# THE EFFECT OF ARECOLINE ON DRE-CALUX AND CYP1A1 ACTIVATION IN HUMAN HEPATOMA CELLS

Wang YF[1], Chao HR[2], Chen HD[3] and Tsou TC[4]

[1]Department of Bioenvironmental Engineering, Chung Yuan Christian University, 320, Taiwan, [2]Department of Environmental Science and Engineering, National Pingtung University of Science and Technology, 912, Taiwan, [3] Sustainable Environment Research Center, National Cheng Kung University, 701, Taiwan, [4]Division of Environmental Health and Occupational Medicine, National Health Research Institutes, 350, Taiwan

## Introduction

There are 2 to 2.8 million people (one-tenth of the total population) with a betel quid chewing habit in Taiwan. Areca nut contains a lot of polyphenols and various alkaloids particularly for arecoline that has been recognized as cytotoxicity and genotoxicity in vitro. Arecoline is defined as a mutagenic chemical in bacterial and cellular tests(1) to induce the proliferation of buccal mucosal fibroblast and collagen(2). It's interested to understand more about the effect of arecoline on activation of DRE-CALUX and CYP1A1 enzyme activity in human hepatoma cell (Huh7) lines.

Dioxin-responsive element Chemical Activated LUciferase eXpression (DRE-CALUX) bioassay has been recently introduced and tested in both Environmental Protection Administration (EPA) and Bureau of Food and Drugs Analysis of Department of Health (DOH) in Taiwan. CALUX bioassay is a more rapid and lower-cost screening method particularly for fast-screen identification of highly dioxin-contaminated samples from environment and food compared with HRGC/HRMS. Recently, the CALUX bioassay has been used in detection of the dioxin-TEQ levels in both biota and environment in some developed countries. However, some problems are left to be overcome while applying CALUX bioassay. Uncertainty of false-negative or false-positive result was part of these.

Few studies were focused on the induction of cotreatment with arecoline and 2,3,7,8-TCDD in human hepatoma and its recombinant cells. The effect of arecoline on activation of aryl hydrocarbon receptor (AhR)-mediated gene expression by the 2,3,7,8-TCDD-induced CYP1A1 in human hepatoma (Huh-7) and a dioxin-responsive element(DRE)-mediated Chemical Activated LUciferase eXpression (CALUX) cells were examined in this study.

## Materials and Methods

A DRE-CALUX cells, Huh7-DRE-Luc cells, by stable transfection of Huh7 with a DRE-driven firefly luciferase reporter plasmid (4xDRE-TATA-Luc), were established. Different concentrations of arecoline (50, 100, 200 and 300 μM) with 10nM TCDD were cotreatment in cells for 24hr. The luciferase activity in Huh7-DRE-Luc cells were measured by Luciferase Assay System (Promega, Masison, WI, USA).

The EROD activity was determined as previously described[3] using 5μM of ethoxyresorufin in DMEM medium as the substrate of CYP1A1 enzyme, in the presence of 1.5mM of salicylamide to inhibit conjugating enzymes. After incubation for 30 min at 37℃, fluorescence was measured by using a Fluoroskan multi-well fluorescence plate reader (Labsystems), with excitation at 530 nm and emission at 590 nm. Resorufin standard curve were used to convert fluorescence to pmole of resorufin formed. The CYP1A1 activity was defined as resorufin formation (in pmol) per 100 min of reaction time (pmol resorufin/100 min).Cell viability was determined by MTT assay, following the procedure previously described[4]. The differences between the untreated controls and the arecoline-treated data were examined by the nonparametric Mann-Whiney *U* test.

**Results and Discussion**

**Induction of DRE-CALUX by 2,3,7,8-TCDD in Huh7-DRE-Luc cells**

The Huh7-DRE-Luc cells for DRE-CALUX bioassay were performed that various 2,3,7,8-TCDD concentrations were treated with the genetically recombinant cells for 24 h and a dose–response induction of DRE-CALUX activity was shown in Fig. 1. This DRE-CALUX bioassay used a semi-logarithmic dose–response curve as a standard curve with a sigmoid appearance. The detection limit for 2,3,7,8-TCDD was 2.5 pM.

**Cytotoxic effect of arecoline on Huh7 cells**

Prior to addressing cadmium with Huh7 cells, it is essential to define the non-toxic range of arecoline for Huh7 cells, the parental cells of Huh7-DRE-Luc cells, for evaluating the cadmium effects on DRE-CALUX activation by 2,3,7,8-TCDD. To analyze the cytotoxic effect of arecoline, Huh7 cells were treated with different concentrations of arecoline for 24 h and then survival rate was determined with MTT assay. As shown in Fig. 2, treatments with arecoline ≤100 μM caused no significant effect on cytotoxicity of Huh7 cells ($p>0.05$), whereas treatment with arecoline ≥ 100 μM had slightly cytotoxic effects on Huh7 cells ($p<0.05$). There were not significant differences in relative survival rate values in arecoline of 100 μM in comparison to those of 200 and 300 μM ($p>0.05$).

**Effect of arecoline on DRE-CALUX induction by 2,3,7,8-TCDD**

To address the arecoline effect on the DRE-CALUX induction by 2,3,7,8-TCDD, Huh7-DRE-Luc cells were treated with 10nM 2,3,7,8-TCDD in the presence of different concentrations of arecoline. As shown in Fig. 3, co-treatments with 10 nM TCDD and arecoline ≥ 100 μM at least increased 1.5 folds of relative luciferase activity compared to treatment with 10 nM TCDD alone ($p<0.001$).

**Inhibition of arecoline on CYP1A1 induction by 2,3,7,8-TCDD**

EROD assay was used to investigate the effect of arecoline on CYP1A1 enzyme activation induced by 10nM 2,3,7,8-TCDD. The result indicated that arecoline inhibited the TCDD-induced EROD activation in a

dose-dependent manner (Table 1). The result indicated that arecoline ≥ 100 μM could cause significant decreases in EROD activity ($p$<0.05).

The results showed that arecoline increases the induction of luciferase gene expression mediated by 2,3,7,8-TCDD, but inhibits the TCDD-induced CYP1A1 activation. It's interesting to report a non-AhR-binding compound, arecoline, strongly and sensitively enhances the gene expression of luciferase activity in DRE-CALUX cells, but significantly inhibits the CYP1A1 enzyme activation in human hepatoma cells. Furthermore, the arecoline inhibited AhR mRNA expression with no direct effect on CYP1A1 enzyme activity. The observed inhibitory effect of arecoline on CYP1A1 activation was not due to the up-regulation of AhRR or direct inhibitory effect on CYP1A1 in the present study. According to our previous study, we found that sodium arsenite inhibited the 2,3,7,8-induced activation of DRE-CALUX and EROD, but arecoline had the inverse results of induction activities between DRE-CALUX bioassay and CYP1A1 enzyme activation. In conclusion, this finding indicated the differential effect of arecoline on the endogenous dioxin-responsive CYP1A1 and on a stably transfected DRE-driven reporter in human hepatoma cells. The present study suggests that induction of DRE-CALUX alone does not necessarily parallel endogenous gene expression, and that the reporter assay may detect interactions that are not functional in vivo. However, more experiments warrants further investigation in the future while applying the DRE-CALUX.

**Acknowledgements**

This work was supported by grants from National Health Research Institutes (Taiwan) (EO-095-PP-04) and Environmental Resource Management and Research Center (ERMRC), National Cheng Kung University (H96-303).

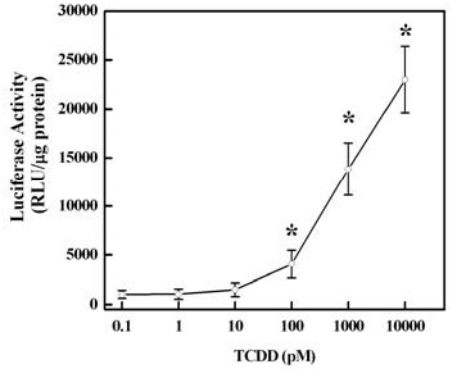


Fig. 1. Dose–response induction of luciferase activity by 2,3,7,8-TCDD. Huh7 cells at ≈ 90% confluence were incubated with 2,3,7,8-TCDD for 24 h. The measurements of luciferase activity are shown as means ± S.D., n=3. *p < 0.05, compared with the treatment with 0.01pM TCDD.

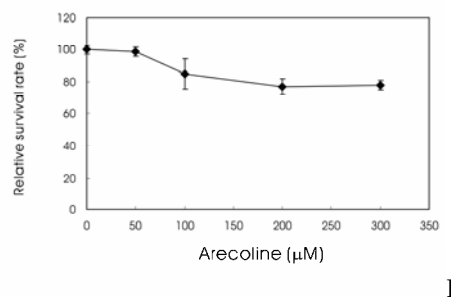


Fig 2. Cytotoxic effects of arecoline on Huh7 cells. The data are presented as percentage of survival rate as compared to that of untreated control. *$p$<0.05, **$p$<0.01, and ***$p$<0.001, compared with the control.

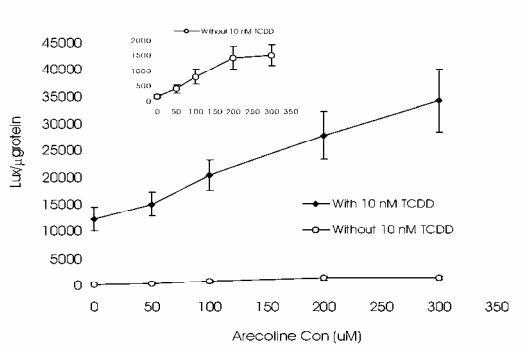


Fig 3.Huh7-DRE-Luc cells were treated with arecoline in the present or absent of 10 nM TCDD for 24 hours. The data are expressed as the relative luciferase activities (%) as compared with that of 10 nM TCDD-treated control (100%). **$p$<0.01 and ***$p$<0.001, compared with 10 nM TCDD only.

Table 1. Inhibitory effect of arecoline/cadmium (II) on TCDD-induced CYP1A1 activation in Huh7 cell (*p* mole resorufin/100 min)

| Arecoline concentration | 0 μM | 50μM | 100 μM | 200 μM | 300 μM |
|---|---|---|---|---|---|
| 10 nM TCDD + arecoline (n=7) | | | | | |
| Mean ± SE | 73.96 ± 10.0 | 64.60 ± 4.91 | 62.31 ± 5.51 | 49.17 ± 3.19 | 35.52 ± 3.97 |
| 95% C.I. [a] | 64.71-83.21 | 49.56-79.64 | 45.43-79.19 | 39.41-58.92 * | 23.37-47.67 ** |
| Arecoline alone (n=8) | | | | | |
| Mean ± SE | 17.01 ± 1.32 | 15.30 ± 1.09 | 15.05 ± 1.32 | 12.24 ± 1.27 | 9.10 ± 2.11 |
| 95% C.I. | 13.35-20.67 | 12.28-18.32 | 11.38-18.71 | 8.75-15.75 | 3.07-15.12 |

[a] 95 C.I. means 95% of confidence interval

* $p$<0.05, ** $p$<0.01, *** $p$<0.001

References
1. Ashby J, Styles JA, Boyland E. *Lancet* 1979;1:112.
2. Chang Y.C., Tai K.W., Cheng M.H., Chou L.S. and Chou M.Y., *J. Oral. Pathol. Med.* 1998; 27:68.
3 Kennedy S.W., Jones S.P., Anal. Biochem. 1994; 222:217.
4 Tsou T.C., Yeh S.C., Tsai F.Y. and Chang L.W. *Chem. Res. Toxicol.* 2004;17:208.