# EFFECT OF NICOTINE AND ITS METABOLIZED FORM ON PRIMARY MOUSE EMBRYONIC FIBROBLAST CELL LINE (PMEF) *IN VITRO*

HATHAIRAT LEKATANA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Oral Biology - (Type 2.1)
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Thesis entitled "Effect of nicotine and its metabolized form on primary mouse embryonic fibroblast cell line (PMEF) *in vitro* "

By Hathairat Lekatana

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Oral Biology - (Type 2.1) of Naresuan University

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| **Title** | EFFECT OF NICOTINE AND ITS METABOLIZED FORM ON PRIMARY MOUSE EMBRYONIC FIBROBLAST CELL LINE (PMEF) *IN VITRO* |
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## ABSTRACT

Nicotine is the principal alkaloid found in tobacco and 70%-80% is converted to cotinine. Like nicotine, cotinine can rapidly pass through the placenta from mother to child by transplacental transfer which occurs throughout pregnancy. Epidemiological research has found a statistically significant association between maternal smoking and the occurrence of oral clefts in newborns. In this study, the PMEF cell line (E13) was selected to represent mesenchymal cells at embryonic day 13 (E13) in mice, which coincides with the elevation of the palatal shelves marking a critical time in palatogenesis. This study aims to investigate the effects of nicotine, cotinine, and their combination on ROS generation, cell viability, cell apoptosis, and apoptosis-related gene expression in PMEF cells *in vitro*. Results showed that nicotine and cotinine had an adverse effect on PMEF cells through decreased cell viability and increased apoptotic cell death in a dose-dependent manner. Nicotine, cotinine, and the combination of them significantly increased the generation of ROS. Overproduction of ROS was closely associated with the number of viable cells, apoptotic cells, and the expression of apoptosis- related PMEF genes, such as CAS3 and P53, which induce apoptosis. Interestingly, this study also found that cotinine enhanced the cytotoxic effect of nicotine treatments by decreasing cell viability, activating cell apoptosis by increasing ROS production and expression of CAS3 and P53.

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Hathairat Lekatana

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# ABBREVATION

| | | |
|---|---|---|
| Apaf-1 | = | Activates factor 1 |
| Bax | = | Bcl2-associated X protein |
| Bcl2 | = | B-cell lymphoma-2 |
| BclB | = | Bcl2-like protein 10 |
| BclW | = | Bcl2-like protein 2 |
| BclXL | = | Bcl-X large |
| Bfl1 | = | Bcl2-related protein A1 |
| BH | = | Bcl2 homology |
| CAS3 | = | Caspase3 |
| CAT | = | Catalase |
| CNCCs | = | Cranial neural crest cells |
| CSC | = | Cigarette smoke condensate |
| CSE | = | Cigarette smoke extraction |
| DISC | = | Death-inducing signalling complex |
| DMEM | = | Dulbecco's modified Eagle medium |
| DMSO | = | Dimethyl sulfoxide |
| GAPDH | = | Glyceraldehyde 3-phosphate dehydrogenase |
| GPXs | = | glutathione peroxidase |
| hOMF | = | human Oral mucous fibroblasts |
| $H_2O_2$ | = | Hydrogen peroxide |
| hPDLCs | = | human Periodontal ligament cells |
| $OH^{\bullet}$ | = | Hydroxyl radical |
| $O2^{\bullet-}$ | = | Superoxide anion |
| Mc11 | = | Myeloid cell leukemia 1 |
| mESCs | = | Mouse Embryonic stem cell |
| MOMP | = | Mitochondrial outer membrane permeabilization |
| MPT | = | Mitochondrial permeability transition |

| | | |
|---|---|---|
| MRC-5 | = | Pulmonary fibroblast cell line |
| MSCs | = | Mesenchymal stem cells |
| MTT | = | 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium |
| nAChRs | = | Nicotinic acetylcholine receptors |
| NSCLC | = | Non-small cell lung cancer cells |
| PBS | = | Phosphate Buffered Saline |
| PMEF | = | Primary mouse embryonic fibroblast |
| RLU | = | Relative luminescence units |
| ROS | = | Reactive oxygen species |
| STE | = | Smokeless tobacco extracts |
| TNF | = | Tumor necrosis factor |
| UCB | = | Umbilical cord blood |

# CHAPTER I

# INTRODUCTION

Smoking, alcohol consumption, and vitamin deficiency during pregnancy are environmental risk factors that have been widely investigated on their adverse effects on fetal development (1-3). It should be noted that at the beginning of pregnancy, the embryo is highly sensitive to its surroundings. Nutrients, chemicals or drugs that are able to pass the placental barrier can have direct impacts on growth and development.

For example, active maternal cigarette smoking impacts in every trimester of pregnancy ranging from increased miscarriages in the first trimester (4) to increased premature delivery and decreased fetal growth in the third trimester (5). Cigarette smoke contains thousands of compounds and some of them are known to impair human reproduction and development resulting in disability (6). Studies in mouse experiment reported that components in tobaccos directly affected the craniofacial development and obviously interrupted morphological process formation (7, 8). Nicotine is the principal alkaloid in tobacco and it comprises approximately 95% of the total alkaloid (9). Adverse effects of nicotine on fetal growth and development have been reported elsewhere.

Also, nicotine is considered as the main teratogenic substance that alters and delays embryonic development (10). Several authors stated that there is an evidence supported the nicotine accumulation in fetal serum and amnionic fluid because slightly higher concentrations of nicotine were found in fetal serum and amnionic fluid than that found in maternal serum (11, 12). Another interesting finding was a dose effect relationship between nicotine intake and smoking-related outcomes of pregnancy (13, 14).

Nicotine is extensively metabolized in liver and approximately 70%-80% of nicotine is converted to cotinine. The cotinine levels are of particular interest as qualitative markers of tobacco use and quantitative indicators of nicotine intake (15). Like nicotine, cotinine can rapidly pass the placenta from mother to child through transplacental transfer occurring throughout pregnancy (16-18).

Cigarette smoke or tobacco alkaloids are not only absorbed locally but may also enter the systemic circulation. Interestingly, evidences have demonstrated the effects of cigarette smoke in biological processes, including inflammation, antioxidant defence and cell apoptosis (19-21). The cytotoxic effects of cigarette smoke are associated with its action to augment the intracellular reactive oxygen species (ROS) level (22) and to diminish protective antioxidant enzymes (23). Effects of ROS depend on the quantity and duration of exposure. At low concentrations, ROS activates cell cycle progression by stimulating signalling cascades and in the case of continued exposure, cell cycles may be arrested. At high concentrations, cell cycle/growth is terminated and cell damage is increased leading to cell apoptosis or necrosis (24).

Moreover, ROS overproduction induces severe mitochondrial dysfunction (25) and leads to mitochondrial structural oxidation, opening of the mitochondrial permeability transition (MPT) pores (26-28). Previous studies revealed that increased ROS generation such as $H_2O_2$ and $O_2^{\bullet-}$ resulted in the release of cytochrome C and induction of apoptosis through the mitochondrial pathway (29, 30).

ROS can alter pro- and anti-apoptotic Bcl2 family proteins and activate pro-apoptotic Bcl2 family proteins which are then translocated into the outer mitochondrial membrane. This process facilitates MOMP by formation of large channels and leakage of pro-apoptotic factors (i.e cytochrome C) into the cytosol and eventually results in apoptotic cell death (31, 32). The roles of ROS in apoptosis is supported by Wang's study. The results showed teroxirone induced cytotoxicity in human non-small cell lung cancer cells (NSCLC) which could involve in P53-associated intrinsic apoptosis pathway and ROS production. In their study, teroxirone induced ROS mediated cytotoxicity and the inhibition of teroxirone using antioxidant pretreatment with N-acetylcysteine diminished ROS and inverted the expression of Bax, Bcl-2 and cytochrome C. These results suggest the roles of ROS as an effective initial mediator in the P53-dependent intrinsic apoptotic pathway (33).

The effects of intracellular ROS induced by tobacco on cell apoptosis have been widely investigated (34). For example, nicotine at a concentration of 6 mM induced ROS level and embryonic apoptosis. The nicotine negatively causes embryonic malformations (35). Moreover, a study in human oral mucous fibroblasts

(hOMF) showed that smokeless tobacco extracts (STE) induced ROS production. STE also induced cell apoptosis by regulating the apoptosis associated proteins of which Bax expression were increased and Bcl2 expression were decreased in a dose-dependent manner (36).

In addition, studies investigated the effects of cigarette smoke extraction (CSE) on cell viability and cell death in mouse embryonic stem cell (mESCs) and the ROS induced by CSE. CSE increased oxidative stress by promoting ROS formation, which resulted in apoptosis of mESCs (37).

Previous studies have been reported the negative effects of cigarette smoke or tobacco alkaloids on mouse embryonic development and various types of cells. However, effects of ROS induced by nicotine (the main teratogenic substance that alters and delays embryonic development), cotinine (the major metabolized form of nicotine) and their combination on cell apoptosis, and the expression of apoptosis-related genes in primary mouse embryonic fibroblast (PMEF) cell line have never been investigated. PMEF is mesenchymal cells that is going to differentiate into various morphological structures and organs of mammalian embryo, especially craniofacial formation. Evidence-based information of the association between nicotine and cotinine, and palatal development can be obtained. This study aims to investigate the effect of nicotine, cotinine and their combination on ROS generation, cell viability, cell apoptosis and apoptosis-related genes expression of PMEF cell *in vitro.*

**Research Objectives**

1. To investigate the effects nicotine, cotinine and their combination on ROS generation
2. To investigate the effects of ROS induced by nicotine, cotinine and their combination on cell apoptosis of PMEF
3. To investigate the effects of ROS induced by nicotine, cotinine and their combination on expression of apoptosis-related genes of PMEF

**Research Significances**

1. Whether or not the effects nicotine, cotinine and their combination on ROS generation can be clarified.

2. Whether or not the effects of ROS induced by nicotine, cotinine and their combination on cell apoptosis of PMEF can be clarified.

3. Whether or not the effects of ROS induced by nicotine, cotinine and their combination on expression of apoptosis-related genes of PMEF can be clarified.

4. Evidence-based information of the association between nicotine, cotinine and their combination, and palatal development can be obtained.

**Research Scope**

This is an *in vitro* study aimed to investigate the effect of ROS generation induced by nicotine, cotinine and their combination on cell apoptosis and apoptosis-related genes expression in primary mouse embryonic fibroblast (PMEF) cell line at E13 stage.

**Keywords**

Apoptosis, Cotinine, Embryonic fibroblast cell, Nicotine, Reactive oxygen species.

**Research Hypothesis**

1. Nicotine, cotinine and their combination alter ROS generation and cell apoptosis, of PMEF.

2. ROS induced by nicotine, cotinine and their combination affects cell apoptosis and apoptosis-related genes expression of PMEF.

# CHAPTER II

# REVIEW OF LITERATURE

**Nicotine and cotinine**

Nicotine is the principal alkaloid in tobacco and it comprises approximately 95% of the total alkaloid. One cigarette contains an average of 10 to 14 mg of nicotine (9). Other than nicotine, numerous alkaloids have been identified at different concentration including nornicotine (27-88 μg), cotinine (9-50 μg), anabasine (3-12 μg), anatabine (4-14 μg), myosmine (9 μg), and 2,3' dipyridyl (7-27 μg), N'-methylanabasine, nicotyrine, nornicotyrine, and nicotine-N'-oxide (38).

Nicotine (3-[1-methyl-2-pyrrolidinyl] pyridine) is a tertiary amine composed of a pyridine and a pyrrolidine ring (Figure 1). It can exist in two different three dimensionally structured shapes, called stereoisomers (R- and S- forms). It must be noted that tobacco contains only (S)-nicotine which is the most pharmacologically active form (39). During smoking, 1 to 1.5 mg of nicotine is absorbed systemically (40) through biological membranes (41), small airways and alveoli of the lung which is rapidly absorbed pathway (42).

Blood or plasma nicotine concentrations during daily smoking range 10-37 ng/ml, and typically peak at the completion of smoking range 19-50 ng/ml (43). Plasma half-life of nicotine after cigarette smoking is approximately 2 h and nicotine may be last 6 to 8 h after cessation of smoking. The longer half-life of nicotine at lower concentrations is most likely due to a slow release of nicotine from tissues such as liver, kidney, spleen, and lung whereas the highest affinity (44).

Nicotine is extensively metabolized in liver. This transformation involves two steps. The first step is mediated primarily by a cytochrome P450 system to produce nicotine-1(5)-iminium ion and 5-hydroxynicotine (45, 46) (Figure 1). The second step is catalysed by a cytoplasmic aldehyde oxidase to produce cotinine; the primary metabolite of nicotine. In humans, approximately 70%-80% of nicotine is converted to cotinine (15).

Cotinine ( [ 5S] -1-methyl-5-[ 3- pyridyl] -pyrrolidin-2-one) is further metabolized and only 17 percent is excreted unchanged in urine (47). The highest concentrations of cotinine are found in liver. Cotinine is also present in the blood of smokers approximately at 250-300 ng/ ml (48, 49). These concentrations are much higher than those of nicotine. The average half-life of cotinine is 16 h which is longer than that of nicotine (50).

The cotinine levels are of particular interest as qualitative markers of tobacco use and quantitative indicators of nicotine intake. Because of the long half-life of cotinine, it has been used as a biomarker for daily intake, both in cigarette smokers and in those exposed to environmental tobacco smoke (15). Since a high correlation among cotinine concentrations measured in plasma, saliva, and urine have been found, measurements of cotinine in any one of these fluids can be used as a marker of nicotine intake (51).

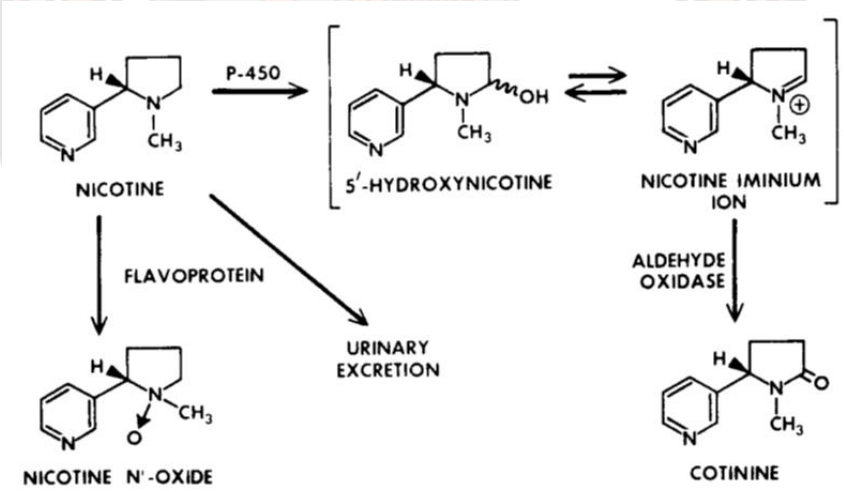


**Figure 1 Major pathways of nicotine metabolism**

**Effects of nicotine and cotinine to embryonic development and cell**

Exposure to tobacco constituents is a risk factor for negative birth outcomes. Adverse effects of nicotine and cotinine on fetal growth/development have been reported elsewhere. Active maternal cigarette smoking has an impact in every trimester of pregnancy, from increased miscarriages in the first trimester (4) to increased premature delivery and decreased fetal growth in the third trimester (5).

In addition, the research of epidemiology found high risk of orofacial cleft of newborns in maternal smokers during pregnancy. In previous studies, meta-analyses found a statistically significant association between maternal smoking and oral clefts (52-54). The evidence associated specifically with orofacial clefts, and smoking tends to support 1.5-fold increased risk of orofacial cleft for maternal smoking compared to non-smoking mother in early pregnancy. This evidence also indicates an exposure-response relationship (55). Moreover, in animal model, it was clear that mouse craniofacial development can be disrupted by exposure to tobacco smoke (8, 10, 56).

According to several studies, nicotine is able to cross the placenta and has been found in amniotic fluid and the umbilical cord blood of neonates (57-59). It was reported that more than 15% of nicotine that mother absorbed during smoking was detected in the fetal circulation within 15–30 minutes. This finding suggested that maternal smoking may directly affect the fetus (11 ). Previous study demonstrated a direct relationship between nicotine concentration and the decreased fetal weight, number of resorptions and malformations, embryotoxicity and intrauterine growth retardation (60 ).

Nicotine is a low molecular weight and has high lipid solubility. As a result, it is absorbed by the mother quickly and easily crosses the placenta into the fetal bloodstream (38, 61). Also, several authors stated that there are evidences for the accumulation of nicotine in fetal serum and amnionic fluid due to slightly higher concentrations of nicotine were found than that found in maternal serum (11, 12). Another interesting finding was a dose effect relationship existed between nicotine intake and smoking-related outcomes of pregnancy (13, 14).

Animal studies confirmed nicotine’s teratogenic effects by disrupting organ development including brain and lungs. This leads to adverse cognitive, emotional, and behavioural outcomes (62). In addition, nicotine is considered as the main

teratogenic substance that alters and delays embryonic development. Consistent nicotine exposure during pregnancy interferes with normal growth and development and palate formation of the fetus in the BALB/c mouse model. Studying the patterns of differential gene expression in response to nicotine exposure throughout pregnancy may provide the key to understanding the impact of nicotine in the genetic and morphological make-up of the developing fetus (10).

Exposure to nicotine in the fetus can be indirectly evaluated by measuring the concentrations of its metabolite (i.e. cotinine) in maternal urine, serum or saliva (63, 64). Due to the specificity and ease of measurement, cotinine offers advantages over other metabolites/toxins in quantifying fetal exposure to cigarette smoking.

Like nicotine, cotinine can rapidly pass-through placenta from mother to child and transplacental transfer occurs throughout pregnancy (16-18). Previous studies investigating third-trimester samples (65, 66) showed a positive linear correlation between maternal and fetal serum cotinine concentration, and the number of cigarettes smoked per day. The fetal cotinine has a great impact on fetal growth and development in a dose-dependent manner. In addition, exposure to smoking during pregnancy is significantly associated with higher cord blood cotinine level.

Abdullah compared the pregnancy outcomes and umbilical cord blood (UCB) cotinine levels between maternal smokers and maternal non-smokers. It was shown that UCB cotinine levels in newborns of maternal smokers were 16.35 (+/-12.84) ng/mL and 0.56 (+/-0.22) ng/mL for the newborns of maternal non-smokers (67).

Many researchers demonstrated that the constituents of cigarette smoke or smokeless tobacco have been shown to cause damage at the cellular level. Also, cigarette smoke or smokeless tobacco are not only absorbed locally but may also enter the systemic circulation. Interestingly, evidence has demonstrated the effects of cigarette smoke or smokeless tobacco in biological processes, including inflammation, antioxidant defence and cell apoptosis. A previous study reported that cigarette smoke condensate (CSC) exposure induced telomere shortening or loss, leading to chromosomal uncertainty, apoptosis, and compromised embryo cleavage in mouse embryonic stem cell (mESCs), which was provoked in telomeres with anomaly (68).

For example, recent study showed that the potential roles of smokeless tobacco extract (STE) that serve in proliferation, cell cycle and apoptosis of human oral mucosa fibroblasts (hOMF) cells. The results indicated that STE increased the rate of cell cycle progression and apoptosis via cell cycle and apoptosis associated proteins (36). Moreover, a study investigated the effects of cigarette smoke extract (CSE) on cell viability and cell death of mouse embryonic stem cells (mESCs) and correlated the oxidative stress induced by CSE with the cell cycle and apoptosis.

Results suggested that cigarette smoke may adversely affect embryonic growth or function. CSEs inhibited cell proliferation by regulating cell cycle-related protein expression and increased oxidative stress by promoting ROS formation, which resulted in apoptosis of mESCs (37). It should be noted that at the beginning of pregnancy, the embryo is highly sensitive to its surroundings, and chemicals that can pass through the placental barrier, such as cigarette smoke, and can cause developmental disability.

As been recognized as the main alkaloid in tobacco, several studies have investigated nicotine and cotinine on cellular and molecular effects. It showed that cotinine is not only the main metabolite of nicotine, but studies also reported the causative effect of cotinine on smoking related disease, such as cancer (2, 42, 69). Furthermore, previous studies investigated the possible direct action of nicotine and cotinine at the level of the embryo. Nicotine concentrations ranged between 0.5 -5 mM and marked inhibition of blastulation was observed at the nicotine concentration of 5 mM. On the other hand, the sensitivity of the embryo to cotinine was low. Studies reported the minimum concentration of cotinine that can disrupt fetal development was 8 mM. However, in the laboratory study, by adding of 0.5 mM nicotine to the media containing 0.8 mM of cotinine, embryos blastulation was inhibited (70).

Moreover, cotinine causes abnormal cell proliferation as demonstrated by increased cell numbers and reactivation of telomerase in a dose dependent manner. Studies demonstrated cotinine's stimulatory effect on vascular smooth muscle cells in vitro at low doses while high doses of cotinine caused a toxic effect (71). High concentration of cotinine may induce malformed at the cranial part of the thoracic neural tube in a chick embryo model (72). In addition, cotinine concentrations (2 mM)

induced marked cell death in pulmonary fibroblast cell line (MRC-5) and showed lower toxicity than nicotine on the MRC-5 cells (73).

Nicotine also affects in cell proliferation of various types of cell especially on mesenchymal derived cells. For example, at concentrations between 1.8 and 3.7 μM, nicotine leads to multiple adverse effects to human embryonic stem cells such as increased cell death (74). The survival and proliferation of human alveolar bone marrow-derived mesenchymal stem cells decreased when the cells were exposed to 5 mM of nicotine (75).

Nicotine in doses ranging from 3.112 μM to 31.125 μM exerted a significant cytotoxicity on human mesenchymal stem cells (MSCs) derived from the human periodontal tissues after 24 h and 48 h (76). In human Wharton's jelly mesenchymal stem cell culture, 5 μM nicotine treatment impaired cell proliferation (77). At the lower concentrations of nicotine, cell proliferation was increased. On the other hand, high concentrations of nicotine at 1 or 10 mM suppressed mESCs (line D3) proliferation and significantly decreased the levels of PCNA, cyclin A, cyclin B and the levels of proteins involved in cell cycle progression (78).

Interestingly, nicotine has special apoptotic effects both *in vitro* and *in vivo*. Some studies have reported that nicotine triggers apoptosis (79-81). Therefore, nicotine can be pro-apoptotic effect depending on the concentration of the substance used, species-related variations in the metabolism of nicotine and the target cells. Previous studies provided an insight into the molecular mechanisms of nicotine pro-apoptotic effects on the liver and kidney. Results showed a significant increase in Bax/Bcl-2 ratio in the nicotine treated mice (82).

Nicotine promoted early apoptosis of osteoblasts was only significantly promoted by treatment with nicotine concentrations of $1x10^4$ and $1x10^3$ mol/l. Nicotine also inhibited mineralized nodule formation in a dose-dependent manner by regulating alkaline phosphatase activity and the expression of osteoblast metabolism-associated genes and proteins (83).

Yu et al. reported the reduced cell viability of human periodontal ligament cells (hPDLCs) after nicotine treatment in a dose dependent manner (84). In addition, study in pulmonary fibroblast cell line (MRC-5) showed the similar results as high nicotine concentrations (2 mM) induced approximately 50% cell death after 24 h (73).

These finding was an evidence that nicotine significantly affected the cell growth rate and apoptosis.

**Apoptosis**

Apoptosis or programmed cell death is an essential role in development, immune response, and normal physiological conditions. It is often found during normal cell turnover, tissue homeostasis, and embryogenesis (85). Apoptosis occurs through an active cellular signalling process triggered by varieties of stimuli such as deprivation of growth/survival factors, exposure to cytotoxic drugs, reactive oxygen species (ROS) or DNA damaging agents, activation of death receptors, and action of cytotoxic cells (86).

Apoptotic cell death is characterised by various morphological and biochemical changes. Morphologically, it is characterized by cell shrinkage followed by the plasma membrane blebbing, nuclear and cytoplasmic condensation, chromatin aggregation, degradation of DNA, partition of cytoplasm and nucleus into membrane bound-vesicles, and formation of apoptotic bodies. *In vitro* observation revealed that the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse (87). These apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells as observed *in vivo* (88).

Apoptosis involves several biochemical features. Induction of apoptosis can occur through two signalling pathways: the extrinsic and intrinsic pathways. Both pathways induce caspase 3 causing fragmentation of DNA at the last step of apoptosis (89). The extrinsic pathway, also called “the death receptor pathway”, is initiated when certain members of the tumor necrosis factor (TNF) receptor family such as Fas death receptor bind to their cognate receptors or ligand, leading to the formation of an intracellular death-inducing signalling complex (DISC) or initiate procaspase 8, procaspase 10. Once activated in the DISC, the initiator caspase activated downstream executioner procaspases to induce apoptosis (90-92) (Figure 2).

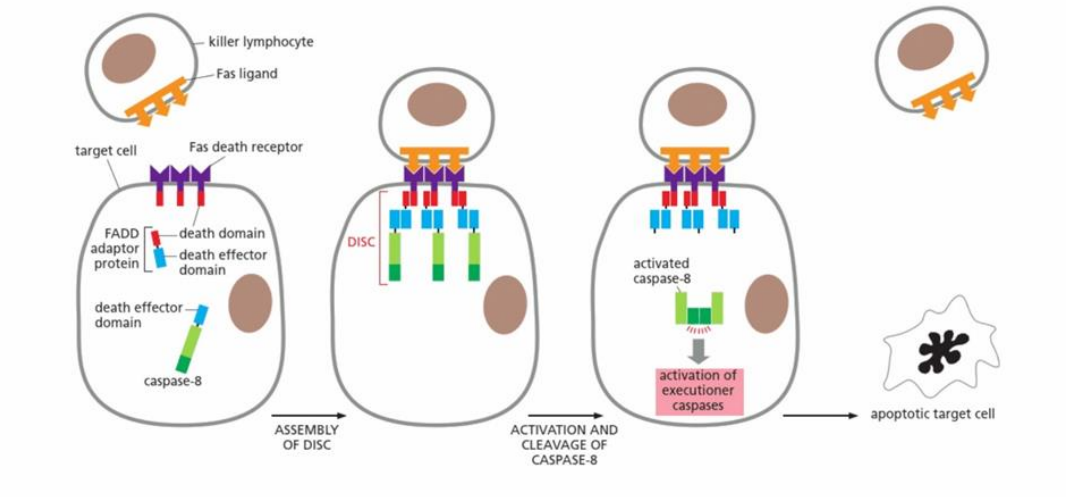


**Figure 2 The extrinsic pathway of apoptosis activated through Fas death receptor**

The intrinsic pathway or "the mitochondrial pathway", is controlled by members of the B-cell lymphoma-2 (Bcl2) family proteins, which regulate mitochondrial outer membrane permeabilization (MOMP). Bcl-2 family proteins have both pro-apoptotic and anti-apoptotic members and the ratio between these two subsets helps determine, in part, the susceptibility of cells to death signals (93).

Once MOMP occurs, mitochondrial intermembrane space proteins such as cytochrome Care detached causing the release of other apoptogenic proteins, including apoptosis inducing factor (94), Smac/Diablo, HtrA2/Omi (serine protease), and endonuclease G. These proteins play crucial roles in the downstream apoptosis-signalling pathway. For example, cytosolic cytochrome C interacts with apoptotic protease and activates factor 1 (Apaf-1) and procaspase 9 to form a complex called apoptosome. This complex facilitates caspase 9 activation and sequentially cleaves and activates the effector caspases 3, 6, and 7 (31, 90, 95, 96) (Figure 3).

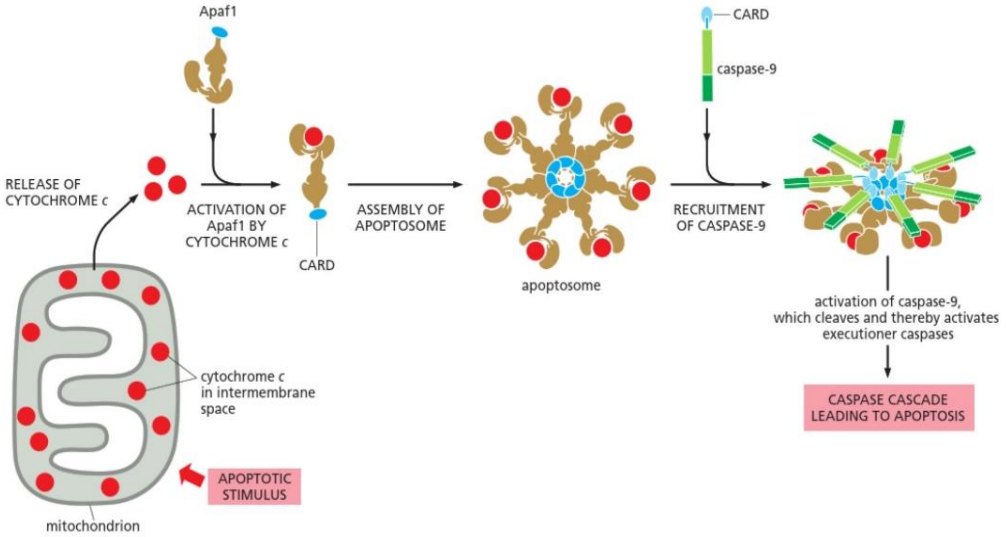


**Figure 3 The intrinsic pathway of apoptosis**

**Bcl2 family proteins (pro-apoptotic or anti-apoptotic members)**

B-cell lymphoma-2 (Bcl2) family proteins are the major mediators of the intrinsic apoptotic pathway by changing the process of MOMP through protein-protein interactions. The Bcl2 family proteins can be partitioned into three groups according to their configuration and intracellular functions as follows: (1) pro-apoptotic Bcl2 family proteins, (2) anti-apoptotic or pro-survival Bcl2 family members and (2) BH3-only proteins. First group, pro-apoptotic Bcl2 family proteins, consists of Bcl2 antagonist/killer and Bcl2-associated X protein (Bax), which are recognized as apoptosis effectors. Bax and Bak comprise of Bcl2 homology (BH) domain 1-3 and can directly MOMP when activated (97). Bak and Bax prevent spontaneous induction of MOMP by binding to anti-apoptotic Bcl2 family proteins (98). In addition, Bax widely expresses in normal tissues whereas Bak expresses in a substantial fraction of cancer such as (99) Hodgkin's disease and aggressive non–Hodgkin's lymphomas (100). Second group of Bcl2 family proteins is anti-apoptotic or pro-survival Bcl2 family members. These proteins are composed of Bcl2, Bcl-X large (BclXL), Bcl2-like protein 2 (BclW), Bcl2-like protein 10 (BclB), myeloid cell leukemia 1 (Mcl1), and Bcl2-related protein A1 (Bfl1) (A1 in mouse). The mechanisms of inhibition are characterized by binding of anti-apoptotic Bcl2 family proteins to BH3-only proteins or Bak/Bax impeding MOMP (101).

Third group is BH3-only proteins such as Bim, Puma, Bid, Bad, Noxa, Bik, Bmf, and Hrk. These polypeptides share 15-25 residue BH3 domain in common with other Bcl2 family proteins (102).

Under normal mitochondria metabolic conditions, electron-transporting complexes also called complex V, FoF1-ATP synthase, coenzyme Q, and cytochrome C carry out oxidative phosphorylation. The a-and b-type cytochromes are inaccessible components of large complexes but cytochrome C is monomeric, freely diffusible in the inner membrane, and in equilibrium between the inner membrane, intermembrane space and cristae (103).

The intrinsic pathway of apoptosis is dependent on Bak and Bax activation by BH3-only proteins. BH3 only proteins such as Bid may promote apoptosis by modifying the structure of Bax leading to its insertion into mitochondrial membranes (104). Bid interaction with Bax, was able to trigger this conformational change in Bax (105). The conformational change leads to the oligomerization of Bax and activation of Bax proteins at specific sites of the mitochondrial outer membrane (106, 107). This circumstance leads to perturbing the permeability of the mitochondrial outer membrane or MOMP which results in the release of cytochrome C into the cytosol and eventually resulting in apoptotic cell death (108).

Moreover, Bax and Bak are directly responsible for breaching the mitochondrial outer membrane, interacting with the mitochondria permeability transition to induce a permeability transition, production of ROS, and in certain settings, releasing of cytochrome C, which sequentially activates a downstream caspase program (109-112).

**Reactive oxygen species (ROS)**

Reactive oxygen species (ROS) can be either oxygen-derived free radicals (i.e. superoxide anion ($O2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$)), or non-radical molecules (i.e. hydrogen peroxide ($H_2O_2$)). ROS can be produced in cells following exogenous and endogenous stimuli. The exogenous agents include chemicals found in tobacco smoke, environmental toxins, medications, UV and radiation (25, 113). The endogenous mechanisms such as immune response, inflammation and any physiological /pathological processes that produce NADPH oxidase complex in cell

membranes, peroxisomes, mitochondria, endoplasmic reticulum and other organelles (114, 115).

Maintaining a balance between oxidants and antioxidants is challenging for any living system in virulent environmental conditions, and a shift in balance could result in accumulation of ROS that further generates “oxidative stress.” The oxidative stress following the imbalance between oxidative and antioxidative defences led to increased ROS levels such as $O_2^{\bullet -}$ and $OH^{\bullet}$ or $H_2O_2$, cause severe cell damage and initiate cell death processes such as apoptosis and/or necrosis (116).

The generation of ROS in cells exists in equilibrium with a variety of antioxidative defences such as superoxide dismutases (117), catalase (CAT), glutathione peroxidase (GPXs) and peroxiredoxins (PRXs) (118). Various antioxidant compounds and enzymes play their roles at respective site and constitute a total defence network system in vivo (119). The effects of antioxidants have been assessed also in human studies and model animals under normal conditions and oxidative stress. The studies confirmed the positive effects of antioxidants and antioxidant rich diets to reduce the level of oxidative stress status in vivo, while others did not show any significant effects of the increase in the antioxidants (120).

However, in the cultured cells respond well to the added antioxidants, the beneficial effect of antioxidants is difficult to observe in normal healthy subjects with sufficient amount of antioxidants (121, 122). Thus, inadequate study design such as choice of antioxidant, its dosage, starting time, duration, and methods of analysis. The choice of subjects may also be critically important. The response may depend on genotype (123, 124).

**Effect of reactive oxygen species on regulators of cell apoptosis**

Effects of ROS on cell is dependent on the quantity and duration of exposure. At low levels, ROS activates cell cycle progression by stimulating signalling cascades and in the case of continued exposure, cell cycles may be arrested. At high concentrations, cell cycle/growth is terminated and cell damage is increased leading to cell apoptosis or necrosis (24).

Normally, mitochondrial outer membrane permeabilization (MOMP) is controlled by the Bcl2 family proteins. The pro-apoptotic Bcl2 family proteins found

in the cytosol (i.e. Bax, Bim, Bak and Bad) form heterodimers with the anti-apoptotic Bcl2 family proteins found in the outer mitochondrial membrane inhibiting their pro-apoptotic properties (32, 125).

ROS can induce changes in pro- and anti-apoptotic Bcl2 family proteins. For example, BH3-only proteins are activated by ROS and then promote cellular expression of pro-apoptotic. Then, activated pro-apoptotic Bcl2 family proteins are translocated to the outer mitochondrial membrane. This process facilitates MOMP by formation of lager channels followed by leakage of pro-apoptotic factors (i.e cytochrome C) into the cytosol and eventually resulting in apoptotic cell death (31, 32).

Furthermore, ROS overproduction induces severe mitochondrial dysfunction (25) and leads to mitochondrial structural oxidation, opening of the mitochondrial permeability transition (MPT) pores (26-28). The increased inner mitochondria membrane permeability allows free passage of molecules (i.e. protons) into the mitochondrial matrix. This results in osmotic swelling of the mitochondrial matrix and compression of vesicles created by infolding of the intercristal space. Increased MOMP accelerates transportation of cytochrome C into cytosol (126, 127).

Afterwards, cytochrome C interacts with Apaf-1 and procaspase 9 and forms a complex called “apoptosome” which sequentially facilitates and activates caspase 3, 6, 7 and 9 (31, 95, 96).

Moreover, excessive cellular levels of ROS also cause damage to mitochondrial membranes by electron mistakenly leaking from the electron transport chain and reacting with $O_2$ to form $O_2^{\bullet-}$. This $O_2^{\bullet-}$peroxide is then converted to $H_2O_2$ and $OH^{\bullet}$, respectively, thus motivating nearby mitochondria to produce more ROS (28, 118, 127).

Structures and functions of membrane permeation channels, exchanger, and pores are under way to confirm ROS roles in an intrinsic apoptotic pathway whether they act together or act separately to promote the mitochondrial events.

Previous studies revealed that increased ROS generation such as $H_2O_2$ and $O_2^{\bullet-}$ resulted in the release of cytochrome C and induction of apoptosis through the mitochondrial pathway (29, 30). A study conducted in HeLa cells also reported that $H_2O_2$ induced apoptosis through the mitochondrial pathway was mediated by p53 (128). $H_2O_2$ also caused initial mitochondrial membrane hyperpolarisation leading to

failure of mitochondrial membrane potential, mitochondrial translocation of Bax and Bad, and cytochrome C release (129).

Moreover, ROS contributes to the endoplasmic reticular stress and DNA damage that changes p53, p21 protein expression. p53 is a key protein mediating cell response to stresses. It was reported that p53 is associated with genes in cell cycle arrest and apoptosis (130). p53 also regulates p21 expression which takes part in cell cycle checkpoints. A previous study publicized that ROS was decisive in initiating apoptotic cell death by activating intrinsic apoptotic pathway and mitochondria signal transducer which contributes to p53-dependent apoptosis (131).

A study conducted in human breast carcinoma MDA-MB-435 cells found elevated ROS level in the ziyuglycoside II treated cells in a dose dependent manner, up-regulated expression of p53, p21 and Bax but down-regulated Bcl-2 expression. This study indicated that ziyuglycoside II induced apoptosis by decreased mitochondrial membrane potential (MMP) as well as increased Bax/Bcl-2 ratio, cytochrome C release and the activity of caspase 3 and caspase 9 (132).

The roles of ROS in apoptosis is also supported by Wang's study. They showed that the teroxirone could induce cytotoxicity in human non-small cell lung cancer cells (NSCLC) which could involve in p53-associated intrinsic apoptosis pathway and ROS production. In their study, teroxirone induced ROS mediated cytotoxicity and the inhibition of teroxirone using antioxidant N-acetylcysteine pretreatment diminished ROS and inverted the expression of Bax, Bcl-2 and cytochrome C. These results suggest the roles of ROS as an effective initial mediator in the p53-dependent intrinsic apoptotic pathway (33).

**Effect of cigarette on reactive oxygen species related to cell apoptosis**

Roles of cigarette on the ROS levels has been widely investigated. The cytotoxic effects of cigarette smoke are associated with its action to augment the intracellular ROS level and diminish protective antioxidants such as superoxide dismutases (117), glutathione peroxidase (GPXs) and catalase (CAT) (22, 23).

In recent years, several studies investigated the role of smokeless tobacco (ST); a tobacco consumed orally or nasally without burning product, on cellular response(133). ST generates free radicals and results in increased oxidative stress, which leads to imbalance between pro- and antioxidants (134, 135).

Moreover, smokeless tobacco extracts (STE) can enter the systemic circulation (136, 137) affecting numerous biological processes, antioxidant protective mechanism and cell apoptosis (138, 139). A recent studies in human oral mucous fibroblasts (hOMF) showed that STE at concentration 200, 400 and 800 μg/ml induced ROS production and that ROS was closely associated with superoxide dismutases (117) and catalase (CAT). These findings indicated that STE inhibited cell proliferation and also induced cell apoptosis by regulating the apoptosis associated proteins; Bax expression levels were increased and Bcl2 expression levels were decreased in a dose-dependent manner (36).

In addition, studies investigated the effects of CSE on cell viability and cell death of mouse embryonic stem cell mESCs and correlated the ROS induced by CSE with the cell cycle and apoptosis. Results suggested that cigarette smoke may adversely affect embryonic cell growth and function. CSEs increased oxidative stress by promoting ROS formation, which resulted in apoptosis of mESCs (37).

Nicotine is a major component of cigarette smoke and patho physiological effects of nicotine are believed to be mediated by nicotinic acetylcholine receptors (nAChRs) in target cell (140, 141). The interaction of nicotine with nAChR has been associated with increased oxidative stress and multiple signaling pathways that regulate the progression, growth, and metastasis of tumors (35, 142).

Several studies have investigated the effects of nicotine related to cell apoptosis. For example, studies reported that nicotine blocks antioxidant enzymes, thereby increasing lipid peroxidation, followed by formation of ROS. ROS in the tissues are able to trigger cytoplasmic membrane damage and DNA fragmentation (143). Elevated ROS is a distinguished cause of the mitochondrial apoptotic pathway and that directly oxidizes DNA and triggers genotoxicity (144, 145).

Moreover, the nicotine negatively causes embryonic malformations. Murine embryos were treated with nicotine at concentration of 0.6- 6 mM. Results showed that nicotine at a concentration of 6mM induced embryonic apoptosis and ROS level.

ROS detection in the developing embryos indicates that nicotine caused oxidative stress and activated apoptosis by increasing intracellular calcium. The study also suggested the role of calcium in apoptosis that inducing of nicotine to nAChRs leads to increased levels of intracellular $Ca^{++}$, which in turn causes $Ca^{++}$ flux into mitochondria (35, 141). This is attended by osmotic swelling and rupture of the mitochondrial membrane and following release of pro-apoptotic proteins into the cytosol eventually causing apoptosis (35, 81, 142). Hence, calcium plays a key role in the initial cell apoptosis or death (146).

# CHAPTER III

# RESEARCH METHODOLOGY

**Population and Samples**

Primary Mouse Embryonic Fibroblast cell line (PMEF)

**Research Variables**

Independent variables: nicotine and cotinine concentration

Dependent variables: viabilities, reactive oxygen species, cell apoptosis and gene alteration

**Research Instrument**

SpectraMax® M3 microplate reader (Molecular Devices, LLC, CA, USA.

Inverted microscope (IX70, Olympus®, Japan)

Nanodrop spectrophotometer (Nanodrop, Thermo Scientific®, USA)

Thermocycler (Veriti™ Thermal Cycler, Foster City, CA, USA)

Roche Light Cycler 480 real time PCR system machine (Roche®, Germany)

Laminar airflow cabinet (ESCO®, USA)

Fluorescence microscope (BX60, Olympus®, Tokyo, Japan)

Pipette (Gilson®, USA)

Culture plates

Pipette tip

**Research materials and chemical agents**

Primary mouse embryonic fibroblast cell line (E13) (Merck®, Kenilworth, NJ, USA)

Cotinine (Sigma®, Saint Louis, MO, USA)

Nicotine (Sigma®, Saint Louis, MO, USA)

Dimethyl sulfoxide (DMSO) (Sigma®, Saint Louis, MO, USA)

Phosphate Buffered Saline (PBS)

Special Dulbecco's modified Eagle medium (DMEM) (Merck®, Kenilworth, NJ, USA)

10% ES cell qualified FBS (Merck®, Kenilworth, NJ, USA)

1% penicillin-streptomycin (Merck®, Kenilworth, NJ, USA)

1% L-glutamine (Merck®, Kenilworth, NJ, USA)

Gelatin solution (Merck®, Kenilworth, NJ, USA)

0.25% trypsin/EDTA solution (Merck®, Kenilworth, NJ, USA)

3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma®, Saint Louis, MO, USA)

Dulbecco's modified Eagle medium (DMEM without phenol red) (Merck®, Kenilworth, NJ, USA)

HT TiterTACSTM Apoptosis Detection Kit (R&D®, USA)

NucleoSpin® RNA Plus (Machery-Nagel GmbH & Co KG)

iScriptTM RT Supermix for RT-qPCR (Bio-Rad®, USA)

LightCycler® 480 SYBR Green I Master (Roche®, Germany)

ROS-Glo $H_2O_2$ Assay (Promega®, Madison, WI, USA)

**Research methods**

**Chemicals**

Nicotine ((-)-Nicotine 36733, Sigma®, Saint Louis, MO, USA) was dissolved in Special Dulbecco's modified Eagle medium (DMEM, Merck®, Kenilworth, NJ, USA) and cotinine ((-)-Cotinine 74003, Sigma®, Saint Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma®, Saint Louis, MO, USA) for 100 mM stock solution followed by dilution in DMEM to a desired concentration prior to use.

**Primary mouse embryonic fibroblast (PMEF) cell line culture and nicotine, cotinine treatments**

Primary mouse embryonic fibroblast (PMEF) cells isolated from CF-1 mouse embryos at day 13 (E13, Merck®, Kenilworth, NJ, USA) were cultured a humidifier incubator (5% $CO_2$ /95% air) at 37°C in Special DMEM supplemented with 10% embryonic stem (ES) cell qualified FBS, 1% penicillin-streptomycin and 1% L-glutamine (Merck®, Kenilworth, NJ, USA). Prior to cell plating, cell culture plates

were coated with gelatin solution (Merck®, Kenilworth, NJ, USA) by adding approximately 3 ml per 25 $cm^2$ of gelatin solution and leaving undisturbed with the lids on in the laminar flow hood at room temperature. After 30 min, the excess gelatin solution was removed. The PMEF cells were subcultured at the exponential growth stage (80%confluence) using 0.25% trypsin/EDTA solution (Merck®, Kenilworth, NJ, USA) and cells at the third -forth passages were used for the experiments.

PMEF cells were treated with 0, 1, 3, 4, 5 mM nicotine, 0, 0.1, 0.5, 1, 5, 10 mM cotinine and 5mM nicotine combined with 0, 0.1, 0.5, 1, 5, 10 mM cotinine. This study in order to test the effect of DMSO on PMEF cell viability, we designed a range of DMSO concentrations (0, 0.001, 0.005, 0.01, 0.05 and 0.1%) were performed as vehicle controls for cotinine treatments, respectively. Previously study suggested that DMSO concentrations should not increase 0.1% are used to study embryotoxic parameters *in vitro* (147, 148). In this study, the result indicated that DMSO are not toxicity to cell after 24 h incubation.

**Cell viability assay**

Optimal concentrations of nicotine and cotinine on cell survival were determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. The 5 x $10^4$ PMEF cells/well were seeded in to 48 well cell culture plate.

PMEF cells were cultured in 300 µl of supplemented Special DMEM. After 18 h, the serum free medium was replaced and cultured for 5 h. PMEF cells were treated with 200 µl the indicated concentrations of nicotine and cotinine.

At 24 h, 300 µl of MTT solution (5 mg/ml, Sigma®, Saint Louis, MO, USA) dissolved in DMEM without phenol red was added to each well and incubated for additional 1 h at 37°C. After that, the MTT solution was removed, and the formazan crystal was solubilized by adding 300 µl DMSO into each well. The absorbance was read at 530 nm on a SpectraMax® M3 microplate reader (Molecular Devices, LLC, CA, USA) and DMSO solution was served as a blank.

**Observation of cell morphology under microscopy**

To assess the effects of nicotine and cotinine on PMEF cell morphology, PMEF cells were observed under an Inverted microscope (IX70, Olympus®, Tokyo, Japan) at 6 and 24 h after nicotine and cotinine treatment. Images were taken using Fluorescence microscope (BX60, Olympus®, Tokyo, Japan).

**Cell apoptosis assay**

To assess the effects of nicotine and cotinine on PMEF cell apoptosis, cells were seeded at 2.5 x $10^4$ cells/well in to 96 well cell culture plate. PMEF cell were cultured in 200 µl of supplemented Special DMEM. After 18 h, the serum free medium was replaced and cultured for 5 h. PMEF cells were treated with 100 µl the indicated concentrations of nicotine and cotinine. At 24 h, apoptotic analysis was performed using a HT Titer TACS™ Apoptosis Detection Kit (R&D®, Minneapolis, MN, USA).

The nicotine and cotinine treated cells were centrifuged at 1000 x g for 3 min and the medium was removed and washed once with 200 µl/well of PBS. Cells were fixed with 200 µl/well of 3.7% buffered formaldehyde for 7 min, centrifuged at 1000 x g for 3 min and the solution was removed and washed twice with 200 µl/well of PBS.

Then, cells were permeabilized with 200 µl/well of 100% methanol for 20 min at room temperature and washed twice with PBS. 50 µl/well of cytonin™ was added and incubated for 15 min at room temperature. Cells were centrifuged at 1000 x g for 3 min and the solution was removed and washed with 200 µl/well of distilled water and centrifuged between each wash. The solution was removed, and cells were covered with 200 µl/well of PBS.

Positive control was treated with 50 µl/well of TAC nuclease and incubated 30 min at 37 ˚C. After incubation, all samples were washed with 200 µl/well of PBS for 2 min and centrifuged between each wash. The cells were quenched with 50 µl/well of 3% hydrogen peroxide and incubated for 5 min at room temperature.

After incubation, the cells were washed with distilled water 200 µl/well and 150 µl/well of TdT labelling buffer were added and incubated 5 min. Then, plates were centrifuged at 1000 g x for 3 min and buffer was removed. Cells were incubated with 50 µl/well of labelling reaction mix (deoxynucleotidyl transferase) at 37°C for 60 min in humidity chamber. After incubation, 150 µl/well of stop buffer were added for stop labelling reaction for 5 min. Cells were centrifuged at 1000 x g for 3 min., and the solution was removed and washed twice with PBS 200 µl/well for 2 min per wash.

The cells were incubated with 50 µl/well of Strep-HRP solution for 10 min at room temperature. After incubation, the cells were washed 4 times with 200 µl/well of PBS 0.1% Tween 20 and centrifuged between each wash. Then, 100 µl/well of TACS-Sapphire substrate were added and incubated for 30 min in the dark at room temperature. The reaction was ceased with 100 µl/well of 0.2 M HCl. The absorbance was read at 450 nm on a SpectraMax® M3 microplate reader (Molecular Devices, LLC, CA, USA).

**Reactive Oxygen Species (ROS) detection**

ROS-Glo $H_2O_2$ Assay (Promega®, Madison, WI, USA) were used to assess the effects of nicotine and cotinine on ROS generation in PMEF. The 2.5 x $10^4$ PMEF cells/well were seeded in to 96 white well cell culture plate. PMEF cell were cultured in 200 µl of supplemented Special DMEM. After 18 h, the serum free medium was replaced and cultured for 5 h. PMEF cells were treated with 20 µl $H_2O_2$ substrate solution and 80 µl the indicated concentrations of nicotine and cotinine.

At 6 h, ROS-Glo $H_2O_2$ assay was performed according to the manufacturer's protocol. ROS-Glo™ detection solution 100 µl was added and incubated at room temperature for 20 min.

For the positive control, at the last 30 min of each treatment, 0.03% hydrogen peroxide ($H_2O_2$, positive control) were incubated for 30 min at 37º C. Subsequently, the positive control wells were washed twice with PBS. The produce generating a luminescent signal that is proportional to $H_2O_2$ concentration and relative luminescence units (RLU) was read at 450 nm on a SpectraMax® M3 microplate reader (Molecular Devices, LLC, CA, USA).

**Gene expression assay by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

The cells were seeded at density of 1 x $10^6$ cells/well in to 6 well cell culture plate. PMEF were cultured in 2500 µl of supplemented Special DMEM. After 18 h, the serum free medium was replaced and cultured for 5 h. PMEF cells were treated with 2000 µl the indicated concentrations of nicotine and cotinine.

At 6 h, the total RNA was extracted using the NucleoSpin® RNA Plus (Macherey-Nagel GmbH & Co KG, Duren, Germany) in accordance with the manufacturer's protocols. The quality and quantity of total RNA extraction was

assessed using the Nanodrop (Thermo Scientific®, Waltham, MA, USA) by calculating the ratio of the absorbance at 260 and 280 nm.

The total RNA was reverse transcribed into the first strand cDNA via a iScript™ reverse transcription Supermix for RT-qPCR (Bio-Rad®, Hercules, CA, USA) according to the manufacturer's protocol. Reverse transcription reactions were performed using the Thermocycler (Veriti™ Thermal Cycler, Foster City, CA, USA) at 25°C for 5 min, 46 °C for 20 min and 95 °C for 1 min.

The relative mRNA expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and evaluated with a LightCycler® 480 SYBR Green I Master (Roche®, Grenzach-Wyhlen, Germany) in accordance with the manufacturer's protocols.

A real time PCR was detected with Roche LightCycler 480 real time PCR system machine (Roche®, Grenzach-Wyhlen, Germany). The cycle conditions were set as pre-incubation at 95˚C for 5 min, denaturation at 95˚C for 10 sec, annealing at 55˚C for 5 sec and elongation 72˚C for 30 sec for a total of 45 cycles. The reactions were run in triplicate. The results were analysed using the $2^{-\Delta\Delta Cq \text{ or } t}$ method. The forward and reverse primers for the target genes and GAPDH are listed below: (82)

BAX

Forward primer: *5'-CTCAAGGCCCTGTGCACTAA-3'*

Reverse primer: *5'-GAGGCCTTCCCAGCCAC-3'*

$BCL_2$

Forward primer: *5'-CTCGTCGCTACCGTCGTGACTTCG-3'*

Reverse primer: *5'-ACCCCATCCCTGAAGAGTTCC-3'*

Caspase3

Forward primer: *5'-TCTGACTGGAAAGCCGAAACTC-3'*

Reverse primer: *5'TCCCACTGTCTGTCTCAATGCCAC-3'*

P53

Forward primer: *5'-GTACCTTATGAGCCACCCGA-3'*

Reverse primer: *5'-AGAAGGTTCCCACTGGAGTC-3'*

GAPDH

Forward primer: *5'-AGAACATCATCCCTGCATCCAC-3'*

Reverse primer: *5'-GTCAGATCCACGACGGACAC*

**Data Analysis**

All experiments were performed in triplicate. Data were analysed using a standard statistical package (SPSS Inc., Chicago, IL, USA) and presented as the means and standard deviation of three identical experiments made in triplicate.

The effects of nicotine, cotinine and their combination on cell viability, ROS generation and cell apoptosis were analysed using one-way ANOVA followed by Tukey's test for multiple comparison. The effects of nicotine, cotinine and their combination on apoptosis-related gene expression were analysed using Kruskal-Wallis followed by Mann-Whitney U test for comparison between group. The level of statistically significant difference was set at $p < 0.05$.

# CHAPTER IV

# RESULTS

**Effect of nicotine and cotinine on cell viability**

Cell viability was determined by MTT assay. Twenty-four hours after nicotine /cotinine treatment was chosen as the time-point for the subsequent experiments. As shown in Figure 4, 5 and 6, the cell viability of PMEF cells was decreased by nicotine, cotinine and 5mM nicotine combined with cotinine in a dose-dependent manner. The effect of nicotine on cell viability of PMEF cells at concentrations of 1, 3, 4 and 5 mM was 106.18%, 60.60 %, 41.46% and 31.41%, respectively (Figure 4). The results indicated that cell viability of PMEF cells was not significantly increased with the treatment of nicotine at 1 mM.

On the other hand, the results indicated that cell viability was significantly decreased with the treatment of nicotine at 3, 4 and 5 mM in comparison to that of the control. Treatment of nicotine at 4 mM and 5 mM was significantly decreased cell viability compared to the 3 mM nicotine concentration. However, no significant difference was found between 4 mM and 5 mM of nicotine.

The PMEF cell viability at cotinine concentrations of 0.1, 0.5, 1, 5 and 10 mM was 55.30%, 42.94%, 41.85%, 35.33% and 35.00%, respectively (Figure 5). In this study, the cell viability was significantly decreased in all cotinine treated groups compared the control. In addition, cell viability of 0.1 mM cotinine treated group was significant higher than the remaining cotinine treated groups. No other significant difference was found.

Cell viability after a combined treatment of 5 mM nicotine and cotinine concentrations at 0, 0.1, 0.5, 1, 5 and 10 mM was 31.36%, 24.64%, 22.13%, 18.71%, 13.03 % and 7.26 %, respectively (Figure 6). The results clearly showed a significant decreasing of cell viability in a concentration dependent relationship compared to the control (5 mM nicotine). In addition, the cell viability was significantly decreased with the combined treatment of 5 mM nicotine and 5 and 10 mM cotinine concentrations in comparison to the 5 mM nicotine combined with 0.1, 0.5, 1 mM

cotinine concentration. In Figure 7, the results indicated that the cell viability was significantly decreased with the combined treatment of 5mM nicotine and cotinine compared to the cotinine treated group (control). This suggested that 5mM nicotine combined with cotinine decreased the cell viability in a dose-dependent manner.

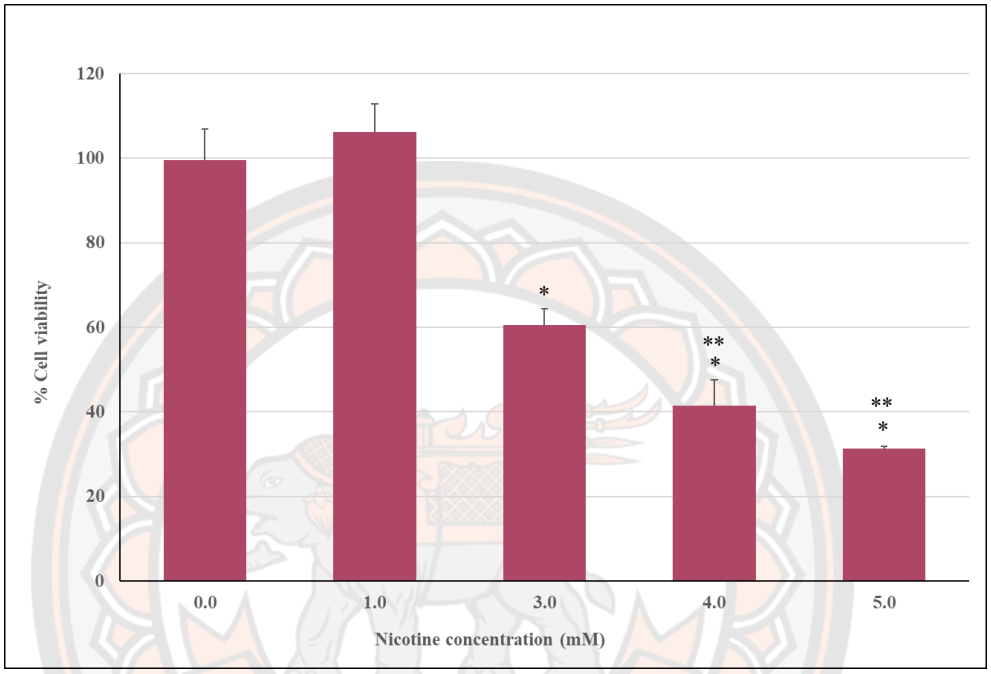


**Figure 4 Cell viability (mean ± SD) of PMEF cells cultured in nicotine (0, 1, 3, 4 and 5 mM) at 24 hours determined using MTT assay**

**Note:** *, ** indicates statistically significant difference compared with the 0 mM (control) and 3 mM of nicotine, respectively ($p < 0.05$)

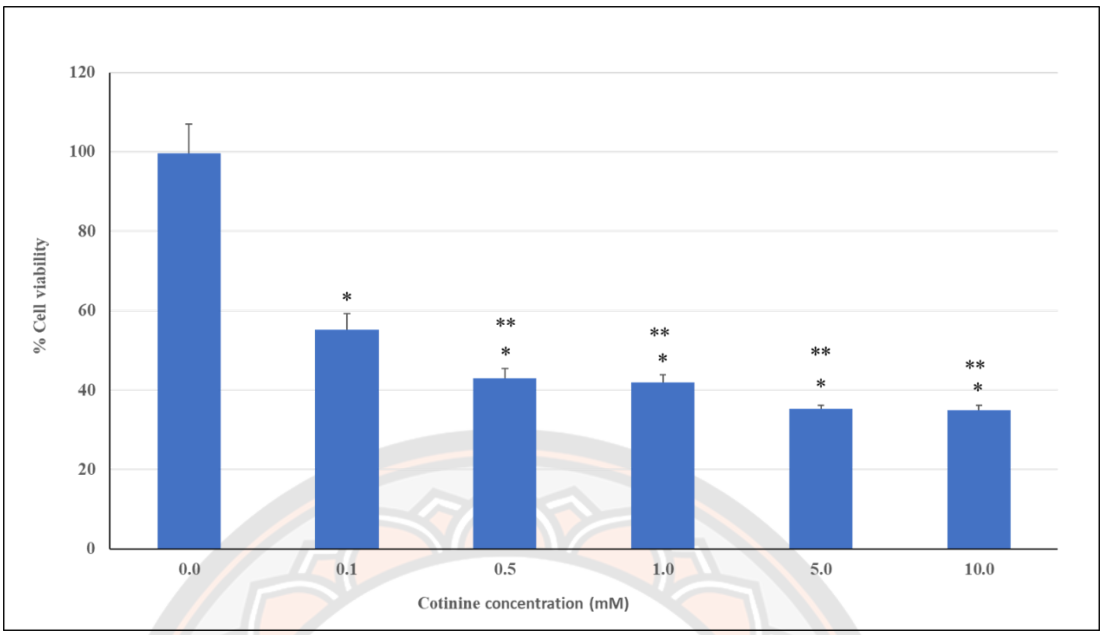


**Figure 5 Cell viability (mean ± SD) of PMEF cells cultured in cotinine (0, 0.1, 0.5, 1, 5 and 10 mM) at 24 hours determined using MTT assay**

**Note:** *, ** indicates statistically significant difference compared with the 0mM (control) and 0.1 mM of cotinine, respectively ($p < 0.05$)

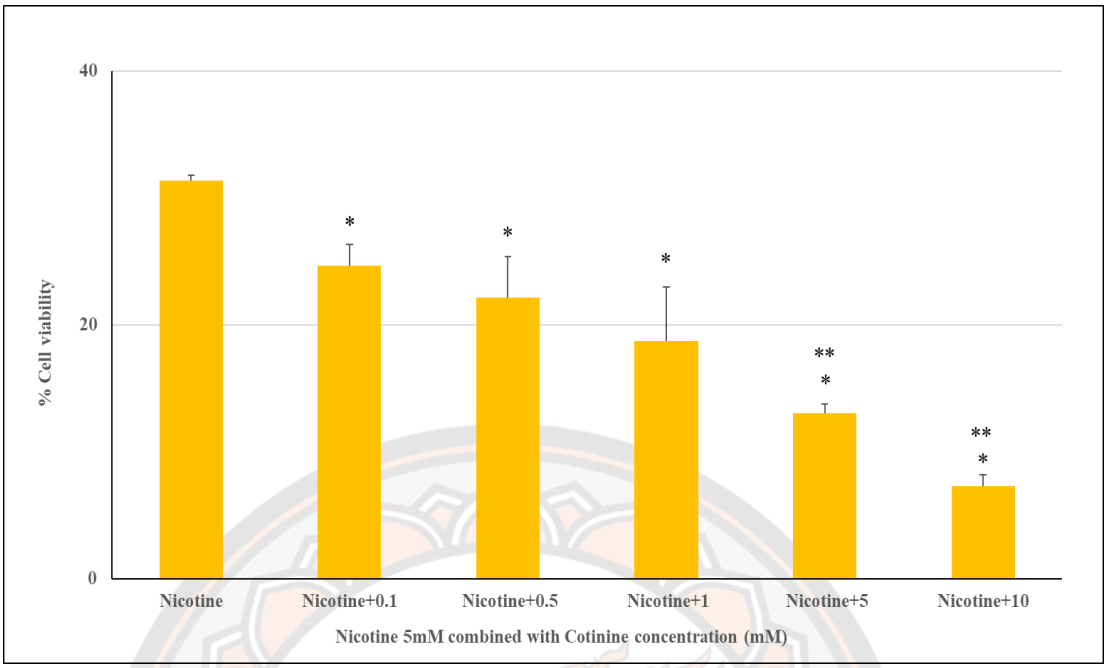


**Figure 6 Cell viability (mean ± SD) of PMEF cells cultured in 5 mM nicotine combined with cotinine (0, 0.1, 0.5, 1, 5 and 10 mM) at 24 hours determined using MTT assay**

**Note:** *, ** indicates statistically significant difference compared with the 5 mM of nicotine (control) and 5 mM nicotine combined with cotinine (0.1, 0.5 and 1 mM), respectively ($p < 0.05$)

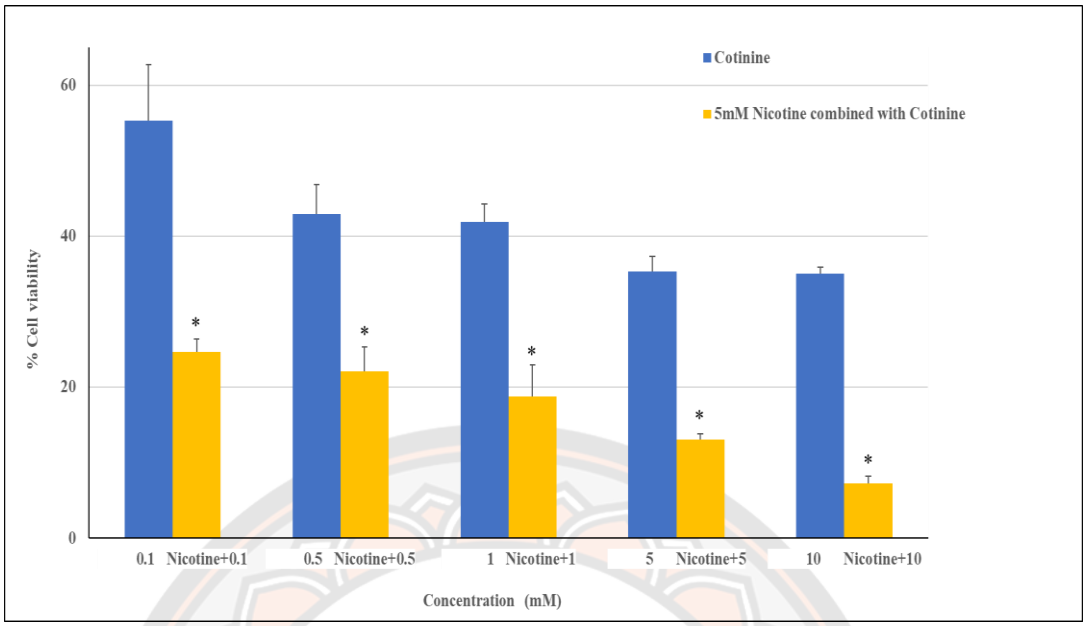


**Figure 7 Cell viability (mean ± SD) of PMEF cells cultured in cotinine (0.1, 0.5, 1, 5 and 10 mM) compared with 5 mM nicotine combined with cotinine (0.1, 0.5, 1, 5 and 10 mM) at 24 hours determined using MTT assay**

**Note:** *indicates statistically significant difference compared with the cotinine (0.1, 0.5, 1, 5 and 10 mM), respectively ($p < 0.05$)

**Effect of nicotine and cotinine on cell morphology**

Morphological changes of cells were determined using Inverted microscope (IX70, Olympus®, Tokyo, Japan) and images were taken using Fluorescence microscope at ×400 magnification (BX60, Olympus®, Tokyo, Japan).

Figure 8 showed PMEF cell morphology at 24 h after nicotine treatment at concentrations of 0, 3, 4 and 5 mM.

Figure 9 showed PMEF cell morphology at 24 h of the control (no treatment) and cotinine treatment at 0.5, 1and 5 mM.

Figure 10 showed PMEF cell morphology at 6 and 24 h of the control (5 mM nicotine) and 5 mM nicotine combined with cotinine at 0.5, 1 and 5 mM.

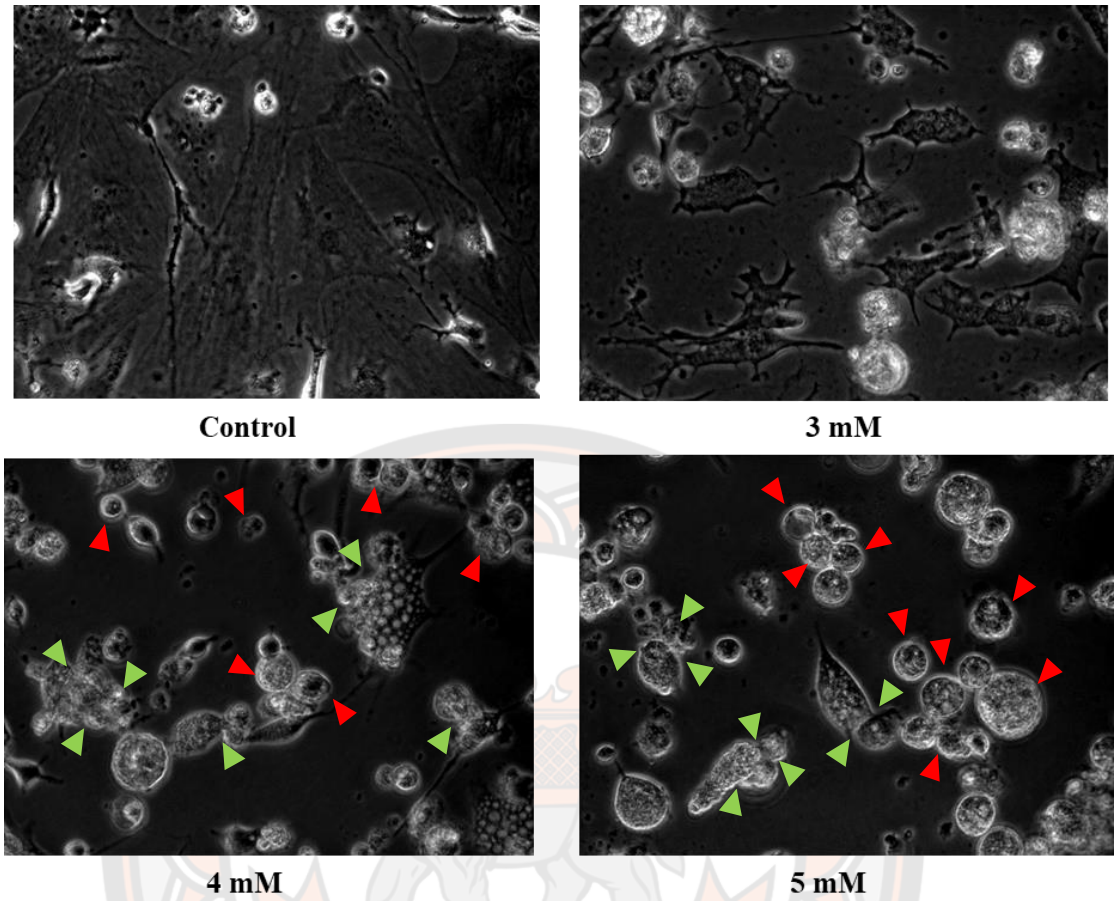


**Figure 8 Morphological with the treatment of nicotine at 3 mM, the cells showed a large amount of cell exhibiting irregular form and in closed contact with neighbouring cells. Treatment with nicotine at 4 and 5 mM showed the cell shrinkage and loose contact to neighbouring cells. Its chromatin condensed followed by blebbing or budding of the plasma membrane (green arrowhead), and fragmentation of the cell into compact membrane-enclosed structures called 'apoptotic bodies' (red arrowhead) (×400 magnification, Fluorescence microscope BX60, Olympus®, Tokyo, Japan).**

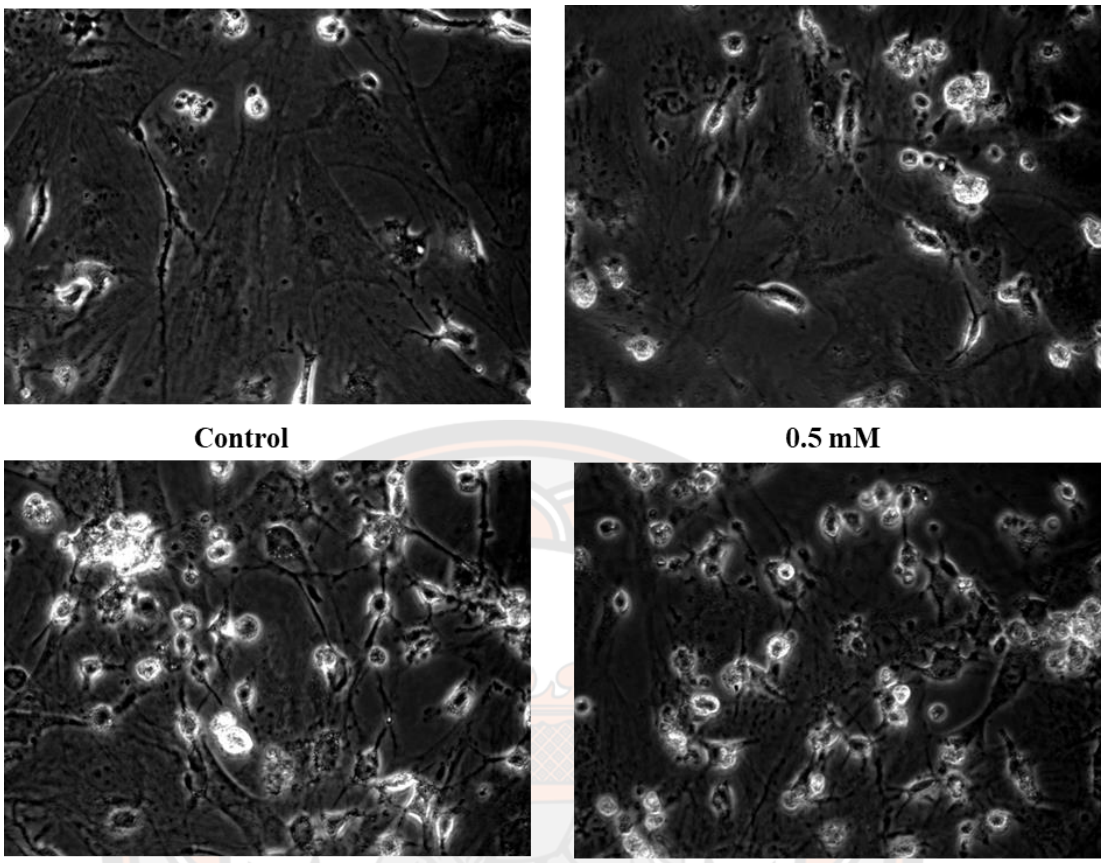


**Figure 9 Treatment with cotinine in all groups showed cell close contact with neighbouring cells, changed in cell shape through shrinking, and an increased degree of condensation in a dose-dependent manner. At 5 mM cotinine cells showed deformation and loosened contact to its neighbouring cells (×400 magnification, Fluorescence microscope BX60, Olympus®, Tokyo, Japan)**

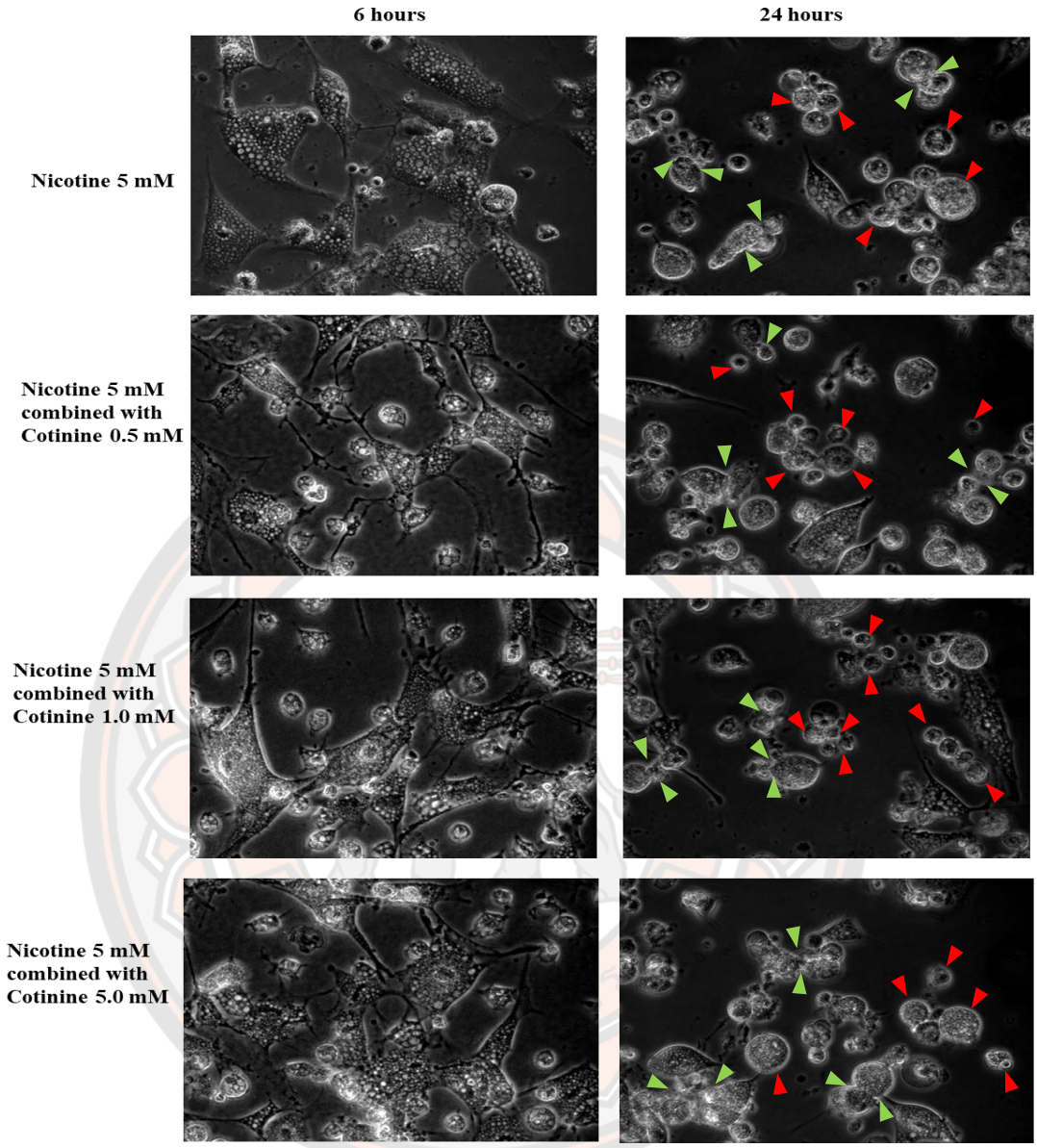


**Figure 10 At 6 h of nicotine at 5 mM, cells exhibited swelling, polymorphic vacuole formation and condensed nuclei. At 5 mM nicotine combined with cotinine at 0.5, 1 and 5 mM, cells displayed an irregular form, close contact with neighbouring cells, and increased the degree of condensation in a dose-dependent manner. At 24 h, all of cell treated groups showed the characterised morphologically of apoptosis, green and red arrowhead indicated plasma membrane blebbing and apoptotic bodies, respectively (×400 magnification, Fluorescence microscope BX60, Olympus®, Tokyo, Japan)**

**Effect of nicotine and cotinine on cell apoptosis**

Cell apoptosis was determined by Cell apoptosis assay. Twenty-four hours after nicotine/cotinine treatment was chosen as the time-point for the subsequent experiments. As shown in Figure 11, 12 and 13, the cell apoptosis of PMEF cells was increased by nicotine, cotinine and 5mM nicotine combined with cotinine in a dose-dependent manner.

The effect of nicotine on cell apoptosis of PMEF cells at concentrations of untreated (0), 3 and 5 mM was 40.17 %, 52.62% and 90.03%, respectively (Figure 11). This result indicated that cell apoptosis was significantly increased with the treatment of nicotine at 3 and 5 mM in comparison to that of the untreated control. Nicotine at 5 mM was also significantly induce cell apoptosis compared to the 3 mM of nicotine. The PMEF cell apoptosis at cotinine concentrations of untreated (0), 0.5, 1 and 5 mM was, 25.64%, 30.44%, 30.53% and 32.54%, respectively. In this study, cell apoptosis was significantly enhanced by cotinine in all cotinine treated groups compared the untreated control. No significant difference was found among cotinine treated groups.

Figure 13 revealed the cell apoptosis of 5 mM nicotine combined with cotinine treatment groups compared to the untreated control. Cell apoptosis of untreated cell and after a combined treatment of 5 mM nicotine and cotinine concentrations of 0, 0.5, 1 and 5 mM was 25.64%, 65.44%, 70.75%, 71.38%, and 75.32%, respectively. The results clearly showed a significant increasing of cell apoptosis in a concentration dependent relationship. This result suggested that 5mM nicotine combined with cotinine increased the cell apoptosis in a dose-dependent manner.

In addition, a combined treatment of 5 mM nicotine and 5 mM cotinine significantly increased cell apoptosis in comparison to the 5 mM nicotine. No other significant differences were found.

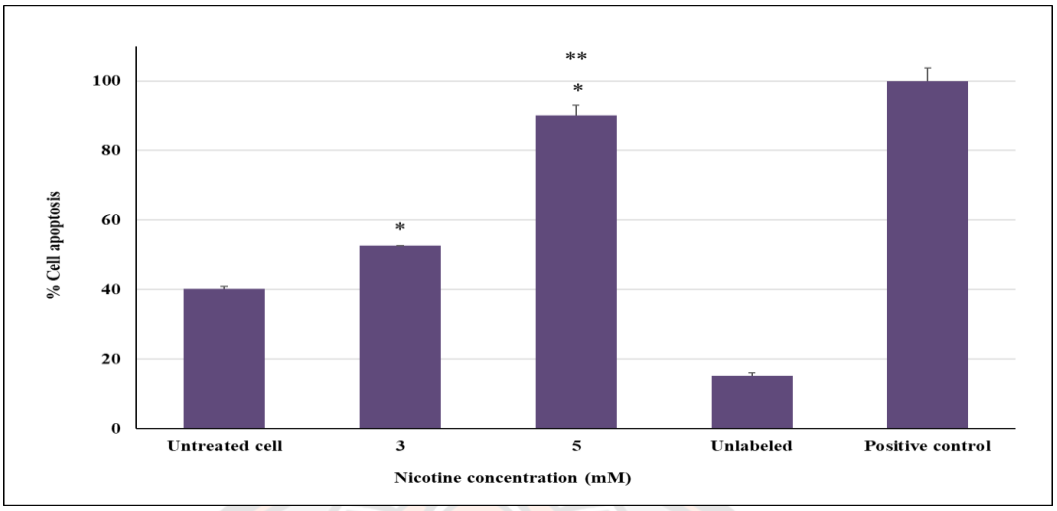


**Figure 11 Cell apoptosis (mean ± SD) of PMEF cells cultured in nicotine (untreated, 3 and 5 mM) at 24 hours determined using HT Titer TACS™.**

**Note:** *, ** indicates statistically significant difference compared with the untreated (control) and 3 mM of nicotine, respectively ($p < 0.05$)

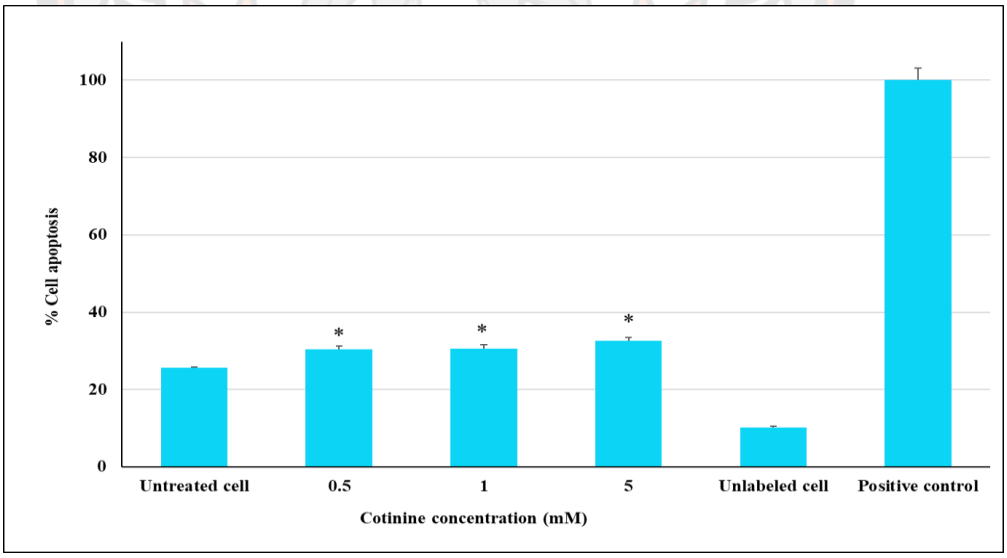


**Figure 12 Cell apoptosis (mean ± SD) of PMEF cells cultured in cotinine (untreated, 0.5, 1 and 5 mM) at 24 hours determined using HT Titer TACS™.**

**Note:** * indicates statistically significant difference compared with the untreated (control) ($p < 0.05$)

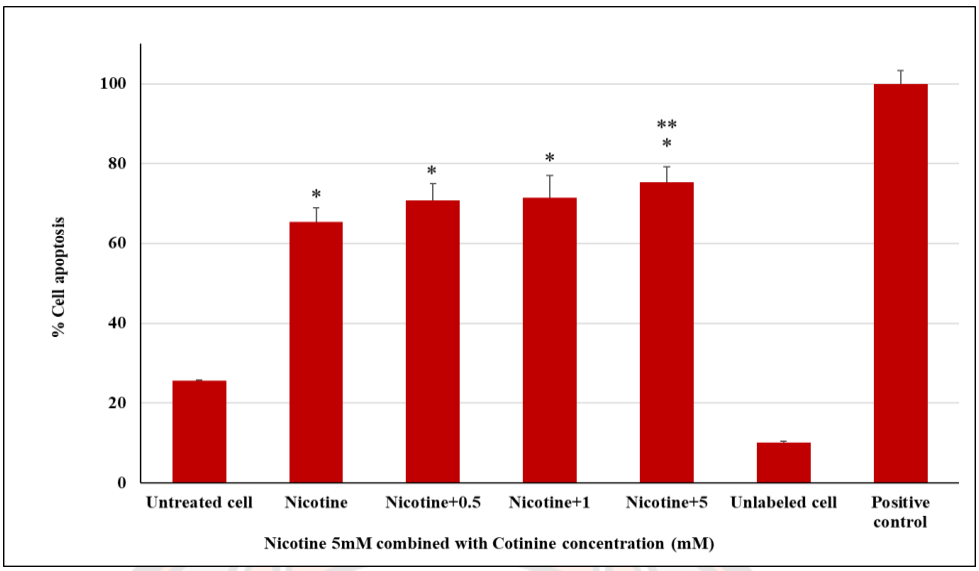


**Figure 13 Cell apoptosis (mean ± SD) of PMEF cells cultured in 5 mM nicotine combined with cotinine (0, 0.1, 0.5, 1 and 5 mM) at 24 hours determined using HT Titer TACS™**

**Note:** *, ** indicates statistically significant difference compared with the untreated (control) and 5 mM of nicotine, respectively ($p < 0.05$)

**Effect of nicotine and cotinine on the generation of ROS**

To determine causative effect of nicotine and cotinine on oxidative stress and ROS generation in PMEF cells, 6 hours after nicotine/cotinine treatment was chosen as the time-point for the subsequent experiments. ROS-Glo $H_2O_2$ assay was used to measure the ROS generation ($H_2O_2$). The luminescence (RLU) was proportional to $H_2O_2$ concentration. High luminescence (RLU) indicates high $H_2O_2$ production in PMEF cells. Positive control was $H_2O_2$.

In this study, the ROS generation in PMEF cells was induced by nicotine, cotinine and 5mM nicotine combined with cotinine in a dose-dependent manner as shown in Figure 14, 15 and 16. ROS generation at nicotine concentrations of 0 (control), 3, 4 and 5 mM was 3156.50, 4311.90, 4789.60 and 5222.86, respectively (Figure 14). The average luminescence (RLU) was gradually increased with the treatment of nicotine. However, the significant difference was found only the concentration of 5 mM compared to the control.

Cotinine induced ROS generation in PMEF cell at concentrations of 0, 0.1, 0.5, 1 and 5 mM was 2065.13, 3298.86, 3499.93, 3661.16 and 3380.10, respectively (Figure 15). Average luminescence (RLU) was significantly increased in all cotinine treated groups compared the control. In addition, average luminescence (RLU) was not significant different among cotinine treated group.

The generation of ROS in PMEF cell after a combined treatment of 5 mM nicotine and cotinine concentrations of 0, 0.1, 0.5, 1 and 5 mM was 4913.46, 5248.6, 5529.03, 4942.83 and 5014.70, respectively and the untreated (control) was 2065.13 (Figure 16). The results clearly showed a significant higher average luminescence (RLU) of the combined treatment compared to the control.

Figure 17 revealed the average luminescence (RLU) of cotinine treated groups (control) in comparison to 5 mM nicotine combined with cotinine treated groups. Results indicated that the average luminescence (RLU) was significantly increased in all combined treatment of 5mM nicotine and cotinine compared to the cotinine treatment alone.

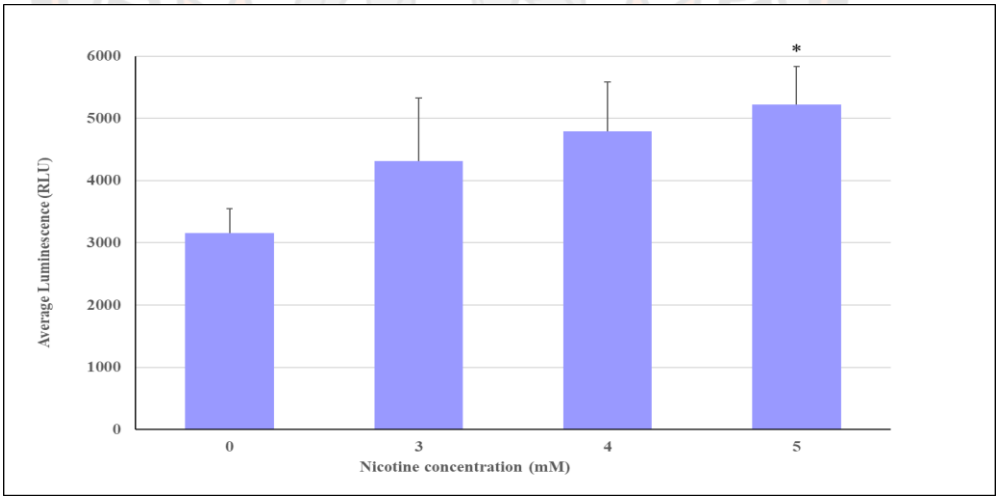


**Figure 14 ROS generation (mean ± SD) of PMEF cells cultured in nicotine (0, 3, 4 and 5 mM) at 6 hours determined using ROS-Glo $H_2O_2$ assay**

**Note:** * indicates statistically significant difference compared with the 0 mM (control) ($p < 0.05$)

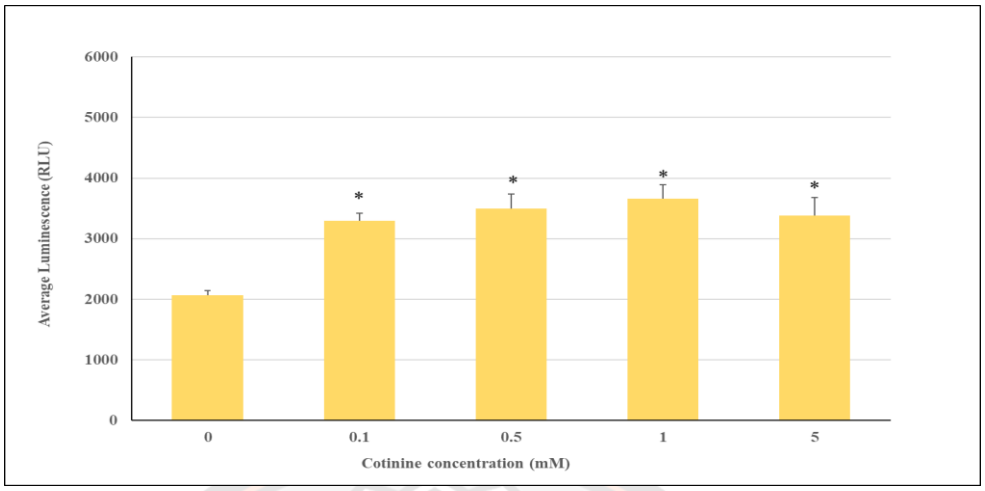


**Figure 15 ROS generation (mean ± SD) of PMEF cells cultured in cotinine (0, 0.1, 0.5, 1 and 5 mM) at 6 hours determined using ROS-Glo $H_2O_2$ assay**

**Note:** * indicates statistically significant difference compared with the 0 mM (control)
($p < 0.05$)

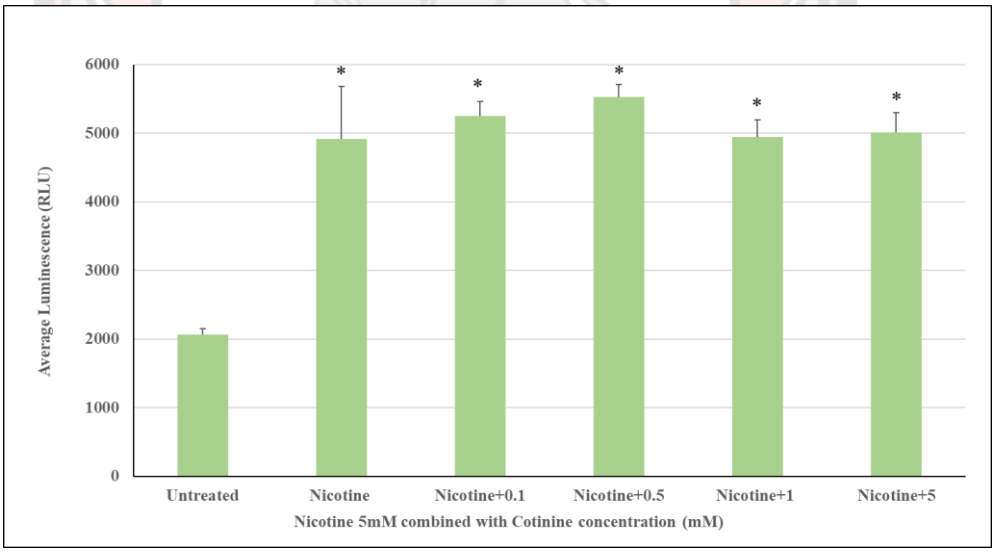


**Figure 16 ROS generation (mean ± SD) of PMEF cells cultured in 5 mM nicotine combined with cotinine concentration (0, 0.1, 0.5, 1 and 5 mM) at 6 hours determined using ROS-Glo $H_2O_2$ assay**

**Note:** * indicates statistically significant difference compared with the untreated (control) ($p < 0.05$)

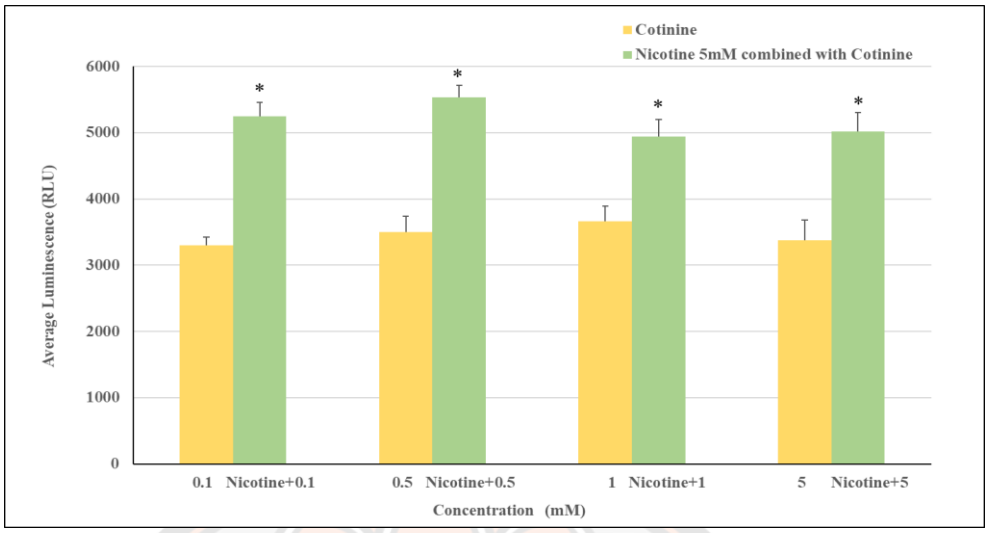


**Figure 17 ROS generation (mean ± SD) of PMEF cells cultured in cotinine (0.1, 0.5, 1 and 5 mM) compared with 5 mM nicotine combined with cotinine (0.1, 0.5, 1 and 5 mM) at 6 hours determined using ROS-Glo $H_2O_2$ assay**

**Note:** * indicates statistically significant difference compared with the cotinine concentration (0.1, 0.5, 1 and 5 mM), respectively ($p < 0.05$)

**Gene expression assay by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

This study also determined the effects of nicotine and cotinine on expression of apoptosis-related genes. To verify expression of apoptosis-related genes, reverse transcription-quantitative polymerase chain reaction was performed on 4 different expressed genes (BAX, BCL2, Caspase3, P53). The results were analysed using the $2^{-\Delta\Delta Cq}$ or t method. The relative mRNA expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Six hours after nicotine/cotinine treatment was chosen as the time-point for the subsequent experiments. Data are reported as mean ± standard deviation of 3 independent experiments Figure 18 showed mRNA expression level of BAX gene. Treatment of 0.5 mM cotinine, 5 mM nicotine and 5mM nicotine combined with 0.5 and 1 mM cotinine increased expression of BAX by 1.47, 1.61, 2.92 and 5.21, respectively, compared to the control. In addition, 5 mM nicotine combined with 0.5

and 1 mM of cotinine significantly increased gene expression compared to the 5 mM nicotine concentration. The mRNA expression level of BCL2 (Figure 19) was increased significantly in comparison to that of the control with the treatment of 0.5 mM, 1 mM cotinine, 5 mM nicotine and 5mM nicotine combined with 0.5 and 1 mM cotinine (3.41, 2.32, 3.51, 5.15 and 10.31, respectively). In comparison to the 5 mM nicotine treatment.

Moreover, mRNA expression levels of Caspase 3 at 0.5, 1 mM cotinine, 5 mM nicotine and 5mM nicotine combined with 0.5, 1 mM cotinine (Figure 20) were 1.04, 1.01, 2.17, 2.67 and 4.33, respectively. The expression of P53 (Figure 21) was 1.09, 1.06, 2.05, 2.79 and 3.15, respectively. Both Caspase 3 and P53 were significantly enhanced in comparison to that of the control with the treatment of 5 mM nicotine and 5mM nicotine combined with 0.5 and 1 mM cotinine.

Furthermore, treatment with 5 mM nicotine combined with 1 mM cotinine were significantly induced Caspase 3 and P53 gene expression compared to the 5 mM nicotine concentration.

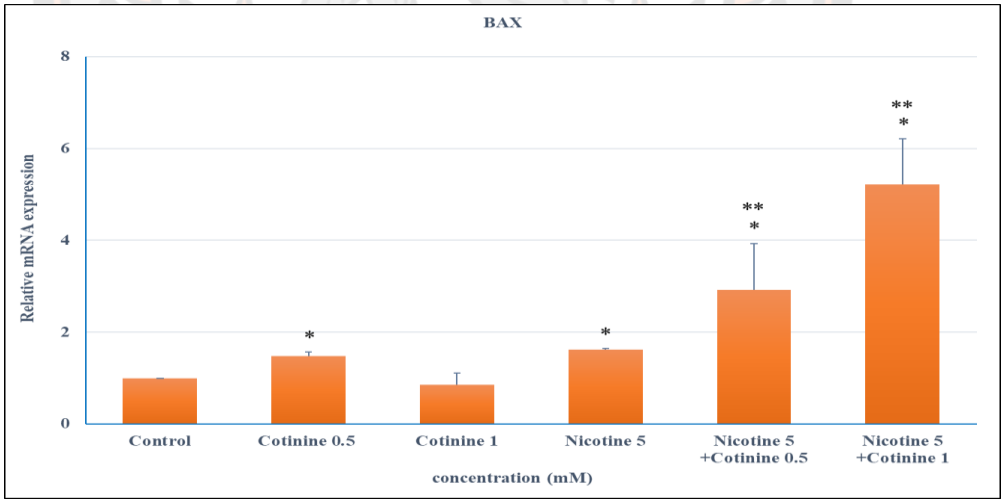


**Figure 18 The relative mRNA expression level of BAX (mean ± SD) in PMEF cells cultured in cotinine (0.5 and 1 mM), nicotine (5 mM) and nicotine 5 mM combined with cotinine (0.5 and 1 mM) at 6 hours determined using RT-qPCR**

**Note:** *, ** indicates statistically significant difference compared with the control and 5 mM of nicotine, respectively ($p < 0.05$)

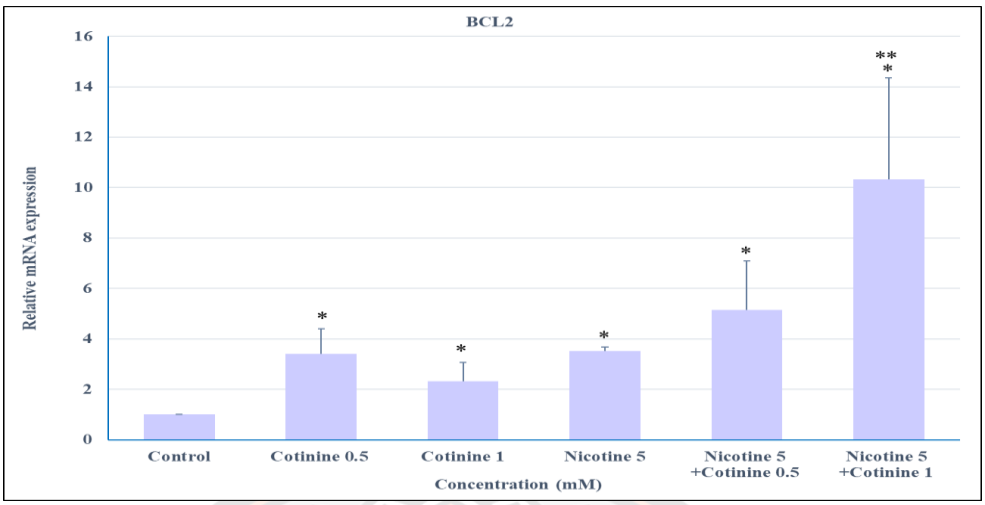


**Figure 19 The relative mRNA expression level of BCL2 (mean ± SD) of PMEF cells cultured in cotinine (0.5 and 1 mM), nicotine (5 mM) and nicotine 5 mM combined with cotinine (0.5 and 1 mM) at 6 hours determined using RT-qPCR**

**Note:** *, ** indicates statistically significant difference compared with the control and 5 mM of nicotine, respectively ($p < 0.05$)

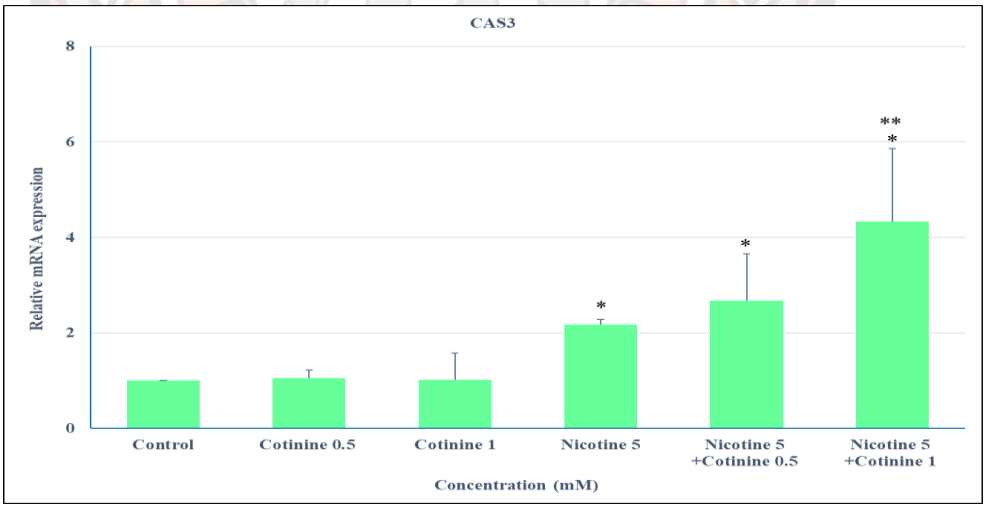


**Figure 20 The relative mRNA expression level of Caspase 3 (mean ± SD) of PMEF cells cultured in cotinine (0.5 and 1 mM), nicotine (5 mM) and nicotine 5 mM combined with cotinine (0.5 and 1 mM) at 6 hours determined using RT-qPCR**

**Note:** *, ** indicates statistically significant difference compared with the control and 5 mM of nicotine, respectively ($p < 0.05$)

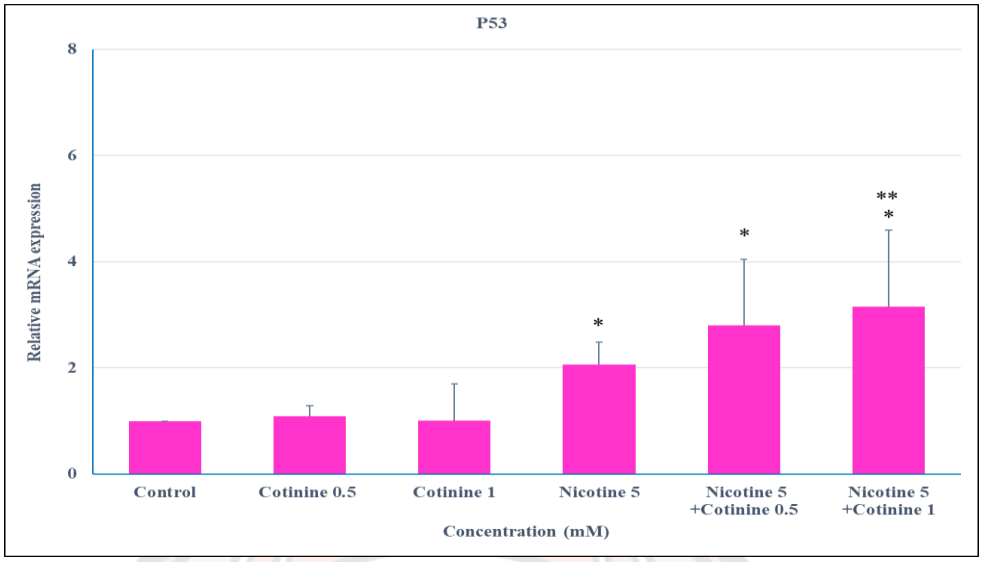


**Figure 21 The relative mRNA expression level of P53 (mean ± SD) of PMEF cells cultured in cotinine (0.5 and 1 mM), nicotine (5 mM) and nicotine 5 mM combined with cotinine (0.5 and 1 mM) at 6 hours determined using RT-qPCR**

**Note:** *, ** indicates statistically significant difference compared with the control and 5 mM of nicotine, respectively ($p < 0.05$)

# CHAPTER V

# DISCUSSION AND CONCLUSION

This study aims to investigate the effect of nicotine, cotinine and their combination on ROS generation, cell viability, cell apoptosis and apoptosis-related genes expression of PMEF cell *in vitro.* PMEF is mesenchymal cells that is going to differentiate into various morphological structures and organs of mammalian embryo, especially craniofacial formation. Previous studies have shown the teratogenic effects of nicotine on maternal smoking. The evidence specifically supported the 1.5-fold increased risk of orofacial cleft from maternal smoking compared to non-smoking mothers in early pregnancy (55).

Nicotine is the substance at which we are interested in this study. Nicotine is the majority, accounting for approximately 95% of tobacco alkaloid content (9). Cigarette smoke or tobacco alkaloids are not only absorbed locally but may also enter through systemic circulation. Interestingly, cigarette smoke can alter biological processes, including inflammation, antioxidant defence, and cell apoptosis (19-21). The cytotoxic effects of cigarette smoke are associated with its action to augment the intracellular ROS level (22) and diminish protective antioxidant enzymes (23). The generation of ROS may induce apoptosis in various cell types.

Therefore, this study further investigated whether nicotine, cotinine and their combination could stimulate ROS generation in PMEF cells. Hydrogen peroxide ($H_2O_2$), a non-radical oxidant of ROS was detected in the study. $H_2O_2$ is stable, long half-life and concentrate as the principal ROS members (149). High concentration of $H_2O_2$ results in cell injury by damaging key cellular molecules such as DNA and lipids (150).

The study was shown, treatment of PMEF cell with nicotine, cotinine and their combination induced the ROS generation. ROS production was gradually increased with the higher concentration of nicotine. However, the significant ROS production was observed only at 5 mM of nicotine treatment compared to the no treatment (Figure 14). All cotinine treated groups significantly induced ROS formation compared to the untreated control (Figure 15). Interestingly, In Figured 16,

a combined treatment of 5 mM nicotine and cotinine clearly evidenced high ROS generation in all doses of their combination. This study also prove that the combined treatment of nicotine and cotinine remarkably promoted ROS production than the cotinine treatment alone (Figure 17). This result is consistent with previous study, ROS can be produced in cells following exposure to exogenous and endogenous stimuli. The exogenous agents include chemicals found in tobacco smoke, environmental toxins, medications, UV, and radiation (25, 113). In recent years, several studies investigated the role of smokeless tobacco (ST), a tobacco product that is either chewed or inhaled without burning, on cellular response (133). ST generates free radicals and increases oxidative stress, which leads to an imbalance between prooxidants and antioxidants (134, 135). For example, a study involving the effects of ST on human oral mucous fibroblasts (hOMF) demonstrated that ST induced reactive oxygen species (ROS) production (117). These findings indicate that ST induces cell apoptosis by regulating apoptosis-associated proteins, including an increase in Bax expression and a decrease in Bcl2 expression in a dose-dependent manner (36). In addition, a study suggested that cigarette smoke may adversely affect embryonic cell growth and function. CSEs increase oxidative stress by promoting ROS formation resulting in apoptosis of mESCs (37). Imbalanced between ROS formation and antioxidants promotes cell apoptosis and death (151).

The nicotine negatively causes embryonic malformations. Murine embryos were treated with nicotine at concentration of 0.6- 6 mM. Results showed that nicotine at a concentration of 6 mM induced embryonic apoptosis and ROS level. ROS detection in the developing embryos indicates that nicotine caused oxidative stress and activated apoptosis by increasing intracellular calcium (152). The result in this study is consistent with previous study. As a result, ROS affect to cell viability and induce cell apoptosis. The nicotine at concentrations (3, 4, and 5 mM) was shown whether cell viability was decreased influenced by nicotine (Figure 4). Nicotine concentration of 4 and 5 mM also significantly reduced cell viability compared to the 3 mM of nicotine.

Apoptotic cells were examined through a light microscope. Regarding morphology in Figure 8, at 3 mM of nicotine treatment showed a large amount of cells exhibiting irregular form and in close contact with neighbouring cells, as well as

an increased degree of condensation. With the higher concentration of nicotine treatment at 4 and 5 mM revealed significant changes in cell shape such as cell shrinkage, deformation and loose contact to neighbouring cells. During this process, cell chromatin condenses and marginates at the nuclear membrane, followed by blebbing or budding of the plasma membrane, and finally fragmentation of the cell into compact membrane-enclosed structures called 'apoptotic bodies', which contain cytosol, the condensed chromatin, and organelles (153-155). The cell morphology is closely related to the amount of cell apoptosis in a dose-dependent manner (Figure11). The results indicated that 5 mM of nicotine may have a strong effect on PMEF cells creating the highest apoptosis among the concentration of treated nicotine. This result close related with previous study which found that the inhibition of blastulation occurred at a nicotine concentration of 5 mM (70). In addition, the survival and proliferation of human alveolar bone marrow-derived mesenchymal stem cells decreased when the cells were exposed to 5 mM of nicotine (75).

In cotinine treated group (Figure 5), cell viability was significantly less than half of the control group in 0.5, 1, 5 and 10 mM of cotinine treatment. This result is consistent with previous study which the minimum concentration of cotinine that can disrupt fetal development was 8 mM (70). Furthermore, 2 mM of cotinine induced marked cell death in pulmonary fibroblast cell line (MRC-5) and showed lower toxicity than nicotine on the MRC-5 cells (73). Therefore, significant doses of cotinine at 0.5, 1, and 5 mM were chosen for cell apoptosis assay. Figure 9 showed PMEF cell morphology at 24 h of the control (no treatment) and cotinine treatment at 0.5, 1 and 5 mM. Treatment with cotinine in all groups showed cells exhibiting close contact with neighbouring cells, change in cell shape through shrinking, and an increased degree of condensation in a dose-dependent manner. However, at 5 mM cotinine cells showed deformation and loosened contact to neighbouring cells. Depicts how cell morphology is closely related to the amount of cell apoptosis (Figure 12) in a dose-dependent manner.

Nicotine and cotinine can rapidly pass the placenta through a child throughout pregnancy (16-18). Among smoke products, nicotine is considered as the main teratogenic substance that alters and delays embryonic development. Previous epidemiological research found a statistically significant association between maternal

smoking and the presence of oral clefts in newborns (156). This study used both 5 mM nicotine combined with cotinine at 0.1, 0.5, 1, 5 and 10 mM to simulate conditions concise maternal smoking. The effect of nicotine combined with cotinine induced apoptosis has never been investigated. The cell viability significantly reduced after a combined treatment. In Figure 6 and Figure 7, the results suggested that a combined treatment decreased the cell viability in a dose-dependent manner. Figure 10 shows cell morphology at 6 h and 24 h after cells exposed to 5 mM nicotine combined with cotinine at 0, 0.5, 1 and 5 mM. At 6 h, cells treated with nicotine at 5 mM (no cotinine) exhibited swelling, polymorphic vacuole formation and condensed nuclei. Cells that exposed to the 5 mM nicotine combined with cotinine at 0.5, 1 and 5 mM displayed an irregular form, close contact with neighbouring cells, and increased the degree of condensation in a dose-dependent manner (153-155). This results showed adverse effect of their combination promoting an apoptotic cell death progression compared to nicotine treated cells. At 24 h, all treated groups exhibited significant changes to cell shape including cell shrinkage, deformation, and loose contact to neighbouring cells. These morphological changes indicated death of cells. During this process, cell chromatin condenses and marginates at the nuclear membrane, followed by blebbing or budding of the plasma membrane, and finally fragmentation into compact membrane-enclosed structures called 'apoptotic bodies', which contain cytosol, the condensed chromatin, and organelles (153-155). Figure 13 quantified cell apoptosis after combined treatment of 5 mM nicotine and cotinine. The results clearly show a significant increase of cell apoptosis in a concentration dependent relationship. Moreover, combined dose on cell apoptosis is dependent on concentration and time which may affect synergistically on PMEF cells. Furthermore, previous studies have investigated the possible direct action of nicotine and cotinine at the embryonic level. The sensitivity of the embryo to cotinine was low. Although studies reported the minimum concentration of cotinine that can disrupt fetal development was 8 mM, in a laboratory study, adding of 0.5 mM nicotine to the media containing 0.8 mM of cotinine inhibited embryonic blastulation (70).

In this study, nicotine and cotinine induced substantial ROS generation. Nicotine and cotinine may have caused the imbalance of ROS content in cells and mediated cell apoptosis because the results of this study showed a positive correlation

between generation of ROS and cell apoptosis. Thus, this study also evaluated nicotine, cotinine and their combination induced ROS effect on expression of apoptosis-related genes. Excess cellular levels of ROS can cause damage to proteins, nucleic acids, lipids, membranes and organelles such as mitochondria (157). Mitochondria also have an important role in triggering and regulating apoptosis. Mitochondrial dependent apoptosis appears to involve the mitochondrial permeability transition pore (MPT) complex (127). Increasing of mitochondrial outer membrane permeabilisation (MOMP) leads to release of pro-apoptotic proteins into the cytosol such as cytochrome C, apoptosis inducing factor (AIF), endonuclease G (endoG) (127, 158). This leads ultimately to apoptotic cell death by both caspase-dependent and -independent mechanisms (146). Previous studies revealed that increased ROS generation such as $H_2O_2$ and $O_2^{\bullet-}$ resulted in the release of cytochrome C and induction of apoptosis through the mitochondrial pathway (29, 159).

ROS was decisive in initiating apoptotic cell death by activating intrinsic apoptotic pathway and mitochondria signal transducer which contributes to p53-dependent apoptosis (131). First, activating intrinsic apoptotic pathway, MOMP is regulated by proteins belonging to the B-cell-lymphoma protein 2 (Bcl-2) family. Under conditions of stress, relative expression of pro- and antiapoptotic Bcl-2 proteins is modified (160). BH3-only Bcl-2 proteins are activated either transcriptionally or post-transcriptionally by ROS and further promote cellular expression of pro-apoptotic proteins. Activated pro-apoptotic proteins are then translocated to the outer mitochondrial membrane. This process facilitates MOMP by formation of lager channels followed by leakage of pro-apoptotic factors (i.e cytochrome C) into the cytosol (31, 32). Afterwards, cytochrome C interacts with Apaf-1 and procaspase 9 and forms a complex called “apoptosome” which sequentially facilitates and activates caspase 3, 6, 7, and 9 (31, 95, 96).

This study showed that nicotine, cotinine and their combination produced a large number of ROS, which caused the imbalance of ROS and induced apoptosis related gene alteration. The RT-qPCR showed that ROS altered the apoptosis-related genes expression such as BAX. The mRNA expression level of BAX, with the treatment of 5 mM nicotine and 5mM nicotine combined with cotinine significantly upregulated in a dose-dependent manner comparison to the control (Figure 18). It has

also been reported that ROS can induce changes in pro-apoptotic Bcl2 family proteins (BAX, BAK). Bax (pro-apoptotic) is directly responsible for breaching the mitochondrial outer membrane, interacting with the mitochondria to induce a permeability transition, producing ROS, and in certain settings, releasing cytochrome C, which sequentially activates a downstream caspase program (109-112). Closely related to CAS3 expression in 5 mM nicotine and a combined 5mM nicotine with cotinine treatments was significantly increased, indicating that PMEF treated cells has been damaged and become in late apoptosis induction. This leads ultimately to apoptotic cell death by caspase-dependent mechanisms (146).

In additions, the mRNA expression level of BCL2 (Figure 19) was increased significantly in all of the treatment groups in comparison to the control. Bcl-2 serves an anti-apoptotic role, inhibiting apoptosis by binding with the BH3 only sensitizers proteins to regulate MOMP (107, 161). In contrast, BH3 only activator proteins directly bind BAX causing a BAX conformational change that leads to the oligomerization of BAX and subsequent activation of apoptosis (162). In this study, treatments of 5 mM nicotine and a combined 5mM nicotine with cotinine increased expression of BAX. This may increase BCL2 expression by restraining the MOMP. The results showed an association of increased expression between BAX and BCL2. Previous studies provided an insight into the molecular mechanisms of nicotine pro-apoptotic effects on the liver and kidney (82). Results showed a significant increase in Bax/Bcl-2 ratio in the nicotine treated mice. However, the result showed contrast with a previous study in hOMF found that ST induced ROS and cell apoptosis by regulating the apoptosis associated proteins. Bax expression was increased and Bcl2 expression was decreased in a dose-dependent manner (36). Therefore, nicotine can have pro-apoptotic effect depending on the concentration of the substance used, species-related variations in the metabolism of nicotine and the target cells (82). However, anti-apoptotic Bcl-2 proteins also contain other genes besides BCL2. This genes such as Bcl-X large (BclXL), Bcl2-like protein 2 (BclW) and Bcl2-like protein 10 (BclB) (101) may reduce expression and result in increased BAX expression. An interesting further study is to determine the effect of nicotine and cotinine on other anti-apoptotic Bcl-2 proteins.

Interestingly, ROS was decisive in initiating apoptotic cell death by activating mitochondria signal transducer which contributes to P53-dependent apoptosis (131).

Closely related to P53 mRNA expression in this study, the mRNA expression level of PMEF treated cells at 5 mM nicotine and combined 5mM nicotine with cotinine was significantly greater than the control. P53 is a key protein mediating cell response to stresses. It has been reported that P53 is associated with genes in cell cycle arrest and apoptosis (130). Previous studies that investigated the effects of nicotine on cell apoptosis via ROS production reported that ROS are able to trigger cytoplasmic membrane damage and DNA fragmentation (143). Elevated ROS is a distinguished cause of the mitochondrial apoptotic pathway which directly oxidizes DNA and triggers genotoxicity (144, 145). In consistent with this study, apoptotic stimuli (ROS) causes DNA damage by induction the expression of P53 and P53 mediated apoptotic signalling which will further affect the mitochondria membrane transition pore complex. When the membrane transition pore complex undergoes apoptotic stimuli, inner mitochondria membrane permeability is increased allowing free passage of molecules (i.e. protons) into the mitochondrial matrix. This cause osmotic swelling of the mitochondrial matrix and compression of vesicles created by infolding of the intercristal space. Then, increased MOMP accelerates transportation of cytochrome C, apoptosis inducing factor (AIF) and endonuclease G (endo) into cytosol to induce apoptosis (126, 127, 158). This leads ultimately to apoptotic cell death by both caspase-dependent and -independent mechanisms (146). This study finding, CAS3 expression in 5 mM nicotine and a combined 5mM nicotine with cotinine treatments was significantly increased closely related to P53 mRNA expression. The result suggested, ROS inducing by nicotine and cotinine was decisive in initiating apoptotic cell death by activating mitochondria signal transducer which contributes to P53-dependent apoptosis (160). This leads ultimately to apoptotic cell death by caspase-dependent mechanisms (146).

Moreover, excessive cellular levels of ROS also cause direct damage to mitochondrial membranes due to electrons leaking from the electron transport chain and reacting with $O_2$ to form $O_2^{\bullet-}$. This $O_2^{\bullet-}$peroxide is then converted to $H_2O_2$ and $OH^{\bullet}$ respectively, thus motivating nearby mitochondria to produce more ROS (28,

118, 127). On the other hand, ROS overproduction can directly contribute to endoplasmic reticular stress and DNA damage that changes P53 expression. The result in this study is consistent with previous study. ROS induced by 5 mM nicotine and a combined 5mM nicotine with cotinine might direct induces severe mitochondrial dysfunction (25) and leads to mitochondrial structural oxidation which opens the MPT pores (26-28). Then, initiating apoptotic cell death by activating mitochondria signal transducer which contributes to P53-dependent apoptosis (131). Adverse effect of ROS production in 5 mM nicotine and 5mM nicotine combined with cotinine treatments in PMEF cells in our study was interesting. The mechanism supported these effect by severe mitochondrial dysfunction, accelerating MOMP thought the opening of the MPT pores and or directly triggers genotoxicity resulting in DNA damage, since we found the associated ROS overproduction and high levels of CAS3 and P53 mRNA expression, and increased expression of BAX and BCL2.

However, the underlying mechanisms of the reported adverse effects of nicotine, cotinine, and their combination on PMEF cells need to be explored in further studies. An interesting future study is to test the incidence occurring in the mitochondria membrane transition pore complex by verifying the mitochondria membrane potential ($\Delta\Psi m$) change. $\Delta\Psi m$ is implicated in many functions such as adding further complexity and diversity to the role of mitochondria in cellular homeostasis, including the ROS generation, MPT pore stabilization (163). The instabilities of $\Delta\Psi m$ have been described (164, 165), and they are occasionally attributed to the oscillations of the MPT (166). Furthermore, at high $\Delta\Psi m$ the mitochondrial respiratory chain becomes a significant producer of ROS (167-169). Given that excessive production of ROS could directly cause apoptosis closely related with this study. In this study, ROS induced $\Delta\Psi m$ change is associated with release of cytochrome C, AIF and other factors, apparently, then induced the cells apoptosis (170). This mechanism is the study of preferable futures.

P21 is a very principal checkpoint gene in the cell cycle. Expression of P21 is induced by DNA damage, which is also regulated by the transcription of P53. In addition, P21 mediates damaged cell repair by stopping DNA synthesis and it is also able to stabilize interactions between cyclin D and Cdk4 (171, 172). Thus, P53 increased expression found in this study may induce expression P21 in early stage

even before initiation of cell apoptosis and may be explore in the future study. In addition, the mRNA expression shown in this study indicates the level of transcriptome. In the future, at the level of the translation of important genes such as P53 should be carried out to prove the function of the protein. If the P53 protein is expressed, it confirms the activity of the expressed gene.

Cells treated with 5 mM nicotine combined with cotinine at 0.5, 1 and 5 mM were chosen for subsequent experiments because the cotinine concentration was low and closely related to UCB cotinine levels in newborns of maternal smokers (67). Although the levels of nicotine used in our study were higher than plasma nicotine, our *in vitro* study was a single dose. The concentration used in this study can serve as the preliminary data to adjust the exposure to be similar to the actual condition. In cigarette smokers, nicotine has high affinity to the organ, resulting in slow release into plasma (43, 44). People who smoke have low levels of nicotine and cotinine in their blood all the time. In the future, we can use the data to design an experiment with a cell test represented *in vivo* condition. The concentration of nicotine and cotinine can be designed similar to those obtained while smoking and the duration of exposure is more frequent instead of giving the test substance only once.

In addition, the relationship between nicotine and cotinine induced ROS and its effect on the antioxidant enzyme within PMEF is interesting. The balance between oxidants and antioxidants is crucial. Imbalance could result in accumulation of ROS and induce cell apoptosis (116). Future studies may be conducted to determine whether antioxidants can reduce ROS levels induced by nicotine and cotinine, possibly providing antioxidants such as vitamin E, vitamin C and carotenoids to reduce the risk of birth defects (173). The effects of antioxidants have been assessed in human studies and model animals under normal conditions and oxidative stress. The studies confirmed the positive effects of antioxidants and antioxidant rich diets to reduce the level of oxidative stress status *in vivo*, and the cultured cells respond well to the added antioxidants (120). PMEF cell line (E13) is mesenchymal cells that is going to differentiate into various morphological structures and organs of mammalian embryo, especially craniofacial formation. At embryonic day 13 (E13) in mouse and the 7th week in humans allowing the palatal shelves to be elevated above the dorsum of the tongue, showing the critical time in palatogenesis (174, 175). Elevation of the

palatal processes or shelves to the horizontal position is caused by cell proliferation which increases volume before palatal processes swing upward. The role of mesenchymal cells is important during that stage (176). Disturbances at any stage of palate development, e.g., defective palatal shelf growth, failed or delayed elevation, and blocked fusion, can result in cleft palate (174, 177). Several environmental factors (e.g., alcohol consumption, tobacco and anticonvulsants) increase the risk of cleft palate (156, 178). However, origin of mesenchymal cells at craniofacial are derived from the neural crest and mesoderm during development. Cranial neural crest cells (CNCCs) give rise to mesenchymal structures, such as neural tissues, cartilage, bone, palate and teeth in the craniofacial region (179). PMEF represents all mesenchyme cells derived from neural crest cells and mesoderm origin at embryonic day 13, thereby it might not be the best representative for palate formation that requires the cells majority derived from CNCCs. Nevertheless, the conditions provide evidence-based information of the association between nicotine and cotinine, and palatal development. It should be noted that at the beginning of pregnancy, the embryo is highly sensitive to its surroundings, and chemicals that can pass through the placental barrier, such as cigarette smoke, and can cause developmental disability.

**Conclusions**

Our study demonstrated that nicotine and cotinine have an adverse effect on PMEF cells as evidenced by decreased cell viability, increased apoptotic cell death in a dose-dependent manner. Overproduction of ROS was found in treatments of nicotine, cotinine, and their combination, suggesting their deleterious effects on PMEF cells. It is possible that ROS overproduction induced by nicotine, cotinine, and their combination could lead to mitochondria dysfunction and DNA damage, resulting in apoptosis through elevated expression of CAS3 and P53 (two apoptosis-related genes), a presumed mechanism of these action is shown in the Figure 22. Our study also found that cotinine enhanced the cytotoxic effect of nicotine treatment by decreasing cell viability, activating cell apoptosis by increasing ROS production and expression of BAX, BCL2, CAS3 and P53.

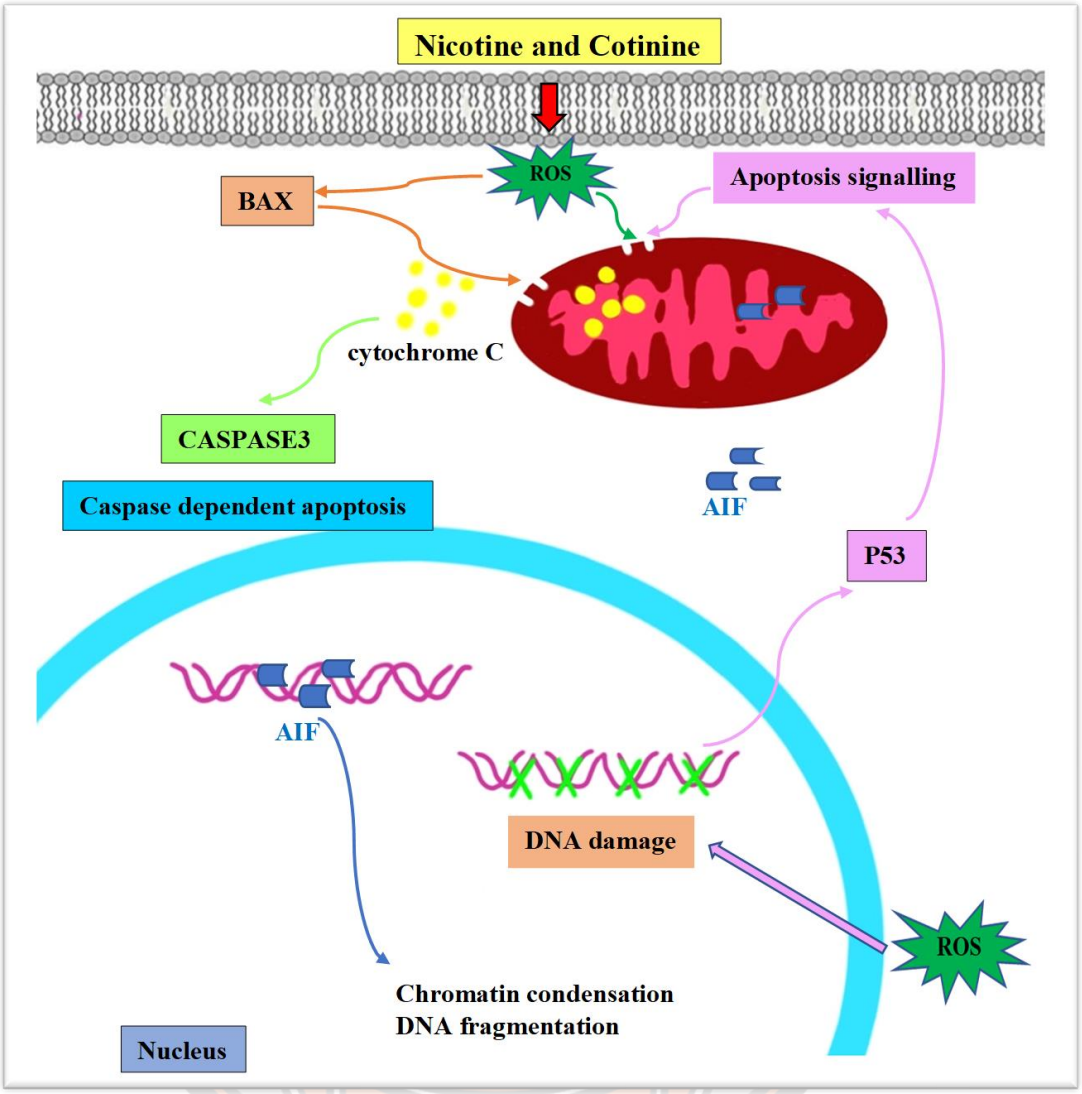


**Figure 22 The propose mechanisms of nicotine and cotinine induced apoptosis on PMEF. Nicotine combined with cotinine increased the ROS generation which resulted in the decreased cell viability, increased cell apopotosis and upregulated expression of BAX and P53. Consequently, the mitochondrial outer membrane permeability was increased and then accelerated the release of cytochrome C into cytoplasm leading to the caspase dependent apoptosis via expression of CAS3**

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# APPENDIX

## Oneway

**Descriptives**

Viability

| Nicotine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 99.56033 | 7.413497 | 4.280185 | 81.14418 | 117.97648 |
| 1.00 | 3 | 106.18340 | 6.615304 | 3.819347 | 89.75008 | 122.61673 |
| 3.00 | 3 | 60.60133 | 3.744706 | 2.162007 | 51.29897 | 69.90370 |
| 4.00 | 3 | 41.46367 | 6.143540 | 3.546974 | 26.20227 | 56.72507 |
| 5.00 | 3 | 31.41733 | .426925 | .246486 | 30.35679 | 32.47787 |
| Total | 15 | 67.84521 | 31.571561 | 8.151742 | 50.36147 | 85.32896 |

**Test of Homogeneity of Variances**

Viability

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 1.897 | 4 | 10 | .188 |

**ANOVA**

Viability

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 13653.347 | 4 | 3413.337 | 113.272 | .000 |
| Within Groups | 301.341 | 10 | 30.134 | | |
| Total | 13954.688 | 14 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Viability

Tukey HSD

| (I) Conc. | (J) Conc. | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 1.00 | -6.623069 | 4.482118 | .597 | -21.37409 | 8.12795 |
| | 3.00 | 38.959000* | 4.482118 | .000 | 24.20798 | 53.71002 |
| | 4.00 | 58.096667* | 4.482118 | .000 | 43.34565 | 72.84768 |
| | 5.00 | 68.143000* | 4.482118 | .000 | 53.39198 | 82.89402 |
| 1.00 | 0 | 6.623069 | 4.482118 | .597 | -8.12795 | 21.37409 |
| | 3.00 | 45.582069* | 4.482118 | .000 | 30.83105 | 60.33309 |
| | 4.00 | 64.719735* | 4.482118 | .000 | 49.96872 | 79.47075 |
| | 5.00 | 74.766069* | 4.482118 | .000 | 60.01505 | 89.51709 |
| 3.00 | 0 | -38.959000* | 4.482118 | .000 | -53.71002 | -24.20798 |
| | 1.00 | -45.582069* | 4.482118 | .000 | -60.33309 | -30.83105 |
| | 4.00 | 19.137667* | 4.482118 | .011 | 4.38665 | 33.88868 |
| | 5.00 | 29.184000* | 4.482118 | .001 | 14.43298 | 43.93502 |
| 4.00 | 0 | -58.096667* | 4.482118 | .000 | -72.84768 | -43.34565 |
| | 1.00 | -64.719735* | 4.482118 | .000 | -79.47075 | -49.96872 |
| | 3.00 | -19.137667* | 4.482118 | .011 | -33.88868 | -4.38665 |
| | 5.00 | 10.046333 | 4.482118 | .240 | -4.70468 | 24.79735 |
| 5.00 | 0 | -68.143000* | 4.482118 | .000 | -82.89402 | -53.39198 |
| | 1.00 | -74.766069* | 4.482118 | .000 | -89.51709 | -60.01505 |
| | 3.00 | -29.184000* | 4.482118 | .001 | -43.93502 | -14.43298 |
| | 4.00 | -10.046333 | 4.482118 | .240 | -24.79735 | 4.70468 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

**Viability**

Tukey HSD[a]

| Nicotine concentrations (mM) | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 5.00 | 3 | 31.41733 | | |
| 4.00 | 3 | 41.46367 | | |
| 3.00 | 3 | | 60.60133 | |
| 0 | 3 | | | 99.56033 |
| 1.00 | 3 | | | 106.18340 |
| Sig. | | .240 | 1.000 | .597 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Viability

| Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 99.56033 | 7.413497 | 4.280185 | 81.14418 | 117.97648 |
| 0.1 | 3 | 55.30500 | 3.917398 | 2.261711 | 45.57364 | 65.03636 |
| 0.5 | 3 | 42.94067 | 2.408275 | 1.390418 | 36.95818 | 48.92315 |
| 1.0 | 3 | 41.85467 | 1.957492 | 1.130158 | 36.99199 | 46.71735 |
| 5.0 | 3 | 35.33833 | .903468 | .521617 | 33.09399 | 37.58267 |
| 10.0 | 3 | 35.00433 | 1.129826 | .652305 | 32.19769 | 37.81098 |
| Total | 18 | 51.66722 | 23.307638 | 5.493663 | 40.07661 | 63.25784 |

**Test of Homogeneity of Variances**

Viability

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 2.359 | 5 | 12 | .104 |

**ANOVA**

Viability

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 9071.121 | 5 | 1814.224 | 132.699 | .000 |
| Within Groups | 164.061 | 12 | 13.672 | | |
| Total | 9235.182 | 17 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Viability

Tukey HSD

| (I) Conc. | (J) Conc. | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | Mean Difference | Std. Error | Sig. | Lower Bound | Upper Bound |
| 0 | 0.1 | 44.255333* | 3.019019 | .000 | 34.11469 | 54.39598 |
| | 0.5 | 56.619667* | 3.019019 | .000 | 46.47902 | 66.76031 |
| | 1.0 | 57.705667* | 3.019019 | .000 | 47.56502 | 67.84631 |
| | 5.0 | 64.222000* | 3.019019 | .000 | 54.08135 | 74.36265 |
| | 10.0 | 64.556000* | 3.019019 | .000 | 54.41535 | 74.69665 |
| 0.1 | 0 | -44.255333* | 3.019019 | .000 | -54.39598 | -34.11469 |
| | 0.5 | 12.364333* | 3.019019 | .014 | 2.22369 | 22.50498 |
| | 1.0 | 13.450333* | 3.019019 | .008 | 3.30969 | 23.59098 |
| | 5.0 | 19.966667* | 3.019019 | .000 | 9.82602 | 30.10731 |
| | 10.0 | 20.300667* | 3.019019 | .000 | 10.16002 | 30.44131 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.5 | 0 | -56.619667* | 3.019019 | .000 | -66.76031 | -46.47902 |
| | 0.1 | -12.364333* | 3.019019 | .014 | -22.50498 | -2.22369 |
| | 1.0 | 1.086000 | 3.019019 | .999 | -9.05465 | 11.22665 |
| | 5.0 | 7.602333 | 3.019019 | .193 | -2.53831 | 17.74298 |
| | 10.0 | 7.936333 | 3.019019 | .163 | -2.20431 | 18.07698 |
| 1.0 | 0 | -57.705667* | 3.019019 | .000 | -67.84631 | -47.56502 |
| | 0.1 | -13.450333* | 3.019019 | .008 | -23.59098 | -3.30969 |
| | 0.5 | -1.086000 | 3.019019 | .999 | -11.22665 | 9.05465 |
| | 5.0 | 6.516333 | 3.019019 | .322 | -3.62431 | 16.65698 |
| | 10.0 | 6.850333 | 3.019019 | .277 | -3.29031 | 16.99098 |
| 5.0 | 0 | -64.222000* | 3.019019 | .000 | -74.36265 | -54.08135 |
| | 0.1 | -19.966667* | 3.019019 | .000 | -30.10731 | -9.82602 |
| | 0.5 | -7.602333 | 3.019019 | .193 | -17.74298 | 2.53831 |
| | 1.0 | -6.516333 | 3.019019 | .322 | -16.65698 | 3.62431 |
| | 10.0 | .334000 | 3.019019 | 1.000 | -9.80665 | 10.47465 |
| 10.0 | 0 | -64.556000* | 3.019019 | .000 | -74.69665 | -54.41535 |
| | 0.1 | -20.300667* | 3.019019 | .000 | -30.44131 | -10.16002 |
| | 0.5 | -7.936333 | 3.019019 | .163 | -18.07698 | 2.20431 |
| | 1.0 | -6.850333 | 3.019019 | .277 | -16.99098 | 3.29031 |
| | 5.0 | -.334000 | 3.019019 | 1.000 | -10.47465 | 9.80665 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

**Viability**

Tukey HSD[a]

| Cotinine concentrations (mM) | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 10.0 | 3 | 35.00433 | | |
| 5.0 | 3 | 35.33833 | | |
| 1.0 | 3 | 41.85467 | | |
| 0.5 | 3 | 42.94067 | | |
| 0.1 | 3 | | 55.30500 | |
| 0 | 3 | | | 99.56033 |
| Sig. | | .163 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Viability

| Nicotine 5mM + Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 3 | 31.36467 | .406592 | .234746 | 30.35464 | 32.37470 |
| 1.0 | 3 | 18.71367 | 4.270327 | 2.465475 | 8.10559 | 29.32175 |
| 5.0 | 3 | 13.03267 | .751500 | .433879 | 11.16584 | 14.89950 |
| 0.1 | 3 | 24.64467 | 1.705877 | .984889 | 20.40703 | 28.88230 |
| 0.5 | 3 | 22.13867 | 3.232579 | 1.866331 | 14.10849 | 30.16884 |
| 10.0 | 3 | 7.26833 | .903468 | .521617 | 5.02399 | 9.51267 |

**Descriptives**

Viability

| Nicotine 5mM + Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 3 | 31.36467 | .406592 | .234746 | 30.35464 | 32.37470 |
| 1.0 | 3 | 18.71367 | 4.270327 | 2.465475 | 8.10559 | 29.32175 |
| 5.0 | 3 | 13.03267 | .751500 | .433879 | 11.16584 | 14.89950 |
| 0.1 | 3 | 24.64467 | 1.705877 | .984889 | 20.40703 | 28.88230 |
| 0.5 | 3 | 22.13867 | 3.232579 | 1.866331 | 14.10849 | 30.16884 |
| 10.0 | 3 | 7.26833 | .903468 | .521617 | 5.02399 | 9.51267 |
| Total | 18 | 19.52711 | 8.278415 | 1.951241 | 15.41035 | 23.64387 |

**Test of Homogeneity of Variances**

Viability

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 2.731 | 5 | 12 | .071 |

**ANOVA**

Viability

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1098.763 | 5 | 219.753 | 39.784 | .000 |
| Within Groups | 66.283 | 12 | 5.524 | | |
| Total | 1165.047 | 17 | | | |

**Multiple Comparisons**

Viability

Tukey HSD

| (I) Conc. | (J) Conc. | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 1.0 | 12.651000* | 1.918958 | .000 | 6.20537 | 19.09663 |
| | 5.0 | 18.332000* | 1.918958 | .000 | 11.88637 | 24.77763 |
| | 0.1 | 6.720000* | 1.918958 | .039 | .27437 | 13.16563 |
| | 0.5 | 9.226000* | 1.918958 | .004 | 2.78037 | 15.67163 |
| | 10 | 24.096333* | 1.918958 | .000 | 17.65070 | 30.54196 |
| 1.0 | 0 | -12.651000* | 1.918958 | .000 | -19.09663 | -6.20537 |
| | 5.0 | 5.681000 | 1.918958 | .097 | -.76463 | 12.12663 |
| | 0.1 | -5.931000 | 1.918958 | .078 | -12.37663 | .51463 |
| | 0.5 | -3.425000 | 1.918958 | .508 | -9.87063 | 3.02063 |
| | 10.0 | 11.445333* | 1.918958 | .001 | 4.99970 | 17.89096 |
| 5.0 | 0 | -18.332000* | 1.918958 | .000 | -24.77763 | -11.88637 |
| | 1.0 | -5.681000 | 1.918958 | .097 | -12.12663 | .76463 |
| | 0.1 | -11.612000* | 1.918958 | .001 | -18.05763 | -5.16637 |
| | 0.5 | -9.106000* | 1.918958 | .005 | -15.55163 | -2.66037 |
| | 10.0 | 5.764333 | 1.918958 | .090 | -.68130 | 12.20996 |
| 0.1 | 0 | -6.720000* | 1.918958 | .039 | -13.16563 | -.27437 |
| | 1.0 | 5.931000 | 1.918958 | .078 | -.51463 | 12.37663 |
| | 5.0 | 11.612000* | 1.918958 | .001 | 5.16637 | 18.05763 |
| | 0.5 | 2.506000 | 1.918958 | .777 | -3.93963 | 8.95163 |
| | 10.0 | 17.376333* | 1.918958 | .000 | 10.93070 | 23.82196 |
| 0.5 | 0 | -9.226000* | 1.918958 | .004 | -15.67163 | -2.78037 |
| | 1.0 | 3.425000 | 1.918958 | .508 | -3.02063 | 9.87063 |
| | 5.0 | 9.106000* | 1.918958 | .005 | 2.66037 | 15.55163 |
| | 0.1 | -2.506000 | 1.918958 | .777 | -8.95163 | 3.93963 |
| | 10.0 | 14.870333* | 1.918958 | .000 | 8.42470 | 21.31596 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10.0 | 0 | -24.096333* | 1.918958 | .000 | -30.54196 | -17.65070 |
| | 1.0 | -11.445333* | 1.918958 | .001 | -17.89096 | -4.99970 |
| | 5.0 | -5.764333 | 1.918958 | .090 | -12.20996 | .68130 |
| | 0.1 | -17.376333* | 1.918958 | .000 | -23.82196 | -10.93070 |
| | 0.5 | -14.870333* | 1.918958 | .000 | -21.31596 | -8.42470 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

**Viability**

Tukey HSD[a]

| Nicotine 5mM + Cotinine concentrations (mM) | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 10.0 | 3 | 7.26833 | | | |
| 5.0 | 3 | 13.03267 | 13.03267 | | |
| 1.0 | 3 | | 18.71367 | 18.71367 | |
| 0.5 | 3 | | | 22.13867 | |
| 0.1 | 3 | | | 24.64467 | |
| 0 | 3 | | | | 31.36467 |
| Sig. | | .090 | .097 | .078 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Viability | Cotinine 0.1mM | 3 | 55.30500 | 3.917398 | 2.261711 |
| | Nicotine5mM+Cotinine 0.1mM | 3 | 24.64467 | 1.705877 | .984889 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Viability | Equal variances assumed | 1.629 | .271 | 12.429 | 4 |
| | Equal variances not assumed | | | 12.429 | 2.732 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Viability | Equal variances assumed | .000 | 30.660333 | 2.466849 |
| | Equal variances not assumed | .002 | 30.660333 | 2.466849 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Viability | Equal variances assumed | 23.811264 | 37.509403 |
| | Equal variances not assumed | 22.355893 | 38.964774 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Viability | Cotinine 0.5mM | 3 | 42.94067 | 2.408275 | 1.390418 |
| | Nicotine5mM+ Cotinine 0.5mM | 3 | 22.13867 | 3.232579 | 1.866331 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Viability | Equal variances assumed | .423 | .551 | 8.938 | 4 |
| | Equal variances not assumed | | | 8.938 | 3.697 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Viability | Equal variances assumed | .001 | 20.802000 | 2.327327 |
| | Equal variances not assumed | .001 | 20.802000 | 2.327327 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Viability | Equal variances assumed | 14.340304 | 27.263696 |
| | Equal variances not assumed | 14.126191 | 27.477809 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Viability | Cotinine 1mM | 3 | 41.85467 | 1.957492 | 1.130158 |
| | Nicotine5mM+ Cotinine 1mM | 3 | 18.71367 | 4.270327 | 2.465475 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Viability | Equal variances assumed | 1.122 | .349 | 8.532 | 4 |
| | Equal variances not assumed | | | 8.532 | 2.805 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Viability | Equal variances assumed | .001 | 23.141000 | 2.712162 |
| | Equal variances not assumed | .004 | 23.141000 | 2.712162 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Viability | Equal variances assumed | 15.610831 | 30.671169 |
| | Equal variances not assumed | 14.160023 | 32.121977 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Viability | Cotinine 5mM | 3 | 35.33833 | .903468 | .521617 |
| | Nicotine5mM+ Cotinine 5mM | 3 | 13.03267 | .751500 | .433879 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Viability | Equal variances assumed | .250 | .643 | 32.876 | 4 |
| | Equal variances not assumed | | | 32.876 | 3.872 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Viability | Equal variances assumed | .000 | 22.305667 | .678480 |
| | Equal variances not assumed | .000 | 22.305667 | .678480 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Viability | Equal variances assumed | 20.421903 | 24.189430 |
| | Equal variances not assumed | 20.397006 | 24.214327 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Viability | Cotinine 10mM | 3 | 35.00433 | 1.129826 | .652305 |
| | Nicotine5mM+ Cotinine 10mM | 3 | 7.26833 | .903468 | .521617 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Viability | Equal variances assumed | .073 | .801 | 33.208 | 4 |
| | Equal variances not assumed | | | 33.208 | 3.815 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Viability | Equal variances assumed | .000 | 27.736000 | .835217 |
| | Equal variances not assumed | .000 | 27.736000 | .835217 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Viability | Equal variances assumed | 25.417067 | 30.054933 |
| | Equal variances not assumed | 25.372156 | 30.099844 |

## Oneway

**Descriptives**

Cell apoptosis

| Nicotine concentrations (mm) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 40.17941 | .757272 | .437211 | 38.29825 | 42.06058 |
| Unlabeled | 3 | 15.14400 | .923626 | .533256 | 12.84959 | 17.43842 |
| 3.00 | 3 | 52.62040 | .106232 | .061333 | 52.35650 | 52.88429 |
| 5.00 | 3 | 90.03777 | 3.029915 | 1.749322 | 82.51105 | 97.56450 |
| Positive control | 3 | 100.00000 | 3.656579 | 2.111127 | 90.91655 | 109.08345 |
| Total | 15 | 59.59632 | 32.656721 | 8.431929 | 41.51163 | 77.68101 |

**Test of Homogeneity of Variances**

Cell apoptosis

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 4.958 | 4 | 10 | .018 |

**ANOVA**

Cell apoptosis

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 14882.482 | 4 | 3720.621 | 775.492 | .000 |
| Within Groups | 47.978 | 10 | 4.798 | | |
| Total | 14930.460 | 14 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Cell apoptosis

Tukey HSD

| (I) Conc. | (J) Conc. | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | unlabeled | 25.035411* | 1.788436 | .000 | 19.14952 | 30.92130 |
| | 3.00 | -12.440982* | 1.788436 | .000 | -18.32687 | -6.55509 |
| | 5.00 | -49.858357* | 1.788436 | .000 | -55.74425 | -43.97247 |
| | Positive control | -59.820585* | 1.788436 | .000 | -65.70648 | -53.93469 |
| unlabeled | 0 | -25.035411* | 1.788436 | .000 | -30.92130 | -19.14952 |
| | 3.00 | -37.476393* | 1.788436 | .000 | -43.36228 | -31.59050 |
| | 5.00 | -74.893768* | 1.788436 | .000 | -80.77966 | -69.00788 |
| | Positive control | -84.855996* | 1.788436 | .000 | -90.74189 | -78.97010 |
| 3.00 | 0 | 12.440982* | 1.788436 | .000 | 6.55509 | 18.32687 |
| | Unlabeled | 37.476393* | 1.788436 | .000 | 31.59050 | 43.36228 |
| | 5.00 | -37.417375* | 1.788436 | .000 | -43.30327 | -31.53148 |
| | Positive control | -47.379603* | 1.788436 | .000 | -53.26549 | -41.49371 |
| 5.00 | 0 | 49.858357* | 1.788436 | .000 | 43.97247 | 55.74425 |
| | Unlabeled | 74.893768* | 1.788436 | .000 | 69.00788 | 80.77966 |
| | 3.00 | 37.417375* | 1.788436 | .000 | 31.53148 | 43.30327 |
| | Positive control | -9.962229* | 1.788436 | .002 | -15.84812 | -4.07634 |
| Positive control | .00 | 59.820585* | 1.788436 | .000 | 53.93469 | 65.70648 |
| | Unlabeled | 84.855996* | 1.788436 | .000 | 78.97010 | 90.74189 |
| | 3.00 | 47.379603* | 1.788436 | .000 | 41.49371 | 53.26549 |
| | 5.00 | 9.962229* | 1.788436 | .002 | 4.07634 | 15.84812 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

Cell apoptosis

Tukey HSD[a]

| Nicotine concentrations (mm) | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| Unlabeled | 3 | 15.14400 | | | | |
| Control (0) | 3 | | 40.17941 | | | |
| 3.00 | 3 | | | 52.62040 | | |
| 5.00 | 3 | | | | 90.03777 | |
| Positive control | 3 | | | | | 100.00000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Cell apoptosis

| Cotinine concentrations (mm) | | | | | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Std. Error | Lower Bound | Upper Bound |
| Control (0) | 3 | 25.64966 | .096246 | .055568 | 25.41057 | 25.88875 |
| Unlabeled | 3 | 10.20212 | .288739 | .166704 | 9.48485 | 10.91939 |
| 0.50 | 3 | 30.44594 | .799965 | .461860 | 28.45872 | 32.43317 |
| 1.00 | 3 | 30.53417 | 1.034649 | .597355 | 27.96396 | 33.10438 |
| 5.00 | 3 | 32.54732 | .842270 | .486285 | 30.45501 | 34.63964 |
| Positive control | 3 | 100.00000 | 3.224254 | 1.861524 | 91.99051 | 108.00949 |
| Total | 18 | 38.22987 | 29.466060 | 6.945217 | 23.57674 | 52.88300 |

**Test of Homogeneity of Variances**

Cell apoptosis

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 2.377 | 5 | 12 | .102 |

**ANOVA**

Cell apoptosis

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 14734.411 | 5 | 2946.882 | 1369.760 | .000 |
| Within Groups | 25.817 | 12 | 2.151 | | |
| Total | 14760.227 | 17 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Cell apoptosis

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Conc | (J) Conc | Mean Difference | Std. Error | Sig. | Lower Bound | Upper Bound |
| 0 | Unlabeled | 15.447546* | 1.197605 | .000 | 11.42489 | 19.47020 |
| | 0.50 | -4.796278* | 1.197605 | .017 | -8.81894 | -.77362 |
| | 1.00 | -4.884504* | 1.197605 | .015 | -8.90716 | -.86185 |
| | 5.00 | -6.897658* | 1.197605 | .001 | -10.92032 | -2.87500 |
| | Positive control | -74.350337* | 1.197605 | .000 | -78.37300 | -70.32768 |
| Unlabeled | 0 | -15.447546* | 1.197605 | .000 | -19.47020 | -11.42489 |
| | 0.50 | -20.243824* | 1.197605 | .000 | -24.26648 | -16.22117 |
| | 1.00 | -20.332050* | 1.197605 | .000 | -24.35471 | -16.30939 |
| | 5.00 | -22.345204* | 1.197605 | .000 | -26.36786 | -18.32254 |
| | Positive control | -89.797883* | 1.197605 | .000 | -93.82054 | -85.77522 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.50 | 0 | 4.796278* | 1.197605 | .017 | .77362 | 8.81894 |
| | Unlabeled | 20.243824* | 1.197605 | .000 | 16.22117 | 24.26648 |
| | 1.00 | -.088226 | 1.197605 | 1.000 | -4.11088 | 3.93443 |
| | 5.00 | -2.101380 | 1.197605 | .525 | -6.12404 | 1.92128 |
| | Positive control | -69.554058* | 1.197605 | .000 | -73.57672 | -65.53140 |
| 1.00 | 0 | 4.884504* | 1.197605 | .015 | .86185 | 8.90716 |
| | Unlabeled | 20.332050* | 1.197605 | .000 | 16.30939 | 24.35471 |
| | 0.50 | .088226 | 1.197605 | 1.000 | -3.93443 | 4.11088 |
| | 5.00 | -2.013154 | 1.197605 | .567 | -6.03581 | 2.00951 |
| | Positive control | -69.465833* | 1.197605 | .000 | -73.48849 | -65.44317 |
| 5.00 | 0 | 6.897658* | 1.197605 | .001 | 2.87500 | 10.92032 |
| | Unlabeled | 22.345204* | 1.197605 | .000 | 18.32254 | 26.36786 |
| | 0.50 | 2.101380 | 1.197605 | .525 | -1.92128 | 6.12404 |
| | 1.00 | 2.013154 | 1.197605 | .567 | -2.00951 | 6.03581 |
| | Positive control | -67.452679* | 1.197605 | .000 | -71.47534 | -63.43002 |
| Positive control | 0 | 74.350337* | 1.197605 | .000 | 70.32768 | 78.37300 |
| | Unlabeled | 89.797883* | 1.197605 | .000 | 85.77522 | 93.82054 |
| | 0.50 | 69.554058* | 1.197605 | .000 | 65.53140 | 73.57672 |
| | 1.00 | 69.465833* | 1.197605 | .000 | 65.44317 | 73.48849 |
| | 5.00 | 67.452679* | 1.197605 | .000 | 63.43002 | 71.47534 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

Cell apoptosis

Tukey HSD[a]

| Cotinine concentrations (mM) | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Unlabeled | 3 | 10.20212 | | | |
| 0 | 3 | | 25.64966 | | |
| 0.50 | 3 | | | 30.44594 | |
| 1.00 | 3 | | | 30.53417 | |
| 5.00 | 3 | | | 32.54732 | |
| Positive control | 3 | | | | 100.00000 |
| Sig. | | 1.000 | 1.000 | .525 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mea Sample Size = 3.000.

## Oneway

**Descriptives**

Cell apoptosis

| Nicotine 5mM+Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Untreated cell | 3 | 25.64966 | .096246 | .055568 | 25.41057 | 25.88875 |
| unlabele | 3 | 10.20212 | .288739 | .166704 | 9.48485 | 10.91939 |
| 0 | 3 | 65.44755 | 3.560141 | 2.055448 | 56.60367 | 74.29143 |
| 0.5 | 3 | 70.75714 | 4.280621 | 2.471417 | 60.12349 | 81.39079 |
| 1.0 | 3 | 71.38274 | 5.606369 | 3.236839 | 57.45575 | 85.30973 |
| 5.0 | 3 | 75.32884 | 3.873942 | 2.236622 | 65.70544 | 84.95225 |
| Positive control | 3 | 100.00000 | 3.224254 | 1.861524 | 91.99051 | 108.00949 |
| Total | 21 | 59.82401 | 29.567145 | 6.452080 | 46.36520 | 73.28281 |

**Test of Homogeneity of Variances**

Cell apoptosis

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 1.793 | 6 | 14 | .173 |

**ANOVA**

Cell apoptosis

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 17308.470 | 6 | 2884.745 | 229.663 | .000 |
| Within Groups | 175.851 | 14 | 12.561 | | |
| Total | 17484.321 | 20 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Cell apoptosis

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Conc | (J) Conc | Mean Difference | Std. Error | Sig. | Lower Bound | Upper Bound |
| Untreated | unlabeled | 15.447546* | 2.893763 | .002 | 5.56654 | 25.32855 |
| | 0 | -39.797883* | 2.893763 | .000 | -49.67889 | -29.91688 |
| | 0.50 | -45.107475* | 2.893763 | .000 | -54.98848 | -35.22647 |
| | 0.10 | -45.733077* | 2.893763 | .000 | -55.61408 | -35.85207 |
| | 5.00 | -49.679179* | 2.893763 | .000 | -59.56018 | -39.79818 |
| | Positive control | -74.350337* | 2.893763 | .000 | -84.23134 | -64.46933 |

| | | | | | | |
|---|---|---|---|---|---|---|
| unlabeled | Untreated | -15.447546* | 2.893763 | .002 | -25.32855 | -5.56654 |
| | 0 | -55.245428* | 2.893763 | .000 | -65.12643 | -45.36442 |
| | 0.50 | -60.555021* | 2.893763 | .000 | -70.43602 | -50.67402 |
| | 1.00 | -61.180622* | 2.893763 | .000 | -71.06163 | -51.29962 |
| | 5.00 | -65.126724* | 2.893763 | .000 | -75.00773 | -55.24572 |
| | Positive control | -89.797883* | 2.893763 | .000 | -99.67889 | -79.91688 |
| 0 | Untreated | 39.797883* | 2.893763 | .000 | 29.91688 | 49.67889 |
| | unlabeled | 55.245428* | 2.893763 | .000 | 45.36442 | 65.12643 |
| | 0.50 | -5.309593 | 2.893763 | .548 | -15.19060 | 4.57141 |
| | 1.00 | -5.935194 | 2.893763 | .428 | -15.81620 | 3.94581 |
| | 5.00 | -9.881296* | 2.893763 | .050 | -19.76230 | -.00029 |
| | Positive control | -34.552454* | 2.893763 | .000 | -44.43346 | -24.67145 |
| 0.50 | Untreated | 45.107475* | 2.893763 | .000 | 35.22647 | 54.98848 |
| | unlabeled | 60.555021* | 2.893763 | .000 | 50.67402 | 70.43602 |
| | 0 | 5.309593 | 2.893763 | .548 | -4.57141 | 15.19060 |
| | 1.00 | -.625602 | 2.893763 | 1.000 | -10.50661 | 9.25540 |
| | 5.00 | -4.571704 | 2.893763 | .696 | -14.45271 | 5.30930 |
| | Positive control | -29.242862* | 2.893763 | .000 | -39.12387 | -19.36186 |
| 1.00 | Untreated | 45.733077* | 2.893763 | .000 | 35.85207 | 55.61408 |
| | unlabeled | 61.180622* | 2.893763 | .000 | 51.29962 | 71.06163 |
| | 0 | 5.935194 | 2.893763 | .428 | -3.94581 | 15.81620 |
| | 0.50 | .625602 | 2.893763 | 1.000 | -9.25540 | 10.50661 |
| | 5.00 | -3.946102 | 2.893763 | .811 | -13.82711 | 5.93490 |
| | Positive control | -28.617260* | 2.893763 | .000 | -38.49826 | -18.73626 |
| 5.00 | Untreated | 49.679179* | 2.893763 | .000 | 39.79818 | 59.56018 |
| | unlabeled | 65.126724* | 2.893763 | .000 | 55.24572 | 75.00773 |
| | 0 | 9.881296* | 2.893763 | .050 | .00029 | 19.76230 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0.50 | 4.571704 | 2.893763 | .696 | -5.30930 | 14.45271 |
| | 1.00 | 3.946102 | 2.893763 | .811 | -5.93490 | 13.82711 |
| | Positive control | -24.671158* | 2.893763 | .000 | -34.55216 | -14.79015 |
| Positive control | Untreated | 74.350337* | 2.893763 | .000 | 64.46933 | 84.23134 |
| | unlabeled | 89.797883* | 2.893763 | .000 | 79.91688 | 99.67889 |
| | 0 | 34.552454* | 2.893763 | .000 | 24.67145 | 44.43346 |
| | 0.50 | 29.242862* | 2.893763 | .000 | 19.36186 | 39.12387 |
| | 1.00 | 28.617260* | 2.893763 | .000 | 18.73626 | 38.49826 |
| | 5.00 | 24.671158* | 2.893763 | .000 | 14.79015 | 34.55216 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

Cell apoptosis

Tukey HSD[a]

| Nicotine 5mM+cotinine concentration (mM) | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| unlabeled | 3 | 10.20212 | | | | |
| untreated | 3 | | 25.64966 | | | |
| 0 | 3 | | | 65.44755 | | |
| 0.50 | 3 | | | 70.75714 | 70.75714 | |
| 1.00 | 3 | | | 71.38274 | 71.38274 | |
| 5.00 | 3 | | | | 75.32884 | |
| Positive control | 3 | | | | | 100.00000 |
| Sig. | | 1.000 | 1.000 | .428 | .696 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Luminescence intensity

| Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 2065.13333 | 81.695614 | 47.166985 | 1862.19018 | 2268.07649 |
| 0.1 | 3 | 3298.86667 | 120.607518 | 69.632783 | 2999.26098 | 3598.47235 |
| 0.5 | 3 | 3499.93333 | 235.604782 | 136.026484 | 2914.65861 | 4085.20806 |
| 1.0 | 3 | 3661.16667 | 230.460720 | 133.056559 | 3088.67050 | 4233.66283 |
| 5.0 | 3 | 3380.10000 | 301.795046 | 174.241451 | 2630.39954 | 4129.80046 |
| Total | 15 | 3181.04000 | 617.295687 | 159.385061 | 2839.19304 | 3522.88696 |

**Test of Homogeneity of Variances**

Luminescence intensity

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| .868 | 4 | 10 | .516 |

**ANOVA**

Luminescence intensity

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4892910.809 | 4 | 1223227.702 | 27.685 | .000 |
| Within Groups | 441844.707 | 10 | 44184.471 | | |
| Total | 5334755.516 | 14 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Luminescence intensity

Tukey HSD

| (I) Conc. | (J) Conc | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | Mean Difference | Std. Error | Sig. | Lower Bound | Upper Bound |
| 0 | 0.1 | -1.233733E3 | 171.628418 | .000 | -1798.57655 | -668.89011 |
| | 0.5 | -1.434800E3 | 171.628418 | .000 | -1999.64322 | -869.95678 |
| | 1.0 | -1.596033E3 | 171.628418 | .000 | -2160.87655 | -1031.19011 |
| | 5.0 | -1.314967E3 | 171.628418 | .000 | -1879.80989 | -750.12345 |
| 0.1 | 0 | 1233.733333* | 171.628418 | .000 | 668.89011 | 1798.57655 |
| | 0.5 | -201.066667 | 171.628418 | .767 | -765.90989 | 363.77655 |
| | 1.0 | -362.300000 | 171.628418 | .287 | -927.14322 | 202.54322 |
| | 5.0 | -81.233333 | 171.628418 | .988 | -646.07655 | 483.60989 |
| 0.5 | 0 | 1434.800000* | 171.628418 | .000 | 869.95678 | 1999.64322 |
| | 0.1 | 201.066667 | 171.628418 | .767 | -363.77655 | 765.90989 |
| | 1.0 | -161.233333 | 171.628418 | .875 | -726.07655 | 403.60989 |
| | 5.0 | 119.833333 | 171.628418 | .952 | -445.00989 | 684.67655 |
| 1.0 | 0 | 1596.033333* | 171.628418 | .000 | 1031.19011 | 2160.87655 |
| | 0.1 | 362.300000 | 171.628418 | .287 | -202.54322 | 927.14322 |
| | 0.5 | 161.233333 | 171.628418 | .875 | -403.60989 | 726.07655 |
| | 5.0 | 281.066667 | 171.628418 | .508 | -283.77655 | 845.90989 |
| 5.0 | 0 | 1314.966667* | 171.628418 | .000 | 750.12345 | 1879.80989 |
| | 0.1 | 81.233333 | 171.628418 | .988 | -483.60989 | 646.07655 |
| | 0.5 | -119.833333 | 171.628418 | .952 | -684.67655 | 445.00989 |
| | 1.0 | -281.066667 | 171.628418 | .508 | -845.90989 | 283.77655 |

## Homogeneous Subsets

Luminescence intensity

Tukey HSD[a]

| Cotinine concentrations (mM) | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| 0 | 3 | 2065.13333 | |
| 0.1 | 3 | | 3298.86667 |
| 5.0 | 3 | | 3380.10000 |
| 0.5 | 3 | | 3499.93333 |
| 1.0 | 3 | | 3661.16667 |
| Sig. | | 1.000 | .287 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Luminescence intensity

| Nicotine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 3156.50000 | 395.466851 | 228.322893 | 2174.10588 | 4138.89412 |
| 3.0 | 3 | 4311.90000 | 1021.038574 | 589.496896 | 1775.49957 | 6848.30043 |
| 4.0 | 3 | 4789.60667 | 793.609315 | 458.190551 | 2818.17184 | 6761.04149 |
| 5.0 | 3 | 5222.86667 | 611.776244 | 353.209179 | 3703.13023 | 6742.60311 |
| Total | 12 | 4370.21833 | 1024.438931 | 295.730046 | 3719.32089 | 5021.11578 |

**Test of Homogeneity of Variances**

Luminescence intensity

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|

**Test of Homogeneity of Variances**

Luminescence intensity

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| .624 | 3 | 8 | .620 |

**ANOVA**

Luminescence intensity

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7138226.925 | 3 | 2379408.975 | 4.320 | .043 |
| Within Groups | 4405999.435 | 8 | 550749.929 | | |
| Total | 1.154E7 | 11 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Luminescence intensity

Tukey HSD

| (I) Conc. | (J) Conc. | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 3.00 | -1155.400000 | 605.942753 | .297 | -3095.84199 | 785.04199 |
| | 4.00 | -1633.106667 | 605.942753 | .102 | -3573.54866 | 307.33532 |
| | 5.00 | -2.066367E3 | 605.942753 | .037 | -4006.80866 | -125.92468 |
| 3.00 | .00 | 1155.400000 | 605.942753 | .297 | -785.04199 | 3095.84199 |
| | 4.00 | -477.706667 | 605.942753 | .858 | -2418.14866 | 1462.73532 |
| | 5.00 | -910.966667 | 605.942753 | .478 | -2851.40866 | 1029.47532 |
| 4.00 | .00 | 1633.106667 | 605.942753 | .102 | -307.33532 | 3573.54866 |
| | 3.00 | 477.706667 | 605.942753 | .858 | -1462.73532 | 2418.14866 |
| | 5.00 | -433.260000 | 605.942753 | .888 | -2373.70199 | 1507.18199 |
| 5.00 | .00 | 2066.366667* | 605.942753 | .037 | 125.92468 | 4006.80866 |
| | 3.00 | 910.966667 | 605.942753 | .478 | -1029.47532 | 2851.40866 |
| | 4.00 | 433.260000 | 605.942753 | .888 | -1507.18199 | 2373.70199 |

**Multiple Comparisons**

Luminescence intensity

Tukey HSD

| (I) Conc. | (J) Conc. | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 | 3.00 | -1155.400000 | 605.942753 | .297 | -3095.84199 | 785.04199 |
| | 4.00 | -1633.106667 | 605.942753 | .102 | -3573.54866 | 307.33532 |
| | 5.00 | -2.066367E3 | 605.942753 | .037 | -4006.80866 | -125.92468 |
| 3.00 | .00 | 1155.400000 | 605.942753 | .297 | -785.04199 | 3095.84199 |
| | 4.00 | -477.706667 | 605.942753 | .858 | -2418.14866 | 1462.73532 |
| | 5.00 | -910.966667 | 605.942753 | .478 | -2851.40866 | 1029.47532 |
| 4.00 | .00 | 1633.106667 | 605.942753 | .102 | -307.33532 | 3573.54866 |
| | 3.00 | 477.706667 | 605.942753 | .858 | -1462.73532 | 2418.14866 |
| | 5.00 | -433.260000 | 605.942753 | .888 | -2373.70199 | 1507.18199 |
| 5.00 | .00 | 2066.366667* | 605.942753 | .037 | 125.92468 | 4006.80866 |
| | 3.00 | 910.966667 | 605.942753 | .478 | -1029.47532 | 2851.40866 |
| | 4.00 | 433.260000 | 605.942753 | .888 | -1507.18199 | 2373.70199 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

Luminescence intensity

Tukey HSD[a]

| Nicotine concentrations (mM) | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| .00 | 3 | 3156.50000 | |
| 3.00 | 3 | 4311.90000 | 4311.90000 |
| 4.00 | 3 | 4789.60667 | 4789.60667 |
| 5.00 | 3 | | 5222.86667 |
| Sig. | | .102 | .478 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## Oneway

**Descriptives**

Luminescence intensity

| Nicotine 5mM +Cotinine concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Untreated | 3 | 2065.13333 | 81.695614 | 47.166985 | 1862.19018 | 2268.07649 |
| 0 | 3 | 4913.46667 | 770.007697 | 444.564151 | 3000.66151 | 6826.27182 |
| 0.1 | 3 | 5248.60000 | 212.655872 | 122.776925 | 4720.33353 | 5776.86647 |
| 0.5 | 3 | 5529.03333 | 182.917368 | 105.607391 | 5074.64140 | 5983.42526 |
| 1.0 | 3 | 4942.83333 | 251.907847 | 145.439063 | 4317.05955 | 5568.60712 |
| 5.0 | 3 | 5014.70000 | 286.001311 | 165.122934 | 4304.23336 | 5725.16664 |
| Total | 18 | 4618.96111 | 1235.072991 | 291.109496 | 4004.77376 | 5233.14846 |

**ANOVA**

Luminescence intensity

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2.428E7 | 5 | 4856969.343 | 35.387 | .000 |
| Within Groups | 1647043.247 | 12 | 137253.604 | | |
| Total | 2.593E7 | 17 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Luminescence intensity

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Conc | (J) Conc | Mean Difference | Std. Error | Sig. | Lower Bound | Upper Bound |
| Untreated | 0 | -2.848333E3 | 302.493641 | .000 | -3864.38554 | -1832.28113 |
| | 0.1 | -3.183467E3 | 302.493641 | .000 | -4199.51887 | -2167.41446 |
| | 0.5 | -3.463900E3 | 302.493641 | .000 | -4479.95220 | -2447.84780 |
| | 1.0 | -2.877700E3 | 302.493641 | .000 | -3893.75220 | -1861.64780 |
| | 5.0 | -2.949567E3 | 302.493641 | .000 | -3965.61887 | -1933.51446 |
| 0 | Untreated | 2848.333333* | 302.493641 | .000 | 1832.28113 | 3864.38554 |
| | 0.1 | -335.133333 | 302.493641 | .869 | -1351.18554 | 680.91887 |
| | 0.5 | -615.566667 | 302.493641 | .379 | -1631.61887 | 400.48554 |
| | 1.0 | -29.366667 | 302.493641 | 1.000 | -1045.41887 | 986.68554 |
| | 5.0 | -101.233333 | 302.493641 | .999 | -1117.28554 | 914.81887 |
| 0.1 | Untreated | 3183.466667* | 302.493641 | .000 | 2167.41446 | 4199.51887 |
| | 0 | 335.133333 | 302.493641 | .869 | -680.91887 | 1351.18554 |
| | 0.5 | -280.433333 | 302.493641 | .932 | -1296.48554 | 735.61887 |
| | 1.0 | 305.766667 | 302.493641 | .906 | -710.28554 | 1321.81887 |
| | 5.0 | 233.900000 | 302.493641 | .967 | -782.15220 | 1249.95220 |
| 0.5 | Untreated | 3463.900000* | 302.493641 | .000 | 2447.84780 | 4479.95220 |
| | 0 | 615.566667 | 302.493641 | .379 | -400.48554 | 1631.61887 |
| | 0.1 | 280.433333 | 302.493641 | .932 | -735.61887 | 1296.48554 |
| | 1.0 | 586.200000 | 302.493641 | .427 | -429.85220 | 1602.25220 |
| | 5.0 | 514.333333 | 302.493641 | .556 | -501.71887 | 1530.38554 |
| 1.0 | Untreated | 2877.700000* | 302.493641 | .000 | 1861.64780 | 3893.75220 |
| | 0 | 29.366667 | 302.493641 | 1.000 | -986.68554 | 1045.41887 |
| | 0.1 | -305.766667 | 302.493641 | .906 | -1321.81887 | 710.28554 |
| | 0.5 | -586.200000 | 302.493641 | .427 | -1602.25220 | 429.85220 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 5.0 | -71.866667 | 302.493641 | 1.000 | -1087.91887 | 944.18554 |
| 5.0 | Untreated | 2949.566667* | 302.493641 | .000 | 1933.51446 | 3965.61887 |
| | 0 | 101.233333 | 302.493641 | .999 | -914.81887 | 1117.28554 |
| | 0.1 | -233.900000 | 302.493641 | .967 | -1249.95220 | 782.15220 |
| | 0.5 | -514.333333 | 302.493641 | .556 | -1530.38554 | 501.71887 |
| | 1.0 | 71.866667 | 302.493641 | 1.000 | -944.18554 | 1087.91887 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

Luminescence intensity

Tukey HSD[a]

| Nicotine 5mM +Cotinine concentrations (mM) | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| Untreated | 3 | 2065.13333 | |
| 0 | 3 | | 4913.46667 |
| 1.0 | 3 | | 4942.83333 |
| 5.0 | 3 | | 5014.70000 |
| 0.1 | 3 | | 5248.60000 |
| 0.5 | 3 | | 5529.03333 |
| Sig. | | 1.000 | .379 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Luminescence intensity | Cotinine 0.1mM | 3 | 5248.60000 | 212.655872 | 122.776925 |
| | Nicotine 5mM +Cotinine 0.1mM | 3 | 3298.86667 | 120.607518 | 69.632783 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Luminescence intensity | Equal variances assumed | .586 | .487 | 13.813 | 4 |
| | Equal variances not assumed | | | 13.813 | 3.166 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Luminescence intensity | Equal variances assumed | .000 | 1949.733333 | 141.148495 |
| | Equal variances not assumed | .001 | 1949.733333 | 141.148495 |

**ndependent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Luminescence intensity | Equal variances assumed | 1557.842284 | 2341.624383 |
| | Equal variances not assumed | 1513.569857 | 2385.896809 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Luminescence intensity | Cotinine 0.5 mM | 3 | 5529.03333 | 182.917368 | 105.607391 |
| | Nicotine 5mM +Cotinine 0.5mM | 3 | 3499.93333 | 235.604782 | 136.026484 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Luminescence intensity | Equal variances assumed | .048 | .837 | 11.783 | 4 |
| | Equal variances not assumed | | | 11.783 | 3.769 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Luminescence intensity | Equal variances assumed | .000 | 2029.100000 | 172.209540 |
| | Equal variances not assumed | .000 | 2029.100000 | 172.209540 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Luminescence intensity | Equal variances assumed | 1550.969666 | 2507.230334 |
| | Equal variances not assumed | 1539.160372 | 2519.039628 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Luminescence intensity | Cotinine 1mM | 3 | 4942.83333 | 251.907847 | 145.439063 |
| | Nicotine 5mM +Cotinine 1mM | 3 | 3661.16667 | 230.460720 | 133.056559 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Luminescence intensity | Equal variances assumed | .019 | .896 | 6.502 | 4 |
| | Equal variances not assumed | | | 6.502 | 3.969 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Luminescence intensity | Equal variances assumed | .003 | 1281.666667 | 197.120696 |
| | Equal variances not assumed | .003 | 1281.666667 | 197.120696 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Luminescence intensity | Equal variances assumed | 734.371874 | 1828.961459 |
| | Equal variances not assumed | 732.667628 | 1830.665706 |

## T-Test

**Group Statistics**

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Luminescence intensity | Cotinine 5 mM | 3 | 5014.70000 | 286.001311 | 165.122934 |
| | Nicotine 5mM +Cotinine 5mM | 3 | 3380.10000 | 301.795046 | 174.241451 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Luminescence intensity | Equal variances assumed | .000 | .995 | 6.809 | 4 |
| | Equal variances not assumed | | | 6.809 | 3.988 |

**Independent Samples Test**

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Luminescence intensity | Equal variances assumed | .002 | 1634.600000 | 240.053466 |
| | Equal variances not assumed | .002 | 1634.600000 | 240.053466 |

**Independent Samples Test**

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Luminescence intensity | Equal variances assumed | 968.104729 | 2301.095271 |
| | Equal variances not assumed | 967.346058 | 2301.853942 |

**Descriptives**

**mRNA expression (BAX)**

| Concentrations (mM) | | | | | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Std. Error | Lower Bound | Upper Bound |
| Control (0) | 3 | 1.00000 | .000000 | .000000 | 1.00000 | 1.00000 |
| Cotinine 0.5 | 3 | 1.47733 | .147276 | .085030 | 1.11148 | 1.84319 |
| Cotinine 1.0 | 3 | .85357 | .451868 | .260886 | -.26893 | 1.97607 |
| Nicotine 5.0 | 3 | 1.61733 | .043879 | .025333 | 1.50833 | 1.72633 |
| Nicotine 5.0+cotinine 1.0 | 3 | 5.21500 | 1.727222 | .997212 | .92434 | 9.50566 |
| Nicotine 5.0+cotinine 0.5 | 3 | 2.92233 | 1.752215 | 1.011642 | -1.43041 | 7.27508 |
| Total | 18 | 2.18093 | 1.777904 | .419056 | 1.29680 | 3.06506 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| mRNA expression | 18 | 2.18093 | 1.777904 | .537 | 7.151 |
| Group | 18 | 2.5000 | 1.75734 | .00 | 5.00 |

## Kruskal-Wallis Test

**Ranks**

| | Group | N | Mean Rank |
|---|---|---|---|
| mRNA expression | Control (0) | 3 | 4.00 |
| | Nicotine 5.0 | 3 | 10.33 |
| | Cotinine 0.5 | 3 | 8.33 |
| | Cotinine 1.0 | 3 | 3.33 |
| | Nicotine 5 + cotinine 0.5 | 3 | 14.67 |
| | Nicotine 5 + cotinine 1.0 | 3 | 16.33 |
| | Total | 18 | |

**Test Statistics[a,b]**

| | Absorbance |
|---|---|
| Chi-Square | 15.207 |
| df | 5 |
| Asymp. Sig. | .010 |

a. Kruskal Wallis Test

b. Grouping Variable: Group

## Mann-Whitney Test

## mRNA expression (BAX)

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.121 |
| Asymp. Sig. (2-tailed) | .034 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 4.00 | 12.00 |
| | Cotinine 1mM | 3 | 3.00 | 9.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.696 |
| Asymp. Sig. (2-tailed) | .487 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -1.993 |
| Asymp. Sig. (2-tailed) | .046 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -1.993 |
| Asymp. Sig. (2-tailed) | .046 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

**Descriptives**

**mRNA expression (BCL2)**

| Concentrations (mM) | | | | | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Std. Error | Lower Bound | Upper Bound |
| Control (0) | 3 | 1.00000 | .000000 | .000000 | 1.00000 | 1.00000 |
| Cotinine 0.5 | 3 | 3.41033 | 1.716270 | .990889 | -.85312 | 7.67378 |
| Cotinine 1.0 | 3 | 2.32433 | 1.278764 | .738295 | -.85229 | 5.50096 |
| Nicotine 5.0 | 3 | 3.51167 | .276551 | .159667 | 2.82468 | 4.19866 |
| Nicotine 5.0 + cotinine 1.0 | 3 | 10.31533 | 6.992058 | 4.036866 | -7.05390 | 27.68457 |
| Nicotine 5.0 + cotinine 0.5 | 3 | 5.15100 | 3.352198 | 1.935393 | -3.17632 | 13.47832 |

**Descriptives**

**mRNA expression (BCL2)**

| Concentrations (mM) | | | | | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Std. Error | Lower Bound | Upper Bound |
| Control (0) | 3 | 1.00000 | .000000 | .000000 | 1.00000 | 1.00000 |
| Cotinine 0.5 | 3 | 3.41033 | 1.716270 | .990889 | -.85312 | 7.67378 |
| Cotinine 1.0 | 3 | 2.32433 | 1.278764 | .738295 | -.85229 | 5.50096 |
| Nicotine 5.0 | 3 | 3.51167 | .276551 | .159667 | 2.82468 | 4.19866 |
| Nicotine 5.0 + cotinine 1.0 | 3 | 10.31533 | 6.992058 | 4.036866 | -7.05390 | 27.68457 |
| Nicotine 5.0 + cotinine 0.5 | 3 | 5.15100 | 3.352198 | 1.935393 | -3.17632 | 13.47832 |
| Total | 18 | 4.28544 | 4.122442 | .971669 | 2.23540 | 6.33549 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| mRNA expression | 18 | 4.28544 | 4.122442 | 1.000 | 18.350 |
| Group | 18 | 2.5000 | 1.75734 | .00 | 5.00 |

## Kruskal-Wallis Test

**Ranks**

| | Group | N | Mean Rank |
|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 |
| | Nicotine 5.0 | 3 | 10.67 |
| | Cotinine 0.5 | 3 | 9.00 |
| | Cotinine 1.0 | 3 | 7.00 |
| | Nicotine 5 + cotinine 0.5 | 3 | 12.00 |
| | Nicotine 5 + cotinine 1.0 | 3 | 16.33 |
| | Total | 18 | |

**Test Statistics[a,b]**

| | Absorbance |
|---|---|
| Chi-Square | 12.386 |
| df | 5 |
| Asymp. Sig. | .030 |

a. Kruskal Wallis Test

b. Grouping Variable: Group

## Mann-Whitney Test

## mRNA expression (BCL2)

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.121 |
| Asymp. Sig. (2-tailed) | .034 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 3.33 | 10.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 3.67 | 11.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 4.000 |
| Wilcoxon W | 10.000 |
| Z | -.221 |
| Asymp. Sig. (2-tailed) | .825 |
| Exact Sig. [2*(1-tailed Sig.)] | 1.000[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -1.993 |
| Asymp. Sig. (2-tailed) | .046 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

**Descriptives**

**mRNA expression (CAS3)**

| Concentrations (mM) | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control (0) | 3 | 1.00000 | .000000 | .000000 | 1.00000 | 1.00000 |
| Cotinine 0.5 | 3 | 1.04983 | .292792 | .169043 | .32250 | 1.77717 |
| Cotinine 1.0 | 3 | 1.01147 | .989459 | .571265 | -1.44649 | 3.46942 |
| Nicotine 5.0 | 3 | 2.17300 | .196977 | .113725 | 1.68368 | 2.66232 |
| Nicotine5.0 +cotinine 1 | 3 | 4.33833 | 2.649942 | 1.529945 | -2.24449 | 10.92115 |
| Nicotine5.0 +cotinine 0.5 | 3 | 2.67633 | 1.695978 | .979173 | -1.53671 | 6.88938 |
| Total | 18 | 2.04149 | 1.688694 | .398029 | 1.20173 | 2.88126 |

**Kruskal-Wallis Test**

**Ranks**

| | Group | N | Mean Rank |
|---|---|---|---|
| mRNA expression | Control (0) | 3 | 6.00 |
| | Nicotine 5.0 | 3 | 12.67 |
| | Cotinine 0.5 | 3 | 5.00 |
| | Cotinine 1.0 | 3 | 5.00 |
| | Nicotine 5 + cotinine 0.5 | 3 | 12.00 |
| | Nicotine 5 + cotinine 1.0 | 3 | 16.33 |
| | Total | 18 | |

**Test Statistics[a,b]**

| | Absorbance |
|---|---|
| Chi-Square | 12.232 |
| df | 5 |
| Asymp. Sig. | .032 |

a. Kruskal Wallis Test

b. Grouping Variable: Group

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| mRNA expression | 18 | 2.04149 | 1.688694 | .399 | 7.383 |
| Group | 18 | 2.5000 | 1.75734 | .00 | 5.00 |

## mRNA expression (CAS3)

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 4.00 | 12.00 |
| | Cotinine 0.5mM | 3 | 3.00 | 9.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.696 |
| Asymp. Sig. (2-tailed) | .487 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 4.00 | 12.00 |
| | Cotinine 1mM | 3 | 3.00 | 9.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.696 |
| Asymp. Sig. (2-tailed) | .487 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 4.00 | 12.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 3.00 | 9.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.655 |
| Asymp. Sig. (2-tailed) | .513 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -1.964 |
| Asymp. Sig. (2-tailed) | .050 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

**Descriptives**

**mRNA expression (P53)**

| Concentrations (mM) | | | | | 95% Confidence Interval for Mean | |
|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Std. Error | Lower Bound | Upper Bound |
| Control (0) | 3 | 1.00000 | .000000 | .000000 | 1.00000 | 1.00000 |
| Cotinine 0.5 | 3 | 1.09240 | .346996 | .200338 | .23042 | 1.95438 |
| Cotinine 1.0 | 3 | 1.06523 | 1.180527 | .681577 | -1.86736 | 3.99782 |
| Nicotine 5.0 | 3 | 2.05900 | .743050 | .429000 | .21316 | 3.90484 |
| Nicotine 5.0 +cotinine 1.0 | 3 | 3.15600 | 2.488066 | 1.436485 | -3.02470 | 9.33670 |
| Nicotine 5.0 +cotinine 0.5 | 3 | 2.79800 | 2.160363 | 1.247286 | -2.56864 | 8.16464 |
| Total | 18 | 1.86177 | 1.524817 | .359403 | 1.10350 | 2.62005 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|
| mRNA expression | 18 | 1.86177 | 1.524817 | .315 | 6.006 |
| Group | 18 | 2.5000 | 1.75734 | .00 | 5.00 |

**Kruskal-Wallis Test**

**Ranks**

| | Group | N | Mean Rank |
|---|---|---|---|
| mRNA expression | Control (0) | 3 | 5.00 |
| | Nicotine 5.0 | 3 | 13.00 |
| | Cotinine 0.5 | 3 | 7.00 |
| | Cotinine 1.0 | 3 | 5.67 |
| | Nicotine 5 + cotinine 0.5 | 3 | 12.67 |
| | Nicotine 5 + cotinine 1.0 | 3 | 13.67 |
| | Total | 18 | |

**Test Statistics[a,b]**

| | Absorbance |
|---|---|
| Chi-Square | 8.553 |
| df | 5 |
| Asymp. Sig. | .038 |

a. Kruskal Wallis Test

b. Grouping Variable: Group

## Mann-Whitney Test

## mRNA expression (P53)

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.121 |
| Asymp. Sig. (2-tailed) | .034 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 3.00 | 9.00 |
| | Cotinine 0.5mM | 3 | 4.00 | 12.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.696 |
| Asymp. Sig. (2-tailed) | .487 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 4.00 | 12.00 |
| | Cotinine 1mM | 3 | 3.00 | 9.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 3.000 |
| Wilcoxon W | 9.000 |
| Z | -.696 |
| Asymp. Sig. (2-tailed) | .487 |
| Exact Sig. [2*(1-tailed Sig.)] | .700[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Control (0) | 3 | 2.00 | 6.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 5.00 | 15.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | .000 |
| Wilcoxon W | 6.000 |
| Z | -2.087 |
| Asymp. Sig. (2-tailed) | .037 |
| Exact Sig. [2*(1-tailed Sig.)] | .100[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 3.33 | 10.00 |
| | Nicotine 5mM +cotinine 0.5mM | 3 | 3.67 | 11.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 4.000 |
| Wilcoxon W | 10.000 |
| Z | -.221 |
| Asymp. Sig. (2-tailed) | .825 |
| Exact Sig. [2*(1-tailed Sig.)] | 1.000[a] |

a. Not corrected for ties.

b. Grouping Variable: Group

## Mann-Whitney Test

**Ranks**

| | Group | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| mRNA expression | Nicotine 5mM | 3 | 3.33 | 10.00 |
| | Nicotine 5mM +cotinine 1mM | 3 | 3.67 | 11.00 |
| | Total | 6 | | |

**Test Statistics[b]**

| | Absorbance |
|---|---|
| Mann-Whitney U | 4.000 |
| Wilcoxon W | 10.000 |
| Z | -.221 |
| Asymp. Sig. (2-tailed) | .825 |
| Exact Sig. [2*(1-tailed Sig.)] | 1.000[a] |

a. Not corrected for ties.

b. Grouping Variable: Group