科技部補助專題研究計畫成果報告
期末報告

檳榔鹼及其氧化物之代謝與毒性作用機制探討

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 104-2320-B-040-013-
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人 ： 王祖興

計畫參與人員： 博士班研究生-兼任助理人員：李錦裳

中 華 民 國 105 年 10 月 31 日

中 文 摘 要 ： 國際癌症研究機構已將檳榔歸類為人類環境致癌物。以前的研究表明，檳榔鹼是存在於嚼食檳榔者唾液中主要的生物鹼。多年來，研究證據證實，唾液中含有大量檳榔鹼將導致口腔粘膜細胞突變，引發口腔癌。檳榔鹼也誘導肝臟毒性並抑制抗氧化系統。檳榔在胃腸道惡性腫瘤，包括肝癌誘發的證據也都在積累當中，然而，檳榔鹼在正常肝細胞中誘導的損傷的機制仍不清楚。因此，本研究的目的是確定檳榔鹼及其氧化代謝物(檳榔鹼N-氧化物/arecoline N-oxide)在正常大鼠肝臟clone9細胞中的細胞毒性、基因毒性及致突變效應。藉由結晶紫染色、鹼性彗星分析及傷寒沙門氏菌致突變分析，分別檢測細胞毒性、基因毒性和致突變效應。試驗結果證實，在大鼠肝臟clone9細胞中，檳榔鹼N-氧化物比其原始化合物檳榔鹼呈現更強烈的細胞毒性、DNA損傷及致突變性。此外，檳榔鹼N-氧化物在斑馬魚胚胎也比檳榔鹼誘發更強烈的毒性。添加抗氧化劑，如N-乙酰半胱氨酸(N-acetylcysteine)、水溶性維生素E(trolox)和青黴胺(d-penicilamine)，均顯著保護大鼠肝臟clone9細胞免於及所誘導的DNA損傷和活性氧(reactive oxygen species/ROS)產生，這些結果指出，從咀嚼檳榔習慣攝取的檳榔鹼，可被生物體氧化代謝形成，從而增強活性氧產生並導致肝損傷。進一步利用人正常肝細胞WRL68，以及多種藥物代謝重要酵素抑制劑，包含細胞色素P450（cytochrome P450）抑制劑(1-aminobenzotriazole)、含黃素單氧化酶(flavin-containing monooxygenase)抑制劑(methimazole)及單胺氧化酶(monoamine oxidase)抑制劑(selegiline、clorgyline、pargyline)，探討代謝酵素在檳榔鹼N-氧化物誘發ROS增加形成的角色，結果發現細胞色素P450抑制劑及含黃素單氧化酶抑制劑均明顯降低檳榔鹼N-氧化物在人正常肝細胞WRL68所造成之ROS產量增加，但多種單胺氧化酶抑制劑則無此抑制現象。另外發現粒線體靶向抗氧化劑(mitoTEMPO)，也可大幅度降低檳榔鹼N-氧化物在人類正常肝細胞WRL68所誘發的ROS增加形成。綜合上述結果，我們得到下列結論：(1)檳榔鹼在生體中的氧化物(檳榔鹼N-氧化物)，比原始化合物檳榔鹼更具毒性。(2)檳榔鹼N-氧化物在肝細胞中增加ROS產生量可能與其毒性增加有密切相關。(3)檳榔鹼N-氧化物除造成肝細胞毒性外，也造成更為強烈地斑馬魚胚胎的致死率。(4)檳榔鹼N-氧化物在肝細胞造成之ROS產生量增加，需透過胞色素P450及含黃素單氧化酶居間促成。(5)檳榔鹼N-氧化物在肝細胞造成之ROS產生量增加必須依賴粒腺體。

中 文 關 鍵 詞 ： 檳榔鹼; 檳榔鹼N-氧化物; 大鼠肝臟clone 9細胞; 人類肝臟WRL 68細胞; DNA鏈斷裂; ROS; 細胞色素P450; 含黃素單加氧酶; 粒線體

英 文 摘 要 ： The IARC has classified the areca nut as a human environmental carcinogen. Previous studies have shown that arecoline is the major alkaloid present in the saliva of areca nut chewers. Evidences over the years have shown that saliva containing a large content of arecoline leads to mutation of oral mucosa cells, resulting in oral cancer. Arecoline also induces liver toxicity and depresses the

antioxidant system. Evidence is also accumulating implicating the areca nut in the development of other gastrointestinal malignancies, including liver carcinoma. However, the mechanism responsible for arecoline-induced damage in normal liver cells remains uncharacterized. Therefore, the purpose of this study was to determine the cytotoxic, genotoxic, and mutagenic effects of arecoline and its oxidative metabolite, arecoline N-oxide (ARNO), in clone 9 cells. The cytotoxic, genotoxic, and mutagenic effects were detected by crystal violet staining, alkaline comet assay, and Salmonella typhimurium mutagenicity test, respectively. The results showed that ARNO exerted higher cytotoxicity, DNA damage, and mutagenicity than its parent compound arecoline in clone 9 cells. Furthermore, ARNO induced higher toxicity to zebrafish embryos than arecoline. The addition of antioxidants, such as N-acetylcysteine, trolox, and penicillamine, significantly protected clone 9 cells from ARNO-induced DNA damage and ROS production. To explore further the role of metabolic enzymes in ARNO-induced ROS production enhancement, we used human normal liver cells WRL68, as well as various types of metabolic enzyme inhibitors, including CYP, FMO and MAO inhibitors. The results showed that both CYP and FMO inhibitors were significantly reduced of ARNO-induced ROS production in WRL68 cells, but the similar phenomenon could not be observed for a variety of monoamine oxidase inhibitors. In addition, ARNO-induced ROS production was significantly reduced by mitoTEMPO in WRL68 cells. Collectively, we can make the following conclusions: (1)ARNO is more toxic than its original compound, arecoline. (2)The enhancement of ROS production in rat liver clone 9 cells by ARNO is closely related to its high toxicity. (3) ARNO also shows significantly higher zebrafish embryonic lethality. (4)ARNO-induced ROS production is mediated by CYP & FMO in WRL68 cells. (5)ARNO-induced ROS in WRL68 cells is via a mitochondria-dependent pathway.

英文關鍵詞：arecoline; arecoline N-oxide; rat liver clone 9 cells; WRL68 cells; DNA strand break, ROS, cytochrome P450, flavin-containing monooxygenase, mitochondria

1. Introduction

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 (IARC, 2012; Gupta & Ray, 2004). The areca nut in BQ has been recognized as a Group I carcinogen to humans by the International Agency for Research on Cancer (IARC, 2004) of the World Health Organization. Case-control studies from India, Pakistan, and Taiwan have reported that BQ use, specifically without tobacco, is a risk factor for oral cancer (Jacob et al., 2004; Warnakulasuriya et al., 2002). Besides oral cancer, BQ chewing is an independent risk factor for cirrhotic hepatocellular carcinoma (Wu et al., 2009; Chung et al., 2008; Tsai et al., 2004). There is an additive interaction between BQ chewing and chronic hepatitis B and/or hepatitis C virus infection (Jeng et al., 2009). 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 (Lin et al., 2014).

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 (Hu & Chao, 2012; Bhattacharjee & Sharan, 2004). 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 (Goswami & Ahmed, 1956). Furthermore, a recent report indicated that arecoline *N*-oxide, 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) (Lin et al., 2011). 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 arecoline *N*-oxide in rat liver clone 9 cells and zebrafish larvae.

2. Materials and methods

2.1. Chemicals

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

2.2. Arecoline *N*-oxide preparation

Arecoline *N*-oxides were prepared according to the published method (Nery et al., 1971) with little modification. A solution of arecoline (2.44 g) in ether (10 mL) was stirred in a water-ice bath and treated with aq. 33% (w/v) peroxyacetic acid (4.56 g) dropwise for 30 min. 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 three 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. Clone 9 cell culture

Rat liver clone 9 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum containing 100 μg/mL penicillin-streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

2.4. Cytotoxicity assay

Cytotoxicity was determined by using the crystal violet staining assay, as in our previous study (Tu et al., 2013). Briefly, cells ($2x10^4$ 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, CA) 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 arecoline *N*-oxide (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 by using the alkaline comet assay as previously described (Chen et al., 2004). Damage was quantified as tail moment of at least 50 cells per sample by 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 pre-cultured overnight. On the next day, the cells were pre-incubated 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 arecoline *N*-oxide (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 by using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). The fluorescence of intracellular DCF was also observed and photographed by fluorescence microscopy (Nikon E400, Tokyo, Japan) coupled with a digital camera.

2.8. Zebrafish embryonic toxicity test

Fertilized embryos were obtained from adult AB strain zebrafish (*Danio rerio*) bred and maintained in our laboratory following standard conditions. Embryos at the same developmental stage (3 hours post fertilization) were collected and rinsed in embryo water. Embryos were examined under a dissecting microscope, and those that had developed normally and had reached the blastula stage were selected for the subsequent exposure experiments. Thirty fertilized eggs were transferred to a new 6-cm dish containing 2 mL solution with and without different concentrations of arecoline or arecoline *N*-oxides (31.25-500 μM) for 24 hours. Three replicates were run for each concentration.

The viability of embryos after treatment is based on the transparency of the embryo. When cells of the embryo lose their ability to regulate membrane permeability, the embryo becomes opaque. Thus, opaque embryos were considered dead. Survival was calculated as the percentage of viable embryos of the total number of embryos for each treatment group.

2.9. Liquid chromatography–mass spectrometry analysis

Fifteen milliliters of each suspension was collected and then injected into an HLB SPE column for cleanup. The column was first eluted with 2 mL of methanol three times. Next, the combined eluents were concentrated by 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% (v/v) 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/min; *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.

2.10. Adult zebrafish swimming behavior analysis

After 58 days of arecoline or arecoline N-oxide exposure, single zebrafish was placed in fresh media solution and transferred to a 0.5-liter glass beaker. Locomotor activity was recorded during 5 min either by a top-view or side-view camera, and analyzed by video tracking software. Total distance (cm) and time spent in upper half or lower half was measured as described by Capiotti et al., 2013.

2.11. Statistical analysis

Results are expressed as means ± SEs from at least three independent experiments. Data were analyzed by using one-way analysis of variance (SAS Institute, Cary, NC, USA). The significance of the difference among group means was determined by Student's *t*-tests; P values < 0.05 were taken to be statistically significant.

3. Results

3.1. Arecoline- and arecoline *N*-oxide-induced cytotoxicity

The cytotoxic effects of arecoline and arecoline *N*-oxide were evaluated in rat liver clone 9 cells by crystal violet staining. Treatment with either arecoline or arecoline *N*-oxide for 24 hours resulted in cell death in a dose-dependent manner (Figure 1). The cytotoxicity of arecoline *N*-oxide was eight times that of arecoline. The IC_{50} values of arecoline and arecoline *N*-oxide were 500 μM and 62.5 μM, respectively.

3.2. Arecoline- and arecoline *N*-oxide-induced DNA damage

The potential of arecoline and arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide-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 arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide 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 arecoline *N*-oxide, 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 arecoline *N*-oxide-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 arecoline *N*-oxide-induced ROS production in clone 9 cells (Figures 5 and 6, respectively). Collectively, these results not only confirm that ROS production plays an important role in arecoline *N*-oxide-induced DNA damage but also indicate that the copper-ion-catalyzed reaction is also involved in arecoline *N*-oxide-induced ROS production.

3.3. Arecoline-induced zebrafish embryo toxicity

Survival rates for zebrafish embryos treated with arecoline- and arecoline *N*-oxide for 24 hours are shown in Figure 7. As shown in Figure 7A, arecoline treatment for 24 hours at a concentration range of 31.25 μM to 250 μM did not cause

death of zebrafish embryos. However, in the same dose range, arecoline *N*-oxide treatment for 24 hours caused significant death of zebrafish embryos. The dosage of arecoline and arecoline *N*-oxide at which 50% of the zebrafish embryos died was more than 500 μM and 125 μM, respectively (Figure 7B). When zebrafish embryos were treated with 500 μM arecoline *N*-oxide, all embryos died 3 hours after exposure, whereas 500 μM arecoline did not cause significant embryo death even after 24 hours of exposure (data not shown). These results revealed that oxidation of arecoline to arecoline *N*-oxide increased intracellular ROS production and cytotoxic and embryotoxic potencies.

3.4. 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 8 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 arecoline *N*-oxide and the dimer of arecoline *N*-oxide, 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 (Giri et al., 2006 and 2007). Our findings indicated that the arecoline *N*-oxide 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.

3.5. Effect of arecoline N-oxide on adult zebrafish swimming behavior

Photographs of adult zebrafish chronically exposed to drugs and drug-free control

in a 2-liter glass beaker were shown in figure 8. The nose-up angle between the longitudinal body axis of zebrafish and horizontal line was increased by both arecoline and arecoline N-oxide treatment for 58 days. The tilt angle of zebrafish treated with 50 μM arecoline, 25 μM arecoline N-oxide, 50 μM arecoline was 10.7°, 21.3° and 27.7°, respectively compared to the control group (6.8°)

The total distance travelled was significantly decrease from 23.9 m to 9.4 m and 12.2 m in zebrafish exposed to 50 μM and 100 μM arecoline, respectively (Figure 9). In comparison with the arecoline, a marked decrease in total distance traveled by zebrafish was also observed after treatment with arecoline N-oxide. The value of total distance travelled by zebrafish was decreased to 3.3 m and 3.9 m in the group of 25 μM and 100 μM arecoline N-oxide, respectively (Figure 9). Besides the reduction of total travel distance, zebrafishs exposed to arecoline and arecoline N-oxide in general showed a significantly decrease in the time spent in the upper portion of the test tank when compared with the control group (89 s). In addition, zebrafishs treated with arecoline and arecoline N-oxide we observed as significant increase in the time spent in the lower zone of the test beaker when compared with the control group (150 s) (Figure 10). These results indicate that increase in time spent in the lower zone may also account for the reduced zebrafish locomotor activity by arecoline and arecoline N-oxide. Interestingly, in the group of 50 μM arecoline N-oxide had greatly increased the total distance travelled compared to control zebrafish. In addition to increase the total distance travelled, 50 μM arecoline N-oxide treated zebrafish spent significantly more time in the upper zone of the test beaker. These findings indicate that both arecoline and arecoline N-oxide can cause either a decrease in locomotor activity or induced anxiolytic-like behaviors and increased locomotor activity depending on the treatment concentration in adult zebrafish.

3.6. Effect of drug metabolizing enzyme and arecoline-N-oxide-induced ROS production

In this study, we have shown that arecoline N-oxide, a major metabolite of arecoline, is more toxic than the parent compound, including intracellular ROS production. In order to further investigate whether drug metabolic enzymes are involved in the enhancement production of ROS by arecoline N-oxide, we examined the inhibition action of drug metabolic enzymes on arecoline N-oxide-induced ROS production in human hepatic cell line WRL-68, including cytochrome P450, flavin-containing monooxygenase, and monoamine oxidase inhibitor. The results were shown in figure X. Arecolin N-oxide induced ROS production in WRL68 cells was strongly blocked by addition of cytochrome P450 inhibitor (1-aminobenzotriazole) and flavin-containing monooxygenase inhibitor (methimazole). On the contrary, the monoamine oxidase inhibitors (pargyline, selegiline, and clorgyline) did not sifnificantly affect on arecoline N-oxide-induced ROS production in WRL68 cells. Furthermore, treatment with mitochondria-targeted antioxidant mitoTEMPO was also strongly inhibited arecoline N-oxide-induced ROS production in WRL68 cells (Figure 11). These results suggest that arecoline N-oxide-induced ROS production may be via a CYP/FMO-mediated metabolism and mitochondria-dependent pathway in human liver cell line WRL68.

4. Discussion

4.1. Arecoline cytotoxicity

Our present findings showed that oxidation of arecoline highly enhanced its cytotoxicity and mutagenicity in clone 9 liver cells. 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 (Ullah et al., 2014; Li et al., 2014; Zhou et al., 2013; Tseng et al., 2012; Chen and Chang, 2012; Shih et al., 2010; Chiang et al., 2007; Chang et al., 2001b; Jeng et al., 1999). However, sensitivity to arecoline varies depending on cell type. Comparative studies of the cytotoxic effect of arecoline have shown that arecoline is much less cytotoxic in two different human fibroblast cell lines HGF-1 and Hel than in human umbilical vein endothelial cells and human keratinocyte cells (Li et al., 2014; Chiang et al., 2007). 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 the other studies listed in Table 2. Thus, the liver may be one of the most sensitive organs to arecoline and BQ exposure. This possibility is supported by a recent report showing that arecoline exposure can cause serious hepatotoxicity in mice (Zhou et al., 2014). Furthermore, a large population-based study confirmed the independent dose-response relationship of betel chewing with increasing risk for either hepatocellular carcinoma or liver cirrhosis (Wu et al., 2009). Arecoline has been shown to be rapidly metabolized in mouse liver and to form at least 11 metabolites, including arecoline *N*-oxide (Giri et al., 2006; Patterson & Kosh, 1993). Thus, one possibility is that BQ chewing leads to preferential damage to the liver owing to the conversion of arecoline to a more toxic

metabolite, arecoline *N*-oxide.

4.2. Arecoline ROS production and DNA damage

The detailed mechanisms by which arecoline or BQ causes several kinds of cancer, metabolic disease, cardiovascular disease, and type 2 diabetes remain unclear. Several previous studies have shown that arecoline- or BQ-induced intracellular ROS production play 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 (Yen et al., 2011), (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 (Ji et al., 2012), (3) elevation of transglutaminase-2 expression mediates fibrosis in BQ chewing-associated oral submucous fibrosis via ROS (Lee et al., 2015), and (4) elevated expression of the transcription factor snail mediates tumor progression in BQ chewing-associated oral squamous cell carcinoma via ROS production (Lee et al., 2013).

In the present study, we found that the typical antioxidant NAC could potently block arecoline *N*-oxide-induced DNA strand breaks in rat liver clone 9 cells (Figure 3). In addition, co-treatment with NAC, Trolox, and penicillamine could prevent arecoline *N*-oxide-induced ROS production these cells (Figures 4-6). In fact, several other previous studies have also shown that areca nut extract and arecoline increase ROS production in various cell lines and that this effect can be effectively attenuated by antioxidants, such as catalase, NAC, and glutathione (Chen et al., 2014; Yen et al., 2011; Shih et al., 2010). In general, the harmful effects of ROS on the cell are often found as DNA damage, lipid peroxidation, and protein oxidation. ROS-induced DNA damage is believed to contribute to carcinogenesis. These results also mean that

arecoline- and arecoline *N*-oxide-induced ROS production may initiate BQ carcinogenesis. Although the major sites of ROS production induced by arecoline or their metabolites are still unclear and require further investigation, a previous report revealed that NADPH oxidase-derived ROS may play an important role in arecoline- and arecoline-*N*-oxide-stimulated ROS production in cells (Shih et al., 2010). Collectively, these findings not only confirm again the central importance of ROS for BQ toxicity but also indicate that enhancement of ROS production by arecoline *N*-oxide increases the risk of arecoline toxicity.

Another interesting finding in the present study was that copper ions may be involved in arecoline *N*-oxide-induced ROS production because the addition of penicillamine (a membrane-impermeable copper chelator) could strongly block arecoline *N*-oxide-induced ROS production in rat liver clone 9 cells. This finding further indicates that extracellular copper ion influx may be involved in arecoline *N*-oxide-induced ROS production. A recent report provided evidence that the epithelial atrophy in oral submucous fibrosis is mediated by copper(II) and arecoline of areca nut (Khan et al., 2015). Furthermore, the copper levels in commercial products are significantly higher than raw areca nuts and this phenomenon is highly associated with the increasing prevalence of oral submucous fibrosis (Mathew et al., 2014). These reports indirectly support our view that the copper-catalyzed Fenton reaction may contribute to ROS production and the toxicity of arecoline and its metabolite arecoline *N*-oxide. However, further investigations are needed to fully understand the role of copper ions in arecoline *N*-oxide-induced ROS production and toxicity.

4.3. Arecoline metabolism

We showed that arecoline *N*-oxide was the main oxidation product induced by in

vitro oxidation with peracetic acid. Besides in vitro chemical oxidation, arecoline can also be efficiently formed by both flavin-containing monooxygenase-catalyzed oxidation (Giri et al., 2006) and in vivo metabolic processes in rat (Nery, 1971). Eleven metabolites of arecoline have been identified. They are arecaidine, arecoline *N*-oxide, arecaidine *N*-oxide, *N*-methylnipecotic acid, *N*-methylnipecotylglycine, arecaidinylglycine, arecaidinylglycerol, arecaidine mercapturic acid, arecoline mercapturic acid, and arecoline *N*-oxide mercapturic acid, together with nine unidentified metabolites (Giri et al., 2006). The urinary excretion of arecoline-derived metabolites within 12 hours after arecoline treatment in rat comprises arecoline *N*-oxide (7.4-19.1%), *N*-methylnipecotic acid (13.5-30.3%), arecadine (7.1-13.1%), and unchanged arecoline (0.3-0.4%) (Giri et al., 2006). Although the related contributions of each of these metabolites to toxicity remain largely unknown, the findings of this study suggest that the enzyme-mediated oxidative metabolism of arecoline into arecoline *N*-oxide is particularly notable for its role in toxicity and carcinogenesis. This possibility is supported by the evidence that increased risks of cirrhosis and hepatocellular cancer are found in BQ chewers free of hepatitis B/C infection (Wu et al., 2009).

4.4. Arecoline embryo toxicity

We also showed that arecoline within the dose range of from 31.25 to 250 μM did not cause significant acute toxicity in zebrafish embryos when treated for 24 hours. However, its dominant oxidized product in vitro, arecoline *N*-oxide, significantly enhanced the toxic effect in zebrafish embryos compared with the parent compound arecoline. The mechanism for the enhanced toxic effect of arecoline *N*-oxide in zebrafish embryos is still unclear. However, we believe that increased ROS production may play a significant role. To our understanding, only a few studies have

shown that arecoline can induce embryo/developmental toxicity in zebrafish embryos (Peng et al., 2015; Chang et al., 2004), but the doses in general were higher than what we used in this study. These reports also pointed out that oxidative stress resulting from protein thiol depletion and mitochondrial damage plays an important role in arecoline-induced developmental toxicity in zebrafish embryos. These findings provide a possible explanation that arecoline *N*-oxide indirectly promotes mitochondrial damage and/or protein thiol depletion to increase intracellular ROS production and to enhance high embryotoxicity. Nevertheless, further investigation will be conducted to confirm the differential ability to induce mitochondrial dysfunction and protein oxidation between arecoline *N*-oxide and its parent compound arecoline.

4.5. Conclusions

In conclusion, we have shown that arecoline *N*-oxide, a major arecoline oxidation metabolite, exerts higher cytotoxicity, mutagenicity, and clastogenicity than its parent compound arecoline in rat liver clone 9 cells. The higher toxicity of arecoline *N*-oxide can be simply explained by its induction of ROS. Furthermore, arecoline *N*-oxide-induced oxidative damage may also simultaneously induce an influx of extracellular copper ion and in turn enhance ROS production and toxicity. In fact, a higher toxicity of arecoline *N*-oxide occurs not only in rat liver clone 9 cells but also in zebrafish embryos. This suggests that the high toxicity of arecoline *N*-oxide is likely universal. Collectively, the toxic effects of the metabolite arecoline *N*-oxide in vivo should be carefully considered to minimize the health risks of BQ chewing. It may be possible to prevent BQ-associated lesions by supplementation with thiol-containing small molecules for simultaneous free radical scavenging and copper chelation. Nevertheless, further studies will be required to determine whether

thiol-based biofunctional antioxidants can reduce BQ-induced oral submucous fibrosis and oral cancer in either animal models or humans. The best way to avoid the adverse effects of BQ certainly is to stop chewing BQ now.

Acknowledgement

This study was supported by a grant from the National Science Council, ROC (MOST 104-2320-B-040-013).

References

Bhattacharjee, C., Sharan, R.N., 2008. Aqueous extract of betel nut-induced adducts on pMTa4 DNA acquires stability in the presence of Na+ and K+ ions. Mol. Med. Rep. 1(3), 435-441.

Chang, B.E., Liao, M.H., Kuo, M.Y., Chen, C.H., 2004. Developmental toxicity of arecoline, the major alkaloid in betel nuts, in zebrafish embryos. Birth Defects Res. Part-A Clin. Mol. Teratol., 70(1), 28-36. http://dx.doi.org/10.1002/bdra.10136

Chang, M.C., Ho, Y.S., Lee, P.H., Chan, C.P., Lee, J.J., Hahn, L.J., Wang, Y.J., Jeng, J.H., 2001a. 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 22(9), 1527-1535

Chang, Y.C., Hu, C.C., Tseng, T.H, Tai, K.W., Lii, C.K., Chou, M.Y., 2001b. Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. J Oral Pathol Med. 30(8):458-64. http://dx.doi.org/10.1034/j.1600-0714.2001.030008458.x

Chang, Y.C., Lii, C.K., Tai, K.W., Chou, M.Y., 2001c. Adverse effects of arecoline and nicotine on human periodontal ligament fibroblasts in vitro. J. Clin. Periodontol. 28(3), 277-282.

Chen, H.W., Chien, M.L., Chaung, Y.H., Lii, C.K., Wang, T.S., 2004. Extracts from cigarette smoke induce DNA damage and cell adhesion molecule expression through different pathways. Chem. Biol. Interact. 150(3), 233-241. http://dx.doi.org/10.1016/j.cbi.2004.09.014

Chen, I.C., Chiang, W.F., Chen, P.F., Chiang, H.C., 2014. STRESS-responsive deacetylase SIRT3 is up-regulated by areca nut extract-induced oxidative stress in human oral keratinocytes. J. Cell. Biochem. 115(2), 328-339. http://dx.doi.org/10.1002/jcb.24667

Chen, Y.J., Chang, L.S., 2012. Arecoline-induced death of human leukemia K562 cells is associated with surface up-modulation of TNFR2. J. Cell. Physiol. 227(5), 2240-2251. http://dx.doi.org/10.1002/jcp.22963

Chiang, S.L., Jiang, S.S., Wang, Y.J., Chiang, H.C., Chen, P.H., Tu, H.P., Ho, K.Y., Tsai, Y.S., Chang, I.S., Ko, Y.C., 2007. Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling. Toxicol. Sci. 100(1), 66-74. http://dx.doi.org/10.1093/toxsci/kfm201

Chung, Y.T., Chen, C.L., Wu, C.C., Chan, S.A., Chi, C.W., Liu, T.Y.,2008. Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid

chewing. Toxicol. Lett. 183(1-3), 21-27. http://dx.doi.org/10.1016/j.toxlet.2008.09.013

García-Algar, O., Vall, O., Alameda, F., Puig, C., Pellegrini, M., Pacifici, R., Pichini, S., 2005. Prenatal exposure to arecoline (areca nut alkaloid) and birth outcomes. Arch. Dis. Child Fetal Neonatal Ed., 90(3), F276-277. http://dx.doi.org/10.1136/adc.2004.061325

Giri, S., Idle, J.R., Chen, C., Zabriskie, T.M., Krausz, K.W., Gonzalez, F.J., 2006. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. Chem. Res. Toxicol. 19(6), 818-827. http://dx.doi.org/10.1021/tx0600402

Giri, S., Krausz, K.W., Idle, J.R., Gonzalez, F.J., 2007. The metabolomics of (+/-)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. Biochem. Pharmacol. 73(4), 561-573. http://dx.doi.org/10.1016/j.bcp.2006.10.017

Goswami, R., Ahmed, A., 1956. Study of arecoline contents in Assamese areca nut. Ind. J. Med. Sci. 10, 656-658.

Gupta, P.C., Ray, C.S., 2004. Epidemiology of betel quid usage. Ann. Acad. Med. Singapore 33(4 Suppl), 31-36.

Hu, C.W., Chao, M.R., 2012. Direct-acting DNA alkylating agents present in aqueous extracts of areca nut and its products. Chem. Res. Toxicol. 25(11), 2386-2392. http://dx.doi.org/10.1021/tx300252r

IARC (International Agency for research on Cancer), 2004. Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. IARC monographs on the evaluation of carcinogenic risks to humans. Vol. 85. Lyon, France.

IARC (International Agency for research on Cancer), 2012. A review of human carcinogens-Biological agents. Vol. 100B, Lyon, France.

Jacob, B.J., Straif, K., Thomas, G., Ramadas, K., Mathew, B., Zhang, Z.F., Sankaranarayanan, R., Hashibe, M., 2004. Betel quid without tobacco as a risk factor for oral precancers. Oral Oncol. 40(7), 697-704. http://dx.doi.org/10.1016/j.oraloncology.2004.01.005

Jeng, J.E., Tsai, H.R., Chuang, L.Y., Tsai, J.F., Lin, Z.Y., Hsieh, M.Y., Chen, S.C., Chuang, W.L., Wang, L.Y., Yu, M.L., Dai, C.Y., Chang, J.G., 2009. 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) 88(6), 349-357. http://dx.doi.org/10.1097/MD.0b013e3181c10477

Jeng, J.H., Tsai, C.L., Hahn, L.J., Yang, P.J., Kuo, Y.S., Kuo, M.Y., 1999. Arecoline cytotoxicity on human oral mucosal fibroblasts related to cellular thiol and esterase

activities. Food. Chem. Toxicol. 37(7), 751-756. http://dx.doi.org/10.1016/S0278-6915(99)00050-2

Ji, W.T., Yang, S.R., Chen, J.Y., Cheng, Y.P., Lee, Y.R., Chiang, M.K., Chen, H.R., 2012. 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. 103(7), 1221-1229. http://dx.doi.org/10.1111/j.1349-7006.2012.02294.x

Khan, I., Pant, I., Narra, S., Radhesh, R., Ranganathan, K., Rao, S.G., Kondaiah, P., 2015. Epithelial atrophy in oral submucous fibrosis is mediated by copper (II) and arecoline of areca nut. J. Cell. Mol. Med. *19*(10), 2397–2412. http://doi.org/10.1111/jcmm.12622.

Lee, S.S., Chen, Y.J., Tsai, C.H., Huang, F.M., Chang, Y.C., 2015. Elevated transglutaminase-2 expression mediates fibrosis in areca quid chewing-associated oral submucocal fibrosis via reactive oxygen species generation. Clin. Oral Investig. http://doi.org/10.1007/s00784-015-1579-0.

Lee, S.S., Tsai, C.H., Yu, C.C., Chang, Y.C., 2013. Elevated snail expression mediates tumor progression in areca quid chewing-associated oral squamous cell carcinoma via reactive oxygen species. PLoS One. 8(7), e67985. http://dx.doi.org/10.1371/journal.pone.0067985

Li, M., Gao, F., Zhou, Z.S., Zhang, H.M., Zhang, R., Wu, Y.F., Bai, M.H., Li, J.J., Lin, S.R., Peng, J.Y., 2014. Arecoline inhibits epithelial cell viability by upregulating the apoptosis pathway: implication for oral submucous fibrosis. Oncol. Rep. 31(5), 2422-2428. doi: 10.3892/or.2014.3091

Lin, K.H., Lin, C.Y., Liu, C.C., Chou, M.Y., Lin, J.K., 2011. Arecoline *N*-oxide: its mutagenicity and possible role as ultimate carcinogen in areca oral carcinogenesis. J. Agric. Food Chem. 59(7), 3420-3428. http://dx.doi.org/10.1021/jf104831n

Lin, S.H., Liao, Y.S., Huang, S.H., Liao, W.H., 2014. Relationship between betel quid chewing and risks of cardiovascular disease in older adults: a cross-sectional study in Taiwan. Drug Alcohol Depend. 141, 132-137. http://dx.doi.org/10.1016/j.drugalcdep.2014.05.020

Lin, S.H., Chiou, S.J., Ho, W.T., Chuang, C.T., Chuang, L.Y., Guh, J.Y., 2016. Arecoline-induced pro-fibrotic proteins in LLC-PK1 cells are dependent on c-Jun N-terminal kinase. Toxicology 344-346, 53-60.

Mathew, P., Austin, R.D., Varghese, S.S., Manojkumar, A.D., 2014. 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. 8(1), 247-249. doi: 10.7860/JCDR/2014/8042.3932

Nery, R., 1971. The metabolic interconversion of arecoline and arecoline 1-oxide in

the rat. Biochem. J. 122(4), 503-508. http://dx.doi.org/10.1042/bj1220503

Patterson, T.A., Kosh, J.W., 1993. Elucidation of the rapid in vivo metabolism of arecoline. Gen. Pharmacol. 24(3), 641-647. http://dx.doi.org/10.1016/0306-3623(93)90224-L

Paul, K., Moitra, P.K., Mukherjee, I., Maity, C., Ghosal, S.K., 1999. Teratogenicity of arecoline hydrobromide on developing chick embryos: a preliminary report. Bull. Environ. Contam. Toxicol. 62(3), 356-362. http://dx.doi.org/10.1007/s001289900882

Peng, W.H., Lee, Y.C., Chau, Y.P., Lu, K.S., Kung, H.N., 2015. Short-term exposure of zebrafish embryos to arecoline leads to retarded growth, motor impairment, and somite muscle fiber changes. Zebrafish 12(1), 58-70. http://dx.doi.org/10.1089/zeb.2014.1010

Shih, Y.T., Chen, P.S., Wu, C.H., Tseng, Y.T., Wu, Y.C., Lo, Y.C., 2010. 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. 49(10), 1471-1479. http://dx.doi.org/10.1016/j.freeradbiomed.2010.07.017

Tsai, J.F., Jeng, J.E., Chuang, L.Y., Ho, M.S., Ko, Y.C., Lin, Z.Y., Hsieh, M.Y., Chen, S.C., Chuang, W.L., Wang, L.Y., Yu, M.L., Dai, C.Y., 2004. Habitual betel quid chewing and risk for hepatocellular carcinoma complicating cirrhosis. Medicine (Baltimore) 83(3), 176-187. http://dx.doi.org/10.1097/01.md.0000126971.80227.a4

Tseng, S.K., Chang, M.C., Su, C.Y., Chi, L.Y., Chang, J.Z., Tseng, W.Y., Yeung, S.Y., Hsu, M.L., Jeng, J.H., 2012. Arecoline induced cell cycle arrest, apoptosis, and cytotoxicity to human endothelial cells. Clin. Oral Investig. 16(4), 1267-1273. http://dx.doi.org/10.1007/s00784-011-0604-1

Tu, C.Y., Chen, Y.F., Lii, C.K., Wang, T.S., 2013. Methylglyoxal induces DNA crosslinks in ECV304 cells via a reactive oxygen species-independent protein carbonylation pathway. Toxicol. In Vitro 27(4), 1211-1219. http://dx.doi.org/10.1016/j.tiv.2013.02.011

Ullah, M., Cox, S., Kelly, E., Boadle, R., Zoellner, H., 2014. Arecoline is cytotoxic for human endothelial cells. J. Oral Pathol. Med. 43(10), 761-769. http://dx.doi.org/10.1111/jop.12186

Warnakulasuriya, S., Trivedy, C., Peters, T.J., 2002. Areca nut use: an independent risk factor for oral cancer. BMJ. 324(7341), 799-800. http://dx.doi.org/10.1136/bmj.324.7341.799

Wu, G.H., Boucher, B.J., Chiu, Y.H., Liao, C.S., Chen, T.H., 2009. 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. 12(1), 129-135. http://dx.doi.org/10.1017/S1368980008002073

Yen, C.Y., Lin, M.H., Liu, S.Y., Chiang, W.F., Hsieh, W.F., Cheng, Y.C., Hsu, K.C., Liu, Y.C., 2011. Arecoline-mediated inhibition of AMP-activated protein kinase through reactive oxygen species is required for apoptosis induction. Oral Oncol.47(5), 345-351. http://dx.doi.org/10.1016/j.oraloncology.2011.02.014

Zhou, J., Sun, Q., Yang, Z., Zhang, J., 2014. The hepatotoxicity and testicular toxicity induced by arecoline in mice and protective effects of vitamins C and e. Korean J. Physiol. Pharmacol. 18(2), 143-148. http://dx.doi.org/10.4196/kjpp.2014.18.2.143

Zhou, Z.S., Li, M., Gao, F., Peng, J.Y., Xiao, H.B., Dai, L.X., Lin, S.R., Zhang, R., Jin, L.Y., 2013. Arecoline suppresses HaCaT cell proliferation through cell cycle regulatory molecules. Oncol. Rep. 29(6), 2438-2444. doi: 10.3892/or.2013.2360.

Figure legends

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 three independent experiments.

Figure 2. 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 three independent experiments.

Figure 3. 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 three 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.

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.

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.

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.

Figure 7. Arecoline- and arecoline *N*-oxide-induced zebrafish embryo toxicity. (A) Groups of 30 zebrafish embryos after 3 hours of fertilization were exposed to arecoline or arecoline *N*-oxide for 24 hours and then dead embryos were observed and imaged under a dissecting microscope. The dead embryos turned white as indicated by their blurry yolks. Embryo survival was expressed as percentage of control. (B) Quantification of arecoline and arecoline N-oxide-induced zebrafish embryo toxicity from experiments shown in panel A. Data are mean ±SE from three independent experiments.

Figure 8. 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 9. Representative photo about the posture of the zebrafish and the location of its body in space after exposure to different doses of arecoline or arecoline N-oxide for 58 days.

Figure 10. Effect of arecoline and arecoline N-oxide on the swimming behavior of adult zebrafish from top-vew. Zebrafish were chronically exposed to different doses of arecoline (AR) or arecoline N-oxide for 58 days. After treatment, zebrafish was transferred into a 0.5-liter glass beaker (1 fish per beaker) and swimming behavior was recorded from top view and analyzed by a digital video camera and motion tracking software over a 5-min period. (A) Typical representative trace images of zebrafish swimming behavior. (B) Quantification of the total distance traveled by a zebrafish during 4 minute periods.

Figure 11. Effect of arecoline and arecoline N-oxide on the swimming behavior of adult zebrafish from side view. Zebrafish were chronically exposed to different doses of arecoline (AR) or arecoline N-oxide for 58 days. After treatment, zebrafish was transferred into a 0.5-liter glass beaker (1 fish per beaker) and swimming behavior was recorded from side view and analyzed by a digital video camera and

motion tracking software over a 5-min period. (A) Typical representative trace images of zebrafish swimming behavior. (B) Quantification of the total distance traveled by a zebrafish during 4 minute periods. Zone1: the upper portions above the midline of the tank; zone 2: the lower portions under the midline of the tank.

Figure 12. 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 mitochondria-targeted antioxidant (mito-TEMPO), inhibitors of cytochrome P450 (1-aminobenzotriazole, ABT), flavin-containing monooxygenase (methimazole, MMI), and monoamine oxidase (selegiline, clorgyline, pargyline, imipramine) 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. Data was represent as mean ± SD of three independent experiments.

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

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

[a] AR: arecoline; [b] ARNO: arecoline *N*-oxide; [c] 4NQO; 4-nitroquinoline-1-oxide; [d] NaN3: sodium azide. Data from the average of three determinations (mean ± standard error).

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
Zebrafish embryo	24 h, 500 μM	Microscopic examination	No cytotoxicity	This study
Pig kidney epithelial cell (LLC-PK1)	24 h, 100 – 500 μM	MTT assay	No cytotoxicity	Lin et al., 2016
Human umbilical vein endothelial cell (HUVEC)	24 h, 940 μM	LDH leakage assay	50%	Ullah et al., 2014
Human immortalized keratinocyte (HaCaT)	24 h, 500 μM	MTS assay	50%	Li et al., 2014
Human embryonic lung fibroblast (HEL)	24 h, 529 μM	MTT assay	30%	Zhou et al., 2013
Human endothelial cell (EAhy 926)	24 h, 800 μM	MTT assay	31%	Tseng et al., 2012
Human leukemia cell (K562)	24 h, 1000 μM	MTT assay	40%	Chen & Chang, 2012
Rat primary cortical neurons	24 h, 200 μM	MTT assay	27%	Shih et al., 2010
Primary human gingival fibroblasts	24 h, 423 μM	LDH leakage assay	40%	Chiang et al., 2007
Primary human oral mucosal cells	24 h, 847 μM	Alamar blue assay	53%	Chang et al., 2001
Human	24 h, 254 μM	MTT assay	33%	Chang et

periodontal ligament fibroblast (PDLF)				al., 2001c
Human oral KB carcinoma cell	5 d, 120 μM	MTT assay	75%	Chang et al., 2001a
Human oral mucosal fibroblast	5 d, 400 μM	Crystal violet staining assay	63%	Jeng et al., 1999

Figure 1

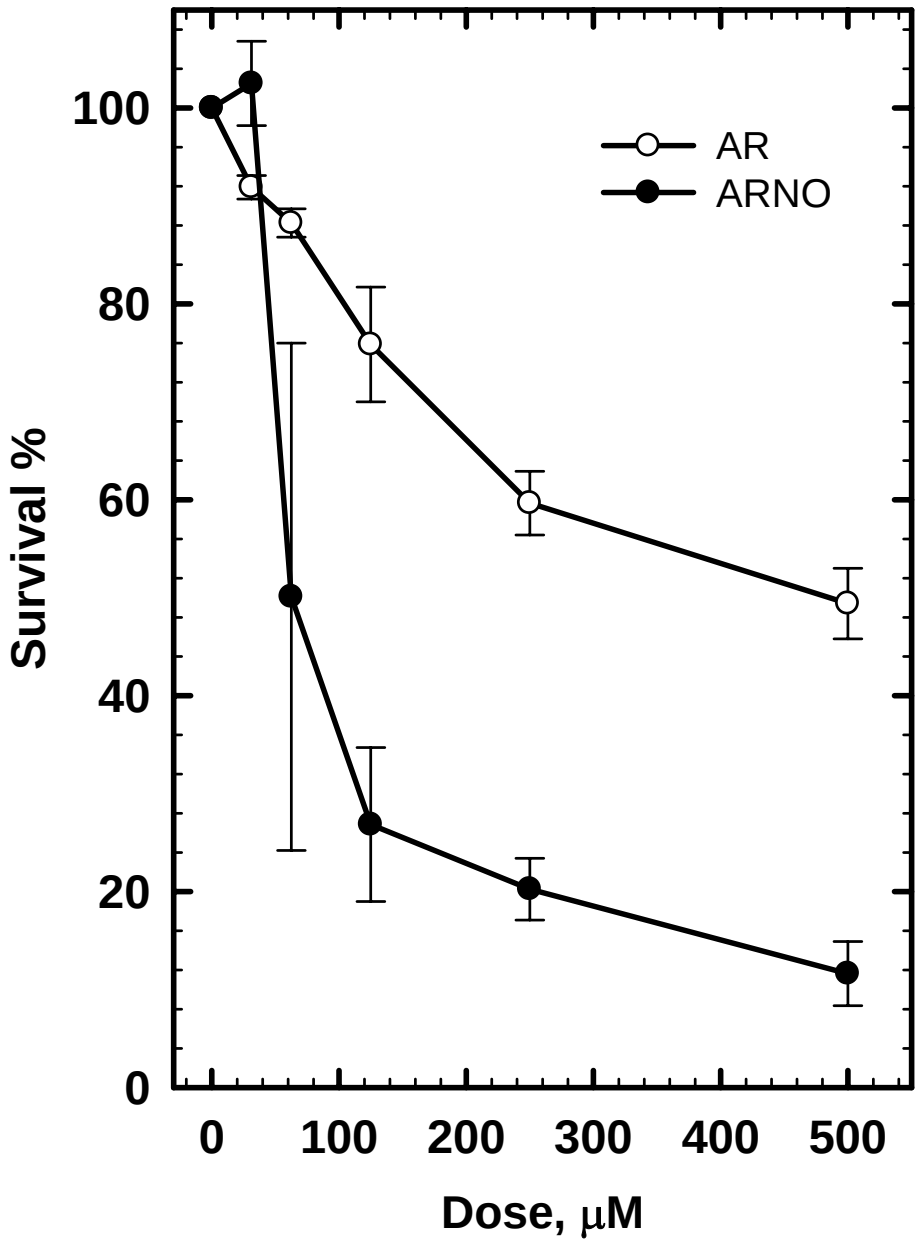


Figure 2

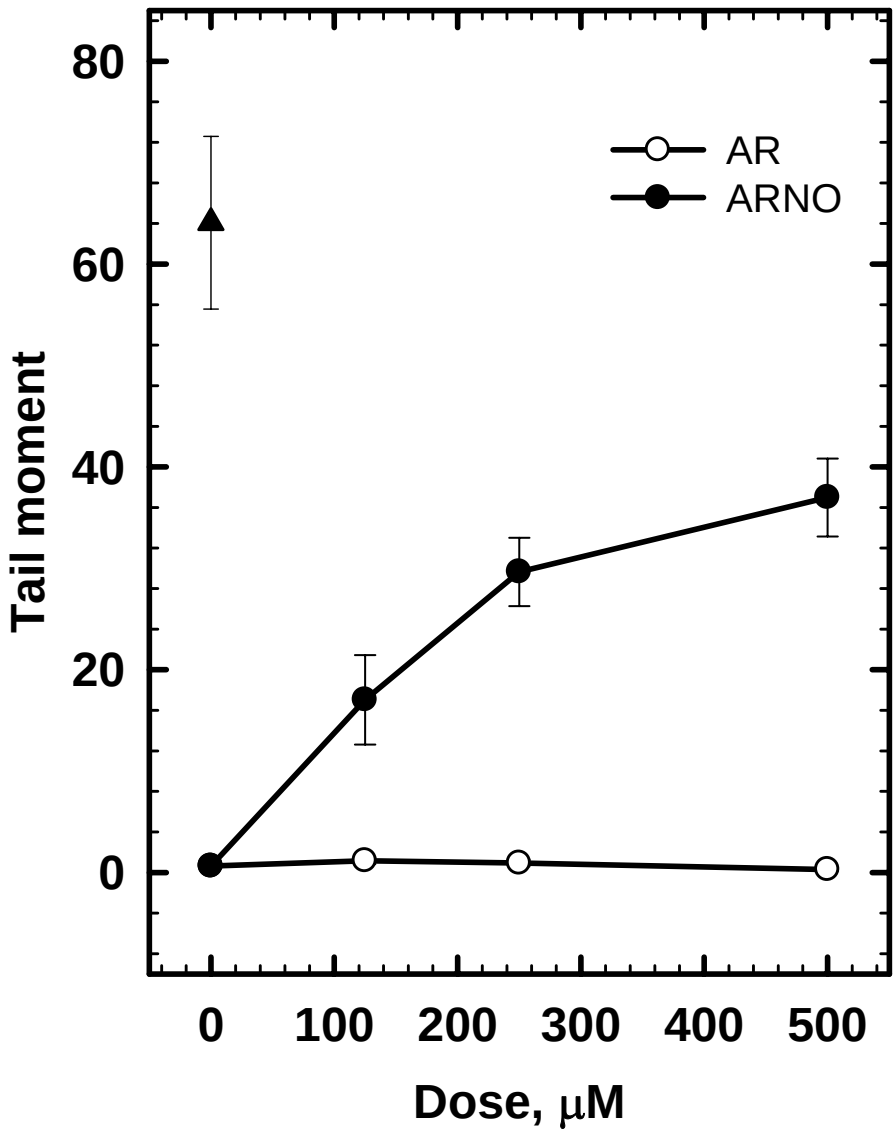


Figure 3

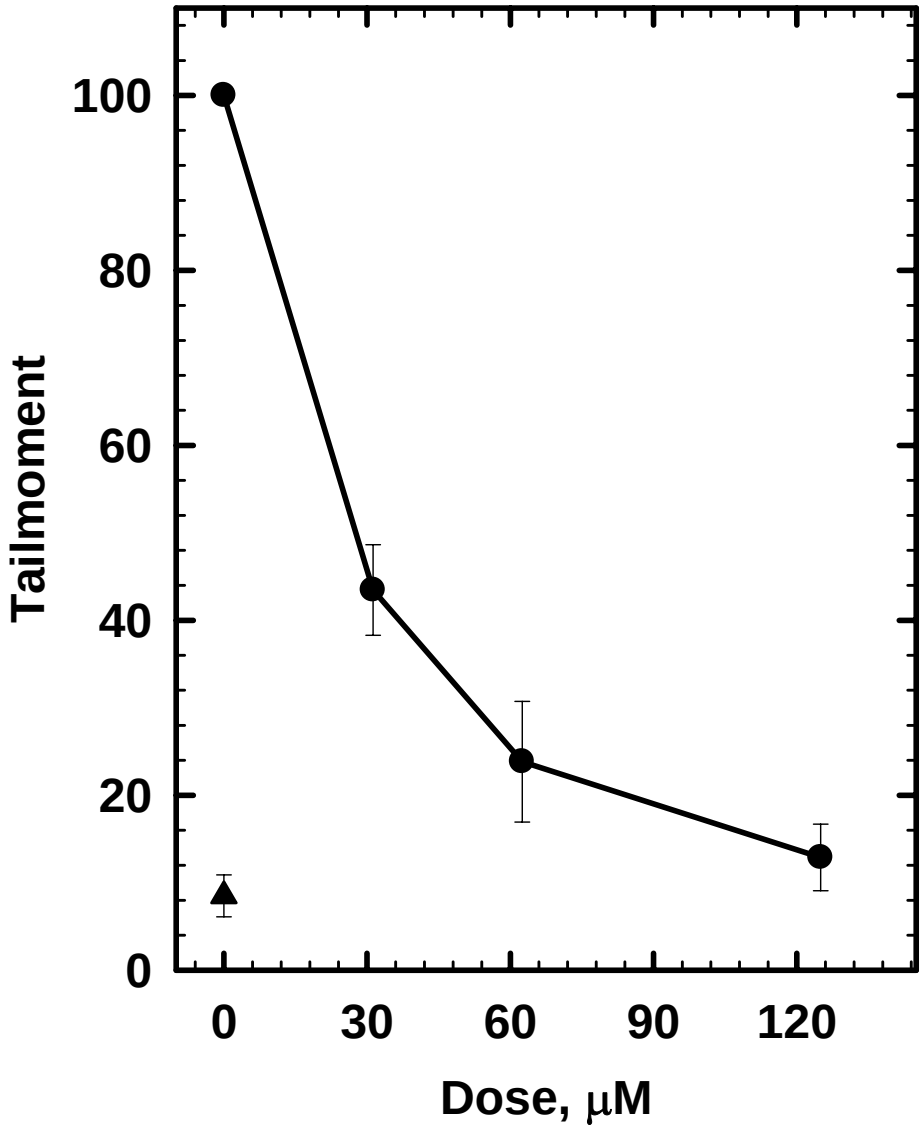


Figure 4

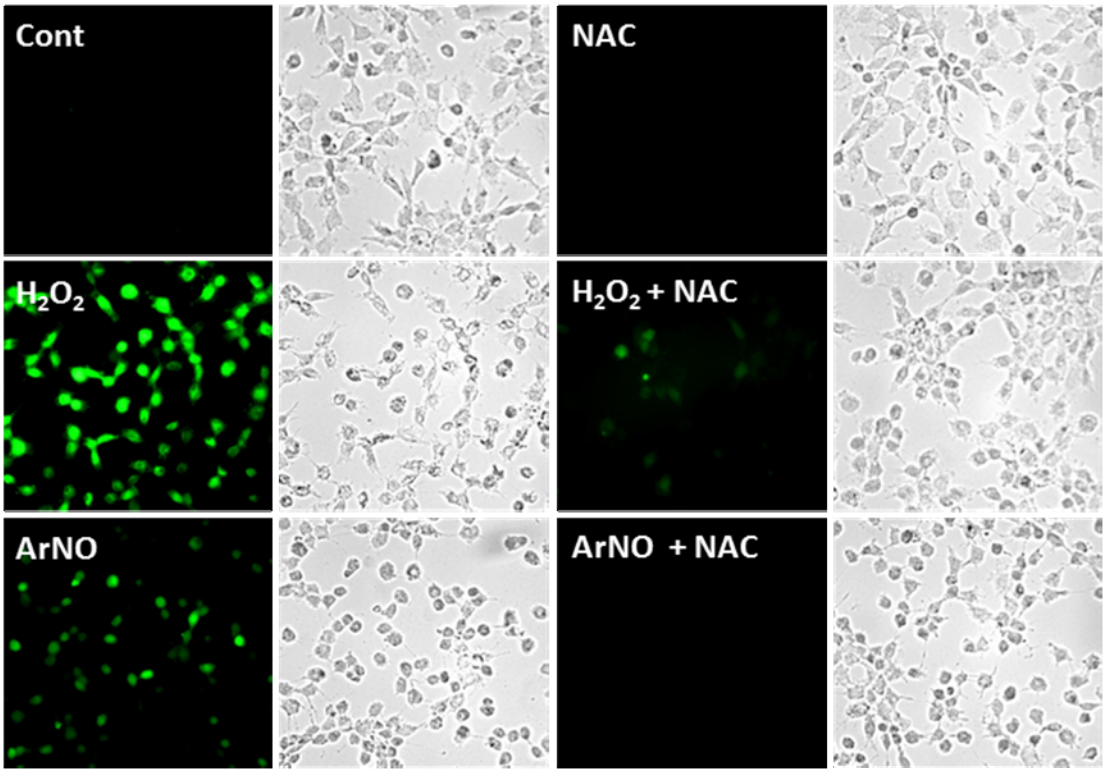


Figure 5

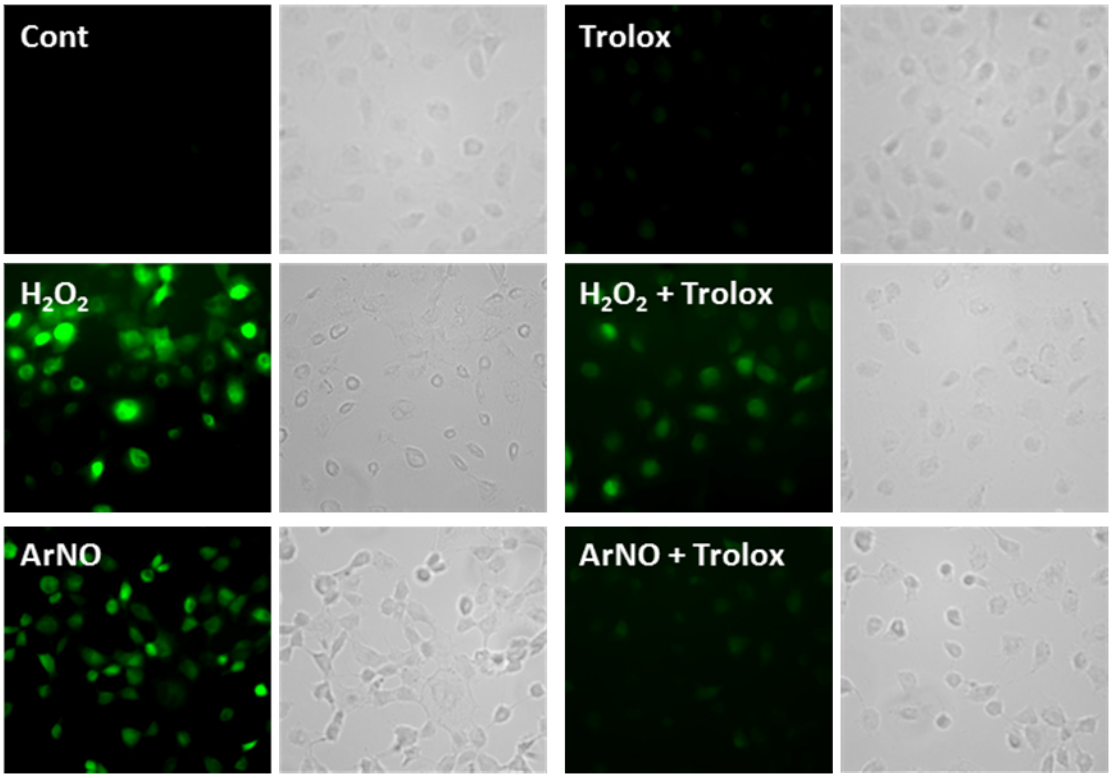


Figure 6

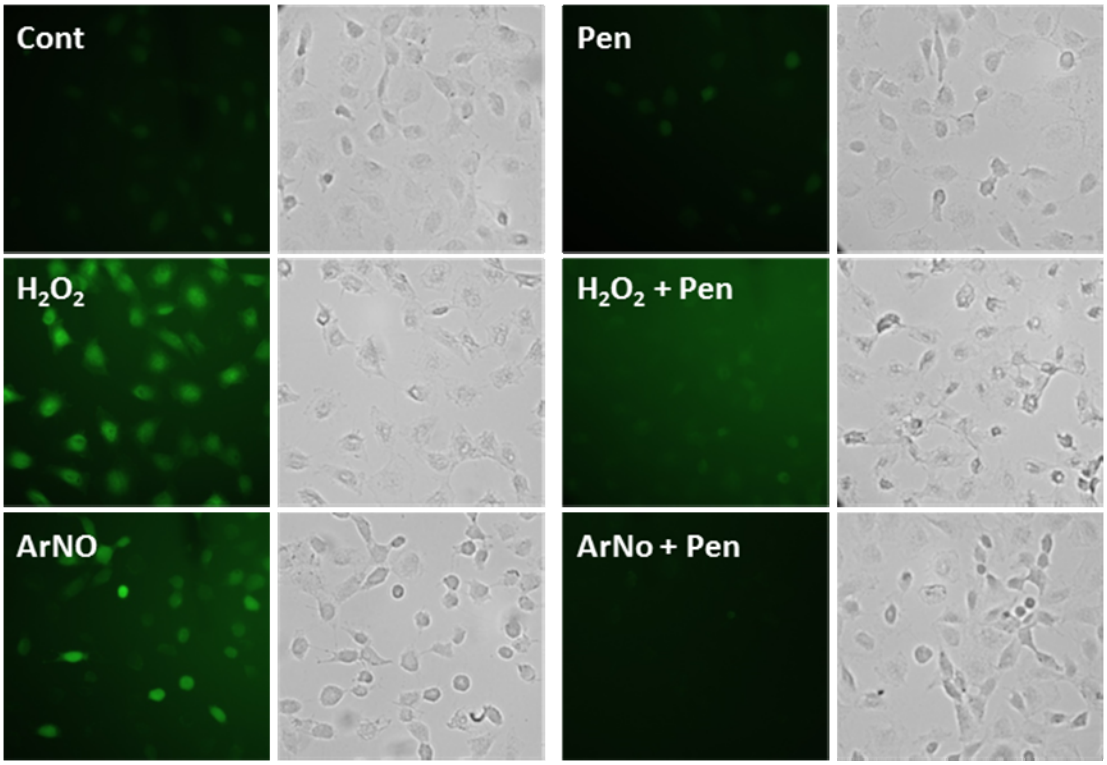
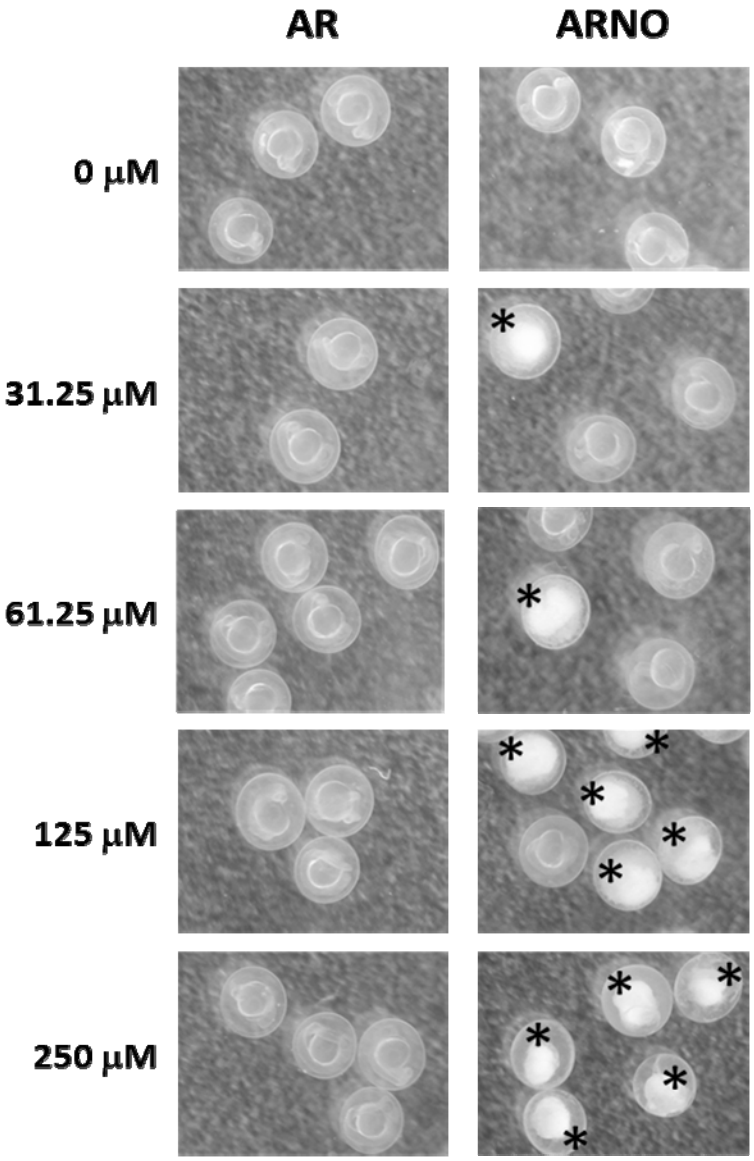


Figure 7

(A)



(B)

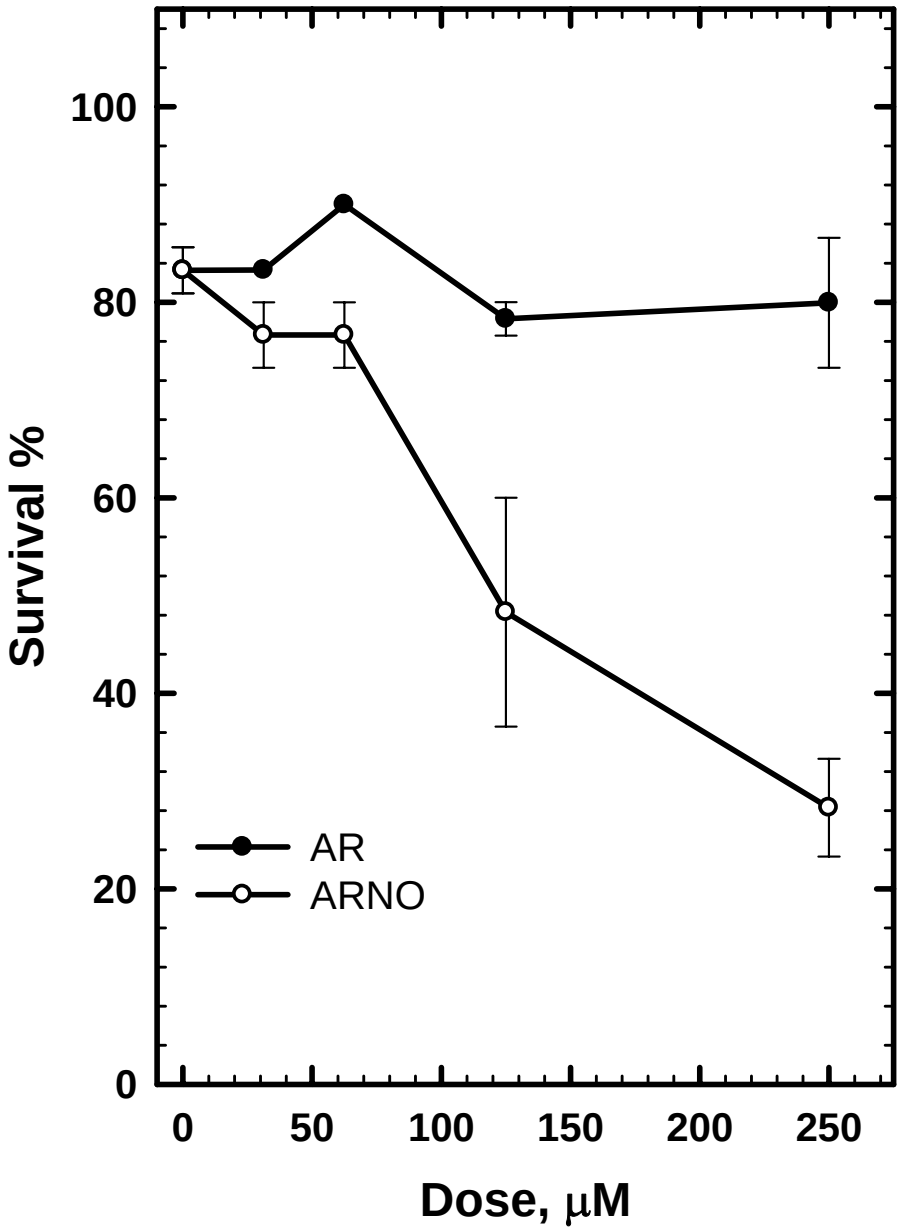

100
80
60
40
20
0
Survival %
0
50
100
150
200
250
Dose, μM
AR
ARNO

Figure 8

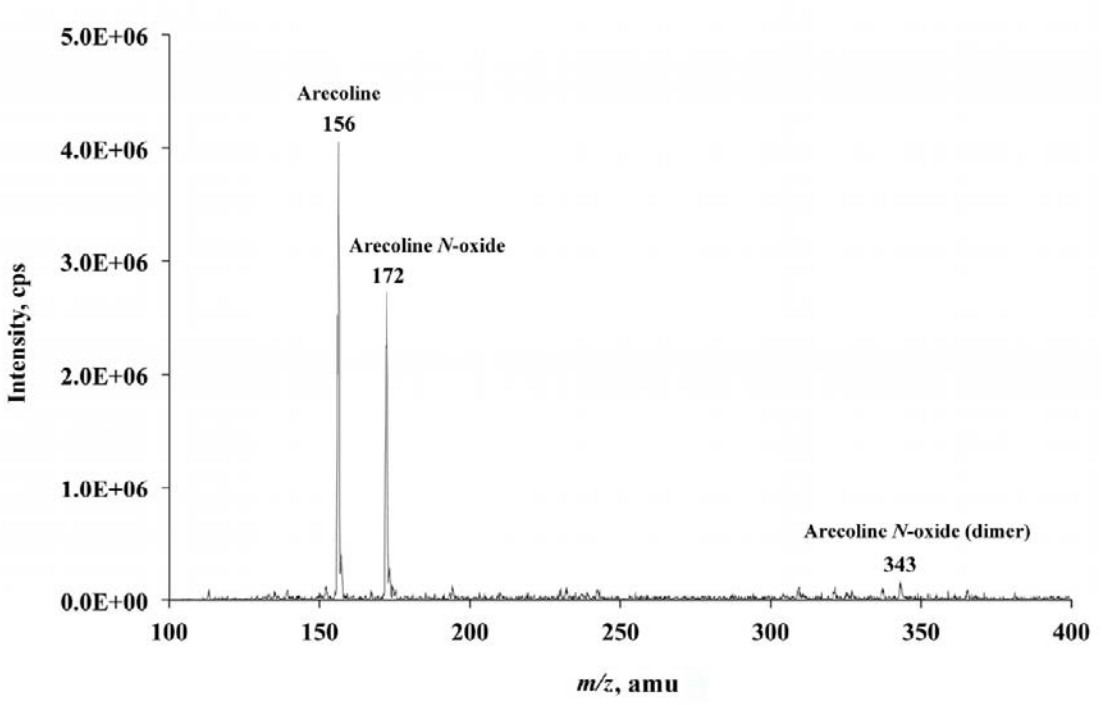

5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
Intensity, cps
Arecoline
156
Arecoline N-oxide
172
Arecoline N-oxide (dimer)
343
100
150
200
250
300
350
400
m/z, amu

Figure 9

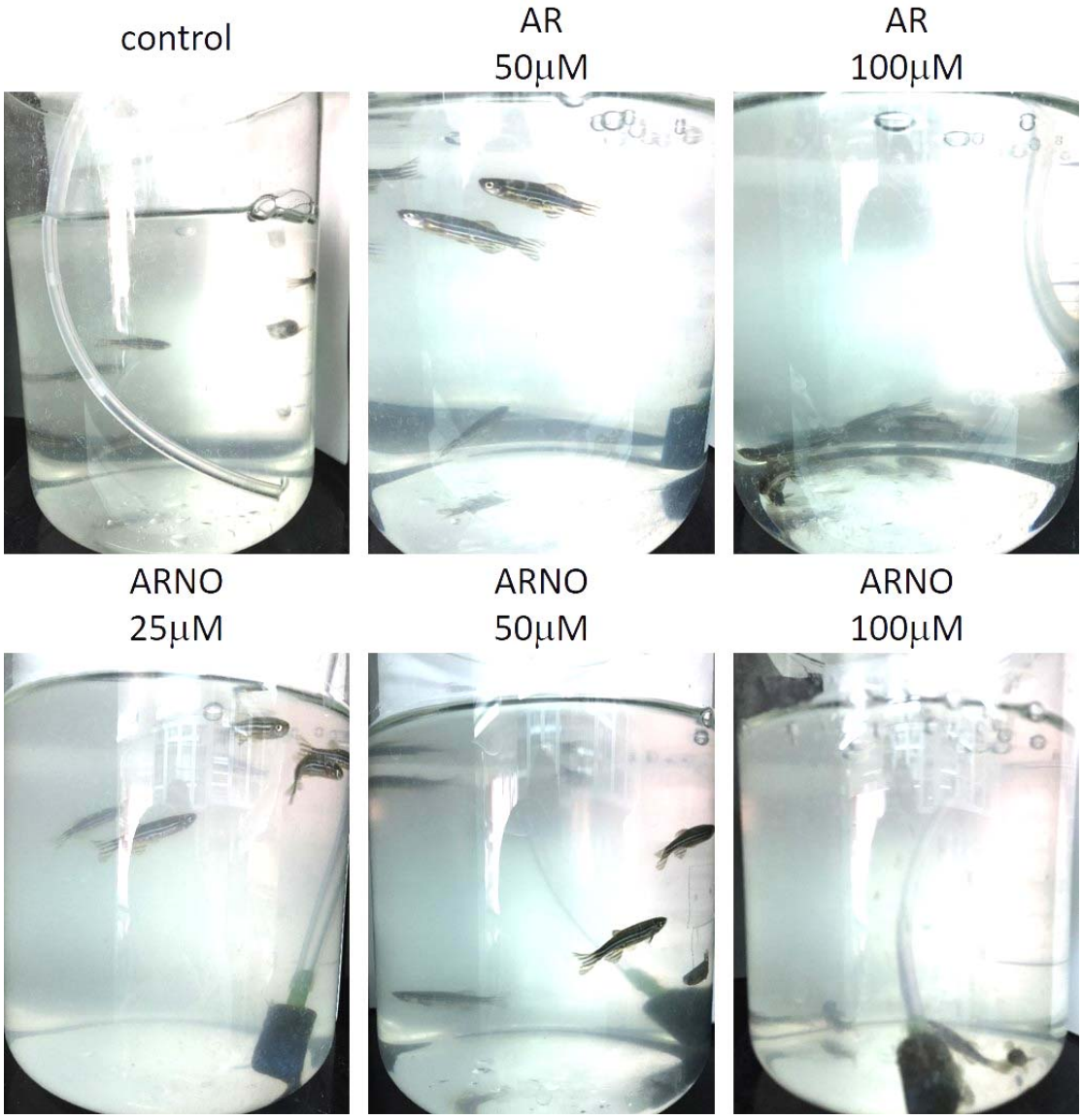
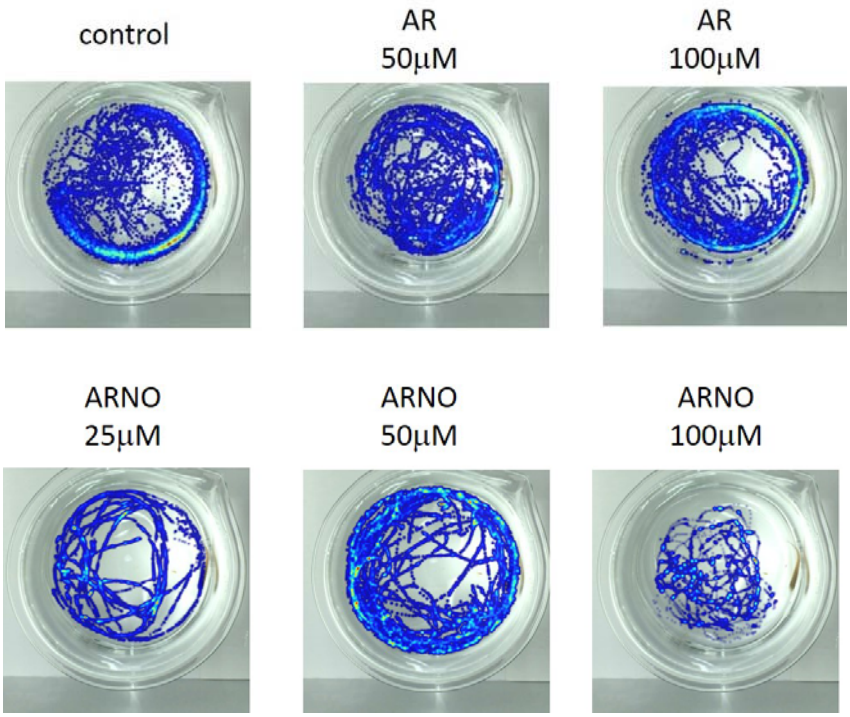


Figure 10

(A)


control
AR
50μM
AR
100μM
ARNO
25μM
ARNO
50μM
ARNO
100μM

(B)

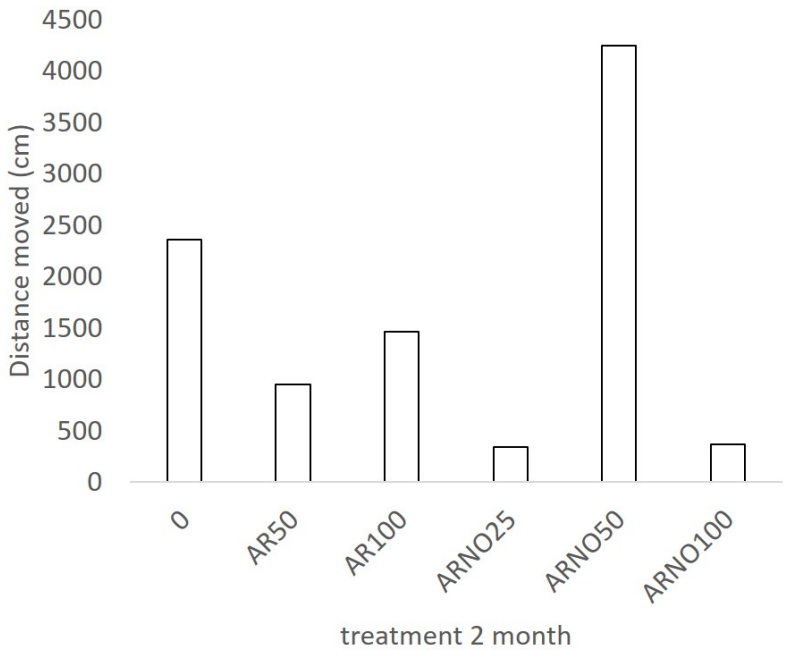
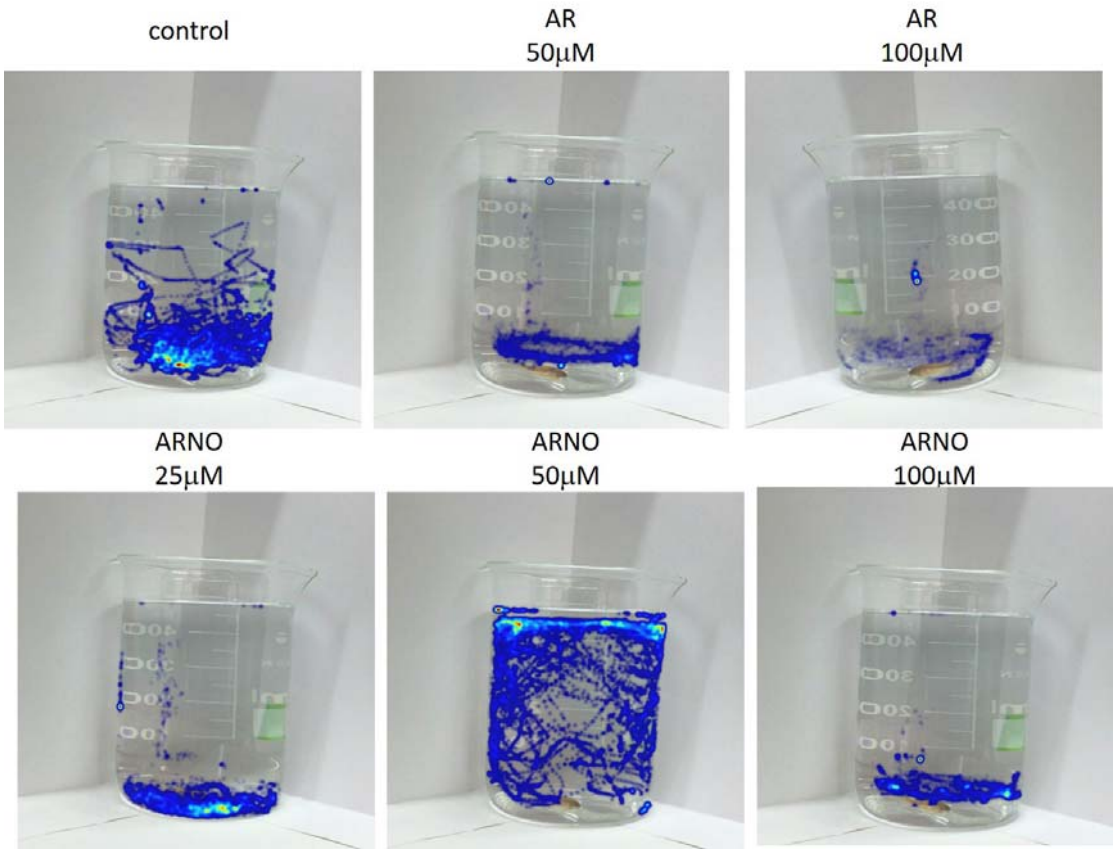

4500
4000
3500
3000
2500
2000
1500
1000
500
0
Distance moved (cm)
0
AR50
AR100
ARNO25
ARNO50
ARNO100
treatment 2 month

Figure 11


control
AR
50µM
AR
100µM
ARNO
25µM
ARNO
50µM
ARNO
100µM

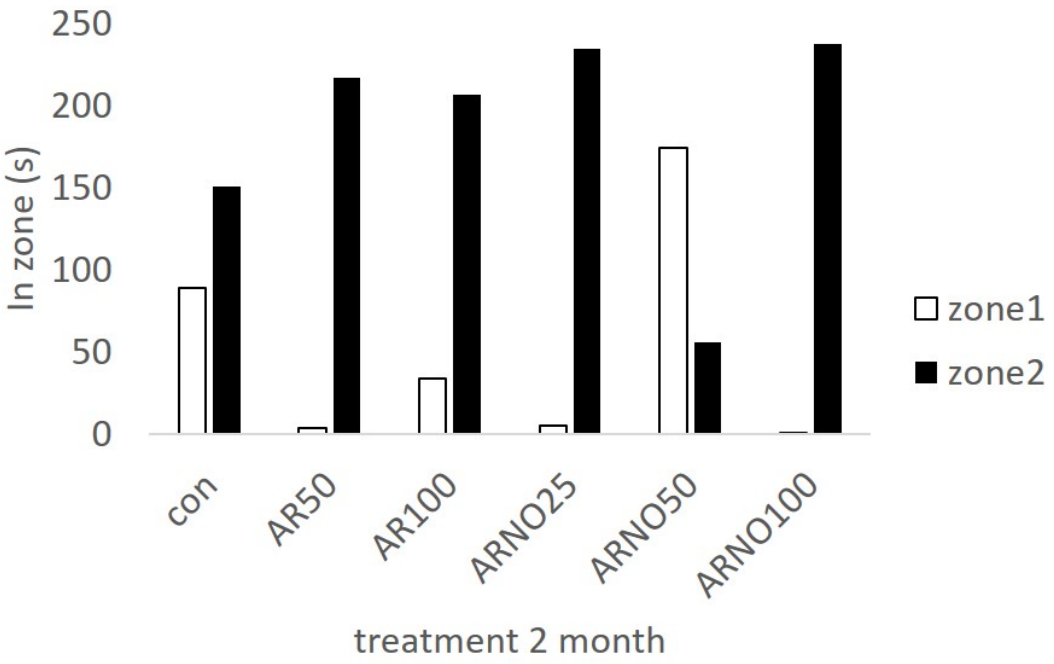
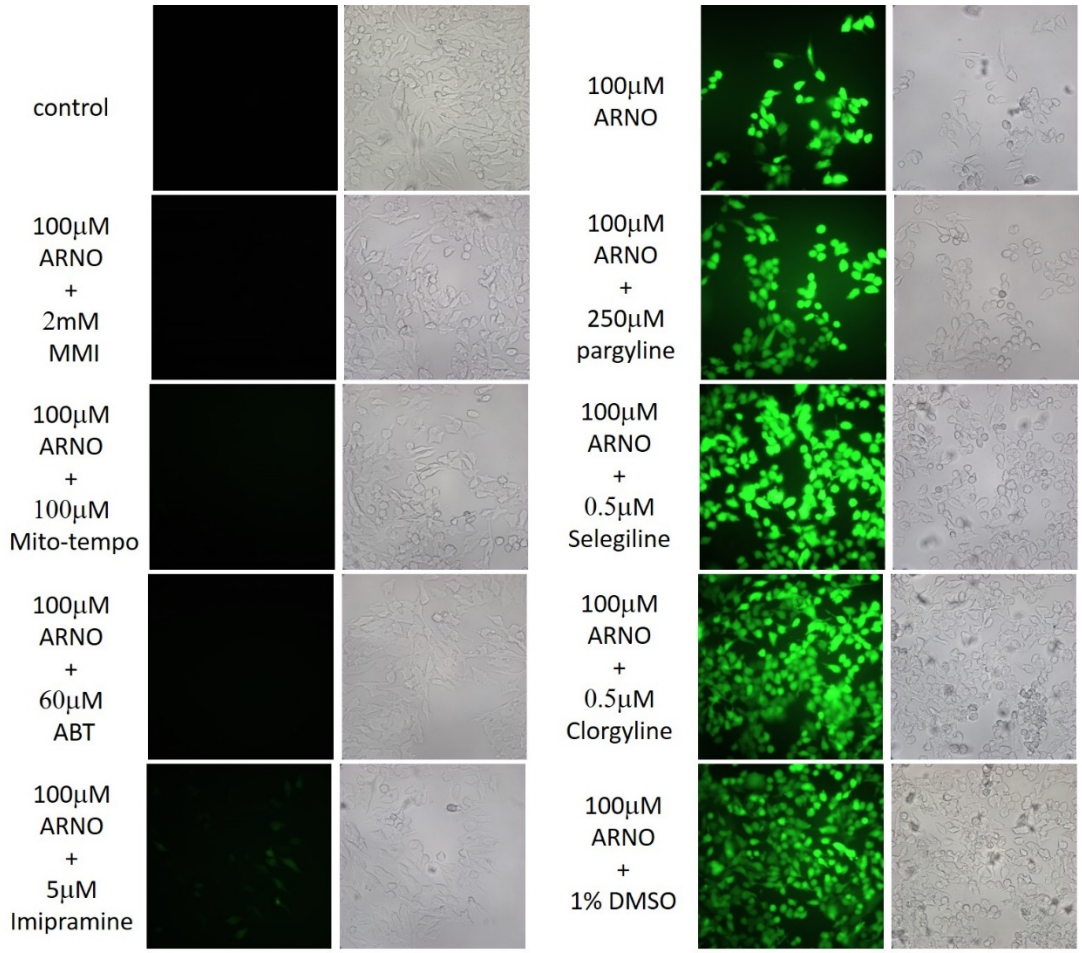
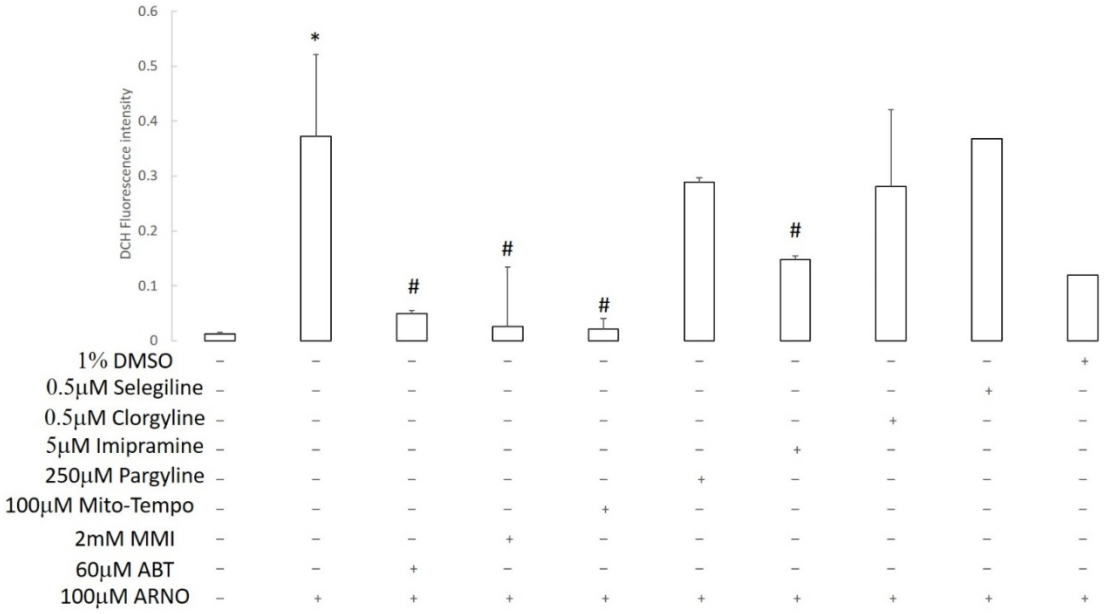

250
200
150
100
50
0
In zone (s)
con
AR50
AR100
ARNO25
ARNO50
ARNO100
treatment 2 month
zone1
zone2

Figure 12


control
100μM ARNO
100μM ARNO + 2mM MMI
100μM ARNO + 250μM pargyline
100μM ARNO + 100μM Mito-tempo
100μM ARNO + 0.5μM Selegiline
100μM ARNO + 60μM ABT
100μM ARNO + 0.5μM Clorgyline
100μM ARNO + 5μM Imipramine
100μM ARNO + 1% DMSO


DCH Fluorescence intensity
0.6
0.5
0.4
0.3
0.2
0.1
0
*
#
#
#
#
1% DMSO
0.5μM Selegiline
0.5μM Clorgyline
5μM Imipramine
250μM Pargyline
100μM Mito-Tempo
2mM MMI
60μM ABT
100μM ARNO

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/31

科技部補助計畫	計畫名稱: 檳榔鹼及其氧化物之代謝與毒性作用機制探討
	計畫主持人: 王祖興
	計畫編號: 104-2320-B-040-013- 學門領域: 營養保健

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：王祖興	**計畫編號：**104-2320-B-040-013-
計畫名稱：檳榔鹼及其氧化物之代謝與毒性作用機制探討	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	1		協助期末報告之數據整理與分析
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）		1.本研究成果將參與2016全國生物科技暨健康產業學術研討會壁報論文發表 2.本研究成果已撰寫完成準備投稿			

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本研究利用斑馬魚模式及肝細胞株模式，發現檳榔鹼的氧化代謝產物(arecoline N-oxide)的細胞毒性及胚胎毒性，都比原合物檳榔鹼提高很多。在肝細胞模式也進一步証實檳榔鹼氧化代謝產物的毒性提高，與大幅度增加細胞內ROS產生量有密切關係，在過程中更牽涉到重要藥物代謝酵素的協調，包括CYP及FMO，但MAO則不參與，除此之外，粒線體也在此ROS產生過程中扮演重要角色，這些數據在了解嚼食檳榔如何造成標的細胞毒性，進而造成肝臟病變及口腔癌都是重要且新的數據，在學術上有良好的創新性及持續研討價值，對檳榔造成健康危害的防治也可提供新穎的思考方向。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）
   1.檳榔鹼氧化物(arecoline N-oxide)在斑馬魚幼胚胎造成明顯毒殺性。2.檳榔鹼氧化物及檳榔鹼(arecoline)在成年斑馬魚造成明顯的游泳行為改變。
   3.檳榔鹼氧化物在大鼠及人類肝臟細胞造成明顯ROS產量增加。此現象中牽涉CYP及FMO代謝酵素的參與，並且是一粒線體依賴途徑。