestion of oat cookies made of natural oat flour.

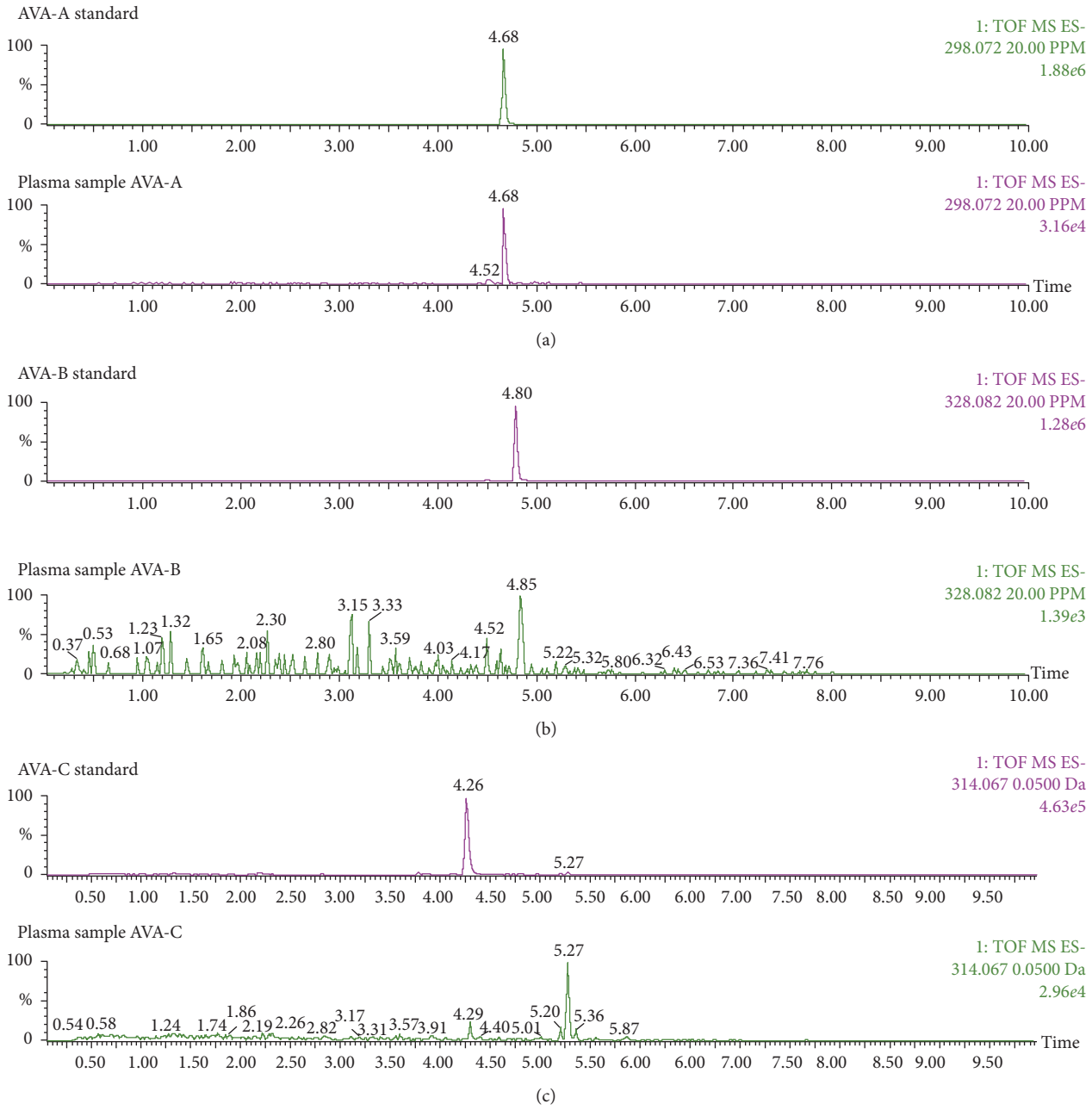

Figure 1: Plasma AVA (bottom) and AVA standard (top) TOF-MS chromatographs. (a) The retention times for AVA-A standard and plasma AVA-A were both 4.68 min. (b) The retention times for AVA-B standard and plasma AVA-B were 4.80 min and 4.85 min, respectively. (c) The retention times for AVA-C standard and plasma AVA-C were 4.26 min and 4.29 min, respectively.

H-AVA group was found longer T_{max} and higher C_{max} than L-AVA group in this study. Chen et al. [11] first reported bioavailability of AVAs in humans after consumption of 1 g AEM and found that C_{max} was 112.1 ng/ml, 31.6 ng/ml, and 28.1 ng/ml for AVA-A, AVA-B, and AVA-C, respectively. The discrepancies on plasma concentrations were mainly due to the doses of AVA-A, AVA-B, and AVA-C in AEM, which were 9-fold, 4-fold, and 4-fold higher than our H-AVA cookies made of natural oat flour. Other pharmacokinetic properties such as T_{max} and $T_{1/2}$, however, were similar between the two studies.

H-AVA group exhibited a transient plateau in AVA-B concentration around 30 min to 1 h, which might be related to high absorption of AVA-B after oat flour cookie consumption. Moreover, the absorption profile of AVA-B differed from that of AVA-A and AVA-C, with the highest C_{max}, longest $T_{1/2}$, and shortest T_{max}. These differences might be related to the different hydroxycinnamic moieties between AVA-A (-H), AVA-B (-OCH_3), and AVA-C (-OH). That is, as AVA-B is more hydrophobic than AVA-A and AVA-C, this may lead to a slower elimination rate [11]. However, Koenig et al. [12] found that AVA-B showed the fastest

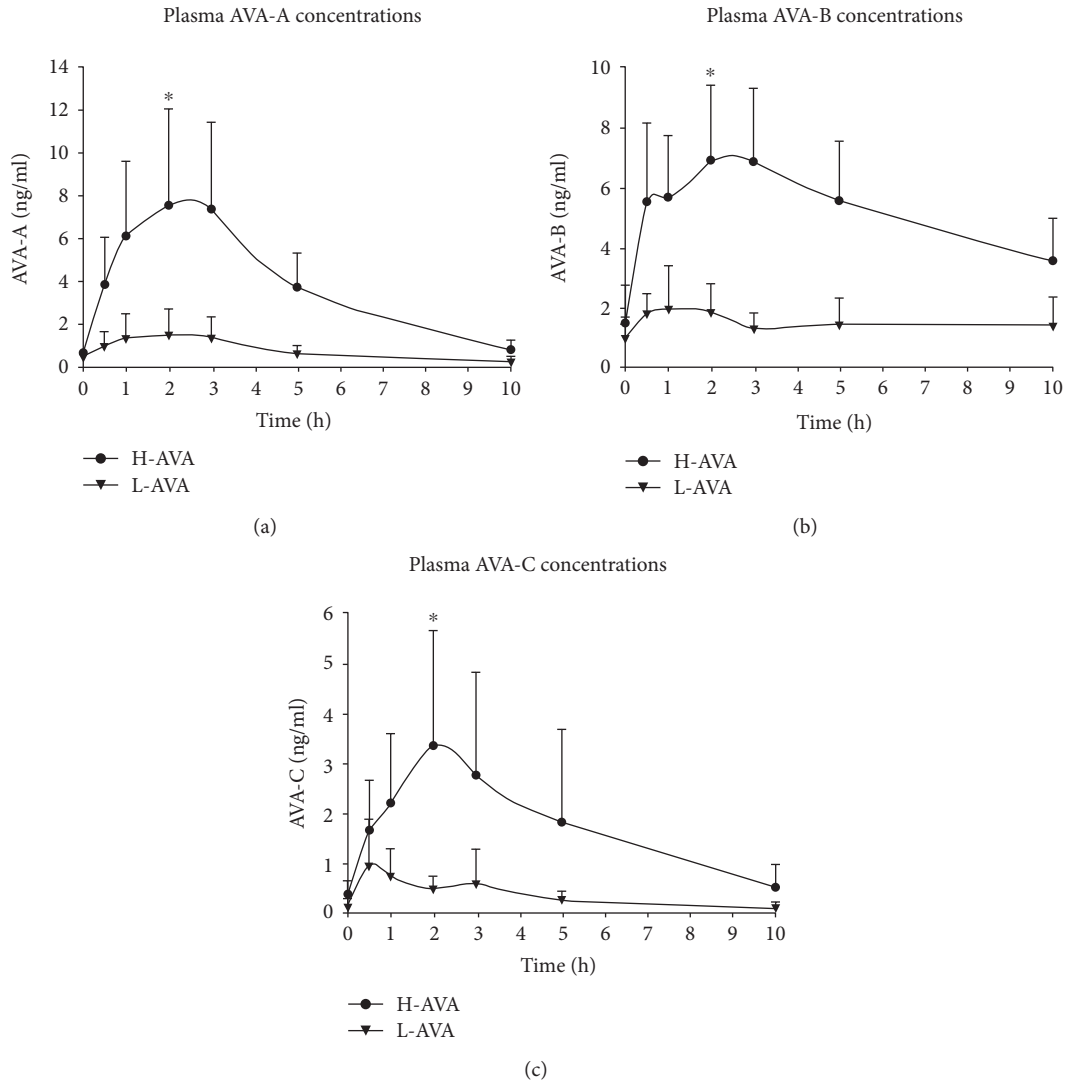

Figure 2: Changes in plasma AVA concentration over time. (a), (b) and (c) represent AVA-A, AVA-B and AVA-C, respectively. Data shown as mean ± SD at each time point. Mean AUC_{0-t} was compared between H-AVA and L-AVA for AVA-A, AVA-B, and AVA-C. $^{*}P < 0.001$, H-AVA versus L-AVA

Table 3: Pharmacokinetic properties of AVAs.

	C_{max} (ng/ml)		T_{max} (h)		$T_{1/2}$ (h)	
	H-AVA	L-AVA	H-AVA	L-AVA	H-AVA	L-AVA
AVA-A	8.39 ± 4.2^{c}	$1.98 \pm 1.3^{*}$	2.50 ± 1.2	1.80 ± 1.3	2.16 ± 0.6^{b}	2.44 ± 0.8
AVA-B	8.44 ± 2.3^{c}	$2.43 \pm 1.1^{*}$	2.04 ± 0.9	1.50 ± 1.5	4.23 ± 0.8^{ac}	4.60 ± 2.7
AVA-C	4.26 ± 2.0^{ab}	$1.33 \pm 0.7^{*}$	2.29 ± 1.0	1.32 ± 1.0	2.71 ± 1.2^{b}	2.87 ± 1.4

$^{*}P < 0.001$ versus H-AVA; $^{a}P < 0.05$ versus AVA-A within H-AVA or L-AVA group; $^{b}P < 0.05$ versus AVA-B within H-AVA or L-AVA group; $^{c}P < 0.05$ versus AVA-C within H-AVA or L-AVA group.

elimination rate in rats. This discrepancy between human and rat studies might be due to species differences in phase I and II metabolism [17].

Although participants received a higher dose of AVA-C than AVA-A, plasma concentrations of AVA-C were much lower than those of AVA-A. Likewise, AVA-C showed lower absorption than AVA-A and AVA-B, suggesting its metabolism may be different from the traditional liver phase II pathway. Wang et al. showed that oat AVA-C can be biotransformed and metabolized by mouse and human microbiota into reduction product dihydroavenanthramide-(DH-) 2c, hydrolysis product caffeic acid (CA), and

Table 4: Plasma absorption of AVAs.

	Mean AUC_{0-t} (h × ng/ml)		Mean K_{el} (h^{-1})		Dose (ng × 10^6)	
	H-AVA	L-AVA	H-AVA	L-AVA	H-AVA	L-AVA
AVA-A	42.56 ± 17.9[bc]	8.50 ±4.4*[b]	0.35 ± 0.1[b]	0.32 ± 0.1[b]	5.09	0.57
AVA-B	53.17 ± 12.1[ac]	6.31±1.6*[ac]	0.17 ± 0.1[ac]	0.20 ± 0.1[ac]	8.67	0.95
AVA-C	19.81 ± 12.7[ab]	3.88±2.0*[b]	0.30 ± 0.1[b]	0.30 ± 0.2[b]	7.85	1.55

*$P < 0.001$ versus H-AVA; [a]$P < 0.05$ versus AVA-A within H-AVA or L-AVA group; [b]$P < 0.05$ versus AVA-B within H-AVA or L-AVA group; [c]$P < 0.05$ versus AVA-C within H-AVA or L-AVA group.

reduction of hydrolysis product DH-CA [18]. The hydrolysis of AVA-C could also explain why plasma concentrations of AVA-C were lower than those of AVA-A and AVA-B. In this study, we also observed that concentrations of plasma caffeic acid (an AVA-C hydrolysis product), but not concentrations of AVA-A and AVA-B hydrolysis products, increased after oat cookie consumption (data not shown), which might explain the relatively low plasma concentration of AVA-C. In addition, the pattern of differences in AUC for AVA-A and AVA-B between H-AVA and L-AVA groups is not consistent with the doses received, which might also be evidence for metabolic variations between different AVAs. Further studies are required to confirm this hypothesis.

We found that L-AVA group had shorter T_{max} for AVA-A, AVA-B, and AVA-C compared with H-AVA group, indicating that AVA absorption might be dose-dependent. L-AVA group showed relatively flat time-dependent concentration curves compared with H-AVA group, with plasma AVA concentrations fluctuating around the MS detection limit (~1 ng/ml), indicating that L-AVA group could serve as an appropriate control group compared with H-AVA group. In addition, variations in plasma AVA concentrations among individual participants were relatively large, reaching 50% SD, especially 1–3 h after oat cookie consumption. This variation is similar to that reported in a study on the bioavailability of AVA after acute consumption of "false malted" oat bran, which is high in endogenous AVA [13]. In addition, we also compared the pharmacokinetic parameters between male and female subjects within H-AVA and L-AVA groups (results are not shown) and found some significant differences between female and male subjects, especially in L-AVA group. Female subjects tend to have higher C_{max}, mean AUC, and shorter $T_{1/2}$ than male subjects, indicating female subjects might have higher absorption profile over time and faster absorption rate on AVA than male subjects. However, to clarify these phenomena or mechanisms, further investigations with a more sensitive equipment, more time points, and higher AVA doses are required to compare the dose-response curves.

One limitation in this study was the lack of determination on absolute and relative bioavailability of AVAs. The bioavailability was not calculated because we did not have intravenous AVA doses or other comparable biological products for oral intake. In conclusion, we found that AVAs consumed from cookies made with natural oat flour are absorbed in humans. AVA-B demonstrated the slowest elimination rate and longest $T_{1/2}$, perhaps due to its more hydrophobic hydroxycinnamic moiety. Also, AVA-C was estimated to have the lowest relative absorption compared to AVA-A and AVA-B, possibly due to its biotransformation or hydrolysis process. These data demonstrated that AVAs are bioavailable to human, and its previously proved anti-inflammatory and antioxidant effects make it a useful natural phytochemical in sports science and chronic disease prevention. Further investigations are needed to determine the bioavailability of other AVA compounds and the mechanisms contributing to the different absorption and elimination profiles of various AVAs.

Disclosure

The views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo Inc. An earlier version of this work was presented as a poster at the Journey Through Science Day Project 2015, and the Experimental Biology 2016 Meeting.

Conflicts of Interest

This study was sponsored by PepsiCo Nutrition. Jodee Johnson and YiFang Chu are full-time employees of PepsiCo Inc., which manufactures oatmeal products under the brand name Quaker Oats. YiFang Chu and Jodee Johnson claim conflicts of interest.

Authors' Contributions

Li Li Ji, YiFang Chu, and Jodee Johnson designed the research; Tianou Zhang, Jing Shao, and Yike Gao conducted the research; Tianou Zhang, Jing Shao, and Chi Chen analyzed the data; Tianou Zhang analyzed data and wrote the paper; and Chounghun Kang and Dan Yao provided laboratory technical support. All authors read and approved the final manuscript.

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Review Article

Treatment of the Fluoroquinolone-Associated Disability: The Pathobiochemical Implications

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Long-term fluoroquinolone-associated disability (FQAD) after fluoroquinolone (FQ) antibiotic therapy appears in recent years as a significant medical and social problem, because patients suffer for many years after prescribed antimicrobial FQ treatment from tiredness, concentration problems, neuropathies, tendinopathies, and other symptoms. The knowledge about the molecular activity of FQs in the cells remains unclear in many details. The effective treatment of this chronic state remains difficult and not effective. The current paper reviews the pathobiochemical properties of FQs, hints the directions for further research, and reviews the research concerning the proposed treatment of patients. Based on the analysis of literature, the main directions of possible effective treatment of FQAD are proposed: (a) reduction of the oxidative stress, (b) restoring reduced mitochondrion potential $\Delta\Psi_m$, (c) supplementation of uni- and bivalent cations that are chelated by FQs and probably ineffectively transported to the cell (caution must be paid to Fe and Cu because they may generate Fenton reaction), (d) stimulating the mitochondrial proliferation, (e) removing FQs permanently accumulated in the cells (if this phenomenon takes place), and (f) regulating the disturbed gene expression and enzyme activity.

1. Long-Term Adverse Reactions Caused by Fluoroquinolones

Fluoroquinolones (FQ) belong to the group of broad-spectrum antibiotics, effective for both gram-negative and gram-positive bacteria. The most frequently prescribed drugs are ciprofloxacin (CIP), norfloxacin (NOR), and levofloxacin (LEV). FQs employ their antibacterial effect by preventing bacterial DNA from unwinding and duplicating which takes place by inhibition of bacterial topoisomerase and gyrase. For the last three decades, FQs played an important role in treatment of serious bacterial infections, especially hospital-acquired infections. However, due to the possibility of serious side effects, these drugs are not currently first-line medicines and their use becomes more restrictive and limited. FQs should be reserved for those who do not have alternative treatment options.

In 2016, the US Food and Drug Administration (FDA) updated warnings, using next "black box" for oral and injectable FQs. The authors showed that FQs, when used systemically, are associated with disabling and potentially permanent serious side effects. These side effects can involve the disruption of tendons, joins, muscles, nerves, nervous system disturbances, and even induction of type 2 diabetes. Due to the increasing number of reports about FQ toxicity and long-term complications, FDA has introduced significant restrictions on their use in recent years, particularly in children and in people aged 65 years.

1.1. Tendon Rupture. FQs are associated with a significant risk of tendonitis and tendon rupture. Stephenson et al. [1] showed in their review the incidence of tendon injury among those taking FQs to be between 0.08 and 0.2%. In 2014, Lewis and Cook [2] proved that FQ-related tendinopathy is a complication of treatment with this family of antibiotics and it is usually linked with 1 or more synergistic factors: male sex, age, renal disease, rheumatic disease, coprescription of corticosteroid, and physical activity. For this reason, some sport medicine specialists have advised avoidance of FQs for athletes. Some authors, for example, [3, 4], proved that chronic renal disease, concomitant use of corticosteroids, and age > 60 years are known risk factors for FQ-induced tendinopathies. Concluding, FQs are associated with an increased risk of tendinitis and tendon rupture. This risk is further increased in those over age 60, in kidney, heart, and lung transplant recipients, and with use of concomitant steroid therapy.

1.2. Nervous System Disturbances. Taking FQs is associated with their neurotoxicity as well [5–8]. The main symptoms being correlated to FQ treatment include insomnia, restlessness, and, rarely, seizure, convulsions, and psychosis [9–11]. Many reports point to chronic persistent peripheral neuropathy to be generated by FQs [12–18]. Cohen [19] showed that a possible association between FQ and severe, long-term adverse effects involving the peripheral nervous system as well as other organ systems is observed.

1.3. Cardiotoxicity. Stahlmann and Riecke [20] showed that FQs prolong the heart's QT interval by blocking voltage-gated potassium channels. In some cases, this can be a life-threatening condition because prolongation of the QT interval can lead to torsades de pointes, a life-threatening arrhythmia. Statistically, significant risk factors for clinically significant changes in QTs were hypokalemia and a left ventricular ejection fraction < 55%.

1.4. Hepatotoxicity and Nephrotoxicity. The other adverse reactions generated by FQs include hepatotoxicity [21] and nephrotoxicity [22]. Golomb et al. [23] reported a case-series study and showed the potential occurrence of serious, persistent, and delayed multisymptom serious side effects apparently triggered by FQ use causing severe functional compromise and disability in previously vigorous, healthy individuals. In this study, Golomb et al. described patients who developed new-onset symptoms during and following FQ use. Domains of serious and persistent sequels included the better-recognized tendon and muscle issues but extended to the well-reported but still often unappreciated potential for cognitive, psychiatric, peripheral nervous, and gastrointestinal issues as well as endocrine issues.

1.5. Diabetes Mellitus. Telfer [24] conducted interesting study about FQ intake and development of type 2 diabetes mellitus (T2DM). They hypothesized that FQs induce an intracellular Mg^{2+} deficit that can lead to insulin resistance. Their data suggests that FQ exposure predisposes an individual to develop diabetes. He also showed a strong correlation between the increase in FQ application in the US in years 1990–2012 and the increase in T2DM incidences in subsequent years which suggests a large part of T2DM to be maybe generated by FQ exposure.

1.6. FQ-Associated Disability. In 2016, Kaur et al. [8] conducted basic science and clinical investigations of a newly identified adverse drug reaction, termed FQ-associated disability (FQAD). They proved that severe toxicities that develop when cancer patients receive supportive care drugs such as FQs are important, yet difficult to understand, detect, and to communicate to clinicians. Their findings supported recommendations of the FDA's advisory committee. Revision of FQ-product labels should be considered to include prominent descriptions of a newly identified FQ-associated long-term toxicity.

Concluding, patients with impairments of the CNS (e.g., epilepsy or arteriosclerosis), prolongation of the QT, elderly persons, and individuals with concurrent use of glucocorticoids or chronic renal diseases should not be treated with FQs. FQs are contraindicated in children because they cause destruction of the immature joint cartilage in animals. The use in pediatrics is restricted to life-threatening infections.

2. Oxidative Stress

One of the main effects generated by FQs in cells is connected with the oxidative stress (OS). Thus, a brief review about OS is presented below.

The main aspect of OS involves the overdosed leakage of electrons from the electron transport chain (ETC). In the normal, healthy state, the Krebs cycle (KC) supplies hydrogen from glyco- and lipolysis in the form of $NADH_2$ and $FADH_2$ from KC to ETC. ETC separates hydrogen into protons and electrons. Protons are transported into mitochondrial intermembrane space (IMS) generating proton gradient, and mitochondrial membrane potential $\Delta\Psi_m$ across the inner membrane and electrons is transported into oxygen. This complex process is a masterwork of the evolution because 4 electrons must enter the oxygen simultaneously in order to produce 2 water molecules. In the case of the block in the electron transport and the oxygen not to be fully reduced in one step, the reactive oxygen species (ROS) are created, which might be able to generate OS.

The total physiological electron leakage earlier estimated to be about 1% of electrons supplied to ETC [25, 26] currently has been estimated in favourable conditions to be 0.1–0.5% in rest [27–29] and 0.01–0.03% in the exercise [30, 31]. Assuming the oxygen consumption by the human body in the rest to be about 500 g (which corresponds the basal metabolic rate of 1920 kcal/24 h), the total electron current in all human mitochondria can be easily estimated to be about 70 Amperes. The 0.1% leakage denotes the leakage electron current (LEC) to be about 0.07 Amperes. In the case of physical or mental exercise, both the metabolic rate and LEC increase several times; however, recent studies show that LEC does not depend so strictly on the metabolic rate [30]. Disturbing the precise mechanism of electron flow through ETC causes nearly always the increased leakage which can increase LEC even up to 10% (~7 A in the rest). Majority of

toxins joining ETC may do this leak. Because the increased OS is the side effect of the exercise state and OS is one of the limits of the exercise, the increase in LEC during the rest may reduce the reserve for the OS increase during the exercise. The patient may feel tired even during small physical or mental exercise.

O_2^- is the first oxygen radical created by LEC. Joining the second electron causes the generation of H_2O_2, and joining the third electron to H_2O_2 creates a very dangerous hydroxyl radical OH^*. H_2O_2 is relatively stable and works as a cellular sensor of the OS state and is involved in the cellular metabolism regulation [30, 32]. Brand [30] has recently detected 11 sites in ETC where electrons leak from ETC generating OS. Brand and coworkers propose new postulate that the rate of electron leak does not depend on the electron flow rate but on the redox state (electron pressure) of the given site of leakage. Thus, ETC blockers increase the leak in sites before the block and decrease the leak behind the block. Majority of the sites generate the leak to the mitochondrial matrix and only two sites to both sides of inner mitochondrial membrane: the sites III_{Qo} connected with complex III and G_Q connected with glycerol 3-phosphate dehydrogenase [30]. Brand et al. suggest also that the increase in O_2^- production from complex III that signals hypoxia to the HIF-1α signaling system in cells is probably caused by indirectly changed metabolite concentrations that deliver more electrons to the ETC and leads to higher electron leak to oxygen. This is of high importance because as will be discussed later in this paper, HIF-1α signaling is blocked in FQ patients disturbing strongly very important energy production regulatory pathway.

The last radical being created during the OS is OH^*. It is very dangerous because its lifetime is very short (10^{-9} s) and the cell does not possess any enzymes removing it. The overdosed production of OH^* is generated especially by Fenton reaction or in the ischemia state in which, because of lack of the oxygen, all the electrons create ROS. This massive ROS production induces the death of the ischemic cell.

The relatively high physiological LEC forced the evolution to create the mechanisms carrying against free radicals. The first and, thus, most important barrier is the enzyme SOD2 (MnSOD, mitochondrial superoxide dismutase) which annihilates O_2^-—the first molecule of the O_2-radical chain ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$). H_2O_2 is next removed by catalase or glutathione peroxidase. Meanwhile, it comes out of mitochondria and regulates, for example, redox-dependent Kv1.5 channels in the cell membrane [32]. This mitochondrion-ROS-Kv channel axis is now recognized as basis of an important O_2-sensing mechanism in many tissues [33].

The simplified scheme of the electron leakage from ETC is presented in Figure 1.

2.1. The Role of Mitochondrial Permeability Transition Pores (PTP) in the Regulation of Energy Production. The mitochondrial permeability transition pore (PTP) is a large protein complex placed in the outer mitochondrial membrane being precisely regulated by many factors [34, 35]. It consists mainly of voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D (CypD). In order to make the cell function properly, the degree of the opening state of this complex must be precisely fitted to the actual physiological state of the cell. If the complex is open, the nonselective traffic between the IMS and cytosol of small-charged particles, water, and substances up to 1.5 kDa takes place. ADP can enter mitochondria to produce ATP but the protons leak from IMS to cytoplasm reducing the mitochondrial potential $\Delta\Psi_m$ from −140 mV to about −110 mV and contributing to apoptosis. If the complex is closed, ADP cannot enter mitochondria to produce ATP and $\Delta\Psi_m$ growths from −140 to −160 mV. The decrease in $\Delta\Psi_m$ is a characteristic for OS state, and the increase in $\Delta\Psi_m$ is a characteristic for some types of cancers [32]. This observation explains the ability of FQs to treat cancers [36–41].

The main factors that regulate PTP state [34] are as follows:

(a) Opening PTP

(i) $[Ca^{2+}]_{mit}$: concentration of mitochondrial Ca^{2+}

(ii) Reduced $\Delta\Psi_m$ (positive loop)

(iii) Free radicals (oxidative stress)

(iv) Inorganic phosphate (only with Ca^{2+})

(v) Some apoptotic factors

(b) Closing PTP

(i) Acidic pH (a part of cancer state is lactate accumulation in the cell)

(ii) ATP, ADP and NADH

(iii) Mg^{2+}

Hexokinase II (HKII), mitochondrial creatine kinase (CK), benzodiazepine receptor (PBR), and Bcl-2-family members (Bcl-2, Bcl-xL, and Bax) are putative regulatory components.

It can be observed that OS contributes to opening PTP and reducing $\Delta\Psi_m$. Reduced $\Delta\Psi_m$ causes further opening of PTP and the decrease in energy production. The final step and physiological sense of this positive loop are the induction of the apoptosis. However, if the apoptosis induction is not reached, the new balance between factors being in the common regulatory loop finds a new equilibrium point which can be far away from that optimal one. The "positive loop" regulation of PTP points to its high sensitivity to different factors controlling it and suggests the regulation of PTP to be one of the most important points of energy production control. Some positive regulatory loops are presented in Figure 2. According to the control theory, such positive loops denote that the reaction to small stimulus may be strongly magnified. The influence of FQs on the detailed regulation of PTP is the urgent topic for further research.

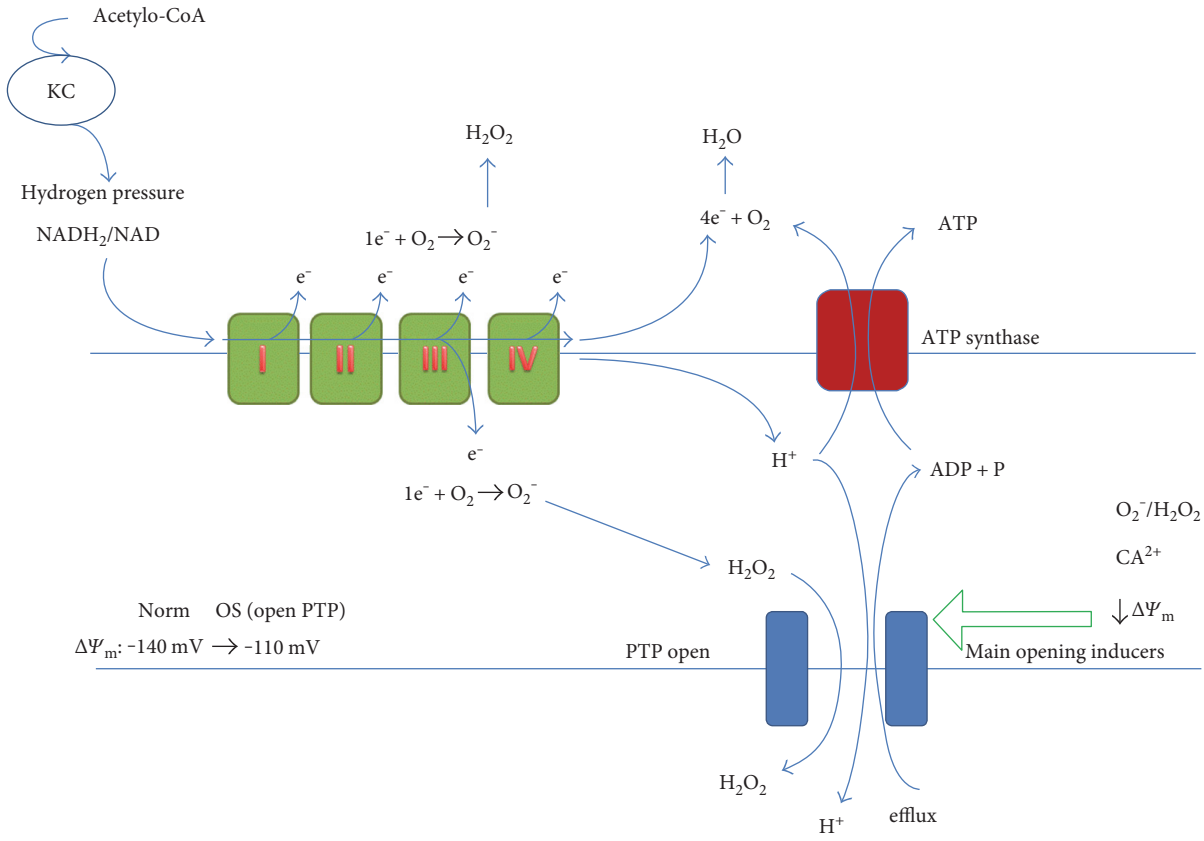

Figure 1: The schematic presentation of the ATP production system. The acetyl-CoA supports Krebs cycle to produce $NADH_2$. Hydrogen from $NADH_2$ (and $FADH_2$) enters the cytochrome chain. Some electrons leak before they reach oxygen generating O_2^- and next H_2O_2. H_2O_2 comes away from mitochondria and works as a redox signaling molecule. The amount of leaking electrons depends mainly on $NADH_2$/NAD ratio (hydrogen pressure). The degree of PTP opening must be precisely regulated and depends on many factors, for example, O_2^-/H_2O_2, Ca^{2+}, and $\Delta\Psi_m$.

2.2. How the Cell Adapts to OS State. Increased OS state is characterized first by increased H_2O_2 level in the cell. In the physiological state, H_2O_2 level informs the cell and nucleus about the mitochondrial energy production state because, in the physiological conditions, it is connected with the metabolic rate of the cell. In the case of disturbed metabolic regulation (e.g., mitochondrial toxins), increased OS and open PTP, ATP production is reduced. The main process that consumes ATP in the cell is the Na/K pump that removes Na^+ from the cell, pumps K^+ to the cell, and generates the negative membrane potential of the cell. The work of Na/K pump is the most fundamental process of life because it consumes 20% to 50% of ATP and generates strongly negative charge inside the cell. Many other transports that take place across the cell membrane work as Na^+ cotransport, for example, amino acids, phosphates, Ca^{2+}, and boric acid. One molecule of Na^+ cotransport contributes to about 30% of ATP/ADP Gibbs free energy; thus, it can be treated as "small energy bricks" when compared to ATP as a large one. The lack of ATP reduces the work of Na/K pump reducing negative membrane potential ΔV and K^+ concentration in the cell. However, H_2O_2 generated during OS activates the opening of redox-dependent Kv1.5 channels in the cell membrane [32] contributing to the further efflux of K^+ from the cell. The escaping K^+ increases temporarily the negative potential inside the cell. It can be concluded that restoring the negative cell potential is more important for the cell than keeping high K^+ concentration in the cell.

On the other hand, in the case of reduced ATP production, the lowered ΔV opens voltage-gated Ca^{2+} channels which causes Ca^{2+} influx into the cell [32]. Increased $[Ca^{2+}]_i$ activates calcineurin. Activated calcineurin shifts NFAT (nuclear factor of activated T-cells) to the nucleus where it inhibits Kv1.5 potassium channel production. Lowered amount of Kv1.5 channels reduces K^+ efflux. Concluding, in the OS state, Kv1.5 channels are more open; however, their amount is reduced. Two opposite mechanisms regulating the membrane potential and K^+ efflux find some new equilibrium. This mechanism seems to be the natural adaptation process to the increased metabolic rate of the cell generating physiological OS. In the state of increased metabolic rate, the concentration of K^+ (and most probably Mg^{2+}) is reduced, the concentration of Ca^{2+} and H_2O_2 (and most probably Na^+) is higher, and the membrane potential is also reduced. If the healthy cell returns from the increased metabolic rate to the resting state, all these parameters return to their optimal values. This return depends on the ability of the mitochondria to produce ATP which drives Na/K pump.

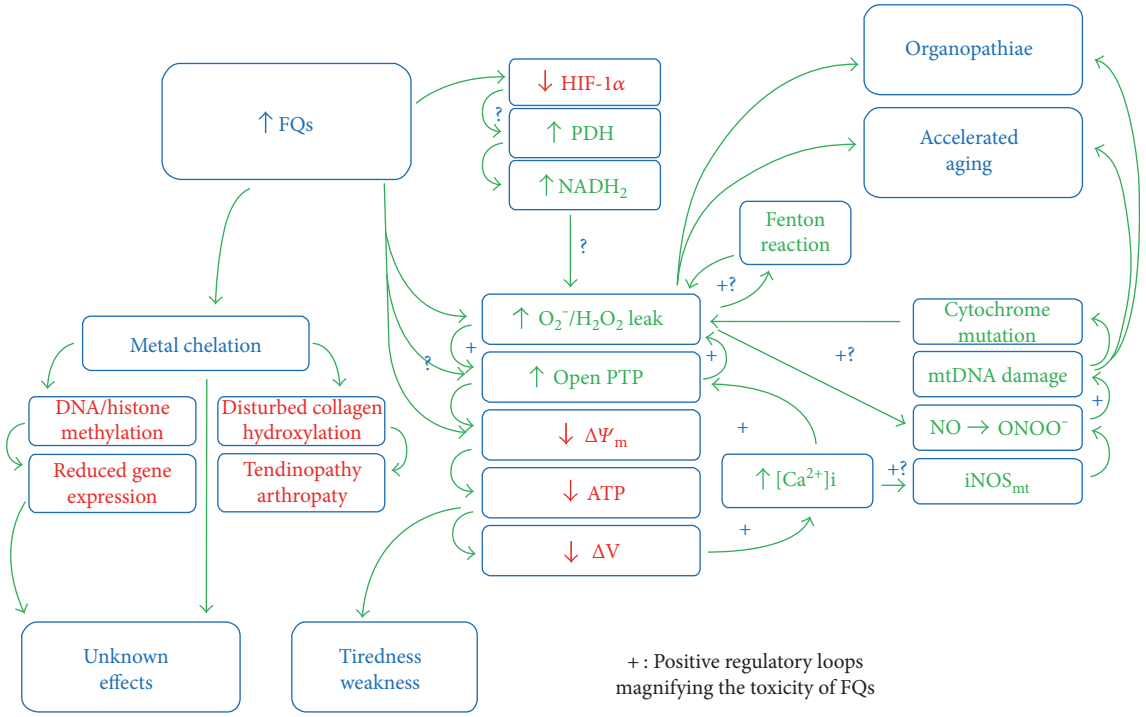

Figure 2: The main ways of FQ toxicity. The positive regulatory loops magnifying the toxicity of FQs are marked with "+." The "?" signs denote the possible but not confirmed effects of FQ toxicity.

In the case of the permanent OS or disturbed state of PTP being inadequately open, this return cannot take place because the ATP production is lowered. The cell being in the resting state comes to the state which can be called "permanent stress adaptation." In the case of the necessity to increase the metabolic rate, further increase in metabolic rate is difficult because of the lack of physiological adaptation reserve. The final effect for the patient is the feeling of "the lack of energy." Many other regulative processes take place, of course, as OS adaptation. However, the above-described changes belong in authors' opinion, to the primary regulatory axes.

3. Molecular Mechanisms of FQ Toxicity

Good understanding of the OS state is very important for understanding the consequences of OS generation by FQs. From the therapeutic point of view, the important question concerns the molecular mechanisms leading FQs to generate OS, because they determine the possible effective treatment of this state. Many people are waiting for the understanding of the FQ toxicity mechanisms and treatment methods. Beside OS, epigenetic effects of FQs are of high importance as well. The epigenetic effects may depend on the methylation of DNA and/or histones; however, ROS contribute also to epigenetic changes [42]. Some authors point also to the similarity of bacterial and mitochondrial DNA, both existing in circular super-twisted helices and gyrase-like enzymes being postulated to be responsible for the organization of mitochondrial DNA, suggesting the possible direct effect of FQs to mitochondrial DNA leading to the disturbed mitochondrion regeneration and division [43, 44]. The changes in the cytoskeleton were observed also after FQ treatment [45], and cytoskeleton has been demonstrated to be strictly connected with energy dissipation and organization in mitochondria [46–49]. The most important elements of FQ toxicity are presented in Figure 2. The positive regulatory loops magnifying the toxic effects are marked with "+." Let us analyze the most important aspects of the molecular activity of FQs in the cell, being reported until now.

3.1. Chelating Bivalent Cations and Proteins by FQs. FQs are the group of chemical compounds called zwitterions. Zwitterion is a neutral molecule with both positive and negative electrical charges on its opposite sides. This feature makes them possible to create strong complexes with both protein anions and positively charged bivalent cations. The protonation constants for the acidic and base part of the FQ molecules were estimated as $pK_1 = 8.2–8.5$ and $pK_2 = 5.6–6.2$. These values denote that the dissociation coefficients are equal to about 90–95% in neutral intracellular conditions of pH = 7.0 for both the acidic and base side of the molecule [50]. The structure of the exemplary FQ is presented in Figure 3.

FQs possess two main sites for metal chelate formation. The first one, represented by the carbonyl and carboxyl groups in neighboring positions, is the most common coordination mode in the quinolone chelates [50]. Quinolones bind divalent cations as Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and Co^{2+}, forming chelates with 1 : 1 or 1 : 2 metal : FQ stoichiometry or trivalent cations (e.g., $A1^{3+}$ and Fe^{3+}), forming chelates with 1 : 1, 1 : 2 or 1 : 3 metal : FQ stoichiometry. The constant values for CIP chelates have been measured to decrease in the following order: $Al^{3+} > Fe^{3+} > Cu^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$

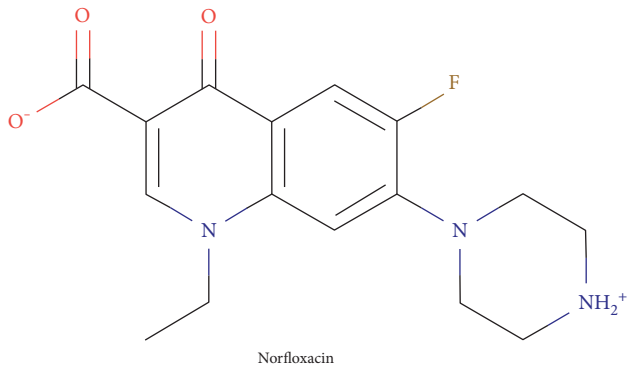

FIGURE 3: The exemplary FQ—norfloxacin and its zwitterion structure.

[51]. For NOR chelates, the relation is quite similar: $Fe^{3+} > Al^{3+} > Cu^{2+} > Fe^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$. Let us observe that presented research [50, 51] did not analyze Se^{2+} ions which are also of high importance in the cell, because Se^{2+} is the cofactor of glutathione peroxidase removing H_2O_2. The examination of the ability of FQs to generate Se^{2+}-FQ complexes is the important aim for further research.

Seedher and Agarwal [52] analyzed the ability of 5 cations: Fe^{3+}, Al^{3+}, Zn^{2+}, Cu^{2+}, and Mg^{2+} to create the complexes with four FQs and human serum proteins using fluorescence UV absorption spectroscopy. They measured the association constants to be of the order of 10^2–10^4 for the FQ-Me^{n+} interaction. The interaction was the highest for Al^{3+} and lowest for Mg^{2+}. At a Me^{n+}/drug ratio of 1 : 1, approximately 50%–73% of metal ion was bound per mole drug in most cases. Seedher's results indicate that chelate formation with bivalent metals can cause significant alteration in the human serum-FQ-binding affinity.

Koga [53] measured the FQ concentration ratio between intra- and extracellular fluid in human polymorphonuclear leukocytes using high-performance liquid chromatography. This c_i/c_{out} ratio oscillated between 2.2 to 8.2. In another paper, Pascual et al. [54] presented c_i/c_{out} gradient for trovafloxacin (TRV) to be about 9 for extracellular concentrations ranging from 0.5 to 25 ug/mL. Assuming net charge of the FQ molecules being close to 0, the expected ratio for the soluble fraction is expected to be close to 1 : 1. These results point to the ability of FQs to create complexes with different intracellular molecules which do not participate to the soluble fraction of FQs in the cell.

The other important feature of FQs has been presented by Andriole et al. [55]. Namely, they estimated the minimum solubility of FQs in neutral pH. They pointed that this class of molecules is characterized by very high melting point, generally > 200°C, which indicates that the crystal forms are very stable.

All these FQ features strongly support the thesis that FQs can survive in the cell for a long time contributing to chronic, long-term adverse reaction in patients treated with FQs. The question, to what extent this phenomenon takes place and if it contributes to chronic symptoms of FQAD, remains unclear.

Metal ion chelating seems to be the most fundamental feature of FQs which probably leads to all other observed toxic effects. The antibacterial effect is connected with chelating Mg^{2+} which disturbs the gyrase and topoisomerase interaction with DNA. However, the Mg^{2+} is described to create weaker chelates with FQs than other important ions like $Fe^{2/3+}$, $Cu^{1/2+}$, Zn^{2+}, and Mn^{2+}.

The well-described activity of FQs which concerns the Fe^{3+} chelating was examined by Badal et al. [56]. They examined that three FQs, NOR, CIP, and enrofloxacin (ENR), are the powerful $Fe^{2/3+}$ chelators comparable with deferoxamine, a clinically useful Fe-chelating agent. He showed that $Fe^{2/3+}$chelating by FQs leads to epigenetic effects through inhibition of α-dependent dioxygensases (DOXG) that require Fe as a cofactor. Three important DOXGs were analyzed: jumonji domain histone demethylase, TET DNA demethylase, and collagen prolyl 4-hydroxylase. The activity of all 3 enzymes was reduced by FQs in micromolar concentrations leading to accumulation of methylated histones and DNA and to inhibition of proline hydroxylation. The IC_{50} concentrations for Fe-chelation were equal to about 52 ± 20 uM for CIP, 44 ± 15 uM for NOR, 41 ± 20 uM for ENR, and 360 ± 25 uM for deferoxamine. These results point to high ability of FQs to absorb $Fe^{2/3+}$ and reduce the activity of enzymes which use $Fe^{2/3+}$ as a cofactor. The results suggest also the possibility that the tendon FQ toxicity is dependent on lack of collagen proline hydroxylation which changes significantly the mechanical properties of the collagen. Some other studies report, however, the association of the enhanced extracellular matrix metalloproteinases [51, 52], mainly collagenases expression [53], to be associated with FQ-induced tendinopathy.

It must be pointed that cytochromes are the other important proteins which use $Fe^{2/3+}$ in hem groups as a cofactor. Thus, the question arises if the reduced free $Fe^{2/3+}$ concentration in the cell contributes to ETC inhibition, electron leakage, and/or OS. The question arises, as well, which cellular effects are connected with Cu^{2+} and Zn^{2+} ions, because Cu^{+} is also an important cytochrome cofactor and Zn^{2+} is important due to high number of enzymes (about 300 total) being cofactored by this element and the total cytoplasm concentration of Zn^{2+} is similar and even higher than that of $Fe^{2/3+}$. Valko et al. [57] present that redox-inert zinc (Zn^{2+}) is the most abundant metal in the brain and an essential component of numerous proteins involved in biological defense mechanisms against oxidative stress. The depletion of zinc may enhance DNA damage by impairing DNA repair mechanisms.

Some effects of Mg^{2+} chelation are also well described, especially with respect to the cartilage damage. Mg^{2+} is an important intracellular ion being a cofactor of about 300 enzymes. Shakibaei et al. [58] showed that effects of individual dose of ofloxacin give identical effects in cartilage observed in electron microscopy as Mg^{2+}-deficient diet suggesting that quinolone-induced arthropathy is probably caused by a reduction of functionally available Mg^{2+} in cartilage. Similar results were observed during cultivation of chondrocytes in Mg-free medium [45, 59–61]. The supplementation of Mg^{2+} accompanying the FQ treatment restored

to some degree the cartilage lesions [60, 61], however, did not restore the reduced cell division [61]. This suggests that other mechanisms are involved in cell division reduction after FQs [44]. Mg^{2+} deficiency in immature dogs induced similar clinical symptoms as quinolone treatment: distinct alterations in chondrocytic fibronectin staining and their ultrastructure [62, 63]. The effect of Mg^{2+} supplementation had a two-way effect on FQ-treated cultured chondrocytes: the number of cells adhering to culture support was increased and cell morphology was comparable to that of control cells. This suggests that addition of Mg^{2+} restores extracellular Mg^{2+}-dependent intercell interactions.

On the other hand, dietary Mg^{2+} is also presented to reduce intestinal FQ absorption [64–70] and it is also postulated to be in relation to diabetes type 2 and FQ treatment [24].

Summing up, the number of enzymes possessing reduced activity due to their ion-cofactor chelation is probably long and it is the important topic for further research. The separate problem consists the chronicity of ion chelation by FQs. The presented research does not describe the chronic state of FQAD but the phenomena taking place during FQ application. It must be analyzed as to which degree persistent ion chelation takes place at FQAD patients.

3.2. Oxidative Stress Generated by FQs. Many papers point to the feature of FQs to generate oxidative stress in the cells. The molecular mechanisms leading to OS state differ probably in details for different FQs depending on different abilities to chelate subsequent metal ions and on possible different abilities to change enzyme activity in the ion-independent manner.

An example of ion-independent FQ activity was presented by Qin and Liu [71]. They analyzed the influence of CIP and ENR on the erythrocytic catalase (CAT), a vital enzyme involved in OS reduction (CAT reduces H_2O_2 to O_2 and H_2O). The cellular tests firstly confirmed an enhanced oxidative stress in FQ-treated erythrocytes in the form of the GSH content depletion and decrease in CAT activity with CIP effect to be more harmful than ENR. Next, spectroscopic computations showed the FQ-binding places to CAT takes place mainly through electrostatic forces. Binding of two FQs not only caused the conformational and microenvironmental changes of CAT but also inhibited its molecular activity, which was consistent with the cellular activity measurements. On the other hand, the treatment with danofloxacin [72] increased antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT), suggesting that the ability of subsequent FQs to change the activity of different antioxidative enzymes can vary significantly.

Many papers present the existence of OS induced by FQs, for example, Pouzaud et al. [73] measured the redox status change of immortalized rabbit tendon cells as a response to pefloxacin, ofloxacin, LEV, and CIP. All FQs showed moderate cytotoxicity on tendon cells after 24 h and more severe, significant toxicity after 72 h. The intracellular redox potential has been reduced slightly but significantly after 72 h even at concentrations 1 uM (~10 uM is the therapeutic one) and strongly reduced at concentrations 1 mM (100x higher than therapeutic ones). ROS production has increased slightly (~25%) but significantly at therapeutic conditions and strongly (~150%) at 100 uM (10x higher than therapeutic ones). The intracellular GSH concentration was reduced by 20–50% even at 0.01 uM concentrations (1000x smaller than therapeutic ones), and the collapse (decrease in 50–90%) in GSH was observed at 1 mM. The question arises if the GSH depletion is connected only with the increased H_2O_2 generation or also with a reduced activity of GSH-reductase which restores the reduced form of GSH ($GSSG + NADPH_2 \rightarrow 2GSH + NADP$).

It is important to observe that the rapid increase in the toxicity takes place after the given concentration of FQs is reached which is only slightly higher than the therapeutic one. The substantial increase in the ROS production which can lead to serious consequences begins at concentrations being approximately 10x higher than that of the therapeutic ones. This factor is, however, estimated with low precision because no intermediate concentrations were examined between 0.01, 0.1, 1, 10, 100, and 1000 uM. Assuming that some people are charged with reduced ROS annihilation ability (e.g., as presented in [21]), this toxicity limit may occur at lower, therapeutic concentrations. This experiment stays also in the agreement with clinical observations that the FQAD takes place especially in patients who were treated with higher FQ doses, by a longer period or with FQ series repeated a few times in a short period of time.

In the other paper, Pouzaud et al. [74] observed in vitro in rat tendons the increased ROS production and reduced GSH concentration. Similar results were also showed by Yu et al. [72].

Some papers point to detailed FQ effects on different enzymes. Hsiao et al. [21] found that TRV-induced OS on heterozygous SOD2 (+/−) deficiency mice was higher than on the normal mice. Hepatic protein carbonyls were increased by 2.5-fold, and hepatic mitochondrial aconitase activity was decreased by 20% in mutant, but not in wild-type mice. Because aconitase is a major target of peroxynitrite, they determined the extent of nitrotyrosine residues in hepatic mitochondrial proteins (peroxynitrite $ONOO^-$ id formed by the combination of O_2^- and NO). TRV significantly increased nitrotyrosine in SOD2 (+/−) mice only. TRV increased also the production of mitochondrial NO in immortalized human hepatocytes. Similarly, mitochondrial Ca^{2+} was increased by TRV, suggesting Ca^{2+}-dependent activation of mitochondrial NOS activity. Furthermore, the transcript levels of the mtDNA-encoded gene Cox2/mtCo2 were decreased in SOD2 (+/−) mice only, while the expression of nDNA-encoded mitochondrial genes was not significantly altered in both genotypes, suggesting selective effects on mtDNA expression. These data indicate that TRV enhances hepatic mitochondrial peroxynitrite stress at increased basal O_2^- levels, leading to the disruption of critical mitochondrial enzymes and gene regulation.

Another important information can also be found in [21]. OS generation by FQs may take place at lower FQ concentrations in some people being charged with lower ability to reduce OS. The reasons of reduced OS barrier

may be different; however, the most important reasons seem to be the heterozygous mutations, trace element deficiencies, and charge of the cells with other toxins contributing to OS.

Kumbhar et al. [75] reported gatifloxacin (GAT) to produce retinal damage in rabbits and significant alteration in the antioxidant status indicated by the decreased activity of superoxide dismutase and decreased levels of blood glutathione with a concomitant increase in the activity of catalase, glutathione peroxidase, and glutathione S-transferase enzymes. The levels of malondialdehyde were also elevated. The effects were dose-dependent. Talla and Veerareddy [76] examined the OS parameters in the blood after CIP, LEV, and GAT therapy on SOD3 (extracellular), glutathione, plasma antioxidant status, and lipid peroxides evaluated at 53 patients on different dosage regiments up to 5 days. The significant elevation of lipid peroxide was observed in patients treated with CIP and LEV. The substantial depletion in SOD3 and glutathione was observed especially in CIP patients. All three FQs reduced the plasma antioxidant status, but especially CIP and LEV.

Liu et al. [77] determined the effect of ENR on the release of lactate dehydrogenase (LDH), reactive oxygen species (ROS), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), malondialdehyde (MDA), mitochondrion membrane potential ($\Delta\Psi_m$), and apoptosis in the hepatic cell line of grass carp. The doses of 50, 100, and 200 ug/mL increased the LDH release and MDA concentration, induced cell apoptosis, and reduced the $\Delta\Psi_m$ compared to the control. The highest dose of 200 ug/mL also significantly reduced T-AOC.

All the above-presented experiments show the increased OS state after FQ treatment. The changes in enzyme activity vary between experiments, tissues, and kinds of FQs suggesting possible variability of the common relations. What seems to be important is the reduction in SOD activity which is the first-line barrier against O_2^-. New experiments must estimate these changes in details; however, more attention must be paid to mitochondrial MnSOD which cares against mtDNA damage by O_2^- generated by leaking electrons from ETC to mitochondrial matrix. The increased activities of some anti-OS enzymes seem to be the OS adaptation processes.

3.3. Reduction of Mitochondrial $\Delta\Psi_m$ Potential by FQs. One of the cellular symptoms present in the FQ-charged cells is the reduction of the mitochondrial potential $\Delta\Psi_m$ [37, 38, 77–79]; however, the detailed mechanism of this phenomenon remains unknown. Since the main mitochondrion uncoupling factor is the PTP, the reasons of the reduced $\Delta\Psi_m$ should be searched between factors that regulate PTP opening. The first possibility is the OS by itself being generated by FQs. ROS can induce VDAC oligomerization (the main part of PTP) to yield a megachannel creation which are postulated to create large holes being able to release cytochrome c to cytoplasm. O_2^--induced apoptosis has been found to be inhibited by DIDS or anti-VDAC antibodies (VDAC blockers), which suggests that O_2^- increases VDAC-dependent permeabilization of the outer mitochondrial membrane [80, 81].

One of the proteins which can support PTP opening is translator protein (TSPO), called also peripheral-type benzodiazepine receptor or isoquinoline-binding protein. TSPO is predominantly located on the surface of the mitochondria where it is postulated to physically associate with VDAC-ANT. It has been suggested that TSPO may activate PTP opening, causing $\Delta\Psi_m$ reduction and leading to apoptosis [80, 81].

Some authors suggest that epileptogenic activity of FQs possibly relates to GABA-like structure of some FQs which may allow them to act as GABA antagonists [82, 83]. Since TSPO is also a benzodiazepine receptor, similar interaction may maybe also take place between FQs and TSPO leading to opening PTP.

The problem of the PTP opening is probably, however, more complicated and it is in relation with many other factors involved in energy production and apoptosis induction in the cell. The broad reviews about regulation of PTP and VDAC (the main PTP protein) are presented in [34, 35].

The other way to reduce $\Delta\Psi_m$ is the opening of UCP channels which generate the proton leak through the inner mitochondrial membrane. UCP regulation is a separate problem and possible way for treating FQAD patients. The review of this problem is published by Divakaruni et al. [84].

3.4. Does Fenton Reaction Take Place in FQAD Patients? The next problem that is connected especially with Fe^{2+} and Cu^+ ions is the possibility of the Fenton reaction (FR) to be generated on $Fe^{2/3+}$ and $Cu^{1/2+}$ ions, but maybe on the FQ-Fe^{2+} complexes as well. Fenton reaction consists in the conversion of O_2^- and H_2O_2 into strongly dangerous OH^* radical; it takes place on $Fe^{2+/3+}$ and $Cu^{1+/2+}$ ions and consists of 2 steps:

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^* + OH^-$

(2) a. $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$ or (2) b. $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + 2\ H^+$

This reaction increases strongly the effects of OS in the cell and if it is too intense, it may contribute to cell death. The question arises as to what degree does the Fenton reaction magnify initial OS effects.

There is no evidence that would prove such reaction to take place in FQAD patients; however, four theses could be postulated for further research: (a) The reaction is increased just due to the increased substrate concentration (O_2^- and H_2O_2) for FR. (b) The reaction intensity is reduced with respect to that expected due to the reduced Fe^{2+} level in the cell. This can take place due to reduced membrane potential and reduced ability Fe^{2+} to be pulled into the cell. (c) The reaction is magnified due to the increased ability of FQ-Fe^{2+} complexes to generate OH^* in FR reaction by itself. (d) The reaction is magnified due to upregulation of Fe-transporting protein which increases Fe^{2+} concentration in the cell. Similar upregulation has been detected in *Streptococcus pneumoniae* by Ferrandiz and de la Campa [85, 86]. They observed the upregulation of the genes of the fat DCEB operon involved in iron (Fe^{2+} and Fe^{3+}) uptake. In accordance, they observed an attenuation of LEV lethality in

iron-deficient media. However, the bacterial gene regulation cannot be directly compared to that mammalian one. On the other hand, electro-Fenton reaction is described to perform degradation of LEV in experimental conditions [87, 88]; however, it seems to be of low probability for such reaction to take place at in vivo conditions.

3.5. Changes in Gene Expression and Enzyme Activities after FQ Treatment. Besides OS aspects connected with FQAD, some papers point to other effects of FQ toxicity. Fox et al. [89] measured reverse-transcriptase quantitative polymerase chain reaction analyses on total RNA isolated from supraspinatus tendon of rats. They showed the significant upregulation of IL-1b mRNA, tumor necrosis factor (TNF), matrix metalloproteinases MMP-3 (30x increase), MMP-13 (7x), and the tissue inhibitor of metalloproteinases- (TIMP-) 1 (4x) in the FQ-treated rats. FQ-treated groups showed significantly less fibrocartilage and poorly organized collagen at the healing enthesis compared with control animals.

Aranha et al. [41] measured the gene expression effects of CIP on prostate carcinoma and healthy control cells. Treatment of prostate cancer cells with CIP resulted in a dose- and time-dependent inhibition of cell growth (70–100% with 50–400 ug/mL). Cells were arrested at the S and G2/M phases, and apoptosis was induced. The cyclin-dependent kinase (CDK) inhibitor p21/WAF1 was downregulated 12 h following CIP in treatment which can lead to rapid CDK2 activation and caspase-induced apoptosis. There was also observed significant increase in the Bax/Bcl-2 ratio with translocation of proapoptotic Bax to mitochondria and activation of caspase-3. Let us recall that Bax, Bcl-2, and capsace-3 are involved in PTP opening state.

Badal et al. [56] showed that NOR, CIP, and ENR as iron chelators lead to epigenetic effects through inhibition of alpha-ketoglutarate-dependent dioxygenases that require iron as a cofactor. Three dioxygenases were examined in HEK293 cells treated with FQ. At micromolar concentrations, these antibiotics inhibited jumonji domain histone demethylases, TET DNA demethylases, and collagen prolyl 4-hydroxylases, leading to the accumulation of methylated histones and DNA and inhibition of proline hydroxylation in collagen. These effects may explain FQ-induced nephrotoxicity and tendinopathy. Let us observe that the changes in DNA and histone methylation state cause strong and broad epigenetic effects being difficult to predict.

Liang et al. [90] measured the subchronic toxic effects of NOR on a swordtail fish by measuring mRNA expression of cytochrome P450 1A (CYP1A), cytochrome P-450 3A (CYP3A), glutathione S-transferase (GST), P-glycoprotein (P-gp), and their corresponding enzyme activities. Results showed that NOR significantly affected the expression of CYP1A, CYP3A, GST, and P-gp genes in swordtails. The gene expressions were, however, more responsive to NOR exposure than their corresponding enzyme activities. The analyzed enzymes are very important because they express the ability to catalyze detoxification of xenobiotic substrates, including FQs. The possible reduction of its activity in humans may be of high importance, because FQs undergo biotransformation in the liver from approximately 50 percent for pefloxacin to about 6 percent for ofloxacin [91]. Although glucuronide conjugates have been identified as minor metabolites for some agents, most metabolic reactions involving quinolones occur through microsomal oxidative mechanisms at the cytochrome P-450 site. These metabolic alterations involve the piperazinyl moiety and usually result in compounds with significantly less microbiologic activity than the parent drugs. However, the conclusions from fish cannot be directly transferred to humans and the results suggest the possibility of the delayed toxicity to be connected with reduced detoxification induced by itself.

Similar results of P-450 inhibition by FQs were found in chicken by Shlosberg [92] and Granfors et al. [93]. Regmi et al. pointed to the inhibiting effect of FQs in dogs on P-450 1A but not on P-450 3A [94, 95].

3.6. FQs and HIF-1α. Dioxygenase inhibition by FQs was predicted by Badal et al. [56] to stabilize transcription factor HIF-1α by inhibition of the oxygen-dependent hypoxia-inducible transcription factor prolyl hydroxylation. In dramatic contrast to this prediction, HIF-1α protein was eliminated by FQ treatment. Badal and coworkers explored this effect to be caused by inhibition of HIF-1α mRNA translation.

The natural function of HIF-1α system is to change the metabolism of the cell into the anaerobic pathway in order to protect the cell against OS. The conversion of pyruvate to lactate is enhanced by upregulation of the lactate dehydrogenase-A (LDH-A). On the other hand, pyruvate dehydrogenase complex is inhibited by HIF-1α by upregulating pyruvate dehydrogenase kinase (PDK) which inhibits PDH-A. Thus, HIF-1α inhibits pyruvate to enter KC and to produce $NADH_2$. As presented by Brand et al. [96], $NADH_2$/NAD ratio (which can be called "hydrogen pressure") is the main factor determining the escape of electrons from the ETC chain, making this process to high degree independent of the total ETC electron flow. (The term "hydrogen pressure" reflects the natural similarity of ETC to the water flowing through the pipe with small holes. The water escaping through the holes depends on the pressure but not on the water velocity in the pipe.) Thus, in case of the lack of the oxygen in the tissue which is the final recipient of the electrons from $NADH_2$, HIF-1α turns on the side way for hydrogen, not to put it into ETC and produce OS but to produce lactate. The lack of this safety valve (HIF-1α) in FQ patients may cause the shift of the overdosed amounts of hydrogen into ETC causing overdosed electron leak. Assuming this phenomenon to be important in FQ-treated patients, the glyco- and lipolysis inhibitors could be considered as OS reductors. The natural glycolysis inhibitor in the diet is citric acid which inhibits phosphofructokinase (the main regulatory point of glycolysis) and activates fructose 1.6 bisphosphatase which catalyzes the opposite reaction promoting gluconeogenesis and pentose cycle pathway. On the opposite, the vinegar (acetic acid) could be contradicted by FQAD patients because it promotes glyco- and lipolysis both contributing to possible overdosed "hydrogen pressure" and OS.

4. Therapeutic Conclusions

The treatment of the FQAD, especially that lasting for many years, is a very difficult therapeutic problem. The effectiveness of different therapies carried out on patients is rather low. A large number of patients suffers from chronic tiredness, tendinopathies, neuropathies, and lack of sleep, even more than 12 h/24 h. Understanding all the molecular mechanisms of the FQ activity in the cell is the urgent aim for the current science to find methods helping these people.

The main question that arises here concerns the reasons of chronicity of FQAD symptoms which last for many years, sometimes, even after a standard 5-day FQ treatment. 3 reasons can be taken into consideration:

(a) Long-lasting OS destroys the mitochondrial DNA and the newly synthesized proteins creating cytochrome complexes are disturbed in their structure leading to permanent electron leakage and OS.

(b) The complexes of FQ with proteins and cations are so stabile that they exist in the cells by many years disturbing energy production and epigenetics.

(c) Epigenetic changes in gene regulation become persistent many years of FQ application even in the case of lack of FQ in the cell.

The answer, which of these three possible reasons contribute to the chronicity of FQAD symptoms, is of high importance with respect to the problem of effective treatment of this state. The research answering these questions must be performed as fast as possible.

In the case of mtDNA destroying, the treatment is difficult and it must focus on the stimulation of mitochondrial replication. The more destroyed mitochondria must be removed and the less destroyed must replicate in order to substitute for the removed ones and to reduce the total LEC. After many replications, the most healthy mitochondria would dominate the cell. The final effect would depend on the state of the most healthy mitochondrium in the cell. The second possibility is to increase the ratio of cell exchange in the given tissue. The cells with more destroyed mitochondria must be shifted to apoptosis while more healthy cells must substitute them. This process, however, cannot take place in the central nervous system and muscles because the cell exchange is close to zero in these tissues. Also, the collagen exchange is very low causing the tendon regeneration to be a difficult and long-lasting problem.

If new research would confirm the existence of FQs in the cells and mitochondria in the amounts making possible their permanent interactions with proteins and cations even after many years of FQ application, the research must focus on methods on how to remove FQs from strong protein and cation complexes. The simplest way seems to be the application of increased doses of metal cations Fe^{2+}, Cu^{+}, Mn^{2+}, Zn^{2+}, and Mg^{2+} which are natural FQ-competitors for protein-binding sites. It should be pointed that bivalent metal ions enter the cell to some degree due to the negative membrane potential of the cell and, next, enter the mitochondria due to the negative value of mitochondrial potential $\Delta\Psi_m$. The Nernst equation $\Delta V = RT/zF \ln(c_{in}/c_{out})$ defines the equilibrium between the ion concentration gradient and voltage across the membrane. If the membrane voltage $\Delta V/\Delta\Psi_m$ decreases, the concentration equilibrium gradient c_{in}/c_{out} of bivalent ions decreases significantly as well, because the lowered potential may not be able to pull bivalent cations into the cell/mitochondrium up to the required concentration. Table 1 shows the exemplary calculations.

Table 1: The conversion of Nernst equation $c_{in}/c_{out} = \exp(\Delta V\, zF/RT)$ for bivalent ions as Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+} shows strong relation between the actual membrane potential and ability of the cell/mitochondrium to attract and absorb the ions into the cell/mitochondrium. Reduced membrane potential ΔV is a strong factor which hinders entering bivalent cations to the cell. However, the detailed mechanisms of individual ion transport must be analyzed.

$\Delta V/\Delta\Psi_m$	Equilibrium c_{in}/c_{out} gradient ($z = 2$ and $T = 310$ K)
−160 mV	160.000x
−140 mV	36.000x
−110 mV	3.800x
−90 mV	850x
−70 mV	190x
−50 mV	42x
−30 mV	9x

Table 1 shows that the decrease in membrane voltage reduces strongly the ability of the cell to pull X^{2+} ions into the cell/mitochondrium. The question arises as to what extent the transport of X^{2+} ions into the cell is disturbed in FQAD patients. And next, if the possible lower X^{2+} concentration depends only on reduced $\Delta V/\Delta\Psi_m$ or maybe also on the disturbed membrane transport being blocked by FQs joining to metal-binding sites of transport proteins? Every analyzed bivalent cation requires a separate analysis.

The third possible reason of the permanent FQAD state is the permanent disorder in gene expression caused by some positive loop regulations. For example, reduced Fe^{2+} level disturbs oxoglutarate-dependent dioxygenases and increases methylation of DNA and histones which leads to reduced Fe^{2+} absorption to the cell. Many other loops are possible which could cause the chronic state of the patient despite the lack of FQs in the cell. They have to be recognized in order to find the methods restoring the normal regulatory state. This case is the most hopeful for patients, because, for example, the mtDNA damage is rather difficult to be effectively treated and gene expression regulation is difficult, however, possible.

Until detailed knowledge concerning FQ toxicity would be recognized, the following directions in supporting FQAD patients are proposed according to the known and probable mechanisms of FQ toxicity:

(a) Reduction of the oxidative stress: assuming that H_2O_2 is not effectively removed from the cell after FQ treatment, subsequent consequences may occur such as opening Kv1.5 channels, Fenton reaction, peroxynitrite radical creation, opening PTP channels,

decoupling mitochondrial potential, and, finally, breaking down OS barrier. The detailed comparison of different FQs with respect to OS is urgent to determine which FQs are safer in use and which ones are more dangerous. Reduction of OS is a very broad area. There are thousands of natural substances which possess the antioxidant capacity and which are able to reduce free radicals leaked from ETC. One should, however, remember that they work as one to one. It means that, as a rule, they are not reduced in the cell after free radical annihilation in order to work in cycle. They only reduce the size of free radical damage.

Among the antioxidants which enter easily, the mitochondria are the most interesting ones. Lowes et al. [79] show that the mitochondrion-targeted antioxidant MitoQ protects against fluoroquinolone-induced oxidative stress and mitochondrial membrane damage in human Achilles tendon cells. In cells treated with MitoQ, the oxidative stress was lower and mitochondrial membrane potential was maintained.

Simonin et al. [97] report oxidative damage of collagen I to be prevented by coadministration of N-acetylcysteine (150 mg/kg) to the mice. Tsai et al. report similar anticytotoxic effect of resveratrol [98]. Vitamins C and E belong also to this group; however, after they annihilate some radicals, they can be reduced using $NADPH_2$. Some papers point to the ability of vitamin E to reduce the consequences of FQ-induced damage [99]. Vitamin C is presented to possess the ability to protect against lethal gamma photon irradiation in mice—a strong source of OS [100]. Trace elements Zn^{2+}, $Cu^{1/2+}$, Se^{2+}, $Fe^{2/3+}$, and Mn^{2+} are cofactors of important antioxidative enzymes. Selenium supplementation is reported [101] to partially restore oxidative stress and sperm damage in FQ-treated cells.

Mn^{2+} seems to be very important, because it is a cofactor of mitochondrial SOD2 being the first barrier against O_2^- and carrying mtDNA against free radical damage. Thus, the amount of trace elements in the cell must be satisfactory. Detailed research is required for $Fe^{2/3+}$ and $Cu^{1/2+}$ in order to find if their supplementation does not increase Fenton reaction to occur. Citrate and other glycolysis inhibitors may reduce the "hydrogen pressure" on ETC reducing to some degree LEC and OS.

(b) Restoring reduced mitochondrial potential $\Delta\Psi_m$: restoring the reduced mitochondrial potential may be one of the important steps in restoring the proper regulatory balance in FQ-patients; however, it is not a trivial task. On the one hand, reducing OS may contribute to restoring $\Delta\Psi_m$; on the other hand, the reasons of PTP opening seem to be more composed and require advanced research. The points of interest may be reactivating HIF-1α system, reducing intracellular and intramitochondrial Ca^{2+} concentrations, restoring membrane potential ΔV, and restoring intracellular Mg^{2+}, all contributing to PTP closing. Cyclosporine A and metformin are postulated to be able to close PTPs [102, 103] and protect against OS [102, 104–106]; thus, it is an interesting substance for possible FQAD treatment.

(c) Supplementation of uni- and bivalent cations that are chelated by FQs: the role of uni- and bivalent cations was partially discussed in point A. Additionally, the role of Mg^{2+} and K^+ must be presented. Both ions possess high intracellular concentrations (Mg^{2+}: 20x and K^+ 40x higher than extracellular). K^+ is probably removed from the cell in the OS state by opening redox-sensitive Kv1.5 channels. Mg^{2+} is strongly chelated by FQs, but, probably, it also escapes from the cells due to some unrecognized mechanisms. The supplementation must take into consideration the regulatory effect of kidney, which removes overdosed amounts of both cations to the urine. Thus, small but often doses are rather recommended in order to keep a bit higher concentrations of both ions in the blood plasma, which makes it possible to reduce the concentration gradients across the cell membranes and facilitate entering both to the cell.

(d) Supporting the mitochondrial replication in the cell—pulling more damage to apoptosis and proliferation of the more healthy ones: supporting the mitochondrial exchange (removing that destroyed ones and replication of that more healthy ones) is the necessary way in the case of irreversible mtDNA damage. The substance that is postulated to possess the ability to promote the mitochondrial biogenesis is pyrroloquinoline quinone (PQQ) [107, 108]. This substance is also postulated to be OS protective [109].

(e) Removing FQs permanently accumulated in the cells (if this phenomenon takes place): the problem of FQ accumulation is purely present in the available research. Thus, first, it is the urgent topic for establishing if this phenomenon really, and to what extent, takes place. Removing accumulated FQs may undergo in two ways: by cytochromes P-450 in the microsomes and by different processes which can remove the molecule outside the cell. Activating the reduced cytochrome detoxification may be an important point in FQAD patients. On the other hand, ozonation has been described to be an effective method for removing the first generation FQ—flumequine from the liquid water [110]. Thus, ozone therapy can be examined to be a method of FQ degradation in the body.

(f) Regulating the disturbed epigenetics and enzyme activities: every factor presented above contributes to the disturbed gene expression which can contribute to vicious circle-like regulations causing the new regulatory balance lying far away from that optimal one. If it is the main reason of the chronic state of FQAD patients, then there is a big chance to find methods for quick and effective treatment of this state. However, the problem is of high complexity.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

FOXO Transcriptional Factors and Long-Term Living

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Several pathologies such as neurodegeneration and cancer are associated with aging, which is affected by many genetic and environmental factors. Healthy aging conceives human longevity, possibly due to carrying the defensive genes. For instance, FOXO (forkhead box O) genes determine human longevity. FOXO transcription factors are involved in the regulation of longevity phenomenon via insulin and insulin-like growth factor signaling. Only one FOXO gene (FOXO DAF-16) exists in invertebrates, while four FOXO genes, that is, FOXO1, FOXO3, FOXO4, and FOXO6 are found in mammals. These four transcription factors are involved in the multiple cellular pathways, which regulate growth, stress resistance, metabolism, cellular differentiation, and apoptosis in mammals. However, the accurate mode of longevity by FOXO factors is unclear until now. This article describes briefly the existing knowledge that is related to the role of FOXO factors in human longevity.

1. Introduction

Aging is related to the age-dependent impaired functioning of the cells, tissues, organs, and organ systems [1, 2]. This impairment leads to chronic pathologies including neurodegeneration, cardiovascular diseases, and cancer. Owing to these age-associated diseases, the researchers have always been interested in understanding the aging process and delaying the aging for human longevity [3, 4].

Healthy aging is a complex phenotype and an interplay of genetic and environmental factors such as food, exercise, and habits [5, 6]. However, rather than environmental factors, the contribution of genetic factors towards healthy aging is more significant. Thus, intensive studies have been done to investigate the genetic variants associated to human longevity.

Since health status affects the lifespan, the development of chronic diseases is delayed in the long-lived individuals [7–9]. These individuals could be the carriers of the defensive genes, which may be involved in body's defense against vulnerable moieties such as reactive oxygen species [10]. FOXO gene is one of the most crucial defensive genes that are known for ameliorating lifespan [4, 11–16]. Thus, aging can be considered an evolutionary process that is modulated by genetic programming and biochemical processes [17–20]. Figure 1 illustrates various modes of aging as studied in different organisms [18].

As observed in an animal study, the first ever documented mode of aging was deregulated nutrient sensing that involved the insulin and insulin-like growth factor- (IGF-) 1 signaling (IIS) pathway [21]. In IIS pathway, IGF-1 and insulin share and stimulate the same signaling pathways [22–24]. Thus, food that is an important environmental factor involved in IIS pathways must be cautiously used to achieve human longevity [23, 25, 26]. Other components of nutrient sensing pathways are the sirtuins, AMP (adenosine

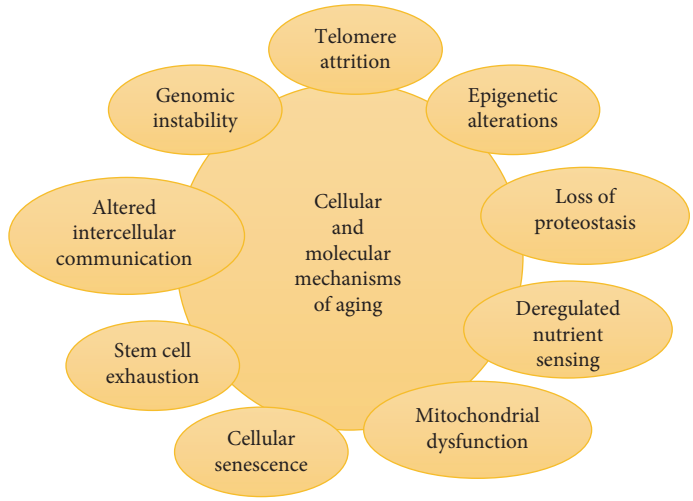

Figure 1: Various modes of aging.

monophosphate) kinase, and the kinase mTOR (molecular target of rapamycin) [21].

2. FOXO Transcription Factors

The IIS pathway is highly influenced by the FOXO proteins [27, 28]. Forkhead box (FOX) transcription factor family was named after the Drosophila forkhead gene. The FOX family contains nineteen subfamilies of FOX genes, FOXA-FOXS, and is described by a highly conserved, winged-helix DNA-binding domain and the forkhead motif [29–32]. Other (O) subfamily of FOX, FOXO, is conserved from *Caenorhabditis elegans* (*C. elegans*) to mammals; only one FOXO gene exists in the invertebrates while mammals have 4 FOXO genes, FOXO1, FOXO3, FOXO4, and FOXO6 [29, 33, 34]. The alternate names FKHR, FKHRL1, and AFX have been used for FOXO1, FOXO3, and FOXO4, respectively [35]. FOXO1, FOXO4, and FOXO6 are overexpressed in the adipose, skeletal, and nervous tissues, respectively, while FOXO3 are excessively found in the spleen, stomach, intestine, kidney, and cardiac tissues [36]. These four genes are involved in the multiple cellular pathways, which regulate proliferation (FOXO1, FOXO3, and FOXO4), oxidative stress resistance (FOXO1 and FOXO3), metabolism (FOXO1 and FOXO3), cellular differentiation (FOXO3), inflammation (FOXO1, FOXO3, and FOXO4), aging (FOXO1, FOXO3, and FOXO4), and apoptosis (FOXO1, FOXO3, and FOXO4) in mammals [29–32, 37–40]. However, the involvement of these four genes in human longevity is still unrevealed.

FOXO proteins act as transcription activators and are suppressed by the IIS pathway [31, 37–40]. Concisely, P13K-AKT-mediated signaling pathway is activated by IGF-1 or insulin. It leads to the serine/threonine kinase AKT-induced phosphorylation of FOXO factors resulting in its nuclear exclusion and inhibition of FOXO-dependent transcription of target genes [41]. On the other hand, cellular stress leads to translocation of FOXO factors into the nucleus and activation of FOXO-dependent transcription. Besides phosphorylation, other posttranslational modifications including ubiquitination and methylation also influence the FOXO-dependent transcription [39, 40]. Thus, the FOXO posttranslational modifications lead to the aggregation of particular FOXO-dependent moieties that regulate various FOXO-dependent gene expressions [39, 42, 43]. In this article, various modes of human longevity involving FOXO transcription factors have been suggested.

3. Role of FOXO in Autophagy

The genes which mediate the intracellular clearance through autophagy and the ubiquitin-proteasome system are also regulated by FOXO factors [40, 44, 45]; thus, it can be suggested that FOXOs function as prolongevity factors. Starvation-induced defects in autophagy and the ubiquitin-proteasome system have been linked with the frailty and early aging (Figure 2) [46–48]. In addition, the genes which mediate the autophagy and mitophagy in muscle cells are also regulated by FOXO factors; it helps the tissues to adapt to starvation [49–51]. Moreover, Webb and Brunet [40] observed the activation of autophagy mechanisms by FOXO1 and FOXO3 in renal tubular cells, neurons, and cardiomyocytes. Since, FOXO factors play a role in proteasome-mediated degradation of short-lived cellular organelles and proteins, a suppressed proteasomal activity results in the aggregation of degraded proteins in the heart, liver, and muscle leading to aging [52, 53]. Additionally, the malfunctioned ubiquitin-proteasome system is a direct or indirect cause of various neurodegenerative diseases, for instance, Alzheimer's disease [40, 54, 55]. FOXO factors act on the neurodegenerative system via the upregulated ubiquitin ligases and by mediating the proteasome's composition [56–59]. However, the direct influence of proteostasis provoked by FOXO factors in mammals is not still disclosed.

4. Role of FOXO in Oxidative Stress

Antioxidant role of FOXO is its most crucial function. Since reactive oxygen species (ROS) produce conserved deteriorating effect on cells and induce aging, FOXOs could be used to

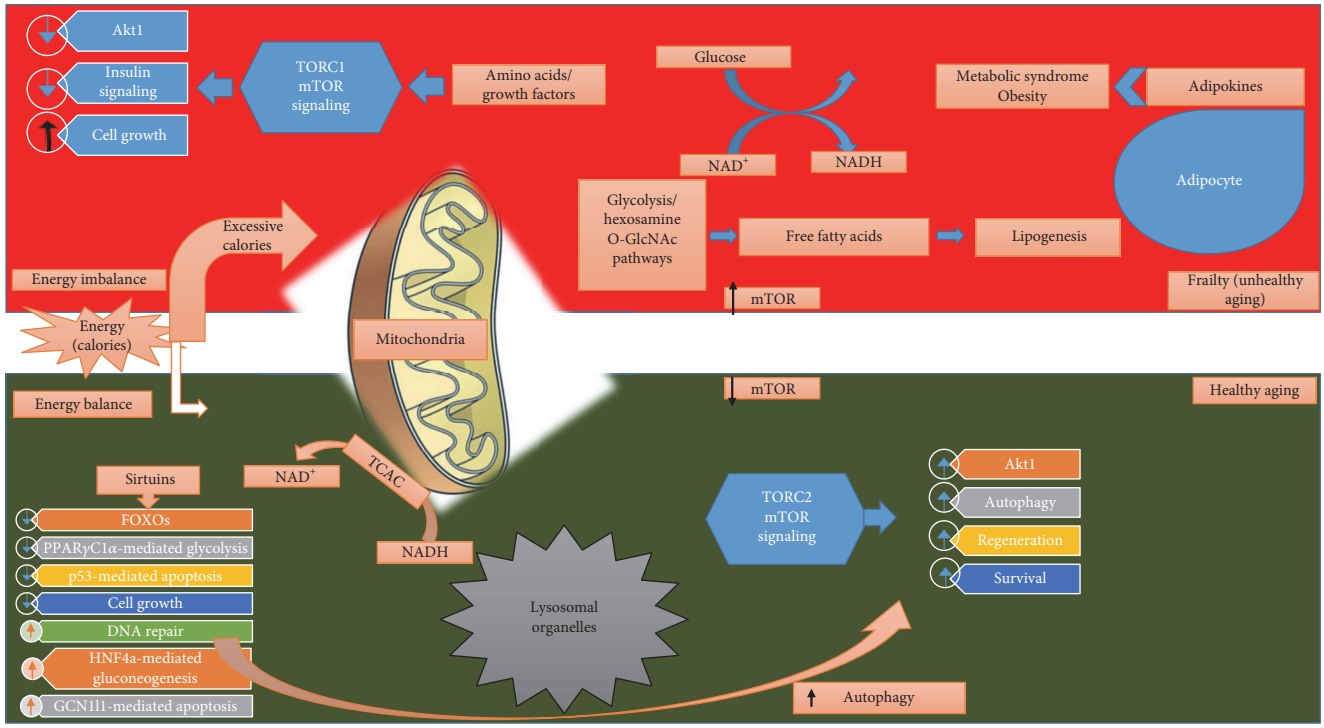

Figure 2: The crucial intracellular pathways targeted by FOXOs are presented here as modes of longevity effects of FOXOs. FOXOs are known to regulate translation of environment-induced stimuli into gene expression. FOXO-mediated longevity (especially through FOXO3) could be due to upregulated target genes pertained to apoptosis, cell cycle arrest, and resistance to stress leading to prevention of aging and age-associated diseases such as cancer and neurodegenerative diseases. The green part of the above figure illustrates cellular redox potential in mitochondria restoring NAD^+. It results in the calorie restriction leading to various processes such as ameliorated autophagy, inhibited mTOR activity, and sirtuin-mediated activation of FOXOs giving rise to long-term living. While the red part of the above figure shows elevated levels of NADH due to excessive calorie intake resulting in lipogenesis, activated mTOR, excessive release of ROS, and suppressed autophagy leading to frailty. TCAC = tricarboxylic acid cycle; CAD = coronary artery disease; PPARγC1α = peroxisome proliferator-activated receptor-γ coactivator 1α; GCN1l1 = general control of amino acid synthesis 1-like 1; HNF4a = hepatocyte nuclear factor 4α; O-GlcNAc = O-linked N-acetylglucosamine.

influence aging by ameliorating the antioxidant potential of cells [60, 61]. ROS act as second messengers in various signaling pathways. An equilibrium in the production and degradation of ROS is necessary for normal cellular functioning, while imbalanced level of ROS results in abnormal functioning of the cells leading to various pathologies such as neurodegenerative diseases and cancer. Oxidative stress regulates FOXO factors, either through detection of cellular redox potential or modifying the upstream FOXO regulatory pathways [62, 63]. Normally, cellular detoxification keeps ROS level in normal range. An impaired cellular detoxification results in oxidative stress. Manganese superoxide dismutase (MnSOD), catalase, and GADD45 are major detoxification enzymes that are regulated by FOXOs [60, 64]. Hence, the inactivation of FOXOs result in the ROS built-up in the cells; it leads to various cellular abnormalities such as the compromised proliferation of normal stem cells but quick proliferation of transformed cells [65, 66].

5. Role of FOXO in Stem Cells

FOXO factors are known to be involved in stem cell biology. Aging is characterized with disequilibrium between removal and regeneration of cells in tissues, since the regeneration capability of adult stem cells is decreased with aging. Knockout hematopoietic stem cell (HSC) mice (mice with FOXO1/3/4-deficient hematopoietic stem cells) showed apoptosis of HSCs as well as termination of repopulation of HSCs. Likewise, FOXO3-deficient mice illustrated the reduced potential of regeneration of cells [67]. The deletion of FOXO factor could lead to exhaustion of the respective stem cell pool [68]. Surprisingly, the HSC compartment was restored in FOXO-deficient mice treated with an antioxidant N-acetylcysteine, proposing that stem cells are disturbed by accumulation of ROS. This finding supports the hypothesis that oxidative stress contribute primarily to aging, while malfunctioned adult stem cells have secondary significance in this context [69]. Besides playing a role in adult stem cells, FOX factors mediate the expression of OCT4 and SOX2 factors associated with stemness. Similarly, FOXO1 factors are pluripotent for human embryonic stem cells (ESC), and the ortholog FOXO1 plays similar role in mouse ESCs [70].

6. FOXO Factors and Long-Term Living

This review article narrates a summary of the prevalent knowledge that is associated to the role of FOXO factors in extending human lifespan. Until now, no study describes

the exact mode of action of FOXOs in human aging. However, some studies on various populations narrate the possible role of FOXO factors in human longevity.

The older the age, larger is the contribution of genetics in lifespan stating genetics as a function of human longevity. Thus, genetics is the basic parameter that discriminates the average-lived population from the centenarians [1, 2, 71, 72]. Thus, the centenarians are rich in specific alleles, which possibly represent the genes contributing to human longevity. These genes are therefore extensively being investigated in current years.

First study of this type narrated the association of human longevity with FOXO3A [73]. This study was performed on 4 genes named as FOXO1A, FOXO3A, SIRT1, and COQ7 and one SNP, named as rs2764264, in long-lived American males of Japanese origin. Only FOXO3A and rs2764264 were observed to have association with human longevity among the studied genes and SNPs [73]. The incidence rate of age-associated pathologies such as cancer and neurodegenerative and cardiovascular diseases in these individuals was also lower than control group. The control group was eleven years younger than test. The significantly lower level of insulin in the control group was also due to the same allele [73].

Subsequently, same association was found in male centenarians from Italy [11], Germany [12], and Denmark [13]. In an Italian study, rs2802288 exhibited the maximum allelic relationship-minor allele frequency. All three studies described the significant association between FOXO3 polymorphism and human longevity. While, the Danish study proposed four new single nucleotide polymorphisms (SNPs) (named as rs9400239, rs2764264, rs479744, and rs13217795) associated with human longevity [13].

In other study, two SNPs from FOXO1A (rs2755209 and rs2755213) and three from FOXO3A (rs4946936, rs2802292, and rs2253310) were analyzed in Chinese centenarians [74]. All the six SNPs were positively and gender-independently linked with long-term survival, except two SNPs from FOXO1A that were negatively linked with longevity in female subjects [74]. The conclusion of study states that there is strong association between FOXO1A and long lifespan in females showing the influence of gender in genetic association to human longevity [74]. Another study in Chinese population reported the gender involvement in the impact of FOXO1A and FOXO3A SNPs independently on human longevity [75–77]. Similar finding showing the importance of genetics in the IIS pathway in long-lived Jews and people of Italy, Japan, and Netherlands has also been reported [78–81]. In addition, human longevity has also been found to be associated with other five FOXO3A SNPs (named as rs2802288, rs2802292, rs1935949, rs13217795, and rs2764264) [15]. Among these five SNPs, rs2802292 and rs2764264 polymorphisms were observed in males only.

The combined effect of FOXO3A and APOE on long-term living has also been reported [2, 16, 82]. Additionally, an increase in the activity of daily life and decrease in the risk of bone fracture in individuals with FOXO3A SNPs were found in long-lived Danish individuals [14]. Conclusively, there is remarkable association between human longevity and FOXO3A SNPs as evident from above cited studies conducted in various populations. However, the translation of FOXO3 gene sequences into phenotypic features that facilitate a long-term living is still unrevealed. Moreover, rather than associating with known SNPs, FOXO3A alleles related to long-term living act as introns [2, 72, 83]. It proposes that these SNPs are expected to influence FOXO3A without affecting protein functionality.

7. Prediction of Mechanisms of FOXO in Long-Term Living

Network pharmacology is a multidisciplinary field that integrates different scientific concepts such as systems biology, cheminformatics, and bioinformatics to explore various novel bioactivities from network-based analysis. For instance, network pharmacology helps us to study gene characteristics and its functions [84], identify therapeutic targets, and explore the mode of action of various drugs [85]. Thus, network pharmacology is used here to predict the possible modes of action of FOXOs in human longevity.

STITCH 4.0 database (http://stitch.embl.de/) [86] has been used to retrieve targets (confidence score > 0.4) of FOXO1, FOXO3, FOXO4, and FOXO6 in the form of protein-protein network (Supplementary data, Figure 3, Table 1 available online at https://doi.org/10.1155/2017/3494289). These protein targets were fetched into Cytoscape, and protein-protein interaction network was constructed to visualize the functionality-associated genes. The functional enrichment analysis was conducted by using the gene ontology terms (GO terms) for annotation of the biological functions of FOXO-related targets. Subsequently, Cytoscape plug-in ClueGO [87] was utilized to analyze FOXO-mediated biological process term (BP term) to explore the biological importance of the specific targets linked to FOXO1, FOXO3, and FOXO4. Overall, the significant enrichment of 14, 16, and 7 GO terms was achieved for FOXO1, FOXO3, and FOXO4 (Supplementary data, Figure 4, Table 2). The effect of FOXOs on these BPs has been reported by some investigators. FOXOs are mainly involved in the regulation of metabolism, regulation of reactive species, and regulation of cell cycle arrest and apoptosis. FOXO1 regulates adipogenesis, gluconeogenesis, and glycogenolysis. Mechanistically, the unphosphorylated FOXO1 binds to the insulin response sequence present in the promoter region of G6P (glucose-6 phosphatase) in the nucleus [88]. It leads to the accelerated transcription resulting in the enhanced production of glucose in the liver. After Akt-mediated phosphorylation, FOXO1 is transferred to the cytoplasm and undergoes ubiquitination and degradation. It leads to the decreased production of glucose in the liver via decreased transcription of G6P leading to the decreased rate of gluconeogenesis and glycogenolysis [89]. Adipogenesis is negatively regulated by FOXO1 through its binding to the promoter region of PPARG (peroxisome proliferator-activated receptor gamma) and inhibiting its transcription [90]. It results in the FOXO1-mediated inhibition of adipogenesis [91]. The initiation of adipogenesis requires the increased levels of PPARG [92, 93]. Moreover, FOXO1

functions as an association between transcription and insulin-mediated metabolic control; thus, FOXO1 is a promising genetic target to manage type 2 diabetes.

FOXO3 probably induces apoptosis either upregulating the genes needed for cell death [94] or downregulating the antiapoptotic factors [95]. In addition, FOXO3 has been found to regulate Notch signaling pathway during the regeneration of muscle stem cells [96]. Moreover, antioxidants are thought to be upregulated by FOXO3 to protect human health from oxidative stress. Additionally, FOXO3 is documented to suppress tumour [97]. Thus, tumour development may occur if FOXO3 is deregulated. Most importantly, FOXO3 are described to play a role in long-term living [12].

FOXO4 is involved in the regulation of various pathways associated to apoptosis, longevity, cell cycle, oxidative stress, and insulin signaling. FOXO4 are associated with longevity through IIS pathway [98, 99]. Finally, mutation-triggered Akt phosphorylation results in the inactivated FOXO4 [100]. It deregulates cell cycle and activates kinase inhibitor involved in cell cycle [101, 102]. It leads to the prevention of tumour progress into G1. These biological processes make us better understand the modes of action of FOXOs.

8. Conclusions

In current years, the rigorous research attention has been focused on the role of FOXO transcription factors in human longevity. In different animal models, numerous studies have been conducted to investigate the signaling pathways involved in the regulation of the FOXO factors. Moreover, the effect of FOXO-mediated processes on the cellular, tissue, or organism level functions has also been discussed. As a result, a pleiotropic nature of FOXOs' effect on longevity is established, since FOXOs participate in a number of cellular functions, including growth, stress resistance, metabolism, cellular differentiation, and apoptosis. From the above discussion, numerous strategies for future research can be predicted. For instance, the triggering of FOXO-mediated processes in the tissues with metabolically different features can be valuable to explore the mechanism of FOXO-mediated longevity. In addition, the human FOXO sequence variations and their effect on the resulting proteins should be studied, the possible findings can also reveal the underlying mechanisms of FOXO-induced health aging. The delay in age-related pathologies including cancer and neurodegenerative diseases and living long life depends on the control of morbidity. It is therefore an exciting area of study to investigate the antiaging compounds; however, their testing in clinical setup would need age markers to assess aging rate. Owing to the potential effect of FOXOs on health issues, the future therapies could be based on the FOXOs.

Conflicts of Interest

There is no conflict of interest among authors over contents of this article.

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Research Article

The Evaluation of Antioxidant and Anti-Inflammatory Effects of *Eucommia ulmoides* Flavones Using Diquat-Challenged Piglet Models

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This study was designed to evaluate the antioxidant and anti-inflammatory effects of *Eucommia ulmoides* flavones (EUF) using diquat-challenged piglet models. A total of 96 weaned piglets were randomly allotted to 1 of 3 treatments with 8 replication pens per treatment and 4 piglets per pen. The treatments were basal diet, basal diet + diquat, and 100 mg/kg EUF diet + diquat. On day 7 after the initiation of treatment, the piglets were injected intraperitoneally with diquat at 8 mg/kg BW or the same amount of sterilized saline. The experiment was conducted for 21 days. EUF supplementation improved the growth performance of diquat-treated piglets from day 14 to 21. Diquat also induced oxidative stress and inflammatory responses and then impaired intestinal morphology. But EUF addition alleviated these negative effects induced by diquat that showed decreasing serum concentrations of proinflammatory cytokines but increasing antioxidant indexes and anti-inflammatory cytokines on day 14. Supplementation of EUF also increased villi height and villous height, crypt depth, but decreased the histopathological score and MPO activity compared with those of diquat-challenged pigs fed with the basal diet on day 14. Results indicated that EUF attenuated the inflammation and oxidative stress of piglets caused by diquat injection.

1. Introduction

Oxidative stress is a common phenomenon in humans and animals that resulted from a large number of biological and environmental factors and stressors [1]. Under normal physiological conditions, there is a balance between the production of oxidants and antioxidants in the biological system [2]. The overwhelming of free radicals could impair this redox balance and thereby results in oxidative stress, including the oxidation of proteins, lipids, and nucleic acids [3, 4]. Many chronic diseases have been reported to be linked with the excessive production of reactive oxygen species (ROS) [5, 6].

Natural compounds present in plants have been reported to exhibit antioxidant activities that interact with ROS/reactive nitrogen species (RNS) and thus terminate chain reaction [7–9]. *Eucommia ulmoides* (EU) (also known as "Du Zhong" in Chinese) contains enriched chemical components such as lignins, iridoids, phenolics, steroids, and flavonoids and, therefore, presents various medicinal properties as a Chinese traditional medicine [8, 10]. The leaf of EU also contains abundant secondary metabolites, such as flavonoids [8, 11, 12]. It has been reported that flavonoids have strong antioxidant activities; it shows direct scavenging free radicals, suppression of proinflammatory cytokines through inhibition of reactive oxygen species and nitric

oxide, decreasing inflammatory genes including cyclooxygenases (COXs) and inducible nitric oxide synthase (iNOS), upregulating antioxidant enzymes, modulating transcription factors such as NF-κB and AP-1, and enhancing the Nrf2 signaling pathway [13–16].

Therefore, the objectives of this experiment were to investigate the antioxidant activities and anti-inflammatory effects of the flavones extracted from the leaves of EU using an oxidative stress piglet model induced by diquat that is a bipyridyl herbicide exerts ability to produce free radicals by redox-cycling metabolism and widely accepted in vivo model of oxidative stress [17–19].

2. Materials and Methods

2.1. EUF Extract. EUF extraction and total flavone content determination were conducted according to the methods of Li et al. [20, 21] at the Department of Medicine, Jishou University (Jishou, Hunan, China). The leaves of EU were shade dried and finely powdered. The extraction was performed using 65% ethanol in Erlenmeyer flasks for 30 min using an ultrasonic cleaner at 50°C, followed by filtration. This extraction process was repeated twice. The filtrate was then concentrated using a rotary vacuum evaporator at 70°C. The concentrated extract solution was dipping degreased using petroleum ether and then purified using a macroporous resin by rinsing the column with distilled water and static absorption for 2 h. The fraction was eluted with the 90% ethanol for 2 h and then sequentially concentrated, washed twice with isopropanol, filtrated, concentrated, and lyophilized. The content of the total flavones in EUF powder was 83.61% analyzed by ultraviolet spectrophotometer methods using rutin as calibration standard [22].

2.2. Animals and Experimental Design. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (2013020).

A total of 96 three-breed crossbred (Duroc × Landrace × Large Yorkshire) piglets weaned at 21 days were randomly assigned to receive 1 of 3 treatments with 8 replicate pens/treatment and 4 piglets/pen. The 3 treatments include basal diet, basal diet + diquat, and 100 mg/kg EUF diet + diquat. The diets were formulated to meet the nutrient requirements for weanling piglets (Table 1). Diquat was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a dose of 8 mg/kg BW was used according to the results reported by Yin et al. [18]. The piglets were housed individually in an environmentally controlled nursery room with hard plastic-slatted flooring. All animals had free access to water. After a 7-day adaptation period, piglets were fed with their respective diets 3 times per day at 8:00, 13:00, and 18:00 for a 21-day period. All piglets were weighed weekly, their average daily gain, daily feed intake, and gain : feed ratio were calculated throughout the entire experiment.

On day 7, the piglets in basal diet + diquat and EUF diet + diquat treatments received an intraperitoneal injection of diquat at 8 mg/kg BW while the piglets on basal diet received the same volume of sterilized saline. On day 14 and 21, 8 piglets (1 pig/pen) were randomly selected and blood samples were collected aseptically from the jugular vein at 2 h after a.m. feeding. Serum samples were obtained by centrifuging blood samples at 2000 ×g for 10 min at 4°C and then immediately stored at −80°C for further analysis. Piglets were anesthetized with sodium pentobarbital and euthanized by jugular puncture. The intestinal samples were collected from the jejunum, ileum, anterior colon, and posterior colon and fixed in 4% formaldehyde for morphology analysis and histopathological grading. Anterior and posterior colonic tissues were immediately snap-frozen in liquid N and stored at −80°C for myeloperoxidase (MPO) activity analysis.

Table 1: Composition of basal diets (as-fed basis).

Ingredients (%)		Chemical composition	
Corn	59.00	Calculated digestible energy, kcal/kg	3510.53
Soybean meal	9.00	Dry matter	84.6
Extruded soybean	8.00	Crude protein	17.5
Fermented soybean meal	5.00	Calcium	0.54
Fish meal	3.00	Total phosphorus	0.68
Whey powder	8.00	Lysine	1.51
Soybean oil	2.00	Methionine	0.39
Sucrose	2.00	Threonine	0.86
Premix[a]	1.00	Tryptophan	0.20
Calcium citrate	0.60		
Calcium hydrogen phosphate	1.00		
Salt	0.30		
98% lysine	0.64		
Threonine	0.15		
Methionine	0.10		
Compound acidifiers	0.21		

[a]Providing the following amounts of vitamins and minerals per kilogram on an as-fed basis: Zn (ZnO), 50 mg; Cu ($CuSO_4$), 20 mg; Mn (MnO), 55 mg; Fe ($FeSO_4$), 100 mg; I (KI), 1 mg; Co ($CoSO_4$), 2 mg; Se (Na_2SeO_3), 0.3 mg; vitamin A, 8255 IU; vitamin D3, 2000 IU; vitamin E, 40 IU; vitamin B1, 2 mg; vitamin B2, 4 mg; pantothenic acid, 15 mg; vitamin B6, 10 mg; vitamin B12, 0.05 mg; nicotinic acid, 30 mg; folic acid, 2 mg; vitamin K3, 1.5 mg; biotin, 0.2 mg; choline chloride, 800 mg; and vitamin C, 100 mg. The premix did not contain additional copper, zinc, antibiotics, or probiotics.

2.3. Detection of Antioxidative Capacity. Serum concentrations of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T-AOC), and GSH were measured using their corresponding assay kits (Nanjing Jiancheng, Nanjing, China) according to manufacturer instructions. In brief, SOD, CAT, and GSH-Px were analyzed by xanthine oxidase-xanthine reaction method, CAT-H_2O_2 reaction method, and reduced glutathione method, respectively. MDA capacity was assayed by 2-thiobarbituric acid method and T-AOC was detected by ferric-reducing/

antioxidant power reaction method. All samples were measured by UV/visible spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan).

2.4. Analysis of Serum Concentrations of Cytokines. Serum concentrations of interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor-beta 1 (TGF-β1), tumor necrosis factor- (TNF-) α, IL-10, and interferon-gamma (IFN-γ) were determined by using Porcine Cytokine Array QAP-CYT-1 (RayBiotech Inc., Guangzhou, China). An array-based multiplex ELISA system was used for quantitative measurement of multiple cytokines according to the manufacturer's protocol. Briefly, 100 μL of sample diluent was added to each well for 30 min to block slides and then decanted. 100 μL of the sample or cytokine standard was added to the plate and incubated overnight at 4°C. The samples were decanted and washed 5 and 2 times with Wash Buffers I and II, respectively. The plate was incubated in 80 μL of the detection antibody cocktail for 2 h and then washed as before. 80 μL Cy3 dye equivalent dye-conjugated streptavidin was added to each well and the plate was incubated in a dark room for 1 h. After being washed 5 times, the slides were placed in the slide washer/dryer and gently washed with Wash Buffers I and II for 15 and 5 min, respectively. The signals were visualized using InnoScan 300 Microarray Scanner (Innopsys, Parc d'Activités Activestre, Carbonne, France) equipped with a Cy3 wavelength (green channel, at an excitation of 532 nm), and the quantitative data analysis was performed using the Quantibody® Q-Analyzer (QAP-CYT-1, RayBiotech Inc.).

2.5. Determination of Serum Diamine Oxidase (DAO) and D-Lactate. The reaction system for the serum concentration of DAO determination included 0.1 mL (4 μg) horseradish peroxidase solution (Sigma-Aldrich, St. Louis, USA), 3 mL PBS (0.2 M, pH 7.2), 0.1 mL O-dianisidine methanol solution (500 μg of O-dianisidine) (Sigma-Aldrich, St. Louis, USA), 0.5 mL sample, and 0.1 mL substrate solution (175 μg of cadaverine dihydrochloride) (Sigma-Aldrich, St. Louis, USA). The processed samples were incubated in an incubator chamber at 37°C for 30 min and measured at 436 nm by UV/visible spectrophotometer-UV-2450 (Shimadzu, Kyoto, Japan) [23]. Serum D-lactate was determined using a D-Lactate Assay Kit (BioVision, Mountain View, San Francisco, USA) in accordance with the manufacturer's instruction [24].

2.6. Intestinal Morphology Evaluation and Histopathological Grading. The jejunal and ileal morphologies were analyzed using hematoxylin eosin staining according to Xiao et al. [23]. Villous height and crypt depth were measured with computer-assisted microscopy (Micrometrics TM; Nikon ECLIPSE E200, Tokyo, Japan).

Histopathological grading of the jejunum, ileum, anterior colon, and posterior colon was performed as described previously [25]. Histological scoring was carried out by a veterinary pathologist using the methods of Huang et al. [25] that ranged from 0 (minimal injury) to 15 (maximal injury) corresponding to four grades that included mononuclear or polymorphonuclear cell infiltration, histological injury, and erosion or epithelial hyperplasia.

2.7. Analysis of Myeloperoxidase (MPO) Activity in the Colon. Colon samples were homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide through a high-pressure homogenizer at 10,000–15,000 rpm at 4°C. The homogenate was centrifuged at $2500 \times r$ at 4°C for 15 min and the supernatant was transferred into PBS (pH 6.0) containing 0.17 mg/mL 3, 3′-dimethoxybenzidine and 0.0005% H_2O_2. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3, 3′-imethoxybenzidine. One unit of enzyme activity is defined as the amount of MPO present that causes a change in absorbance per min at 460 nm and 37°C [26, 27].

2.8. Statistical Analysis. The data of growth performance were performed with an analysis of variance (ANOVA) for repeated measures and others were subjected to ANOVA using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The differences among treatments were evaluated using Tukey's test. Probability values < 0.05 were taken to indicate statistical significance.

3. Results

3.1. Growth Performance. The body weight, average daily gain, average daily feed intake, and gain : feed ratio are shown in Table 2. The body weight on days 0, 7, and 14 were similar among the treatments ($p > 0.05$). The body weight of piglets in basal diet + diquat treatment were lighter ($p < 0.05$) than those in the basal diet and EUF diet + diquat treatments on day 21. Compared with the piglets in basal diet treatment, diquat exposure reduced average daily gain and gain : feed ratio from day 7 to 14 and day 14 to 21, as well as average daily feed intake and gain : feed ratio from day 14 to 21 ($p < 0.05$). However, there were no difference in the average daily gain, average daily feed intake, and gain : feed ratio between piglets of basal diet treatments and EUF diet + diquat treatment ($p > 0.05$). In diquat-treated piglets, dietary EUF increased the average daily gain from day 14 to 21, average daily feed intake from day 7 to 14 and day 14 to 21, and gain : feed ratio from day 7 to 14 ($p < 0.05$).

3.2. Serum Antioxidant Parameters. On day 14, exposure to diquat decreased ($p < 0.05$) the serum concentrations of SOD, GSH-Px, CAT, T-AOC, and GSH in piglets of basal diet treatments. But the supplementation of EUF increased ($p < 0.05$) serum concentrations of SOD, GSH-Px, CAT, T-AOC, and GSH in piglets in EUF diet + diquat treatment compared with those in pigs in the basal diet + diquat treatment. On day 21, no differences ($p > 0.05$) were observed in the serum concentration of SOD, CAT, T-AOC, and GSH among treatments, with the exception that pigs in EUF treatment had greater ($p < 0.05$) GSH-Px than diquat-challenged pigs fed with the basal diet. There was

Table 2: Growth performance of piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Body weight (kg)				
Day 0	6.48 ± 0.32	6.50 ± 0.29	6.49 ± 0.32	0.999
Day 7	8.51 ± 0.35	8.53 ± 0.31	8.52 ± 0.36	0.999
Day 14	10.87 ± 0.19	10.12 ± 0.41	10.64 ± 0.28	0.232
Day 21	14.15 ± 0.35[a]	12.51 ± 0.45[b]	13.89 ± 0.34[a]	0.014
Average daily gain (g/d)				
Day 0 to 7	290.51 ± 6.21	290.17 ± 5.11	290.44 ± 10.46	0.999
Day 7 to 14	336.57 ± 17.47[a]	226.95 ± 27.36[b]	302.26 ± 19.46[a, b]	0.006
Day 14 to 21	468.55 ± 21.35[a]	342.14 ± 28.26[b]	464.71 ± 25.42[a]	0.002
Average daily feed intake (g/d)				
Day 0 to 7	293.45 ± 31.21	300.13 ± 16.31	299.56 ± 10.46	0.970
Day 7 to 14	389.01 ± 12.67[a]	308.17 ± 27.78[b]	351.16 ± 19.43[a]	0.041
Day 14 to 21	680.56 ± 27.43[a, b]	599.05 ± 32.03[b]	706.63 ± 25.65[a]	0.037
Gain : feed (g/g)				
Day 0 to 7	0.99 ± 0.01	0.97 ± 0.02	0.97 ± 0.02	0.647
Day 7 to 14	0.87 ± 0.02[a]	0.74 ± 0.05[b]	0.86 ± 0.02[a]	0.020
Day 14 to 21	0.69 ± 0.02[a]	0.57 ± 0.04[b]	0.66 ± 0.02[a, b]	0.018

[a]Values are the mean ± SEM, $n = 8$ per treatment group. [a-b]Mean values sharing different superscripts within a row differ ($p < 0.05$).

Table 3: Serum concentrations of superoxide dismutase, glutathione peroxidase, catalase, malondialdehyde, total antioxidant capacity, and glutathione in piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Day 14				
Superoxide dismutase, U/mL	104.21 ± 2.12[a]	73.51 ± 6.31[b]	94.87 ± 3.37[a]	<0.001
Glutathione peroxidase, U/mL	359.34 ± 3.12[a]	335.21 ± 4.14[b]	360.85 ± 2.49[a]	<0.001
Catalase, U/mL	7.02 ± 0.21[a]	5.73 ± 0.38[b]	6.97 ± 0.38[a]	0.019
Malondialdehyde, nmol/mL	4.59 ± 0.24	5.76 ± 0.78	4.48 ± 0.36	0.175
Total antioxidant capacity, U/mL	1.25 ± 0.04[a]	0.54 ± 0.01[b]	1.14 ± 0.10[a]	<0.001
Glutathione, mg/L	3.24 ± 0.10[a]	2.43 ± 0.09[b]	3.01 ± 0.15[a]	<0.001
Day 21				
Superoxide dismutase, U/mL	92.18 ± 3.24	82.15 ± 2.16	89.47 ± 4.31	0.116
Glutathione-peroxidase, U/mL	324.25 ± 4.46[a, b]	314.49 ± 5.68[b]	334.15 ± 4.41[a]	0.033
Catalase, U/mL	7.96 ± 0.57	6.97 ± 0.49	7.25 ± 0.68	0.480
Malondialdehyde, nmol/mL	5.42 ± 0.18	5.67 ± 0.49	5.47 ± 0.47	0.900
Total antioxidant capacity, U/mL	1.14 ± 0.08	0.98 ± 0.09	1.21 ± 0.11	0.232
Glutathione, mg/L	3.18 ± 0.15	2.87 ± 0.21	3.14 ± 0.34	0.633

Values are the mean ± SEM, $n = 8$ per treatment group. [a-b]Mean values sharing different superscripts within a row differ ($p < 0.05$).

no difference in the serum concentration of MDA among treatments on days 14 and 21 ($p > 0.05$) (Table 3).

3.3. Serum Profiles of Cytokines. Exposure to diquat increased ($p < 0.05$) serum concentrations of IL-1β, IL-6, IL-8, IL-12, GM-CSF, TNF-α, IL-10, and IFN-γ but decreased ($p < 0.05$) TGF-β1 content when pigs were fed with the basal diet on day 14. Dietary EUF decreased ($p < 0.05$) the serum concentrations of IL-1β, IL-6, IL-8, IL-12, GM-CSF, TNF-α, IL-10, and IFN-γ but increased ($p < 0.05$) serum IL-4 and TGF-β1 compared with diquat-challenged pigs fed with the basal diet on day 14. On day 21, no differences ($p > 0.05$) were observed in serum cytokine concentrations among 3 treatments, except that diquat injection increased ($p < 0.05$) serum TNF-α and IL-10 compared with nonchallenged pigs fed with the basal diet. Supplementation of EUF reduced ($p < 0.05$) serum TNF-α concentration when pigs were challenged with diquat (Table 4).

3.4. Serum Concentrations of D-Lactate and Diamine Oxidase. Diquat exposure increased ($p < 0.05$) serum concentrations of D-lactate and diamine oxidase in pigs fed with

Table 4: Serum profiles of cytokines in piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Day 14 (pg/ml)				
IL-1β	287.23 ± 8.41^{c}	847.24 ± 31.47^{a}	387.47 ± 15.46^{b}	<0.001
IL-4	294.53 ± 20.14^{b}	286.41 ± 19.54^{b}	564.56 ± 35.64^{a}	<0.001
IL-6	21.25 ± 1.34^{c}	257.24 ± 13.24^{a}	54.36 ± 8.68^{b}	<0.001
IL-8	105.45 ± 12.54^{b}	975.64 ± 60.89^{a}	243.57 ± 30.42^{b}	<0.001
IL-12	146.41 ± 9.57^{b}	345.48 ± 35.54^{a}	208.65 ± 24.58^{b}	<0.001
GM-CSF	124.17 ± 10.58^{b}	240.58 ± 27.58^{a}	138.42 ± 16.65^{b}	<0.001
TGF-β1	954.24 ± 23.42^{a}	514.35 ± 42.52^{b}	895.44 ± 28.56^{a}	<0.001
TNF-α	1.56 ± 0.21^{b}	124.25 ± 23.56^{a}	34.45 ± 6.54^{b}	<0.001
IL-10	0.45 ± 0.01^{b}	1.24 ± 0.25^{a}	0.68 ± 0.08^{b}	0.004
IFN-γ	0.06 ± 0.01^{b}	0.58 ± 0.11^{a}	0.21 ± 0.05^{b}	<0.001
Day 21 (pg/ml)				
IL-1β	221.56 ± 11.25	356.45 ± 32.54	215.46 ± 20.56	0.417
IL-4	256.25 ± 25.54	248.36 ± 20.89	324.12 ± 40.56	0.174
IL-6	53.42 ± 6.87	76.25 ± 13.56	64.45 ± 9.58	0.318
IL-8	186.23 ± 19.68	235.56 ± 31.56	206.72 ± 24.56	0.411
IL-12	134.15 ± 12.14	186.84 ± 24.51	164.56 ± 19.45	0.180
GM-CSF	135.12 ± 11.58	169.56 ± 20.45	132.45 ± 12.48	0.188
TGF-β1	817.24 ± 38.42	728.63 ± 56.25	795.42 ± 41.56	0.383
TNF-α	2.87 ± 0.17^{b}	9.54 ± 2.14^{a}	3.57 ± 1.06^{b}	0.005
IL-10	0.38 ± 0.08^{b}	0.75 ± 0.10^{a}	$0.51 \pm 0.10^{a, b}$	0.034
IFN-γ	0.10 ± 0.02	0.19 ± 0.09	0.15 ± 0.09	0.697

Values are the mean ± SEM, $n = 8$ per treatment group. [a-c]Mean values sharing different superscripts within a row differ ($p < 0.05$).

Table 5: Serum concentrations of D-lactate and diamine oxidase in piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Day 14				
D-lactate (mmol/L)	0.74 ± 0.03^{b}	0.98 ± 0.08^{a}	$0.82 \pm 0.04^{a, b}$	0.016
Diamine oxidase, mg/ml	41.16 ± 3.36^{b}	54.21 ± 3.67^{a}	$45.87 \pm 2.14^{a, b}$	0.024
Day 21				
D-lactate, (mmol/L)	0.25 ± 0.02	0.31 ± 0.04	0.29 ± 0.05	0.546
Diamine oxidase, mg/ml	36.15 ± 1.15	39.36 ± 3.10	37.56 ± 2.16	0.615

Values are the mean ± SEM, $n = 8$ per treatment group. [a-b]Mean values sharing different superscripts within a row differ ($p < 0.05$).

the basal diet on day 14; however, this was not the case on day 21. No differences were observed in the serum concentration of D-lactate and diamine oxidase between pigs fed with the basal diet and the EUF diet on day 14 and 21 ($p > 0.05$) (Table 5).

3.5. Jejunal and Ileal Morphology. Diquat challenge reduced ($p < 0.05$) jejunal and ileal villi height and jejunal and ileal villous height, crypt depth, but increased ($p < 0.05$) ileal crypt depth on day 14 when pigs were fed the basal diet. Inclusion of EUF increased ($p < 0.05$) jejunal and ileal villi height and villous height, crypt depth, of diquat-challenged pigs compared with those in the basal diet on day 14. No differences were observed in villous height and crypt depth of jejunum and ileum among 3 treatments on day 21 ($p > 0.05$) (Table 6).

3.6. Histopathological Grading. Diquat exposure increased ($p < 0.05$) the histopathological grading of the jejunum, ileum, anterior colon, and posterior colon on day 14, but no differences ($p > 0.05$) were observed in histopathological grading on day 21. Compared with that in basal diet + diquat treatment, lower histopathological grading of the jejunum, ileum, anterior colon, and posterior colon of piglets in EUF + diquat treatment on day 14 were observed ($p < 0.05$) (Table 7).

3.7. Myeloperoxidase Activity. Diquat challenge increased ($p < 0.05$) the MPO activity in the anterior and posterior colon of piglets on day 14 if they were fed with the basal diet. Dietary EUF supplementation reduced ($p < 0.05$) the MPO activity in the posterior colon compared with diquat-challenged pigs fed with the basal diet. However, no

Table 6: Jejunal and ileal morphology in piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Day 14				
Jejunal villous height (μm)	297.52 ± 7.21^{a}	226.53 ± 21.08^{b}	306.21 ± 27.35^{a}	0.026
Jejunal crypt depth (μm)	93.23 ± 7.14	129.25 ± 18.35	101.25 ± 16.24	0.217
Jejunal villous height: crypt depth	3.19 ± 0.23^{a}	1.75 ± 0.31^{b}	3.02 ± 0.17^{a}	<0.001
Ileal villous height (μm)	240.32 ± 10.56^{a}	164.38 ± 27.32^{b}	237.32 ± 15.25^{a}	0.016
Ileal crypt depth (μm)	69.15 ± 3.25^{b}	86.15 ± 5.43^{a}	$72.43 \pm 9.15^{a, b}$	0.043
Ileal villous height: crypt depth	3.48 ± 0.17^{a}	1.91 ± 0.38^{b}	3.28 ± 0.28^{a}	0.002
Day 21				
Jejunal villous height (μm)	246.32 ± 12.23	216.32 ± 32.12	242.43 ± 15.35	0.578
Jejunal crypt depth (μm)	114.26 ± 9.47	144.39 ± 21.26	113.21 ± 12.74	0.284
Jejunal villous height: crypt depth	2.16 ± 0.24	1.50 ± 0.41	2.14 ± 0.27	0.265
Ileal villous height (μm)	189.32 ± 12.23	154.16 ± 26.36	179.94 ± 13.45	0.084
Ileal crypt depth (μm)	102.13 ± 5.13	125.34 ± 19.44	101.64 ± 11.21	0.372
Ileal villous height: crypt depth	1.85 ± 0.31	1.23 ± 0.31	1.77 ± 0.16	0.232

Values are the mean ± SEM, $n = 8$ per treatment group. $^{a\text{-}b}$Mean values sharing different superscripts within a row differ ($p < 0.05$).

Table 7: Histopathological grading of the jejunum, ileum, and colon in piglets.

Item	Basal diet	Basal diet + diquat	EUF diet + diquat	p value
Day 14				
Jejunum	2.25 ± 0.31^{c}	12.13 ± 0.44^{a}	6.13 ± 0.44^{b}	<0.001
Ileum	3.25 ± 0.45^{c}	9.13 ± 0.55^{a}	5.00 ± 0.46^{b}	<0.001
Anterior colon	4.00 ± 0.38^{c}	10.50 ± 0.76^{a}	6.50 ± 0.57^{b}	<0.001
Posterior colon	4.38 ± 0.63^{c}	11.00 ± 0.65^{a}	7.75 ± 0.75^{b}	<0.001
Day 21				
Jejunum	3.00 ± 0.38	4.50 ± 0.46	3.25 ± 0.49	0.059
Ileum	3.75 ± 0.49	3.88 ± 0.52	3.75 ± 0.49	0.980
Anterior colon	4.50 ± 0.57	4.75 ± 0.53	4.13 ± 0.64	0.748
Posterior colon	4.25 ± 0.65	5.38 ± 0.71	5.00 ± 0.73	0.519

Values are the mean ± SEM, $n = 8$ per treatment group. $^{a\text{-}c}$Mean values sharing different superscripts within a row differ ($p < 0.05$).

difference was observed in the MPO activity among 3 treatments on day 21 ($p > 0.05$) (Figure 1).

4. Discussion

Oxidative stress which resulted in cellular injury and tissue damage has been increasingly recognized as a contributing factor in many chronic diseases such as heart disease, Alzheimer's and Parkinson's diseases, and even cancer [1, 5]. Therefore, inhibition of oxidative stress will be a potential strategy to prevent chronic diseases. There has been considerable interest in the isolation and characterization of antioxidative agents from natural products [6, 8, 9]. The present study is focusing on the antioxidative activity of flavones in the EU leafs that are widely cultivated in China.

In the current experiment, oxidative stress piglet model induced by diquat was used and has been widely used *in vivo* [18, 19]. Diquat has been reported to impair growth performance and nutrient utilization [18, 19]. The reduced gut morphology and growth performance by diquat challenge in the present are in agreement with published report [28]. This is mainly due the disruption in the oxidative balance [18], which is evidenced by the decrease in serum concentrations of SOD, GSH-Px, CAT, T-AOC, and GSH after exposure to diquat injection in piglets of basal diet treatments. In the previous studies, diquat has been demonstrated to increase serum MDA concentration but also to inhibit the activities of SOD and GSH-Px [18, 19]. In addition, the present results of serum cytokine concentrations, intestinal histopathological grading, and MPO activity indicated that diquat evaluates the inflammatory response of weaned pigs, which is consistent with previous research [9, 28, 29].

However, dietary supplementation with EUF showed to alleviate these negative effects induced by diquat. Firstly, EUF significantly elevated the growth performance of weaned piglets and this growth promotion effect of flavonoids also demonstrated in geese, ducks, meat sheep, and

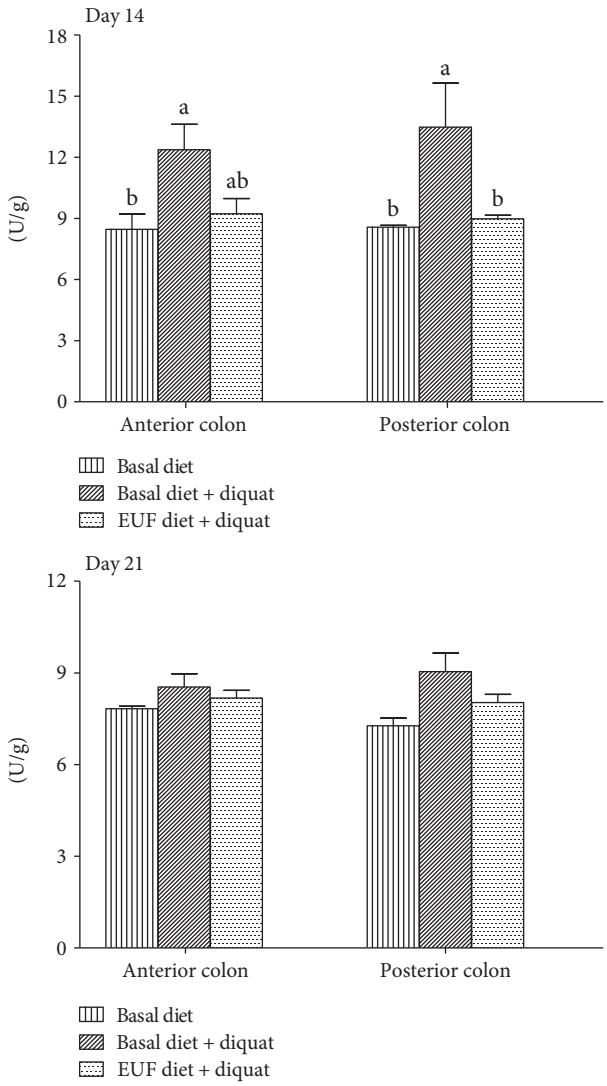

FIGURE 1: Myeloperoxidase activity in the colon of piglets. Values are the mean ± SEM, $n = 8$ per treatment group. [a-b]Mean values sharing different superscripts within anterior colon or posterior colon differ ($p < 0.05$).

fatting pig [30–33]. Therefore, a large scale of performance trial should be conducted to verify the potential of using EUF as a growth promoter for weaned pigs.

The mechanisms of the antioxidant capacity of flavones include suppression of ROS formation by either inhibition of enzymes involved in their production, scavenging of ROS, or upregulation or protection of antioxidant defenses [8]. Diquat could stimulate cellular production of ROS and inhibit the activities of antioxidant enzymes such as SOD and GSH-Px [18, 19, 34]. Antioxidant enzymes including SOD, CAT, and GSH-Px can neutralize toxic oxygen products, thereby normalizing the body homeostasis [2]. SOD converts superoxide radicals into H_2O_2, which is converted into water by GSH-Px and CAT [35]. Oxidative stress results from the failure of antioxidant enzymes to eliminate free radicals [36]. The present results indicated that EUF exerted antioxidant effects at the first line of defense by increasing the level of antioxidant enzymes in the blood to neutralize ROS.

The relationship between oxidative stress and inflammation has been documented by previous research [5, 37]. In the present study, dietary supplementation with EUF significantly alleviated the inflammatory response induced by diquat. Excess antioxidant enzymes in the serum and intestine may be associated with the production of proinflammatory and anti-inflammatory cytokines in the respective locations [38]. Evidences have shown that free radicals especially H_2O_2 and NO may respond as secondary messengers to stimulate proinflammatory cytokines [39, 40]. Serum cytokine profiles could be a used as markers of inflammation [41, 42]. Proinflammatory cytokines such as IL-1β, IL-6, IFN-γ, TNF-α, and IL-1 are released by macrophages upon activation of the NF-κB pathway, a crucial factor responsible for inducing inflammatory response in pathological conditions [43, 44].

It has demonstrated that flavonoids were not absorbed well and their concentrations could be much higher in the lumen of the gastrointestinal tract than were ever achieved in plasma [45]. Therefore, the digestive tract is the major site of antioxidant defense afforded by flavonoids [45, 46]. The EUF improved the morphological structure and barrier function of the intestine in the present study, which is evidenced by higher villous height and lower concentrations of D-lactate and diamine oxidase. As the markers of intestinal integrity, lower serum diamine oxidase and D-lactate contents released from upper villi of the small intestine showed smaller damage of intestinal integrity [47, 48]. The improvement in intestinal barrier function may reflect the alleviation of inflammatory response [49].

In conclusion, flavones extracted from *Eucommia ulmoides* leaf have shown antioxidative activity and anti-inflammatory effects. The results indicated that dietary supplementation with EUF alleviated the growth performance impairment, oxidative stress, inflammatory response, and intestinal damage induced by diquat in piglets. These findings will be helpful for the development of future antioxidant therapeutics and new anti-inflammatory drugs and the application of EUF in piglets. Further studies will be needed to elucidate the molecular mechanisms of EUF-regulating oxidative stress in inflammatory disease.

Abbreviations

CAT: Catalase
DAO: Diamine oxidase
EUF: *Eucommia ulmoides* flavones
GM-CSF: Granulocyte macrophage colony stimulating factor
GSH-Px: Glutathione peroxidase
H_2O_2: Hydrogen peroxide
IFN-γ: Interferon-gamma
IL: Interleukin
MDA: Malondialdehyde
MPO: Myeloperoxidase
NF-κB: Nuclear factor kappa-beta
NO: Nitric oxide
RNS: Reactive nitrogen species
ROS: Reactive oxygen species

SOD: Superoxide dismutase
T-AOC: Total antioxidant capacity
TGF-β1: Transforming growth factor-beta 1
TNF-α: Tumor necrosis factor.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Daixiu Yuan and Tarique Hussain contributed equally to this work.

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