Received: 24 April 2018 | Revised: 28 May 2018 | Accepted: 31 May 2018

DOI: 10.1002/tox.22588

WILEY ENVIRONMENTAL TOXICOLOGY

RESEARCH ARTICLE

CYP450-mediated mitochondrial ROS production involved in arecoline *N*-oxide-induced oxidative damage in liver cell lines

Tsu-Shing Wang[1,2] | Cheng-Ping Lin[1] | Yu-Pong Chen[1] | Mu-Rong Chao[3] | Chien-Chun Li[4] | Kai-Li Liu[4]

[1]Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan

[2]Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan

[3]Department of Occupational Safety and Health, Chung Shan Medical University, Taichung, Taiwan

[4]Department of Nutrition, Chung Shan Medical University, Taichung, Taiwan

Correspondence
Tsu-Shing Wang, PhD, Department of Biomedical Sciences, Chung Shan Medical University, No 110, Sec. 1, Chien-Kuo N. Rd., Taichung, Taiwan.
Email: tsushing@csmu.edu.tw

Funding information
Ministry of Science and Technology, Taiwan, ROC, Grant/Award Number: MOST 104-2320-B-040-013

Abstract

Background: IARC has classified the betel nut as a human environmental carcinogen. Previous studies have found that arecoline (AR) is the major alkaloid present in the saliva of betel quid chewers. Saliva contains a large content of AR which has been further shown to cause mutation of oral mucosa cells, resulting in oral cancer. Whereas, to date, there are only few studies reported the hepatotoxicity associated with arecoline and betel nut chewing. Therefore, the main purpose of this study was to determine the toxic effects of AR and its oxidative metabolite, arecoline *N*-oxide (ARNO), in normal liver cell lines.

Methods: The cytotoxic, genotoxic, and mutagenic effects were detected by crystal violet staining, alkaline comet assay, and Salmonella mutagenicity test, respectively. Measurement of intracellular reactive oxygen species (ROS) generation was determined using the H2-DCFDA assay.

Results: Our results demonstrated that ARNO exerted higher cytotoxicity, DNA damage, and mutagenicity than its parent compound arecoline in liver cells. Antioxidants, such as *N*-acetylcysteine, Trolox, and penicillamine, strongly protected liver cells from ARNO-induced DNA damage and ROS production. Furthermore, co-treatment with Mito-TEMPO also effectively blocked ARNO-induced ROS production in liver cells. Besides antioxidants, co-treatment with 1-aminobenzotriazole and methimazole nearly completely suppressed ARNO-induced ROS production in liver cells.

Conclusions: Our data suggest that arecoline ingested from the habit of chewing betel quid can be primarily oxidized to ARNO, thereby enhancing its toxicity through increased ROS production. Considering the excellent protective effects of both mitochondria-targeted antioxidant and CYP450 inhibitor on ARNO-induced ROS production in liver cells, mitochondria CYP450-mediated metabolism of ARNO may be a key mechanism. Collectively, our results provide novel cellular evidence for the positive connection between habitual betel quid chewing and the risk for liver damage.

KEYWORDS

arecoline, arecoline *N*-oxide, cytochrome P450, DNA strand break, liver cell lines, reactive oxygen species

1 | BACKGROUND

Betel quid (BQ) is a combination of areca nut (*Areca catechu* L.), slaked lime, and *Piper betle* inflorescence or conditional folded in a *Piper betle* leaf. Chewing of BQ is a habit of great antiquity in South Asia, Southeast Asia, and the Pacific Islands, with about 600 million users reported worldwide.[1,2] The areca nut in BQ has been recognized as a Group I carcinogen to humans by the International Agency for Research on Cancer[3] of the World Health Organization. Case-control studies from Asian countries have reported that BQ is a risk factor for oral cancer.[4-7] Besides oral cancer, BQ chewing is an independent risk

Abbreviations: BQ, betel quid; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; LC-MS, liquid chromatography-mass spectrometry; NAC, *N*-acetylcysteine; ROS, reactive oxygen species.

factor for cirrhotic hepatocellular carcinoma.[8-10] There is an additive interaction between BQ chewing and chronic hepatitis B and/or hepatitis C virus infection.[11] In addition, recent studies have shown that BQ chewing or areca nut contents have harmful effects on reproductive function and are associated with obesity and higher risk of cardiovascular disease.[12]

Although the exact mechanism by which betel nut induces adverse health effects has not been clearly elucidated, multiple pathways have been suggested, including enhanced production of reactive oxygen species (ROS) and the formation of DNA adducts.[13,14] These adverse effects may be due to the combined action of the ingredients of the BQ; the active compound accounting for BQ-induced carcinogenicity remains unclear. Previous study has shown that the most abundant active compound of the areca nut is arecoline (1,2,5,6-tetrahydro-1-methylnicotinic acid methyl ester), which may comprise up to 0.8% by weight of the ripe nut.[15] Furthermore, a recent report indicated that arecoline *N*-oxide (ARNO), the active metabolite of arecoline, exerts strong mutagenicity in *Salmonella* test strains and that the mutagenic potency of arecoline is significantly enhanced in the presence of a rat liver activation system (S9 mix).[16] These findings suggest that arecoline and its *N*-oxide metabolite may play a dominant role in BQ-induced cytotoxicity, genotoxicity, and carcinogenesis in vitro and in vivo; however, their relative toxicity is still not well understood. Therefore, the aims of this study were to investigate the properties of arecoline and its oxidized product ARNO in normal liver cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise specified.

2.2 | ARNO preparation

ARNOs were prepared according to the published method[17] with little modification. A solution of arecoline (2.44 g) in ether (10 mL) was stirred in a water-ice bath and 4.56 g of 39% (wt/vol) peroxyacetic acid (Fluka) was added dropwise over a 30 minutes. After the mixture was allowed to stand for 2 hours in a water-ice bath, the oily yellow lower layer that separated was dissolved in ethanol under stirring for 1 hour and precipitated 3 times by the addition of ether. The ether was carefully removed with a micropipette, and then, the yellow viscous oily precipitate of arecoline oxides was dried by lyophilization. Finally, the synthesized product was confirmed by mass spectrometry to give the molecular ion mass (*m/z*) of 171.7.

2.3 | Cell culture

Rat liver clone 9 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Human liver WRL68 cells were cultured in EMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% nonessential amino acid, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human liver WRL-68 and rat liver clone 9 cells were incubated at 37°C in a 5% CO_2 humidified incubator.

2.4 | Cytotoxicity assay

Cytotoxicity was determined using the crystal violet staining assay, as in our previous study.[18] Briefly, cells (2×10^4 cells/well) were seeded in 24-well cell culture plates and drug exposures were made after overnight culture. Cells were treated with or without arecoline or arecoline oxide for 24 hours at concentrations ranging from 31.25 to 1000 μM. After treatment, cells were washed twice with PBS without calcium and magnesium, fixed with 0.3 mL 95% ethanol for 15 minutes, and then stained with 0.3 mL 0.1% crystal violet at room temperature. After 30 minutes, the crystal violet was gently rinsed off with running tap water and the plates were allowed to air-dry. The dye (crystal violet) was extracted from the intact cells with 0.1 M sodium citrate reagent (in 50% ethanol), and the optical density of the solution was measured spectrophotometrically at a wavelength of 540 nm by use of a Molecular Devices (Menlo Park, California) kinetic microplate reader.

2.5 | Mutagenicity assay

Mutagenicity was assessed by the Ames test. The *Salmonella typhimurium* tester strains TA98 and TA100 were kindly provided by Dr. Jen-Kun Lin, Chair Professor (College of Oral Medicine, Chung Shan Medical University, Taichung, Taiwan). The strains were grown overnight from frozen cultures for 12 to 14 hours in Oxoid Nutrient Broth No. 2. To determine chemical mutagenicity, to 2 mL of top agar containing 0.5 mM histidine/biotin were added 0.1 mL of a fresh Salmonella culture, 0.1 mL of various concentrations of arecoline or ARNO (0.125-1 mM), and 0.1 mL a freshly grown culture of a Salmonella tester strain. After thorough mixing, the mixture was spread over the minimal agar plate. After the agar had hardened, the plates were inverted and incubated at 37°C for 48 hours and His^+ revertant colonies were counted manually. All experiments were done in triplicate. The standard mutagens used as positive controls in experiments without the S9 mix were 4-nitroquinoline 1-oxide, 4NQO (5 μg/plate) for TA98 and sodium azide, NaN_3 (5 μg/plate) for TA100.

2.6 | Alkaline comet assay

The alkaline comet assay was used to detect arecoline- and arecoline oxide-induced DNA strand breaks in rat liver clone 9 cells. Cells were treated with various doses of arecoline and arecoline oxide for 2 hours. After treatment, the cells were collected by centrifugation and washed twice in PBS, and DNA damage was measured using the alkaline comet assay as previously described.[19] Damage was quantified as tail moment of at least 50 cells per sample using the software program Comet Assay III (Perceptive Instruments Ltd, Haverhill, UK). Tail moment is defined as the product of tail length and the fraction of DNA in the tail.

2.7 | ROS generation assay

The intracellular generation of ROS was assayed with the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method. DCFH-DA is transported across the cell membrane and cleaved by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound (DCF). Rat liver clone 9 cells were seeded at 8×10^4 cells/well in 4-well chamber slides and precultured overnight. On the next day, the cells were preincubated with 100 μM DCFH-DA in HBSS for 30 minutes and were then incubated in the presence or absence of different doses of arecoline or ARNO (31.25-250 μM) for 2 hours or with 250 μM H_2O_2 for 30 minutes as the positive control. After incubation, cells were washed, and the level of DCF fluorescence was determined using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, California). The fluorescence of intracellular DCF was also observed and photographed by fluorescence microscopy (Nikon E400, Tokyo, Japan) coupled with a digital camera.

2.8 | Liquid chromatography-mass spectrometry analysis

Fifteen milliliters of each suspension were collected and then injected into an HLB SPE column for cleanup. The column was first eluted with 2 mL of methanol 3 times. Next, the combined eluents were concentrated using rotary evaporation (<30°C, 0.05 Mpa) to a volume of approximately 2 mL. The residue eluents (enriched oxidized products of arecoline) were further concentrated to near dryness under a gentle stream of nitrogen, redissolved in 50% (vol/vol) methanol, and analyzed for the oxidized products of arecoline by liquid chromatography-mass spectrometry (LC-MS). The LC-MS analysis was conducted on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) equipped with a TurboIon-Spray source operating in the positive ion electrospray in full-scan mode. The ESI-MS source conditions established were as follows: flow rate from syringe pump, 50 μL/minutes; *m/z* range, 100-400 AMu; needle voltage, 5000 V; nebulizer gas flow, 8; curtain gas flow, 8; declustering potential, 30; focusing potential, 200; entrance potential, 10; dwell time, 0.5 ms; and step, 0.7 Th.

2.9 | Statistical analysis

Results are expressed as means ± SEs from at least 3 independent experiments. Data were analyzed using one-way analysis of variance (SAS Institute, Cary, North Carolina). The significance of the difference among group means was determined by Student's *t*-tests; *P* values <.05 were taken to be statistically significant.

3 | RESULTS

3.1 | Arecoline- and ARNO-induced cytotoxicity and mutagenicity

The cytotoxic effects of arecoline and ARNO were evaluated in rat liver clone 9 cells by crystal violet staining. Treatment with either arecoline or ARNO for 24 hours resulted in cell death in a dose-dependent manner (Figure 1). The cytotoxicity of ARNO was 8 times that of arecoline. The IC_{50} values of arecoline and ARNO were 500 μM and 62.5 μM, respectively. In addition, the mutagenic effect of arecoline and arecoline N-oxide was tested using Salmonella typhimurium TA100 and TA98 in the absence of S9. As shown in Table 1, the mutagenicity of arecoline N-oxide is strikingly higher than arecoline in both strains.

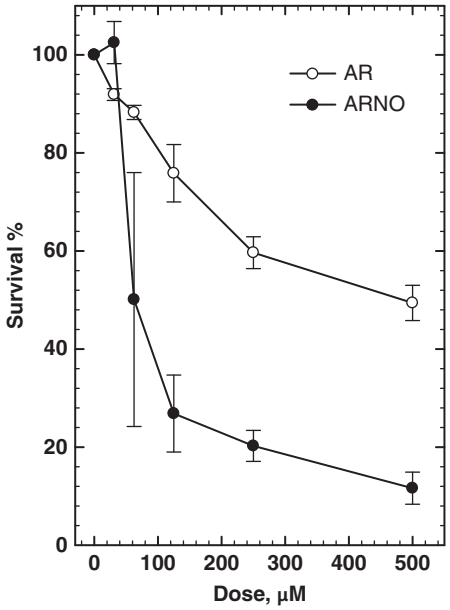

FIGURE 1 Arecoline- and arecoline *N*-oxide-induced cytotoxicity in clone 9 cells. Cells were treated with different doses (0-500 μM) of arecoline (AR, −○-) or arecoline *N*-oxide (ARNO, −•-) for 24 hours and cell density was assessed by crystal violet staining, normalized, and shown as percentages of the cell densities in control. Data are mean ± SE from 3 independent experiments

TABLE 1 Mutagenicity of arecoline and arecoline *N*-oxide in *Salmonella typhimurium* TA 98 and TA 100

	Number of revertants/plate	
Treatments	**TA 98**	**TA 100**
0 μM AR[a]	14 ± 1	168 ± 17
125 μM AR	26 ± 6	170 ± 35
250 μM AR	22 ± 5	182 ± 22
500 μM AR	15 ± 3	165 ± 30
1000 μM AR	23 ± 6	176 ± 18
125 μM ARNO[b]	35* ± 6	230* ± 2
250 μM ARNO	23 ± 4	251* ± 11
500 μM ARNO	26* ± 4	372* ± 42
1000 μM ARNO	53 ± 21	618* ± 132
1.25 μg/mL 4NQO[c]	74* ± 14	---
1.25 μg/mL NaN3[d]	---	1788** ± 321

Data are mean ± SE from 3 independent experiments. Statistically significant increases in the number of revertant colonies compared to the drug-free control.

*$P <$ .05. **$P <$ .01.

[a] AR: arecoline.

[b] ARNO: arecoline *N*-oxide.

[c] 4NQO; 4-nitroquinoline-1-oxide.

[d] NaN3: sodium azide.

3.2 | Arecoline- and ARNO-induced DNA damage

The potential of arecoline and ARNO to induce DNA strand breaks was assessed by the alkaline comet assay. Rat liver clone 9 cells were treated with various doses (31.25 ~ 500 μM) of arecoline or ARNO for 2 hours, after which the alkaline comet assay was conducted immediately. As shown in Figure 2, arecoline treatment for 2 hours did not induce significant DNA strand breaks in rat liver clone 9 cells. By contrast, 2 hours of ARNO treatment significantly induced DNA strand breaks in clone 9 cells in a dose-dependent manner. The mean tail moment for rat liver clone 9 cells treated with 125, 250, and 500 μM ARNO for 2 hours was 17.0 ± 4.4, 29.6 ± 3.3, and 37.0 ± 3.8, respectively. The tail moment of untreated control and H_2O_2 positive control treated (50 μM for 5 minutes) cells was 0.6 ± 0.2 and 64.1 ± 8.5, respectively (data not shown).

To determine whether ROS generation was involved in ARNO-induced DNA strand breaks in clone 9 cells, cells were treated with various doses of *N*-acetylcysteine (NAC, a potent antioxidant) 30 minutes before the addition of 125 μM ARNO and co-incubated for 2 hours. After treatment, DNA damage was evaluated by alkaline comet assay and relative tail moment was expressed as a percentage in comparison with ARNO treatment alone (=100%). The results showed that NAC addition significantly decreased arecoline-*N*-oxide-induced DNA strand breaks in a dose-dependent manner (Figure 3). This finding also supports that ARNO enhances intracellular ROS production and subsequently elevates DNA strand breaks in clone 9 cells.

The production of ROS in clone 9 cells under co-treatment with ARNO and NAC was further examined by fluorescent microscopy with the ROS-sensitive dye DCFH-DA. A significant increase of ROS was observed after 2 hours of treatment with 31.25 μM ARNO, whereas ROS levels were not changed in either the control group or the group treated with 31.25 μM NAC for 2 hours (Figure 4).

In the combination treatment group, NAC almost completely inhibited ARNO-induced ROS production in clone 9 cells (Figure 4). Besides NAC, combined treatment with either Trolox (a water-soluble analog of vitamin E) or D-penicillamine (a copper-chelating agent) for 2 hours also significantly inhibited ARNO-induced ROS production in clone 9 cells (Figures 5 and 6, respectively).

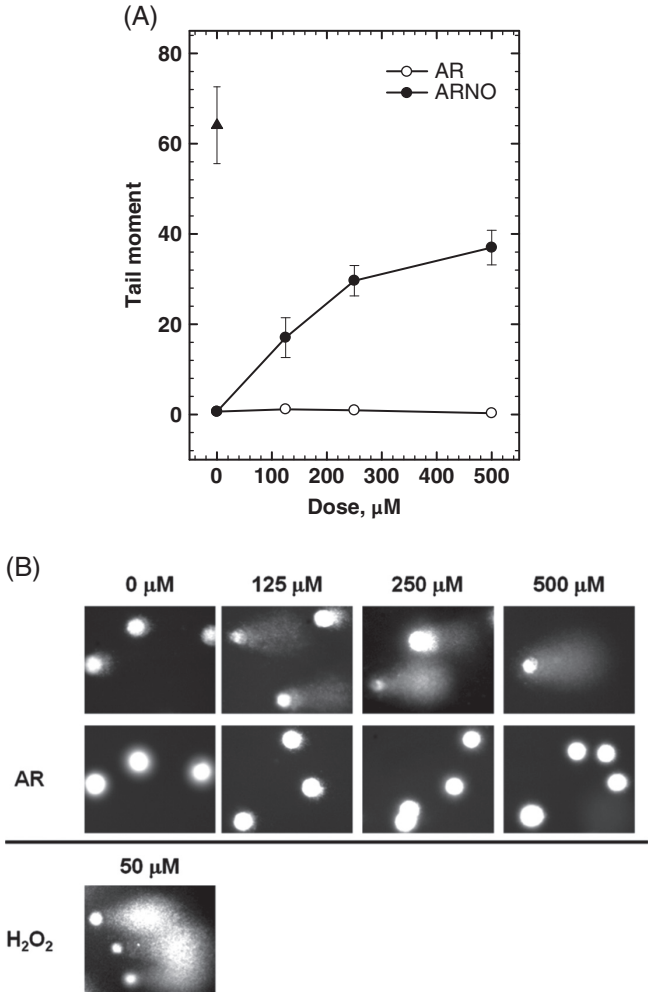

FIGURE 2 (A) Arecoline- and arecoline *N*-oxide-induced DNA damage in clone 9 cells. Cells were treated with different doses (0-500 μM) of arecoline (AR, −○-) or arecoline *N*-oxide (ARNO, −●-) for 2 hours and DNA strand breaks were determined by alkaline comet assay. The DNA damage level is expressed as tail moment. Tail moment induced by H_2O_2 (50 μM) for 5 minutes (solid triangles, −▲-) was used as a positive control. Data are mean ± SE from 3 independent experiments. (B) Representative comet images of panel (A)

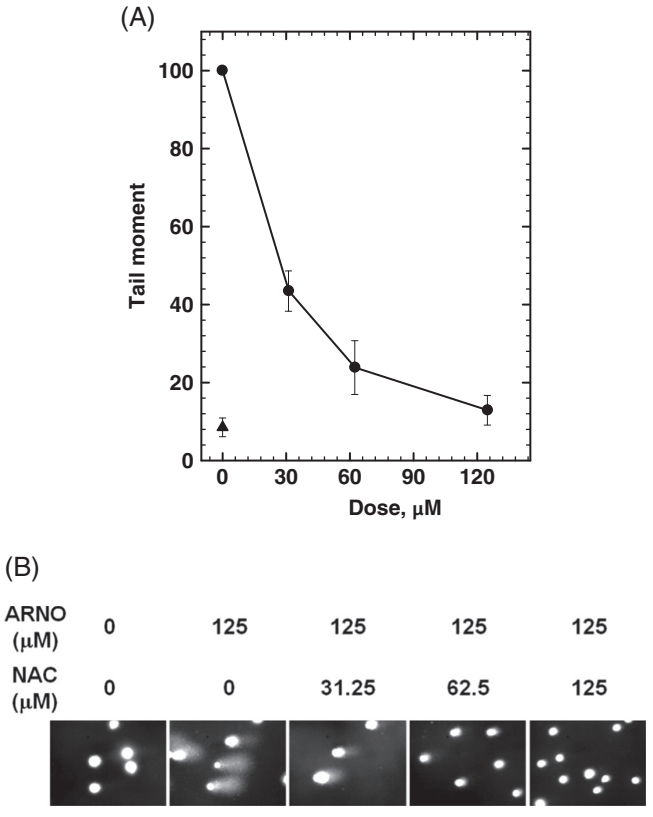

FIGURE 3 (A) Protective effect of *N*-acetylcysteine on arecoline *N*-oxide-induced DNA damage in clone 9 cells. Cells were co-treated with 125 μM arecoline *N*-oxide and different doses of *N*-acetylcysteine (31.25, 62.5, and 125 μM) for 2 hours and DNA strand breaks were determined by alkaline comet assay. The DNA damage level is expressed as tail moment and data are mean ± SE from 3 independent experiments. The mean tail moment of arecoline *N*-oxide is expressed as 100%. Filled triangle (−▲-) represents the mean tail moment of the untreated control relative to the arecoline *N*-oxide-treated group. (B) Representative comet images of panel (A)

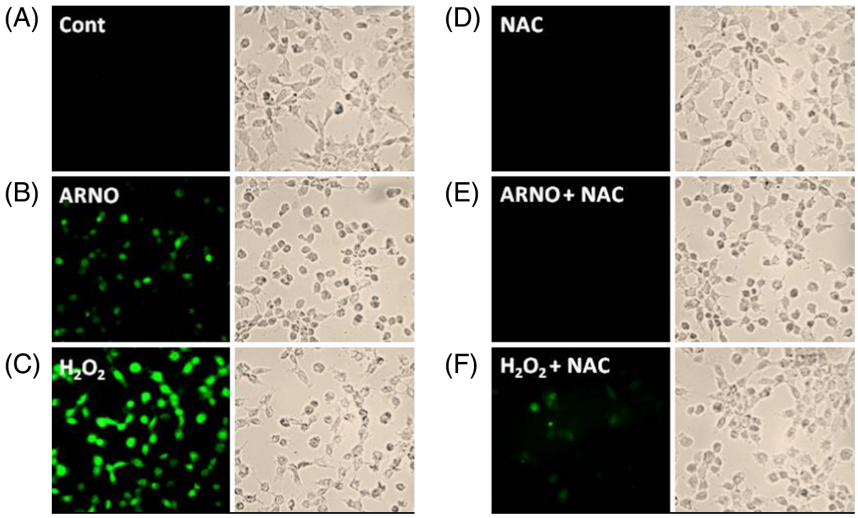

FIGURE 4 Protective effect of *N*-acetylcysteine on arecoline *N*-oxide-induced reactive oxygen species production in clone 9 cells. Cells were co-treated with or without 31.25 μM arecoline *N*-oxide (ArNO) and 31.25 μM *N*-acetylcysteine (NAC) for 2 hours and reactive oxygen species production was detected by fluorescence microscopy using DCFH-DA. Cells were treated with 250 μM H_2O_2 for 30 minutes as a positive control for reactive oxygen species production [Color figure can be viewed at wileyonlinelibrary.com]

Collectively, these results not only confirm that ROS production plays an important role in ARNO-induced DNA damage but also indicate that the copper-ion-catalyzed reaction is also involved in ARNO-induced ROS production.

3.3 | Identification of oxidation products of arecoline

The formation of oxidation products of arecoline catalyzed by peracetic acid in vitro were confirmed and determined by LC-MS. Figure 7 shows the mass spectra of the peracetic acid-catalyzed oxidation products of arecoline. The presence of oxidation products of arecoline was determined from the presence of the protonated ions $[M + H]^+$ of *m/z* 172 and 343, which corresponded to the ARNO and the dimer of ARNO, respectively. The presence of unchanged arecoline was determined from the observation of a protonated ion of *m/z* 156. The protonated ions observed for the ESI/MS analysis of oxidation products of arecoline were consistent with those previously reported.[20,21] Our findings indicated that the ARNO was the dominant oxidized product and was able to induce oxidative stress in vitro, which might be responsible for the toxic effects of arecoline in betel nut in vivo.

3.4 | ARNO metabolism and ROS production

To investigate the role of drug-metabolizing enzymes in ARNO-induced cellular ROS production, we used selective pharmacological inhibitors to investigate the roles of cytochrome P450 (CYP), monoamine oxidases (MAO), and flavin-containing monooxygenases (FMO)

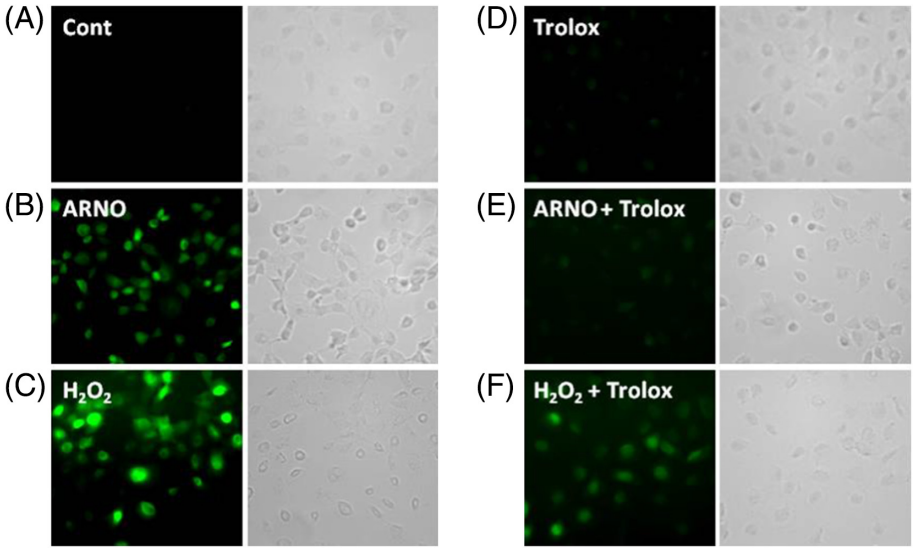

FIGURE 5 Protective effect of Trolox on arecoline *N*-oxide-induced reactive oxygen species production in clone 9 cells. Cells were co-treated with or without 31.25 μM arecoline *N*-oxide (ArNO) and 600 μM Trolox for 2 hours and then reactive oxygen species production was detected by fluorescence microscopy using DCFH-DA. Cells were treated with 250 μM H_2O_2 for 30 minutes as a positive control for reactive oxygen species production [Color figure can be viewed at wileyonlinelibrary.com]

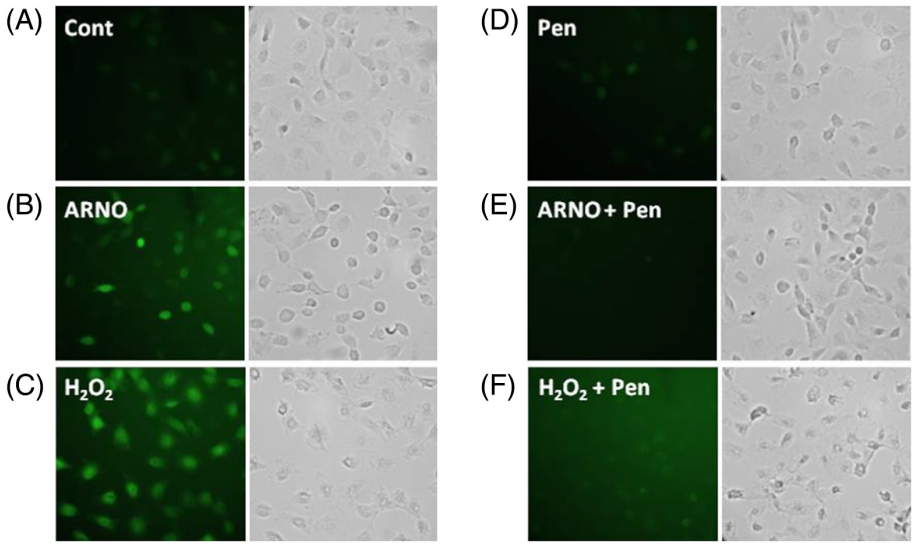

FIGURE 6 Protective effect of penicillamine on arecoline *N*-oxide-induced reactive oxygen species production in clone 9 cells. Cells were co-treated with or without 31.25 μM arecoline *N*-oxide (ArNO) and 500 μM penicillamine (pen) for 2 hours and then reactive oxygen species production was detected by fluorescence microscopy using DCFH-DA. Cells were treated with 250 μM H_2O_2 for 30 minutes as a positive control for reactive oxygen species production [Color figure can be viewed at wileyonlinelibrary.com]

in ARNO-induced ROS in human liver WRL-68 cells. The results were shown in Figure 8. Similar to the finding in rat liver clone 9 cells, exposure of human liver WRL68 cells to 100 mM ARNO for 24 hours resulted in significant ROS production. The addition of mitochondrial-targeted antioxidant Mito-TEMPO totally abolished ROS production induced by RANO in WRL68 cells. In addition, ARNO-induced ROS production in WRL68 cells was potently inhibited both by both methimazole (an inhibitor of FMO) and 1-aminobenzotriazole (an inhibitor of cytochrome P450 enzymes). On the contrary, the monoamine oxidase inhibitors (pargyline, selegiline, and clorgyline) did not exert an apparent inhibitory effect on ARNO-induced ROS production. These findings indicate that ARNO-induce cellular ROS production may be dependent mediated by CYP4540- and/or FMO and -mediated mitochondrial ROS production.

4 | DISCUSSION

We showed that ARNO was the main oxidation product of arecoline (a major alkaloid of the areca nut) induced by oxidation with peracetic acid in vitro. Besides chemical oxidation in vitro, ARNO can also be efficiently formed from arecoline by both in vitro FMO-catalyzed oxidation and in vivo metabolic processes.[17,20] Our present findings further discovered that ARNO appears to be more cytotoxic and mutagenic than then parent compound, arecoline. Previous studies have shown that arecoline induces cytotoxicity in various cell lines, including human endothelial cells, human epithelial cells, human mucosal fibroblasts, and human leukemia cells.[22-30] However, the cytotoxic sensitivity to arecoline varies depending on cell types. Studies have shown that arecoline is much less cytotoxic in different human

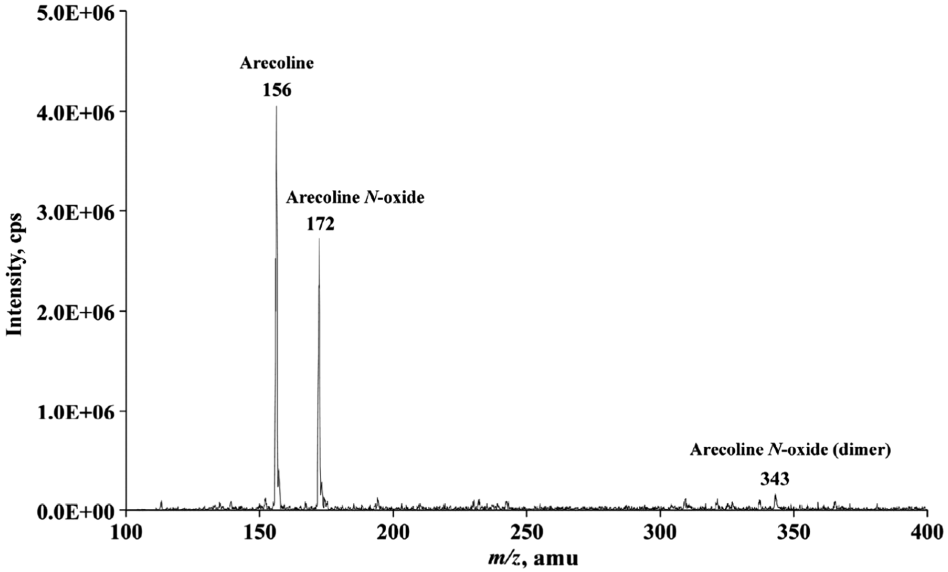

FIGURE 7 Mass spectrum of oxidized products of arecoline induced by peracetic acid in vitro. The lyophilized powder of arecoline oxidation products was dissolved in water and analyzed by mass spectrometry. For details see materials and methods section

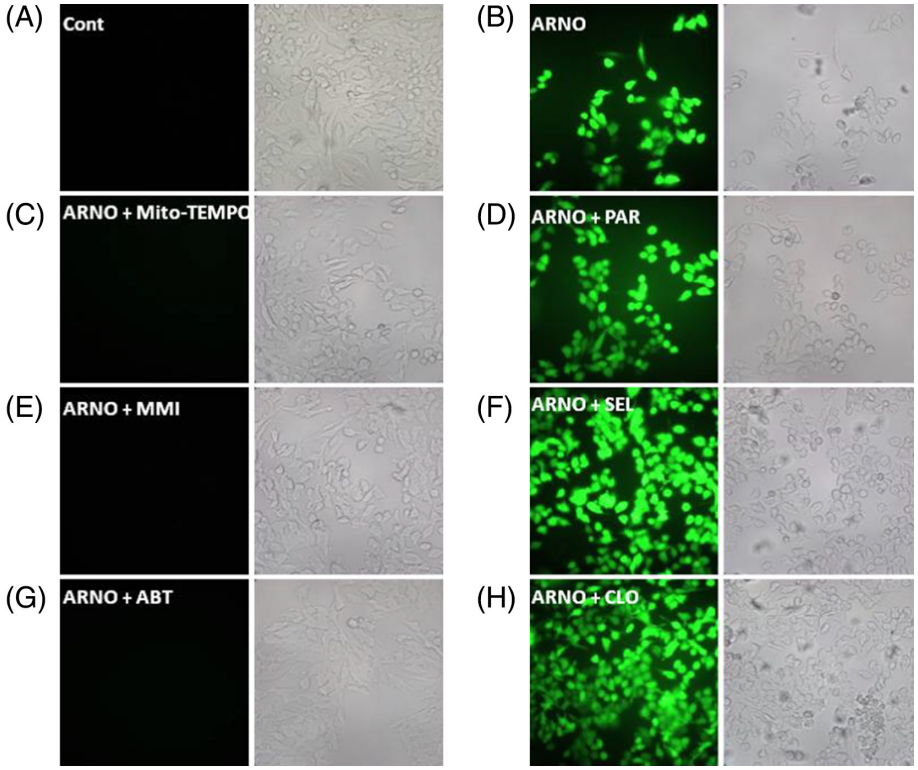

FIGURE 8 Effects of antioxidant and inhibitors of specific metabolic enzymes on arecoline *N*-oxide-induced ROS production in human liver WRL-68 cells. Cells were treated with flavin-containing monooxygenase (methimazole, MMI), mitochondria-targeted antioxidant (Mito-TEMPO), inhibitors of cytochrome P450 (1-aminobenzotriazole, ABT), and monoamine oxidase (imipramine, pargyline, selegiline, clorgyline) for 30 minutes and then co-treated with arecoline *N*-oxide for another 24 hours. After treatment, the levels of intracellular reactive oxygen species (ROS) production were determined by fluorescence microscopy using DCFH-DA probe and fluorescence intensity was quantified by ImageJ [Color figure can be viewed at wileyonlinelibrary.com]

fibroblast cell lines HGF-1 and Hel than in human umbilical vein endothelial cells and human keratinocyte cells.[23,28] In this study, the inhibitory concentration 50% (IC50) of arecoline was around 62.5 μM for 24 hours of treatment in rat liver clone 9 cells, and this dose was lower than the doses used in the different cell types in other studies listed in Table 2. Consequently, liver may be one of the most sensitive organs for toxicity induced by arecoline or BQ chewing. This possibility is supported by a recent report showing that arecoline can cause serious hepatotoxicity in mice,[34] a large population-based study confirmed the independent dose-response relationship of BQ chewing with increasing risk for either hepatocellular carcinoma or liver cirrhosis.[8] Arecoline has been shown that it is rapidly metabolized in mouse liver to form at least 11 metabolites, including ARNO.[20,35] Therefore, BQ chewing leads to preferential damage to the liver owing to the conversion of arecoline to a more toxic metabolite, ARNO is a very real possibility.

Several previous studies have shown that arecoline- or BQ-induced intracellular ROS production plays crucial roles in the toxicity of both arecoline and BQ. Examples include the following: (1) arecoline-mediated inhibition of AMPK (AMP-activated protein kinase) through ROS is required for apoptosis induction,[36] (2) arecoline down-regulates levels of cell-cycle inhibitors (p21 and p27) through the ROS/mTOR complex 1 pathway and may contribute to oral squamous cell carcinoma,[37] (3) elevation of transglutaminase-2 expression mediates fibrosis in BQ-associated oral submucous fibrosis via ROS,[38] and (4) elevated expression of the transcription factor snail mediates tumor progression in BQ -associated oral squamous cell carcinoma via ROS production.[39]

In this study, we found that the typical antioxidant *N*-acetylcysteine (NAC) could completely block ARNO-induced DNA strand breaks in rat liver clone 9 cells (Figure 3). In addition, co-treatment with NAC, Trolox, and penicillamine could prevent ARNO-induced ROS production in the same cell line (Figures 4–6). In fact, similar results have been reported by others.[27,36,40] They show that both arecoline and areca nut extract increase ROS production in various cell lines and this effect can be effectively attenuated by antioxidants, including catalase, NAC, and glutathione. In general, a buildup of ROS in cells may cause damage to DNA, RNA, and proteins, and may cause cell death. Moreover, ROS-induced DNA damage is believed to contribute to carcinogenesis. These results also mean that arecoline- and ARNO-induced intracellular ROS production may initiate BQ carcinogenesis. Although the major sites of ROS production induced by arecoline or their metabolites such as ARNO are still unclear and require further investigation, a previous report revealed that NADPH oxidase-derived ROS may play an important role in arecoline-induced ROS production in cells.[27] Collectively, these findings not only confirm again the central importance of ROS for BQ toxicity but also indicate that enhancement of ROS production by ARNO increase the risks of arecoline and betel nut toxicity.

Although we did not evaluate the role of NADPH oxidase in ARNO-induced ROS production in this study, we found that both FMO and CYP450 inhibitors could completely inhibit ARNO-induced

TABLE 2 Cytotoxic effects of arecoline in different cell types

Cell type	Treating condition	Cytotoxicity assay	Percent cytotoxicity	Reference
Rat liver cell (clone 9)	24 h, 500 μM	Crystal violet staining assay	50%	This study
Human oral mucosal fibroblast	5 days, 400 μM	Crystal violet staining assay	63%	[11]
Human umbilical vein endothelial cell (HUVEC)	24 h, 940 μM	LDH[a] leakage assay	50%	[22]
Human immortalized keratinocyte (HaCaT)	24 h, 500 μM	MTS[b] assay	50%	[23]
Human embryonic lung fibroblast (HEL)	24 h, 529 μM	MTT[c] assay	30%	[24]
Human endothelial cell (EAhy 926)	24 h, 800 μM	MTT assay	31%	[25]
Human leukemia cell (K562)	24 h, 1000 μM	MTT assay	40%	[26]
Rat primary cortical neurons	24 h, 200 μM	MTT assay	27%	[27]
Primary human gingival fibroblasts	24 h, 423 μM	LDH leakage assay	40%	[28]
Primary human oral mucosal cells	24 h, 847 μM	Alamar blue assay	53%	[29]
Pig kidney epithelial cell (LLC-PK1)	24 h, 100-500 μM	MTT assay	No cytotoxicity	[31]
Human periodontal ligament fibroblast (PDLF)	24 h, 254 μM	MTT assay	33%	[32]
Human oral KB carcinoma cell	5 d, 120 μM	MTT assay	75%	[33]

[a] LDH: lactate dehydrogenase.

[b] MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

[c] MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

ROS production in human liver WRL-68 cells (Figure 8). Previous studies have shown that ARNO is formed by a FMO, not by CYP450 in vitro.[20,21] Subsequently, ARNO can be metabolized to at least 13 metabolites in the mouse model.[21] 1-Aminobenzotriazole (ABT), a potent and irreversible inhibitor of CYP450 used in this study significantly suppressed ARNO-induced ROS in WRL-68 cells (Figure 8G). Moreover, FMO inhibitor methimazole (MMI) also exhibited excellently inhibitory effect on ARNO-induced ROS production in WRL-68 cells (Figure 8E) in this study. However, MMI has been reported to exert overlapping inhibitory effect on the activity of multiple CYP450s in human liver microsome.[41] Therefore, according to the inhibitor specificity differences between 1-ABT and MMI, we believed that CYP450s are major ARNO metabolic enzymes than FMO in both human and rat liver cells.

Recently, growing evidence indicates that CYP450s are not only located in the endoplasmic reticulum but also in mitochondria.[42] Furthermore, mitochondria-targeted CYP450s represent an important source of mitochondria ROS and are involved in the metabolism of drugs and carcinogens.[43-45] Interestingly, in this study, we found that mitochondrial-targeted antioxidant Mito-TEMPO as same as CYP450 inhibitor (1-ABT) almost completely inhibited ARNO-induced ROS production in WRL-68 cells (Figure 8C). This finding strongly indicates that the large quantity of the ARNO-induced ROS may be formed directly inside the mitochondria by mitochondria targeted CYP450s. Previous studies show that mitochondria-targeted CYP2E1 and CYP2D6 can play a direct role in xenobiotic metabolism and ROS production in mammalian cells support our assumption that mitochondria-targeted CYP450s-mediated ARNO metabolism and induced to a higher level of ROS production in mitochondria.[44,46] Nevertheless, more investigations need to be done to clarify the role of CYP450 enzymes, especially the mitochondria-targeted CYP450s in ARNO metabolism-mediated toxicity in liver cells.

Another interesting finding in the present study was that copper ions, which may be involved in ARNO-induced ROS production because the addition of penicillamine (a membrane-impermeable copper chelator) could block strongly ARNO-induced ROS production in rat liver clone 9 cells. This finding further indicates that the extracellular copper ion influx may be involved in ARNO-induced ROS production. A recent report provided evidence that the epithelial atrophy in oral submucous fibrosis is mediated by copper (II) and arecoline of betel nut.[47] Furthermore, the copper levels in commercial products are significantly higher than betel nuts and this phenomenon is highly associated with the increasing prevalence of oral submucous fibrosis.[48] These reports indirectly support our finding that the copper-catalyzed Fenton reaction may also contribute to ROS production and the toxicity of ARNO and its parent compound arecoline. Further investigation to discover the role of copper ions in ARNO-induced mitochondrial ROS production by CYP450 in liver cells would be valuable to understand the relationship between the chronic liver disease and BQ chewing.

In conclusion, we have shown that ARNO, a major arecoline oxidation metabolite, exerts higher cytotoxicity, mutagenicity, and clastogenicity than its parent compound arecoline in vitro. The enhancement of toxicity caused by ARNO can be explained by its induction of ROS by mitochondria-targeted CYP450-mediated metabolism. Furthermore, copper ion influx may be associated with ARNO enhancement of ROS production in liver cells. Collectively, the enhanced toxic effects of ARNO, the major metabolite of arecoline should be carefully considered to minimize the health risks of BQ chewing. It may be a more effective way to prevent BQ-associated lesions by supplementation with thiol-containing small molecules for scavenging free radicals and copper ions simultaneously. Nevertheless the best way to avoid the adverse effects of BQ chewing certainly is to quit right now.

ACKNOWLEDGMENT

This study was supported by a grant from the Ministry of Science and Technology, Taiwan, ROC, (MOST 104-2320-B-040-013).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. World Health Organization. *International Agency for Research on Cancer. IARC Monograph on the Evaluation of Carcinogenic Risks to Humans: Betel-Quid and Areca-Nut Chewing and some Areca-Nut-Derived Nitrosamines*. Vol 85. Lyon, France: IARC; 2004.
2. Gupta PC, Ray CS. Epidemiology of betel quid usage. *Ann Acad Med Singapore*. 2004;33:31-36.
3. World Health Organization. *International Agency for Research on Cancer. IARC Monograph on the Evaluation of Carcinogenic Risks to Humans: A Review of Human Carcinogens-Biological Agents*. Vol 100B. Lyon, France: IARC; 2012.
4. Guha N, Warnakulasuriya S, Vlaanderen J, Straif K. Betel quid chewing and the risk of oral and oropharyngeal cancers: a meta-analysis with implications for cancer control. *Int J Cancer*. 2014;135:1433-1443.
5. Gupta B, Johnson NW. Systematic review and meta-analysis of association of smokeless tobacco and of betel quid without tobacco with incidence of oral cancer in South Asia and the Pacific. *PLoS One*. 2014;9:e113385.
6. Jacob BJ, Straif K, Thomas G, et al. Betel quid without tobacco as a risk factor for oral precancers. *Oral Oncol*. 2004;40:697-704.
7. Warnakulasuriya S, Trivedy C, Peters TJ. Areca nut use: an independent risk factor for oral cancer. *BMJ*. 2002;324:799-800.
8. Wu GH, Boucher BJ, Chiu YH, Liao CS, Chen TH. Impact of chewing betel-nut (*Areca catechu*) on liver cirrhosis and hepatocellular carcinoma: a population-based study from an area with a high prevalence of hepatitis B and C infections. *Public Health Nutr*. 2009;12:129-135.
9. Chung YT, Chen CL, Wu CC, Chan SA, Chi CW, Liu TY. Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing. *Toxicol Lett*. 2008;183:21-27.
10. Tsai JF, Jeng JE, Chuang LY, et al. Habitual betel quid chewing and risk for hepatocellular carcinoma complicating cirrhosis. *Medicine (Baltimore)*. 2004;83:176-187.
11. Jeng JE, Tsai HR, Chuang LY, et al. Independent and additive interactive effects among tumor necrosis factor-alpha polymorphisms, substance use habits, and chronic hepatitis B and hepatitis C virus infection on risk for hepatocellular carcinoma. *Medicine (Baltimore)*. 2009;88:349-357.
12. Lin SH, Liao YS, Huang SH, Liao WH. Relationship between betel quid chewing and risks of cardiovascular disease in older adults: a cross-sectional study in Taiwan. *Drug Alcohol Depend*. 2014;141:132-137.
13. Hu CW, Chao MR. Direct-acting DNA alkylating agents present in aqueous extracts of areca nut and its products. *Chem Res Toxicol*. 2012;25:2386-2392.
14. Bhattacharjee C, Sharan RN. Aqueous extract of betel nut-induced adducts on pMTa4 DNA acquires stability in the presence of Na^+ and K^+ ions. *Mol Med Rep*. 2008;1:435-441.
15. Goswami R, Ahmed A. Study of arecoline contents in Assamese areca nut. *Ind J Med Sci*. 1956;10:656-658.
16. Lin KH, Lin CY, Liu CC, Chou MY, Lin JK. Arecoline *N*-oxide: its mutagenicity and possible role as ultimate carcinogen in areca oral carcinogenesis. *J Agric Food Chem*. 2011;59:3420-3428.
17. Nery R. The metabolic interconversion of arecoline and arecoline 1-oxide in the rat. *Biochem J*. 1971;122:503-508.
18. Tu CY, Chen YF, Lii CK, Wang TS. Methylglyoxal induces DNA crosslinks in ECV304 cells via a reactive oxygen species-independent protein carbonylation pathway. *Toxicol In Vitro*. 2013;27:1211-1219.
19. Chen HW, Chien ML, Chaung YH, Lii CK, Wang TS. Extracts from cigarette smoke induce DNA damage and cell adhesion molecule expression through different pathways. *Chem Biol Interact*. 2004;150:233-241.
20. Giri S, Idle JR, Chen C, Zabriskie TM, Krausz KW, Gonzalez FJ. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. *Chem Res Toxicol*. 2006;19:818-827.
21. Giri S, Krausz KW, Idle JR, Gonzalez FJ. The metabolomics of (+/−)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. *Biochem Pharmacol*. 2007;73:561-573.
22. Ullah M, Cox S, Kelly E, Boadle R, Zoellner H. Arecoline is cytotoxic for human endothelial cells. *J Oral Pathol Med*. 2014;43:761-769.
23. Li M, Gao F, Zhou ZS, et al. Arecoline inhibits epithelial cell viability by upregulating the apoptosis pathway: implication for oral submucous fibrosis. *Oncol Rep*. 2014;31:2422-2428.
24. Zhou ZS, Li M, Gao F, et al. Arecoline suppresses HaCaT cell proliferation through cell cycle regulatory molecules. *Oncol Rep*. 2013;29:2438-2444.
25. Tseng SK, Chang MC, Su CY, et al. Arecoline induced cell cycle arrest, apoptosis, and cytotoxicity to human endothelial cells. *Clin Oral Investig*. 2012;16:1267-1273.
26. Chen YJ, Chang LS. Arecoline-induced death of human leukemia K562 cells is associated with surface up-modulation of TNFR2. *J Cell Physiol*. 2012;227:2240-2251.
27. Shih YT, Chen PS, Wu CH, Tseng YT, Wu YC, Lo YC. Arecoline, a major alkaloid of the areca nut, causes neurotoxicity through enhancement of oxidative stress and suppression of the antioxidant protective system. *Free Radic Biol Med*. 2010;49:1471-1479.
28. Chiang SL, Jiang SS, Wang YJ, et al. Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling. *Toxicol Sci*. 2007;100:66-74.
29. Chang YC, Hu CC, Tseng TH, Tai KW, Lii CK, Chou MY. Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *J Oral Pathol Med*. 2001;30:458-464.
30. Jeng JH, Tsai CL, Hahn LJ, Yang PJ, Kuo YS, Kuo MY. Arecoline cytotoxicity on human oral mucosal fibroblasts related to cellular thiol and esterase activities. *Food Chem Toxicol*. 1999;37:751-756.
31. Lin SH, Chiou SJ, Ho WT, Chuang CT, Chuang LY, Guh JY. Arecoline-induced pro-fibrotic proteins in LLC-PK1 cells are dependent on c-Jun N-terminal kinase. *Toxicology*. 2016;344-346:53-60.
32. Chang YC, Lii CK, Tai KW, Chou MY. Adverse effects of arecoline and nicotine on human periodontal ligament fibroblasts in vitro. *J Clin Periodontol*. 2001;28:277-282.
33. Chang MC, Ho YS, Lee PH, et al. Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. *Carcinogenesis*. 2001;22:1527-1535.
34. Zhou J, Sun Q, Yang Z, Zhang J. The hepatotoxicity and testicular toxicity induced by arecoline in mice and protective effects of vitamins C and e. *Korean J Physiol Pharmacol*. 2014;18:143-148.
35. Patterson TA, Kosh JW. Elucidation of the rapid in vivo metabolism of arecoline. *Gen Pharmacol*. 1993;24:641-647.
36. Yen CY, Lin MH, Liu SY, et al. Arecoline-mediated inhibition of AMP-activated protein kinase through reactive oxygen species is required for apoptosis induction. *Oral Oncol*. 2011;47:345-351.
37. Ji WT, Yang SR, Chen JY, et al. Arecoline downregulates levels of p21 and p27 through the reactive oxygen species/mTOR complex 1 pathway and may contribute to oral squamous cell carcinoma. *Cancer Sci*. 2012;103:1221-1229.
38. Lee SS, Chen YJ, Tsai CH, Huang FM, Chang YC. Elevated transglutaminase-2 expression mediates fibrosis in areca quid chewing-associated oral submucocal fibrosis via reactive oxygen species generation. *Clin Oral Investig*. 2016;20:1029-1034.
39. Lee SS, Tsai CH, Yu CC, Chang YC. Elevated snail expression mediates tumor progression in areca quid chewing-associated oral squamous cell carcinoma via reactive oxygen species. *PLoS One*. 2013;8:e67985.
40. Chen IC, Chiang WF, Chen PF, Chiang HC. Stress-responsive deacetylase SIRT3 is up-regulated by areca nut extract-induced oxidative stress in human oral keratinocytes. *J Cell Biochem*. 2014;115:328-339.
41. Guo Z, Raeissi S, White RB, Stevens JC. Orphenadrine and methimazole inhibit multiple cytochrome P450 enzymes in human liver microsomes. *Drug Metab Dispos*. 1997;25:390-393.
42. Ahn T, Yun CH. Molecular mechanisms regulating the mitochondrial targeting of microsomal cytochrome P450 enzymes. *Curr Drug Metab*. 2010;11:830-838.
43. Bansal S, Leu AN, Gonzalez FJ, et al. Mitochondrial targeting of cytochrome P450(CYP) 1B1 and its role in polycyclic aromatic

hydrocarbon-induced mitochondrial dysfunction. *J Biol Chem*. 2014; 289:9936-9951.

44. Bansal S, Liu CP, Sepuri NB, et al. Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *J Biol Chem*. 2010;285:24609-24619.
45. Bajpai P, Sangar MC, Singh S, et al. Metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by mitochondrion-targeted cytochrome P450 2D6: implications in Parkinson disease. *J Biol Chem*. 2013; 288:4436-4451.
46. Sangar MC, Anandatheerthavarada HK, Tang W, et al. Human liver mitochondrial cytochrome P4502D6--individual variations and implications in drug metabolism. *FEBS J*. 2009;276:3440-3453.
47. Khan I, Pant I, Narra S, et al. Epithelial atrophy in oral submucous fibrosis is mediated by copper (II) and arecoline of areca nut. *J Cell Mol Med*. 2015;19:2397-2412.
48. Mathew P, Austin RD, Varghese SS, Manojkumar AD. Estimation and comparison of copper content in raw areca nuts and commercial areca nut products: implications in increasing prevalence of oral submucous fibrosis (OSMF). *J Clin Diagn Res*. 2014;8:247-249.

How to cite this article: Wang T-S, Lin C-P, Chen Y-P, Chao M-R, Li C-C, Liu K-L. CYP450-mediated mitochondrial ROS production involved in arecoline *N*-oxide-induced oxidative damage in liver cell lines. *Environmental Toxicology*. 2018; 33:1029–1038. https://doi.org/10.1002/tox.22588