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三種牙科樹脂之化學成分毒性效應之分析

Investigation of Toxic Effects of Three Dental Resin Chemicals

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三種牙科樹脂之化學成分毒性效應之分析
Investigation of Toxic Effects of Three Dental Resin Chemicals

本論文係施文智君（學號R99422009）在國立臺灣大學牙醫學系、臨床牙醫學研究所完成之碩士學位論文，於民國102年6月11日承下列考試委員審查通過及口試及格，特此證明

口試委員：

系主任、所長

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中文摘要

複合樹脂為現代牙科極為廣泛使用的材料，但是此類材料在聚合之後會釋放出一些未與樹脂結合的化學物質, 可能會導致各種不同的生物毒性反應以及不良的生物效應。二甲基對甲苯胺 (DMPT)、甲基丙烯酸二甲氨基乙酯 (DMAEMA)和二甲氨基苯甲酸乙酯 (DMABEE)，是牙科材料上常用的三種成分，但是因為存在的濃度較低，且在之前的研究中發現其三者屬於中等毒性，因此研究此三種材料的文獻相對較少。但是由於該三種材料在樹脂合成後仍會被釋放出，存在於口腔中的時間會延長，因此對於該化學物質可能造成的毒性作用與機制需要有足夠的瞭解。因此我們以細胞生長、細胞週期分析、活性氧 (ROS) 產生以及去氧核醣核酸 (DNA) 破壞為主要方向，對個別化學物質造成的毒性進行研究。

首先，我們觀察 DMPT 對中國倉鼠卵巢 (CHO-K1) 細胞的影響。DMPT 會抑制 CHO-K1 細胞長期和短期的生長潛力，對於 DNA 破壞的程度也隨著 DMPT 的濃度呈現正相關，而此時在 CHO-K1 細胞中微核的比例也會上升，代表 DNA 破壞的程度也隨著 DMPT 濃度上升而增加。然而在 ROS 的產生上沒有明顯地呈現，這部分同時被印證在加入乙醯基半胱氨酸 (NAC) 作為抑制劑下，細胞生長潛力也無明顯的改變。而對於細胞週期分析上，在不同濃度 DMPT 作用下也沒有明顯的細胞週期休止發生。細胞死亡的方式大部份也集中在壞死的途徑。由此可知 DMPT 造成 CHO-K1 細胞死亡透過的方式可能不同於其他樹脂單體所造成細胞凋亡的方式，而是因為 DNA 損壞之後而造成細胞壞死，而造成細胞死亡的機制可能也非由 ROS 的產生為主要途徑，因此需要透過其他方面的研究來作探討。

DMAEMA 對 CHO-K1 細胞造成的生長抑制和 DMPT 所造成的模式相似，但是於長期的生長抑制更為明顯。但是在 DNA 破壞上，DMAEMA 所造成的效果並不如 DMPT 顯著，只有在高濃度 3.5 mM DMAEMA 下才會有些微的增加。相同的結果也在 ROS 產生的方式被發現，在高濃度 3.5 mM DMAEMA 的作用下，ROS 產生的比率才有增加。而對於細胞週期進行的檢測下，我們發現，從 1.5 mM DMAEMA 作用下，細胞開始停滯在 S 週期，同時在 Sub-G0/G1 週期也有增加，而這部分的細胞可能來自於 G0/G1 週期部分的減少，這部分可顯示細胞凋亡在進行。透過實驗結果的瞭解，我們可以推估因為 DMAEMA 造成 CHO-K1 細胞死亡的原因可能來自於 ROS 的產生，然而其影響的毒性不是相對明顯可能是因為細胞修復的能力還可以對抗 DMAEMA 所帶來負面的影響，然而對於 DNA 損壞的部分不如 DMPT 明顯，可能兩種藥物造成細胞死亡的路徑並不相同。

DMABEE 在三種藥物中，對 CHO-K1 細胞所造成的毒性是最強的，產生毒性的濃度相較於 DMPT 以及 DMAEMA 低很多。只要 0.75 mM DMABEE 就可造成百分之五十的 CHO-K1 生長潛力的下降，而對長期生長潛力影響下在 0.25 mM DMABEE 就開始有顯著的降低。同時，在細胞的形態上也有明顯地影響，在高濃度 DMABEE 的作用下，CHO-K1 細胞會從原本多角形或是圓形的狀態，形成紡錘狀或是絲狀的變化，而這部分的形態變化下，會在加入羧酸酯酶 (CES) 除了可使生長潛力復原以外，也會讓細胞形態回復。在 DNA 損壞的實驗下發現，從

0.25mM DMABEE 的作用開始就有明顯微核比例的增加，而且在 1.0 mM 的作用下，因為細胞破壞的太嚴重，因此無法去計算，可能因為細胞形態改變太大已經不同於正常的 CHO-K1 細胞。而在 ROS 產生上，DMABEE 也有相當程度的影響，隨著藥物濃度增加，ROS 的比例也會上升。然而在 NAC 的作用下卻沒有明顯的抑制作用，推測可能在這時 NAC 無法作為還原劑，而是成為氧化強化劑 (pro-oxidant) 因此造成細胞生長抑制更顯著。而在細胞週期上，大部份的細胞也在 0.25 mM DMABEE 作用下開始停留在 G0/G1 週期，而且搭配細胞死亡分布的比例也可以發現, 在 0.1 mM DMABEE 的作用下，細胞壞死的比例有增加，而在高濃度 1.0 mM 的作用下，晚期凋亡的比例也有提高。這部分的結果可透過因為 ROS 的產生比例增加，進而破壞 DNA，而造成細胞凋亡或是壞死的表徵。

本實驗結果有助於瞭解三種牙科複合樹脂的化學成分毒性發生的機制和濃度上的影響。雖然透過本實驗所檢測的濃度，在正常牙科治療下應不易發生。但是在缺乏厚度的牙本質以及材料聚合不全的狀況下，被釋放出的化學成分即有可能到達本實驗所觀察到的影響，同時因為對於人體組織的破壞上常常是不可逆的，因此對於該化學成分的使用和毒性，應在臨床使用上審慎避免。

關鍵字 ： DMPT，DMAEMA，DMABEE，細胞毒性，細胞週期，ROS，DNA damage

Abstract

Resin-containing products are widely applied in modern dentistry. Chemical substances released from the materials may cause cytotoxicity and adverse biological effects. N,N-Dimethyl-p-toluidine (DMPT), 2-Dimethylaminoethyl methacrylate (DMAEMA) and 4-Dimethylaminobenzoic acid ethyl ester (DMABEE) are three chemicals released from composite resins. Because of moderate toxicity, they were less investigated in previous studies. However, these three chemicals do not bond to composite resin after curing and are released to oral cavities for a long time. Thus, it is necessary to examine their cytoxicity and toxic mechanism. Investigations of cell growth, cell cycle progression, reactive oxygen species (ROS) production and DNA damage are valuable direction to realize the mechanism, thus were applied in the present studies to get insight into the chemicals-induced toxicity.

First of all, we evaluated the effect of DMPT on CHO-K1 cells. DMPT induced both short-term and long-term growth inhibition of CHO-K1 cells. In the mean time, the ratio of micronuclei (MNi) increased in a dose-dependent manner. However, ROS production was not elevated and N-acetyl-cysteine (NAC) could not rehabilitate the growth potential. On cell cycle analysis, after treating with DMPT, there were no obvious arrest at different phases of cell cycle compared to control group. Moreover, the mode of cell death most accumulated in necrosis. According to the results from above, it suggested that ROS production was not the main cause of CHO-K1 cell death and we needed to examine other pathways to explain of DNA damage and cell death.

DMAEMA also produced growth inhibition of CHO-K1 cells in the same pattern compared to DMPT. But, the effect of DNA damage of DMAEMA was weaker than DMPT. Only under 3.5 mM DMAEMA, the amount of MNi rised slightly. Similarly,

ROS production obviously increased when CHO-K1 cells exposed to the highest concentration DMAEMA. However, we found that the cell cycle arrested at S and Sub-G0/G1 phase started from 1.5 mM DMAEMA. This result implied that the cells might get into apoptosis. Thus, first, we could state that DMAEMA could induce CHO-K1 cells death by ROS production, but the ability of self-repairing might alleviate the toxicity of DMAEMA. Secondary, due to different results of DNA damage between DMPT and DMAEMA, we hypothesize that they induced cell death by different mechanism.

Among three drugs, DMABEE was the most toxic to CHO-K1 cells. It elicited growth inhibition of CHO-K1 cells in a dose-dependent manner, and at a much lower concentration compared to DMPT and DMAEMA. In short-term inhibition, 0.75 mM DMABEE induced about 50% down-regulation of growth capacity; in long-term inhibition, there were obvious decrease of growth potential from 0.25 mM DMABEE. Further, in morphology, CHO-K1 cells changed from cuboid- or round-shape to fibroblastic variation under high concentrations of DMABEE. However, the morphologic change could be recovered by pre-treating with carboxylesterases (CES). Similarly, after co-incubating with CES, the growth inhibition of CHO-K1 cells by DMABEE could be prevented. In CBMN assays, the percentage of MNi elevated started from 0.25 mM DMABEE. The cells were severely broken and could not be calculated by treating with 1.0 mM DMABEE. ROS production of CHO-K1 cells also increased in a dose-related manner of DMABEE. But, there was no prevention of growth inhibition after pre-incubating with NAC. We believed that NAC played a role of pro-oxidant instead of anti-oxidant. Thus, the growth inhibition of CHO-K1 cells dramatically increased. The cell cycle got arrested on G0/G1 phase started from treating

with 0.25 mM DMABEE and the cell residing in the quadrant of necrosis and late apoptosis would increase. Theses results indicated that CHO-K1 cells would death through DNA damage by ROS attack and cause cell necrosis or late apoptosis.

The present studies helped us to elucidate the cytotoxic mechanism of these three chemicals leached from composite resins. Although the cytotoxic concentration reported by us might not reached in prudent application. while lacking sufficient thickness of dentin or poor polymerization, the unbound chemical substances could lead potential toxic effect to the pulp tissue.

Key words : DMPT, DMAEMA, DMABEE, cytotoxicity, cell cycle, ROS, DNA damage

Chapter I. Literature Review

1.1 Introduction

Resin composites are widely used as restorative materials in dentistry due to their ease of handling, rapid polymerization, strength, mechanical bonding to enamel and dentin, and esthetic properties. There are several types of resin materials that the dentist can choose in different situations (Reichl, Esters et al. 2006). Thus, more and more chemicals and substances are added into modern dental resins (Allen, Bayne et al. 2001). These resins, however, have several drawbacks such as water sorption, and dissolution of the residual monomers (Ortengren 2000). They could cause hypersensitivity or toxicity to different tissues or even other body organs. Therefore, further studies of cytotoxicity of chemicals leached out from composite resin are essential.

Sphal and coworkers investigated 10 composite resin products and 67 different organic ingredients available in commercial materials. Because of the complex chemical interactions and incomplete monomer-polymer conversion, many substances such as (co)monomers, additives, or polymerization products may be released from composite fillings into the adjacent tissues and oral cavity (Spahl, Budzikiewicz et al. 1998). In several previous studies, it has been found that pulp inflammations may be caused by resin composites (Pashley 1996). Stanley stated that the degree of pulpal alteration may be accompanied by deeper cavities. However, histological studies have reported conflicting results of tissue response to resin-based restoration, and it remains controversial if they are biocompatible with the pulp, periapical and periodontal tissues.

Geurtsen and coworkers (1997), investigated 35 dental resin composite monomers / additives. The data indicated that not only monomers or (co)monomers had cytotoxicity, but several initiators, co-initiators, or additives also showed toxic effects (Geurtsen, Lehmann et al. 1998). Thus, it is important to replace the materials that have been found to have marked cytotoxicity with the substances that are less toxic.

Most studies available today are concerned about dental resin toxicity focused on the major monomers of composite resin such as Bis-GMA, HEMA or TEGDMA. However, there are still some ingredients such as co-initiators / initiators, reducing agents and additives, that could set apart from the elution of residual monomers immediately after placement at salivary surface of filling (Stanterre et al. 2001). To date, there have been few studies evaluating the cytotoxicity of co-initiators / initiators, reducing agents or additives. To elucidate their possible toxic effect and mechanism may help to improve the safety of clinical application of dental composite resin.

1.2 Chemicals (or components) leached out from composite resins

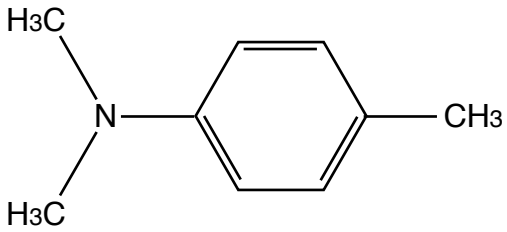
Composite resin materials generally consist of organic polymers and inorganic filler particles that are bonded to a silorane coupling. They are commonly used in restoration and takes place of tooth structures (Allen, Bayne et al. 2001). The setting process is often incomplete and monomers could be unbonded to the restorations, because monomer-polymer conversion rate is from 35% to 77%. Un-polymerized or residual chemicals may be released into oral cavity, affect oral mucosal tissues, diffuse through the dentin to the dental pulp, and even migrate into the bloodstream (Taira, Urabe et al.

1988, Ferracane and Condon 1990). Restorations of the tooth may be degraded into monomers, oligomers and several chemicals through chewing process or salivary sorption (Kodaka, Kobori et al. 1999). In previous studies, lots of elution from resin-based dental restorative materials have been characterized by identification and quantification. The leaching of components from dental composites has a potential impact on both the structural stability and the biocompatibility of the material. The latter is of much greater concern. The concentration of degraded chemicals may be around several μM to mM, however, it is enough to induce some adverse effects. The concentration of resin monomers migrating into dental pulp may reach to 8 mM. Pulp inflammation has been found in deep caries restoration without liner, even in the absence of bacterial leakage. These findings strongly support the inference of cytotoxicity from resin composites (Ferracane 1994, Hofmann, Renner et al. 2002, Yap, Han et al. 2004). According to the instruction for use, these composites contain a variety of substances, such as co-monomers, stabilizers, co-initiators / initiators, etc. It deserves attention that DMPT, DMAEMA and DMABEE performing as reducing agents or co-initiators may be released from composite resins more easily, because they are not bonded to resin composites after polymerization.

1.3 N,N-Dimethyl-p-toluidine (DMPT)

N,N-Dimethyl-p-toluidine (DMPT) is an important reducing agent (RA) or accelerator in modern dental resins. It often combines with a photosensitizer such as camphorquine (CQ) in visible light-cured resins and benzoyl peroxide (BPO) in self-curing resins

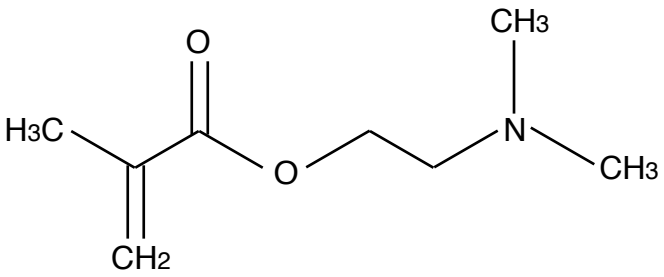
(Taira, Urabe et al. 1988). Because of the mixing ratio in different brands varies significantly with CQ:RA < 1 or > 1, Thus, DMPT is in the vast majority of light-cured dental resins at a concentration between 0.2 to 1.0% w/w (Lee, Pagoria et al. 2007). It is regarded as tertiary amines (Masuki, Nomura et al. 2007). Few previous studies stated that DMPT, in the presence of CQ and emitted by visual light, creates a redox environment in which DNA is attacked and generates oxidative damage. ΦX-174 RF I double-stranded DNA, incubated with CQ/DMPT solution, shows higher DNA damage ratio and has been reduced while co-treating with antioxidants (Lee, Pagoria et al. 2007). DMPT, however, exhibited only moderate cytotoxic effect (the effect dose is between 2.30 ± 0.16 and 4.25 ± 0.06 mM). But this chemical, in general, is found during polymerization or decomposition of the polymerized resin composites (Geurtsen, Lehmann et al. 1998). Thus, it provides a reason for evaluating the long-term effect of DMPT in oral cavity. DMPT toxicity was evaluated by using human gingival fibroblast cell through cell cycle analysis and growth assays, the results indicated the inhibition of human gingival fibroblast cell growth (Masuki, Nomura et al. 2007). On the other hands, there were some studies stated that DMPT are not genotoxic, but the metabolic products of DMPT are estrogenic (Nomura, Ishibashi et al. 2003, Nomura, Teshima et al. 2006). Apart from dental materials, human exposure to DMPT can possibly lead to methemoglobinemia and allergic reactions (Kim, Ghanbari et al. 2007). But, the questions about the biological safety of DMPT is justified and the adverse effects associated with DMPT are not well documented. The structure of DMPT is depicted below.



1.4 2-Dimethylaminoethyl methacrylate (DMAEMA)

2-Dimethylaminoethyl methacrylate (DMAEMA) is used in conjunction with a photo-initiator (typically camphorquinone) as an electron acceptor for forming free radicals in visible light-cured dental restorative materials (Masuki, Nomura et al. 2007). DMAEMA is one kind of methacrylates. Cellular responses to methacrylates include altered growth rates, changes in DNA, RNA and protein synthesis, alterations in synthesis of the major membrane lipids, especially phospholipids, and accumulation of unusual types or amounts of lipids (Schuster, Erbland et al. 1997, Caughman, Schuster et al. 1999). The amount of DMAEMA in commercial resin composites is about 0.5% w/w and it could leach out after incomplete curing resin composites (Moreau, Chappard et al. 1998). The proliferation rate of human gingival fibroblast cell has been down-regulated under treating with DMAEMA. And results of cell cycle analysis suggested that cell in G0/G1 phase increased significantly dependent on the concentration of DMAEMA. The Annexin V-FITC / PI assay showed 5mM DMAEMA induced human gingival fibroblast cell death through the way of necrosis but not apoptosis (Masuki, Nomura et al. 2007). Another articles also reported that DMAEMA was dose-dependent on proliferation of gingival fibroblast by MTT assay (Lapp and Schuster 2002).

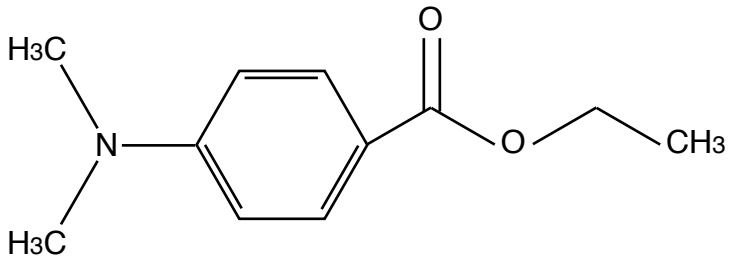
Moreover, at higher DMAEMA concentrations, both cell growth and enzymatic activities of yeast were significantly decreased at all time points (Nomura, Ishibashi et al. 2003). DMAEMA, readily elute from resin materials, could not only affect cell proliferation, but also has been shown to affect cell neutral lipid and phospholipid metabolism. Nevertheless, some previous studies stated that DMAEMA did not show significant genotoxic activity in bioluminescent bacterial genotoxicity test (Nomura, Teshima et al. 2006). Because of doubtful toxicity of DMAEMA in experiment, it should be received more investigation to confirm its cytotoxicity and genotoxicity in detail. The structure of DMAEMA is depicted below.



1.5 4-Dimethylaminobenzoic acid ethyl ester (DMABEE)

4-N,N-Dimethyl amino benzoic acid ethylester (DMABEE), a leachable lipophilic component of polymer-based dental-filling materials and is a co-initiator in pit and fissure sealants (Lygre, Hol et al. 1999). According to current research of cytotoxicity of 35 dental resin composte monomers / additive, DMABEE has been reported to have a moderate cytotoxic effect (the effect dose is between 1.22 ± 0.06 and 1.26 ± 0.06 mM), (Geurtsen, Lehmann et al. 1998). Seiss and coworkers investigated the amount of

DMABEE in elutes from resin composites by GC / MS. They reported that DMABEE released from the resin composites was around 0.11% w/w in water (Seiss, Langer et al. 2009). DMABEE has been shown to interact with cell membrane phospholipids, such as phosphatidylcholine and phosphatidylserine (PS). PS exposure seems to be a universal phenomenon of apoptosis occurring in most if not all cell types independent of the initiating trigger (Lygre, Vorland et al. 2001). Previous studies have shown that polymer-based dental materials induce apoptosis and necrosis. It was therefore of interest to investigate the cytotoxicity of DMABEE. U-937 cell at higher concentrations DMABEE was more likely to induce strong inflammatory tissue reactions (Cimpan, Cressey et al. 2000). Although this was to be expected as in vitro in the absence of phagocytes apoptotic cells undergo postmortem changes similar to necrosis, it seemed to be realized that DMABEE certainly can elicit cellular death. Hence, further in vitro and in vivo studies are required to decipher the mechanisms leading to apoptosis and necrosis by DMABEE. The structure of DMABEE is depicted below.



1.6 Chinese hamster ovary (CHO) cells and CHO-K1 cells

Chinese hamster ovary (CHO) cells are a cell line derived from the ovary of the Chinese hamster (Karthik P. et al. 2007). They are often used in biological and medical research and commercially in the production of therapeutic proteins. They were introduced in the 1960s, and are grown as a cultured monolayer and require the amino acid proline in their culture medium. CHO cells are used in studies of genetics, toxicity screening, nutrition and gene expression, particularly to express recombinant proteins. Today, CHO cells are the most commonly used mammalian hosts for industrial production of recombinant proteins. CHO-K1, used in this study, was purchased from ATCC and derived from the original cell lines in Dr. Puck's laboratory, most likely in the late 1960's. It contains a slightly lower amount of DNA than the original CHO (Tjio and Puck 1958).

1.7 Apoptosis and necrosis

Generally, two types of cell death can occur, namely apoptosis and necrosis (Fadeel and Orrenius 2005). When cytotoxic stimuli are intense, cells may escape from the cell cycle and undergo a process of cell death called apoptosis. It is an active physiological process and usually controlled by several signaling pathways. The cell death of apoptosis is induced rapidly, and the debris is also cleaned quickly by phagocytes. Thereby, an acute inflammatory reaction could be avoided (Savill, Fadok et al. 1993). By contrast, necrosis is so-called “cell murder”, a passive process that follows the

exposure to a gross injury. It may trigger an inflammatory reaction and induce some signal or cell marker, such as IL-6 or CD40 in macrophage. To determine which death process is caused by different chemicals may help to realize the true reaction on cell. Chemicals of dental resin polymerization have been shown to induce cytotoxicity in vitro. However, the mechanism to induce cell death and interfere cell cycle is not fully understood. Against the background, we should evaluate the effects of these chemicals, including their influences on cellular apoptosis and necrosis, cell cycle, and cytotoxicity (Majno and Joris 1995).

1.8 Reactive oxygen species (ROS)

Several studies have revealed that chemicals from resin composites may have the potential of increasing ROS. ROS frequently cause alterations to nucleic acids like base modification, double bands lesions, and strand breaks. It has been speculated that oxidative stress due to ROS is an etiological factor in half of all human cancers (Schweikl, Spagnuolo et al. 2006). ROS, which are also associated with numerous diseases, including the neurodegenerative disorders e.g., Alzheimer's disease and amyotrophic lateral sclerosis, plays a significant role in aging (Beckman and Ames 1997).

Leachable substances from resin-based materials are a likely cause of cellular stress via the formation of ROS. This statement is supported by the finding that such leachable substances may influence the intracellular level of glutathione (GSH) which plays a dominant role in protecting cells against oxidative damage. Some previous articles state

that increased ROS production following mitochondrial GSH depletion, represents a crucial event, which irreversibly commits cells to apoptosis. Recently, resin monomers disrupt the stable redox balance and result in an oxidative cellular damage (Lee, Pagoria et al. 2007). TEGDMA, for example, causes a depletion of GSH in human gingival fibroblast cell. Subsequently, To break the redox system is one way that the production of ROS enhanced cell death (Samuelsen, Dahl et al. 2007).

As reference in numerous studies have revealed, excess ROS results in DNA damage by attacking double strains structure of DNA. Lee and coworkers found that ROS scavengers such as NAC or Vit.C / E can obviously down-regulate the percentage of DNA damage. Moreover, CQ / DMPT also co-stimulated oxidative DNA damage under visible-light irradiation (Lee, Pagoria et al. 2007).

Selective inhibitors of ERK, JNK and p38 may modify the apoptotic response after HEMA and TEGDMA exposure, therefore MAPK pathway could be thought as a route of ROS-induced signaling cascade in cell cycle. TEGDMA has caused accumulation V79 cells in G2 phase and lacked of checkpoint of G1/G0 phase in cell cycle. Likewise, cell cycle of human gingival fibroblast cells may be delayed after treating with HEMA (Samuelsen, Dahl et al. 2007). Apoptosis and cell-cycle arrest have been induced by components of dental adhesive resins such as HEMA and TEGDMA. Thus, it could be assumed that ROS could affect on cell cycle progression.

NAC, as a source of sulfhydryl groups, is a scavenger of ROS and the precursor of GSH. Many adverse effects of ROS in vitro or in vivo can be alleviate while co-treating with materials of resin composites with NAC (Spagnuolo, D'Anto et al. 2006). Schweikl et al stated that NAC, ascorbate and Trolox (Vit. E) could prevent cell from damage caused by HEMA or TEDGMA (Schweikl, Hartmann et al. 2007). Moreover, other anti-

oxidants can play the same role of stopping ROS damage. Vit. C decreased the extent of DNA damage induced by UDMA through pronouncing a protective effect of methylglycol chitosan. Catalase could degrade hydrogen peroxide, one kind of ROS, and recover the cell property. Esterase, an enzyme in saliva, might inhibit ROS over-production (Masgras, Carrera et al. 2012).

Numerous investigations have found that free radical scavengers, such as aspirin, mannitol, or genistein, can effectively reduce the oxidative DNA damage caused by free radicals (Hsu and Li 2002). These studies indicated that the inhibition of ROS-induced oxidative DNA damage is dependent on the concentration of antioxidants. In light of this, we hypothesized that the presence of free radical scavengers will reduce the frequency and severity of oxidative stress caused by DMPT, DMAEMA and DMABEE. Thus, we tested the issue through treating CHO-K1 cells with these toxic chemicals and different radical scavengers.

1.9 Cell cycle

There are four different phases in eukaryotic cell cycle. G0 phase represents a resting phase where the cell has left the cycle and has stopped dividing. Cells increase in size in G1 phase, and it ensure everything is ready for DNA synthesis. DNA replication occurs during S phase. Before mitosis, cells continue to grow and are controlled at G2 checkpoint. Finally, in M phase, the cell gets division into two daughter cells. The successful completion of G1, S and G2 phase of the cell cycle is through several checkpoints. Defects in the checkpoints may affect genomic integrity and induce

irreversible cell damage. There are some proteins, such as p53, CDKs, etc., as signal regulating the cell cycle (Cooper GM. 2000).

Previous studies reported that some chemicals form composite resin may regulate cell cycle. It often induced a significant and sustained accumulation of cells in G0 / G1 phase, compared with the general cell cycle. There was an effect on cell cycle of human gingival fibroblast cell after treating with benzoyl peroxide, camphorquinone, DMAEMA and DMPT (Masuki, Nomura et al. 2007). This implied that the growth inhibitory effect of the polymerization monomers, co-initiators / initiators or additives stemmed from the arrest of DNA replication in the cell cycle. The aim of this study is to investigate the correlation between substances released from commercial resins and cell cycle, and to realize the influence and effects of DMPT, DMAEMA and DMABEE on the phase of CHO-K1 cells accumulation.

1.10 DNA damage

Monomers are released from dental resin materials, and may potentially cause adverse biological effects in mammalian cells. Cytotoxicity and genotoxicity of some of these chemicals have been identified in a vast number of investigations during the last decade. Composite restorative materials are a mixture of polymerized resin components reinforced by inorganic fillers (Schweikl, Spagnuolo et al. 2006). Several studies have shown that monomers and other components were released from these materials into the oral environment even after polymerization. They may induce both cytotoxicity and genotoxicity (Pagoria, Lee et al. 2005, Winter, Pagoria et al. 2005, Lee, Pagoria et al.

2007). Genomic DNA is a molecular target for components of dental resin materials. Genotoxic effects detected in bacteria and mammalian cells are indicative of the interaction between DNA and some monomers or associated metabolites (Jazayeri, Falck et al. 2006). For instance, no induction of gene mutations was detected with Bis-GMA and UDMA. However, DNA damage may have occurred to some extent, because Bis-GMA was tested positive in the DNA synthesis inhibition test. Likewise, no gene mutations were detected with the monomers HEMA and MMA. However, high concentrations of HEMA induced a large number of micronuclei, indicating chromosomal aberrations in vitro (Samuelsen, Dahl et al. 2007).

The molecular mechanisms leading to mutations induced by resin monomers are unclear at present. Nonetheless, there are at least two possibilities for the generation of DNA lesions. First, the spectrum of mutations induced by TEGDMA are similar to those caused in the genome of mammalian cells after exposure to x-rays and various chemical agents. Second, these substances could induce mutations by a secondary mechanism via the generation of ROS, as do agents such as ionizing radiation, UV, and certain chemicals (Vral, Fenech et al. 2011). Thus, the direct way to observe DNA damage is to see if there is any morphologic change on cell nuclei during division.

Biological dosimetry, based on the analysis of micronuclei (MN) in the cytokinesis-block micronucleus (CBMN) assay can be used as an alternative method for scoring dicentric chromosomes in the field of radiation protection (Bull, Beetstra-Hill et al. 2011). In the past, CBMN assay was mainly performed as a method of evaluation DNA damage of radiation. Recently, CBMN assay, combined with a fluorescence hybridization centromere staining technique, becomes a popular method for detecting

DNA damage by scoring MN and nucleoplasmic bridges in the binucleated cells (Fenech, Kirsch-Volders et al. 2011).

Along these issues, substances released from resin composites could induce DNA damage and gene mutation. It is more easily to detect the adverse effect by CBMN assay and apply a reliable method of evaluation severity of genotoxicity.

1. 11 Carboxylesterase (CES) in resin metabolism

Various restorative materials, such as composite resin or glass ionomer cement, could be successfully used to restore cavities. These chemicals, however, directly attach to the pulp or diffuse through dentin layer may induced pulpal inflammation in some treated teeth (Murray, Windsor et al. 2003). Our oral cavity is a complex environment, thus the chemicals of resin composites may not show their cytotoxicity in original form. Sometimes, they affect pulpal or oral mucosal tissue by metabolized form. 2,3-epoxy-2methyl-propionic acid methylester, methacrylate, 2,3-epoxy-2methyl-propionate and bisphenol-A-bis(2,3-dihydroxypropyl) ether which could stimulate ROS and cause unbalanced redox regulation, are reactive intermediates while resin composites are metabolized (Seiss, Marquardt et al. 2009).

Some different esterases in saliva exhibit activities of resin degradation and affect the physical properties (Guillen-Gosalbez and Sorribas 2009). CES are esterases that has ability of detoxification of chemicals with ester, amide or carbamate groups (Sanghani, Sanghani et al. 2009). CES, especially CES2, could prevent human dental pulp cells from toxicity of monomers or hydrolysis caused by methacrylic acid (Durner, Walther et

al. 2010). Moreover, CES certainly preserves cell viability while co-treating BisGMA with porcine esterase in human dental pulp cells (Chang, Lin et al. 2012).

Because DMPT, DMAEMA and DMABEE are chemicals with amide, methacrylate and ester, we ought to evaluate the toxicity and to clarify the effect of these chemicals with CES in CHO-K1 cells.

The aim of this study was to test the hypothesis that the cytotoxicity and genotoxicity on CHO-K1 cells after exposing to DMPT, DMAEMA, or DMABEE, and these adverse reaction could be alleviated by esterase or ROS scavengers such as NAC, catalase.

Chapter II. The purpose and hypothesis of the study

According to the above, DMPT, DMAEMA and DMABEE could be cytotoxic, and may have adverse effects on CHO-K1 cells, a cell line popularly used for cytotoxicity and genotoxicity assays. In previous studies, however, still not explain the mechanism of disturbane of cell growth clearly. Moreover, the result may be inconsistent in different investigations about ROS interference, cell cycle analysis and DNA damage assay.

Therefore, the objectives of our study was to investigate (1) whether DMPT, DMAEMA and DMABEE released form commercial resin composites are cytotoxic and may cause inhibition of cell growth on CHO-K1 cells. (2) Whether ROS may be the main component that induces cell apoptosis and DNA damages on CHO-K1 cells after exposing to DMPT, DMAEMA and DMABEE. (3) Whether NAC, catalase and esterase could be effective to reduce or prevent cytotoxicity or genotoxicity. In order to enhance the safety of using DMPT, DMAEMA and DMABEE, the knowledge of the substance released from resin composites is important and essential.

Chapter III. Materials and Methods

3.1 Chemicals

N,N-dimethy-p-toluidine (DMPT), 2-dimethylaminoethyl methacrylate (DMAEMA) , 4-dimethylaminobenzoic acid ethyl ester (DMABEE), dimethyl-sulphoxide (DMSO), propidium iodide, catalase, N-acetyl-cysteine (NAC), esterase and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma / Aldrich (Sigma Chemical Company, St. Louis, MO, USA). Reagents for cytometry flow were obtained from Becton Dickson (Worldwide Inc. Dan-Jose, CA, USA). Cell-culture medium (F-12) and reagents were from Life Technologies (Gibco, Life Technologies, NY, USA). 4',6-diamidino-2-phenylindole (DAPI) and rhodamine phalloidin for immunofluorence were purchased from Life Technologies (Gibco, Life Technologies, NY, USA). DMPT was dissolved in DMSO as stock in 5 M and diluted to various concentrations in culture medium (1.0, 2.5, 5.0, 7.5 and 10.0 mM). DMAEMA was stored in 5 M by dissolving with DMSO and prepared at 0.5, 1.0, 1.5 2.5 and 3.5 mM in culture medium. DMABEE was soluble at 1 M in DMSO and arranged in different concentration in culture medium (0.1, 0.25, 0.5, 0.75, 1.0 mM). The amount of DMSO in each culture well is equal.

3.2 CHO-K1 cells and cell culture

CHO-K1 cells were purchased from ATCC (Queens Road, Teddington, United Kingdom), and cultured in F-12 nutrient mixture medium, and were incubated at 37°C in water-saturated atmosphere containing 95% air and 5% CO2.

3.3 Evaluation of cell growth

To determine the inhibition of CHO-K1 growth by the chemicals (DMPT, DMAEMA and DMABEE), both MTT assay and cell colony assay were used in this study. An equal number of CHO-K1 cells (1.0×10^6 / well) were seeded in 2 ml of F-12 medium in 6-well plate. The cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO2. For each stock solution of 3 chemicals in DMSO, it was diluted in cell culture medium to obtain a final DMSO concentration of 0.2%. The cells were then treated with various concentrations of 3 chemicals in final concentration of (1) 1.0, 2.5, 5.0, 7.5 and 10.0 mM (DMPT), (2) 0.5, 1.0, 1.5 2.5 and 3.5 mM (DMAEMA), (3) 0.1, 0.25, 0.5, 0.75, 1.0 mM (DMABEE).

For short-term effects, MTT assay is used. After the cultured cells were incubated for 24 hours, 20 μl MTT was added to each well and the plates were incubated for two hours at 37°C. Absorbance at 590 nm was measured with a Dynatech Microwell plate reader (Dynatech Labs, Inc, Chantilly, VA, USA), whereby cell proliferation rate was calculated from the absorbance value.

On the other hand, for long-term effect, colony forming cell (CFC) assay has applied. After the cultured were incubated (200 cells / well) for 24 hours, medium was replaced with F-12 medium containing various chemicals and incubated for 7 days. The colonies were counted (cell numbers > 40) and the ratio was calculated to the control group.

3.4 ROS detection (DCF flow cytometry) / GSH depletion (CMF flow cytometry)

The DCF assay is a method to measure the levels of intracellular ROS. It was first described for micro-plate reader by Wang & Joseph (1999), although the original method had already been discovered in the 60s. Basically, cells were incubated with the pro-fluorescent, lipophilic H2-DCF-DA (dihydrodichlorofluorescein diacetate) which can diffuse through the cell membrane. Inside, the acetate groups are cleaved by cellular esterases so the resulting H2-DCF cannot leave the cells. Reaction with ROS, primarily hydrogen peroxide (H2O2), results in the fluorescent molecule DCF (maximum emission was 530 nm).

Growing 2.5×10^5 CHO-K1 cells in 6-well plate in 2 ml F-12 medium overnight. To add different testing substances to medium and incubate for 24 hours. Then, cells were stained with H2-DCF-DA for 30 minutes and collected by using 10% EDTA-trypsin / PBS and F-12 medium. Each sample was rinsed and resuspended with 250~300 λ PBS on ice. DCF fluorescence was measured at 530 nm using FACSCalibur Flow Cytometry. A total of 10000 cells were analyzed for each sample by Cell Quest software (Becton Dickinson).

CHO-K1 cells (2.5×10^5) were incubated in fresh medium containing DMPT, DMAEMA, DMABEE or DMSO(as negative control) for 24 hours. To evaluate whether the three chemicals may induce ROS production GSH depletion, cells were then stained respectively with CMF-DA for 30 min, detached with trypsin/EDTA, washed with PBS and immediately subjected for flow cytometric analysis.

3.5 Apoptosis and necrosis (PI & Annexin V flow cytometry)

To identify the cells undergoing apoptosis or necrosis, cells were stained with PI and FITC-Annexin V. PI detects cells that have lost their plasma membrane integrity, while Annexin V detects early apoptotic cells based on externalized phosphatidylserine.

Chemicals (DMPT, DMAEMA and DMABEE) of various concentration were added to CHO-K1 cell cultures, which were seeded 2.5×10^6 CHO-K1 cells in 6-well plate overnight, for 24 hours. After incubation, the cells were harvested using EDTA-trypsin and F-12 medium / PBS. The cells were then resuspended in 250 ~ 300 λ binding buffer with 4 μl Annexin V-FITC (Becton Dickson) and 8 μl PI (50 μg / ml) solution. The stained samples were kept on ice and subjected to FACSCalibur Flow Cytometry. FITC fluorescence was collected between 515 and 545 nm, as well as PI fluorescence between 564 and 606 nm. A total of 10000 cells were analyzed for each sample by Cell Quest software (Becton Dickinson).

3.6 Cell cycle (PI flow cytometry)

An equal quantity of 2.5×10^5 CHO-K1 cells were plated in 6-well plate overnight. They were, then, incubated in different concentrations of DMPT, DMAEMA, and DMABEE (as growth assay) for 24 hours.

Cellular DNA content was determined by flow cytometry as described perviously. Attached and floating cells were collected by using EDTA-trypsin and F-12 medium / PBS. The cells were fixed for a day in a 70% ice-cold ethanol solution. After washing with PBS, the cells were treated with RNase (2 mg / ml) and stained with PI (40 μg / ml). The PI-elicited fluorescence of individual cells was measured using a FACSCalibur Flow Cytometry with laser excitation at 488 nm. A total of 10000 cells were analyzed for each sample by ModFit and Cell Quest software (Becton Dickinson).

3.7 DNA damage (CBMN assay with IF)

The cytokinesis-block micronucleus assay (CBMN assay) is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. The extent of DNA damage are scored specifically in once-divided binucleated (BN) cells that include micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss, nucleoplasmic bridges (NPBs), a biomarker of DNA mis-repair and/or telomere end-fusions, and nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/ or DNA repair complexes (Fenech, Kirsch-Volders et al. 2011). The assay is being applied successfully for monitoring of in vivo genotoxicity exposure, in vitro

genotoxicity testing and in diverse research fields, as well as a predictor of normal tissue and tumor radiation sensitivity and cancer risk.

The 18 × 18 mm slides were placed in 6-well plate after sterilization. 1.0×10^5 CHO-K1 cells were seeded in the plate with 2 ml F-12 medium overnight. Before treating with various concentration of substances (DMPT, DMAEMA and DMABEE) for 24 hours, cells were pre-treated with cytochalasin-B, an inhibitor of microfilament ring assembly required for the completion of cytokinesis, for 1 hours. Attached cells were fixed with formaldehyde for 15 minutes, washed with PBS, and rinsed with 2% PBST (Tween 20 and PBS) for 15 minutes. The slides, then, were picked up and added BSA (0.5%) for 45 minutes. Finally, the cells were stained with a mixed solution (BSA and immuno-fluorescence agents (DAPI : rhodamine phalloidin ~ 1 : 3) for 1 hours. DAPI (blue) and rhodamine phalloidin (red) fluorescence were detected by Olympus IX70 Inverted Microscope. A total of 500 BN cells were calculated for each sample.

3.8 Statistical analysis

All experiments were performed at least four times. Statistical analysis of data was performed using the one-way analysis of variance test considering P values less than 5% as significant. All statistical analyses were carried out with SPSS 12.0 and Sigma Plot 10.0.

Chapter IV. Results

4.1 Effects of DMPT, DMAEMA and DMABEE on CHO-K1 cells

4.1.1 Growth assay

DMPT

As shown in Figure 1.1a, DMPT did induce cytotoxicity and growth inhibition in CHO-K1 cells. At concentration of 1.0, 2.5, 5.0, 7.5, 10.0 mM, DMPT inhibited the growth of CHO-K1 cells by 9 to 22% in 24 hours.

The long-term colony survival of CHO-K1 cells significantly decreased under DMPT treatment. Following 24 hours exposure to DMPT and then observation for a week later, colony formation capacity of CHO-K1 cells was suppressed in a dose-related pattern. As depicted in Figure 1.1b, 1.0 to 10.0 mM of DMPT decreased the colony numbers by 12% to 67%.

DMAEMA

DMAEMA induced cytotoxicity and reduced viable cells of CHO-K1 around 20%, especially in higher concentrations (2.5 mM and 3.5 mM). As shown in Figure 1.2a, it showed a trend of increase during 0.5 mM to 1.5 mM.

Colony formation assay of CHO-K1 cells under DMAEMA treatment showed an obvious suppression. As depicted in Figure 1.2b, the cytotoxicity was directly displayed at a high concentration (3.5 mM) as well as the percentage of colony numbers were less than 20%.

DMABEE

DMABEE inhibited the growth of CHO-K1 cells in a dose-dependent manner. After 24 hours, cell viability, as show in Figure 1.3a, was obviously reduced about 50% under 0.75 and 1.0 mM.

DMABEE showed the strongest cytotoxicity on CHO-K1 among three chemicals in a 24 hours MTT assay, however, it seemed that there was a moderate effect in the long-term colony survival of CHO-K1. Under treating with DMABEE, the colony numbers decreased from 13% to 73 % and had little variation in high concentration (0.5, 0.75 and 1.0 mM) as shown in Figure 1.3b.

4.1.2 Morphological alteration of CHO-K1 cells

DMPT

CHO-K1 had mild morphological differences in appearance when grown in F-12 with DMSO as compared to cells exposed to 10.0 mM DMPT for 24 hours. Cells grown in F-12 medium with DMSO were cuboid to round, producing random, loosely arranged distribution (Mendiaz, Mamounas et al. 1986). Cells exposed to DMPT were only slightly different from those grown in the above media, appearing somewhat more round in shape (Fig. 2.1a & b).

DMAEMA

Compared to control group (with DMSO), CHO-K1 cells, exposed to DMAEMA 3.5 mM, seemed to show no difference in cellular morphology (Fig. 2.2a & b).

DMABEE

In contrast, cells grown in F-12 medium with DMABEE were long, spindle-shaped alternation. We have observed that with high concentration of DMABEE, the cells were fibroblastic in appearance with extended cellular processes (Fig. 2.3a & b).

4.1.3 Annexin V-FITC / PI assay

DMPT

We demonstrated the occurrence of DMPT-induced cell death by Annexin V-FITC / PI assay. Flow cytometric analysis with Annexin V-FITC / PI staining showed a 24 hours exposure of CHO-K1 cells to DMPT only slightly fluctuated. The percentages of cells residing in 4 quadrants were not varied in different kinds of concentration of DMPT, namely 2.02% to 3.64% in upper left quadrant (necrotic cells), 1.58% to 3.96% in upper right quadrant (late apoptotic cells) and 0.17% to 0.32 % in lower right quadrant (apoptotic cells) (Fig. 3.1a). However, there were no significant change in the results.

DMAEMA

Similar to DMPT, DMAEMA had no obvious effect on Annexin V-FITC / PI assay of CHO-K1 cells. Compared with control cells at 24 hours, cells exposed to 3.5 mM DMAEMA exhibited mild increasing of necrosis (from 1.35% to 2.27%) and late apoptosis (from 2.88% to 2.93%) (Fig 3.1b).

DMABEE

CHO-K1 cells exposed to DMABEE significantly increased the numbers of necrotic cells (upper left quadrant, from 0.91% to 2.81% with significant difference), resulting in cell death without entering early apoptosis (lower right quadrant, from 0.32% to 0.91% without significant difference) in a dose-dependent manner. Further, the cells exposed to the highest concentration of DMABEE (1.0 mM), an increasing of the amount of cells resided in late apoptosis quadrant was found. Compared with negative control cells, the percentage of late apoptotic cells increased from 1.89% to 6.27% ($p < 0.05$) (Fig. 3.1c).

4.1.4 PI assay

DMPT

CHO-K1 cells treated with DMPT demonstrated growth arrest. A 24 hour exposure of CHO-K1 to different concentrations of DMPT, however, induced no variation on cell cycle distribution (around 45%) (Fig. 4.1a). There was only an elevation on Sub-G0/G1 phase of 10.0 mM DMPT (3.3%), but it did not show significant difference (Fig. 4.2a).

DMAEMA

After treating with DMAEMA, cell cycle of CHO-K1 cells showed a mild increase S phase from 43.9% to 63.7% ($p < 0.05$), and an opposite result was observed on G0/G1 phase (from 42.3% to 26.4% with significant difference) (Fig. 4.1b). Exposure of CHO-K1 cells to 0.5 to 2.5 mM of DMAEMA showed no significant effect on Sub-G0/G1 phase (around 2%). Following exposure to 3.5 mM of DMAEMA for 24 hours, a

dramatic Sub-G0/G1 phase arrest was noted as revealed by increasing percentage to 5.54% ($p < 0.05$) about 2 times of the control group (Fig. 4.2b).

DMABEE

When CHO-K1 cells were incubated with DMABEE for 24 hours, it showed a significant effect on cell cycle progression. A marked G0/G1 phase arrest (from 46.9% to 70.3%) was found from 0.25 mM to 0.75 mM DMABEE. In contrary, the percentage of cells of S phase decreased to 11.63% ($p < 0.05$) in high concentration of DMABEE (Fig. 4.1c). Sub-G0/G1 phase of CHO-K1 cells exposed to DMABEE displayed a smooth elevation and an significant rising was noted at 1.0 mM of DMABEE (12.5%, $p < 0.05$) (Fig. 4.2c).

4.1.5 DCF assay & CMF assay

DMPT

A twenty-four hour exposure to 1.0 mM to 10.0 mM DMPT did not affect intracellular ROS content of CHO-K1 cells significantly. The means of fluorescence of CHO-K1 were shown in Figure 5.1a.

CHO-K1 cells exposed to 1.0-10.0 mM DMPT for 24 hours, there was a mild increase of CMF value. Comparing to control group (DMSO), it only showed significant CMF value of 139.19% on 7.5 mM DMPT. However, there was a declination of CMF value to 103.65% while treating with 10.0mM DMPT (Fig. 5.2a).

DMAEMA

In low concentrations of DMAEMA, it had mild effect the same as DMPT on ROS content of CHO-K1 cells. However, there was significant difference of DCF present value, it showed an increase at 3.5 mM DMAEMA (117.6%, $p < 0.05$) in Figure 5.1b. Interestingly, when CHO-K1 cells exposed to 2.5 mM DMAEMA, DCF has a significant declination (74.3%, $p < 0.05$).

CHO-K1 cells exposed to DMAEMA showed the results of CMF assays as well as DMPT group. Similarly, there was a slight elevation of CMF value when CHO-K1 cells exposed to DMAEMA. Till CHO-K1 cells exposed to 2.5 mM DMAEMA, a significant rising (135.56%) was noted. But, there was a declination on the highest concentration DMAEMA the same as the result of DMPT group (Fig. 5.2b).

DMABEE

24 hours exposure to 0.1 to 0.5 mM of DMABEE did not change the ROS level obviously. However, while the cells were treated with high concentration of DMABEE (0.75 mM and 1.0 mM), the ROS level elevated apparently. Comparing to negative control group, the ROS level obviously increased around 50% at 0.75 mM and 1.0 mM DMABEE (Figure5.1c).

There was a dramatic increase of CMF value while CHO-K1 cells exposed to DMABEE. First, while treating with 0.1 mM DMABEE, the percentage of CMF value (211.19%) showed about 2 times of control group. Further, when CHO-K1 cells were incubated with higher concentration DMABEE (0.5, 0.75, 1.0 mM), the CMF value kept around 250% (Fig. 5.2c).

4.1.6 CBMN assay with IF stain

DMPT

The results of CBMN assay with immunofluorent staining has been reported to be correlated with measuring DNA damage and cytotoxicity. Thus, in the study, the occurrence of DNA damage by different chemicals was demonstrated by CBMN assay.

A 24 hour exposure of CHO-K1 cells to DMPT indicated a slight increase in ratio of the cells with mononucleus (from 19.8% to 27.1%). On the other hand, the ratio of binucleated cells decreased gradually (from 85.5% to 67.6%) (Fig 6.1a). However, the variation was not dependent on the concentration of DMPT, there was only a significant change at the highest concentration. The percentage of MNi increased form 2.9% to 8.3% in a dose-related pattern (Fig 6.1b).

DMAEMA

The ratio of CHO-K1 cells with mononucleus increased slightly without dose-dependent manner (form 8.93% to 20.0%), however, the ratio of the binucleated cells showed a contrary results (from 88.5% to 76.6%) (Fig 6.2a). Although DMAEMA could affect mitosis of CHO-K1 cells, DMAEMA only mildly brought up the percentage of MNi. It showed variation only in the highest concentration of DMAEMA (5.0%) (Fig 6.2b).

DMABEE

After exposure to DMABEE 24 hours and staining with DAPI and rhodamine phalloidin, the ratio of the mononuclear cells increased dramatically in a dose-

dependent manner (from 8.6% to 34.6%). In contrary, the binuclear cells showed an opposite performance (from 89.1% to 64.3%) (Fig 6.3a). As well as the ratio of the mononuclear cells, the percentage of MNi showed a notable rising in a dose-dependent manner. It demonstrated the increasing trend from 2.7% to 7.2%. Furthermore, there was a declination of the amount of MNi cells exposed to 7.5 mM (Fig 6.3b). In the highest concentration of DMABEE (1.0 mM) in the study, however, the viable cell sharply decreased and the cellular morphology got incomplete. It was too hard to observe the DNA damage and measure the nuclear variation. Thus, we did not show the document of CBMN assay of CHO-K1 cells exposed to 10.0 mM DMABEE.

4.2 Effects of NAC, catalase, & carboxylesterase on DMPT-, DMAEMA- or DMABEE-induced cell growth inhibition.

4.2.1 Effect of NAC, catalase and carboxylesterase on CHO-K1 cells

NAC

Theoretically, NAC could decrease the toxic impact that ROS induced on the inhibition of cell growth and proliferation in CHO-K1 cells. After pre-treating with NAC for an hours, CHO-K1 cells exposed to DMPT and DMABEE, however, did not show significant improvement in cell growth inhibition (Fig 7.1a & b). Comparing to control group, there were declinations about 46.75% and 39.51%, respectively, in 1mM and 5mM NAC co-incubation with 10.0 mM DMPT. Moreover, CHO-K1 cells exposed to 1.0 mM DMABEE after pre-treating with 1.0mM / 5.0mM NAC, there were dramatic

declinations in MTT assays. Comparing to control group (1.0mM DMABEE only), the proliferation rate of CHO-K1 cells decreased about 30% to 38%.

The results did not show rehabilitation of the cell growth ability, after incubating with either low or high concentration NAC.

Catalase

In this study, the growth ability would slightly rise while pre-treating with catalase for an hours before incubation with DMPT 10.0 mM or DMABEE 1.0 mM. Treated with DMPT 10.0 mM or DMABEE 1.0 mM, the growth ability of CHO-K1 cells significantly reduced without a concentration-relate manner. While co-treating with various concentration of catalase (500 U or 1000 U), there was a mild increase of proliferation rate about 10% in DMPT and 20% in DMABEE group (Fig 7.2a & b).

Carboxylesterase

When pre-treatment with esterase 1 U/ml or 5 U/ml for an hour and co-incubation with two chemicals (DMPT 10.0 mM and DMABEE 1.0 mM) for 24 hours, the grwth inhibition of CHO-K1 cell might be prevented (Fig 4.3a & c). Especially, the cytoxicity caused by DMABEE could be inhibited obviously, the MTT assays showed that the proliferation rate almost rehabilitated to 94% (Fig 4.3c). However, CHO-K1 cells exposed to DMAEMA co-treating with carboxylesterase did not show significant change (Fig 7.3b).

In morphology, CHO-K1 cells could return to original cuboid or round shape after treating with carboxylesterase.

Chapter V. Discussion

5.1 Morphological and proliferation aberrations

Numerous investigations have revealed that chemicals at milli-molar concentration level eluted from dental restorative material could be found in human tissues or organs (Cetinguc, Olmez et al. 2007). Such concentration are high enough to induce cytotoxicity or genotoxicity. These materials used in dentistry can have harmful effects, manifested mainly as pulp damage or allergic reactions (Schwengberg, Bohlen et al. 2005). In spite of Bis-GMA, TEDGMA and HEMA which are mostly investigated, there are still some initiators / co-initiators, reducing agent and accelerators that should be noted on their toxicity after resins are cured.

In our study, DMPT, DMAEMA and DMABEE were capable of reducing CHO-K1 proliferation rate. Growth assays provided the first evidence that evaluated concentrations of the three substances have chemical and biological effects on CHO-K1 cells. Increasing concentration of DMPT lead to a mild reduction, there was only a 22% declination in cell numbers ($p<0.05$) in a short-term exposure of 24 hours. When CHO-K1 cells were exposed to DMAEMA, the growth assay showed the same results as DMPT. Furthermore, the effective inhibitory concentration (about half cell numbers decreasing) was 1.0 mM for DMABEE ($p<0.05$). Only the results of the growth assays of the cells exposed to DMABEE concurred with the published data. In the previous study, the effective inhibitory concentration demonstrated by Permanent 3T3 Cells, Primary human gingival fibroblasts (HGF), Primary human pulp fibroblasts (HPF), and Primary human periodontal ligament fibroblasts (HPLF) was around 1.22 to 1.26 mM in

treating with DMABEE (Geurtsen, Lehmann et al. 1998). Other two chemicals indicated less cytotoxicity than the results of previous studies. However, the long-term effects of the growth assays (CFC assays) of CHO-K1 cells treated with three chemicals demonstrated more obvious findings. The effective inhibitory concentration were 7.5 mM for DMPT, 2.5 mM for DMAEMA, and 0.5 mM for DMABEE were similar to the published documents. The morphological change was observed in cells incubated with 0.75 and 1.0 mM DMABEE. At these concentrations of DMABEE, CHO-K1 cells were fibroblastic in appearance with extended cellular processes. It might reflect loss of functional organization of CHO-K1 cells as well as the findings of CBMN assays. The morphological change of CHO-K1 cells was also found in Mendiaz research (Mendiaz, Mamounas et al. 1986).

5.2 Cell death induction

The effect of chemicals on cell death was examined by Annexin V-FITC / PI assay. Several studies have indicated that apoptosis may be induced by exogenous oxidants (Lee, Cho et al. 2004). Prior report stated that resin-based dental material caused cell death by apoptosis and necrosis in rat submandibular acinar cells (Roll, Dahl et al. 2004).

In this study, the Annexin V-FITC / PI assay showed that all concentrations of DMPT and DMAEMA used in this research induced cell death without producing an apoptotic sign. In the mean time, the cell cycle of CHO-K1 cells exposed to DMPT did not show obvious G0/G1 phase arrest and the ROS level was not elevated. Thus, DMPT would

cause growth inhibition, but the mechanism was not the same as other monomers, such as HEMA, TEDGMA, UDMA, etc., by causing cell apoptosis. Although DMAEMA would induce cell cycle disturbance, in our study, it could let CHO-K1 cells arrest at G2/M phase. DNA damages, however, were completely repaired in cells with G2/M arrest and show the role of G2/M arrest in giving CHO-K1 cells enough time to repair DNA damages and protect the cells from apoptosis (Lepley, Li et al. 1996). Because of DNA damage repair, the percentage of apoptotic cells decreased as CHO-K1 cells were incubated with DMAEMA. In contrast, CHO-K1 cells exposed to DMABEE, the Annexin V-FITC / PI assay showed an increase of the percentage of cells in upper right quadrant (late apoptosis). The PI flow cytometry assay also showed an increase of CHO-K1 cells arrest in G0/G1 phase, which implied the potential of apoptosis. In the present studies, DMABEE augments cell death via apoptosis and necrosis in human histocytic lymphoma promonocytic cells in a concentration-dependent manner. The results of influence of DMABEE in our study was similar to previous documents (Cimpan, Matre et al. 2005).

5.3 Effect on cell cycle

As a consequence of DNA damage, the progression of cell cycle may arrest in mammalian cells. Since we knew chemicals of dental restorative materials showed genotoxicity, we hypothesize that DMPT, DMAEMA and DMABEE might induce a cell cycle delay on CHO-K1 cells.

CHO-K1 cells exposed to DMPT seemed not to affect cell cycle. Only a mild elevation in Sub-G0/G1 and G0/G1 phase was noted at 10.0 mM DMPT, but it was not statistically significant. In the study of cell exposure to DMAEMA, the cell numbers of Sub-G0/G1 and S phase rised slightly, whereas the percentage of cells in G0/G1 phase barely decreased. The results of cell cycle interfered with DMPT and DMABEE were not similar to the results from Masuki et al (Masuki, Nomura et al. 2007), the article indicated that DMPT and DMAEMA consistently induced G0/G1 cell cycle arrest and necrosis in human gingival fibroblasts. DMABEE, on the other hands, could cause cell cycle arrest in the G0/G1 phase, when CHO-K1 cells exposed to 0.25, 0.5 and 0.75 mM DMABEE. The result of DMABEE was confirmed by CBMN assays for the increasing of MNi. Such disturbances in the cell cycle would be evoked by massive DNA damage and a breakdown in the repair ability. Although the effects of cell cycle arrest by DMABEE have not been elucidated in the previous documents, in our study, it seemed to be more toxic among the three chemicals, especially on cell cycle interference and DNA damage.

5.4 ROS induction

ROS plays an important role in biological and physiological process, however, redundant ROS would be a hazardous factor to cellular structures. It might attack cell membranes, lipids, and DNA double bonds. If the cells can not self-repair, ROS jeopardizes cell survival or induces mutagenic alternations (Valko, Leibfritz et al. 2007). Many resin substances induce cytotoxicity related to ROS as we studied previously.

Thus, we hypothesize that the three chemicals investigated in our study would increase ROS levels. However, we only found that intracellular ROS level increased significantly when CHO-K1 cells were incubated with 0.75 and 1.0 mM DMABEE, at which concentration cell cycle perturbation observed. When CHO-K1 cells treated with DMAEMA, a slight increasing of fluorescence of DCF was noted. However, there seemed to be no variation of ROS level while CHO-K1 cells treating with DMPT at any concentrations, these results were coincident to cell cycle aberration. Therefore, toxicity induced by DMABEE might be speculated to correlate to excess ROS production. Nevertheless, the mechanism of cytotoxicity and genotoxicity caused by DMPT or DMAEMA should be further evaluated, because ROS might be not the major key factor.

5.5 GSH depletion

GSH redox status is vital for biological homeostasis maintenance. CMF fluorescence has been widely used to measure the intracellular levels of reduced GSH. In the experiment, GSH depletion occurred at cells treated with each one of three chemicals. Interestingly, CMF value of CHO-K1 cells treated to both DMPT and DMAEMA, it showed a similar results. The mean GSH value achieved the highest, which was about 1.5 times of control cells at 7.5 mM DMPT or 2.5 mM DMAEMA.The reduced GSH content might induce and indicate apoptosis. The cells that could quickly generate more GSH adapt the toxicant challenge and maintain viability. This might also be explained by the heterogeneity of cells (Chang, Ho et al. 2001). However, there was a slight GSH depletion at the highest concentration of DMPT or DMAEMA, it implied that CHO-K1

cells would start to apoptosis while incubating with high concentration DMPT or DMAEMA.

Intracellular GSH content of individual cell might be crucial for its survival. Thus, if cells contacted with more toxic materials, the GSH level would increase more. As CHO-K1 cells were treated with DMABEE, which was the most cytotoxic drug in this study, GSH level prominently increased. This might indicate that in order to survival, the residual CHO-K1 cells produced more GSH to against the environmental stress.

However, all of this three substances did not induce notable GSH depletion by CMF flow cytometry assay. Possibly ROS accumulation induced by the three chemicals is not be secondary to GSH depletion. Other mechanism should be considered.

5.6 DNA damage

Micronuclei, which mean DNA breakdown, originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. CBMN assay is the preferred method for measuring MNi in cultured human or mammalian cells because scoring is specifically restricted to once-divided binuclear cells, which are the cells that can express MNi (Fenech and Morley 1986). Thus, in our study, we used CBMN assay as a quantitative scoring assessment of DNA damage.

A source of free radicals is required for the initiation of the polymerization process of dental resins. Pagoria et al. showed that these initiating radicals indiscriminately react with molecular oxygen forming ROS, which may damage DNA (Pagoria, Lee et al.

2005). We wanted to analyze the severity of oxidative DNA damage elicited by different chemicals, such as DMPT, DMAEMA and DMABEE.

In our study, the negative control, CHO-K1 cells incubated with DMSO in F-12 medium for 24 hours, contained an average around 2.6% MNi production. On the contrary, the positive control, CHO-K1 cells incubated with mitomycin C in F-12 medium for 24 hours, showed an average around 11.6% MNi production. After incubating with DMPT, the percentage of MNi in BN cells increased in a dose-dependent manner (Fig. 3.1b). The result implied that chromosome of CHO-K1 cell might be attacked by DMPT or the metabolites of DMPT and this kind of DNA damage could not be repaired. DMAEMA is a co-initiator as well as DMPT. However, it showed minor genotoxicity and the MNi ratio increased slightly only at higher concentrations (3.5 mM DMAEMA) comparing to the positive control. Both the results of treating with DMPT and DMAEMA were coincident to the research of Nomura et al. The genotoxicities of DMPT was thought to be moderate and DMAEMA did not appear to be genotoxic (Nomura, Teshima et al. 2006). Among three chemicals used in this study, DMABEE was the most genotoxic and would induce cellular breakdown. An obvious elevation of MNi while incubating CHO-K1 cells with DMABEE were detected. But, treating with 0.75 mM DMABEE, there was a dramatic declination of the percentage of MNi. The reason might be because cells were morphologically altered at the high concentration of DMABEE as well as the figures shown in MTT assays (Fig. 2.3a & b). The cellular image observed under IF staining also displayed a broken cell of skeleton and incomplete nucleus. Moreover, as CHO-K1 treated with 1.0 mM DMABEE, cells were severely disrupted and could not be scored for calculation.

5.7 Inhibitors & enzymes

NAC was regarded as an inhibitor that alleviated genotoxicity and cell cycle arrest induced by monomers, such as TEGDMA and HEMA, and down-regulated ROS level caused by HEMA (Schweikl, Hartmann et al. 2007). Whereas, in our study, either NAC did not decrease growth inhibition and ROS production induced by DMPT and DMABEE. But interestingly, the ROS scavengers NAC could enhance growth inhibition while co-incubating with DMABEE (Fig. 4.1b). This observation supplements data from a previous study. It was found that NAC may obviously behave as antioxidant or pro-oxidant either alone or in combination with vitamin C dependent on the applied concentration. Thus, NAC, about 0.5 to 1.0 mM, produced significantly more DNA damage compared to the exposure of DNA to CQ / DMPT without NAC (Lee, Pagoria et al. 2007).

Catalase is the main element in antioxidant defense system to decompose hydrogen peroxide. However, CHO-K1 cells pre-treated with catalase did not enhance growth ability compared to the cells treated with DMPT or DMABEE alone. The results implied that hydrogen peroxide could not be one of the ROS product induced by DMPT or DMABEE.

In enzymology, the CES are a group of enzymes that catalyze the chemical reaction of hydrolysis of ester- and amide-bond-containing substances. The two substrates of this enzyme are carboxylic ester and water, whereas its two products are alcohol and carboxylate. Saliva contains various types of esterase are from this group belong to the family of hydrolases and act on carboxylic ester bonds. CES reduces the toxicity induced by chemicals with ester, amide or carbamate groups. In our study, the growth

assays of CHO-K1 cells co-treated with three chemicals and CES, there were a dramatic change. While the cells co-incubating with 10.0 mM DMPT, CES alleviated the inhibition of growth assays in a dose dependent manner (Fig. 4.3a). Further, CES showed a more strong ability to reduce the growth inhibition as CHO-K1 cells exposed to 1.0 mM DMABEE without concentration dependent (Fig. 4.3c). Because DMPT belongs to tri-amide family and there are ester group in DMABEE, both of them are objects that can be digested by CES. The statement could explain why the toxicity of DMPT and DMABEE can be down-regulated. CES, however, had less effect on growth inhibition induced by DMAEMA in our study. There were only a little bit of improvement, but it did not show significant correlation. Although DMAEMA owns a carbamate group, the structure is not so typical (hydrogen shifts to methyl group). The structure variation might increase difficulty of DMAEMA metabolization, hence the toxicity of DMAEMA still presented after pre-treating with CES.

5.8 Clinical consideration

Constant salivary flow could provide clearance and rapidly decrease the amount of chemicals delivered from composite resins. While considering dentin thickness or barriers between pulp tissue and dental restorations, it may hard to reach the toxic level of material leached from restoration materials (Chang, Guo et al. 2005). However, as a direct capping material or restoring for deep caries, there were risky to induce pulp inflammation and lead to potential pulpal toxicity (Geurtsen, Spahl et al. 1998). Sometimes, these chemicals may induce DNA damage or be a carcinogen that would

cause severe disease or make tissue condition deterioration or cells death, such as methemoglobinemia and allergic responses (Kim, Ghanbari et al. 2007).

Today, dental composite resins are widely used in modern dentistry. The amount of released components are a lot and the mechanism of their toxicity are not clearly elucidated. The toxic concentration we indicated may not easily be achieved in oral cavities, however, the material used in a confined area, such as in deep caries, retro-filling materials or direct pulp capping, may potentially leach toxin or adverse substances. Therefore, it should have sufficient investigation for reducing risky of pulp inflammation and other tissues damage.

Chapter VI. Conclusion

Resin-base products are widely used in modern dentistry. Chemical substances leached from the materials may potentially cause adverse effects. In order to improve the safety problems, the toxic mechanism and cytoxicity of these chemicals should be investigated. It is reliable to check the mechanism through examination of cell cycle progression, ROS production and GSH alteration. Here we tested three chemicals of composite resins: DMPT, DMAEMA and DMABEE via these aspects, hope to find out the implication for improvement of their clinical security.

DMPT induced both short-term and long-term growth inhibition in CHO-K1 cells in a dose-dependent manners. However, there were not obvious cell cycle deregulation, ROS overproduction / GSH depletion and the cellular distribution was not change significantly by Annexin V-FITC / PI. When the cells were exposed to DMPT, the ratio of MNi was increased. Only through DNA damage could explain the source of cytotoxicity.

DMAEMA produced growth inhibition of CHO-K1 cells in a dose-related manner, especially in long-term growth inhibition. Amount three chemicals, the cytotoxicity of DMAEMA was weaker. Whether ROS production / GSH depletion or DNA damage, there were no obvious results that could explain of cytotoxicity. However, a sub-lethal dose of DMAEMA induced cell cycle disturbance in S phase and G0/G1phase. Therefore, we should find out other mechanisms to explain the toxicity of DMAEMA.

DMABEE elicited growth inhibition of CHO-K1 cells in dose-dependent manner, which may be related to cell cycle perturbation, ROS overproduction and DNA damage. CES and catalase may attenuate toxicity. On the other hand, NAC, as well as a pro-

oxidant, exacerbated cytotoxicity of DMABEE. DMABEE induced cell cycle arrest in G0/G1 phase which could increase the percentage of apoptotic cells evaluated by Annexin V-FITC / PI assay.

The results of this study help to elucidate the toxicity of these chemicals. Many in vitro studies indicate that the dosage of the leachable substances may not reach the toxic level as we reported. However, in some clinical situations, the unbound chemicals may lead to potential toxic effects as we addressed. Thus, for the safety of clinical application, it is important to realize the toxic mechanism of these substances and down-regulate the cytotoxicity as well as possible.

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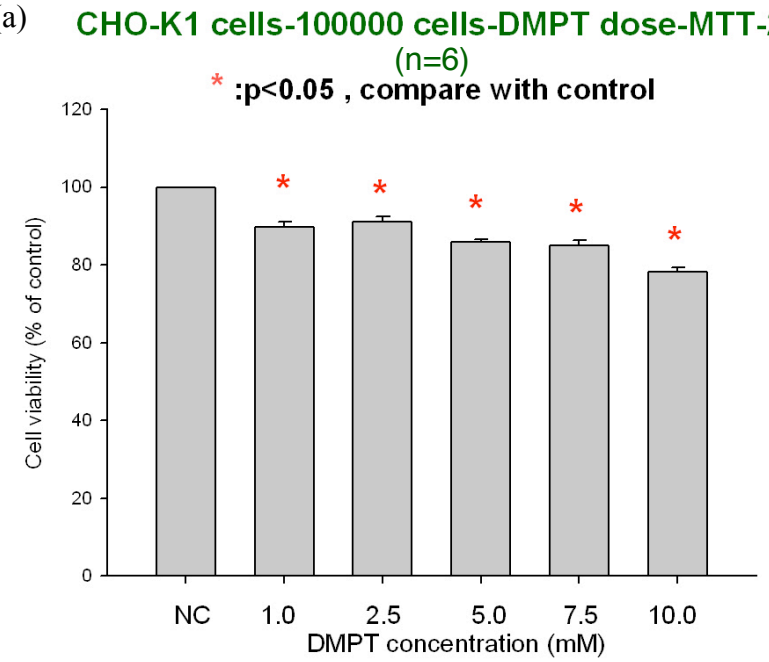
(a)



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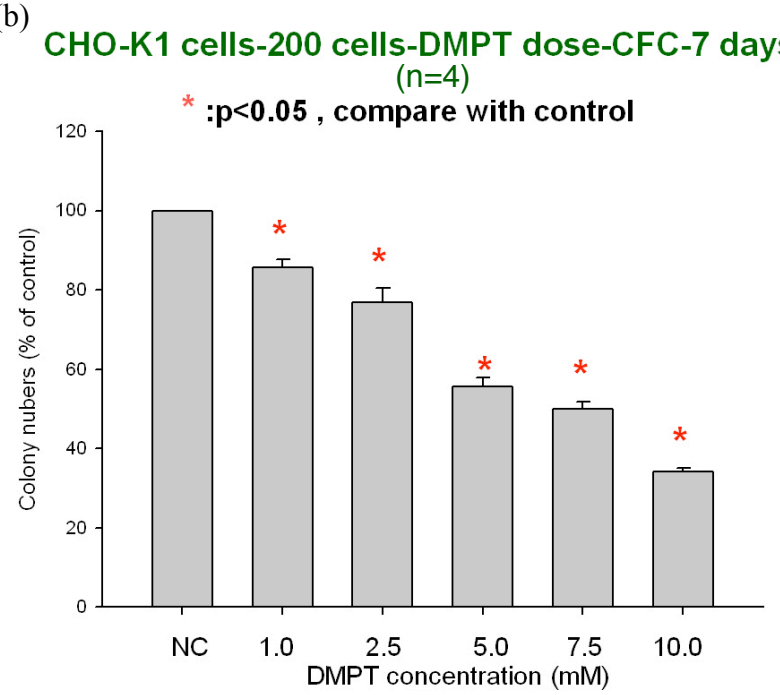
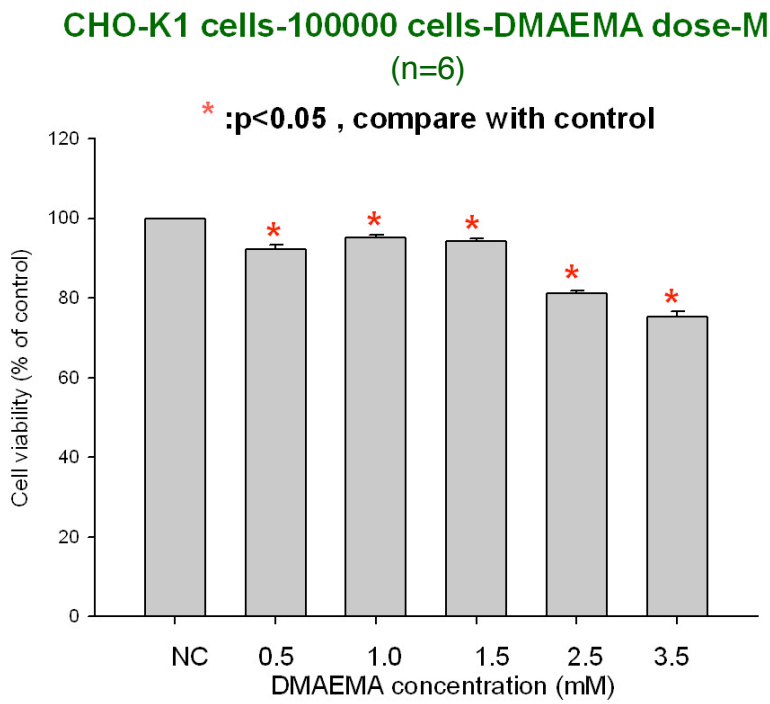


Fig. 1.1a & b Effect of DMPT on the growth of CHO-K1 cells.

(a) Cells (1.0×10^5 cells) in 6-well plate were exposed to DMPT for 24 hours and were measured by MTT assay. (b) Cells (200 cells) in 6-well plate were exposed to DMPT, then were cultured for 7 days and calculated by CFC assay. Results were expressed as percentage of control (mean ± SE). *Denotes marked difference when compared with control.

(a)



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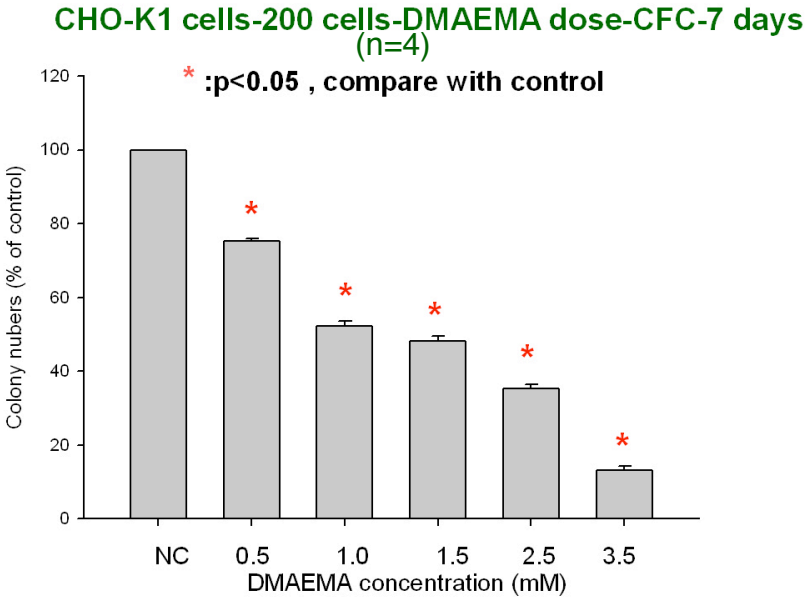
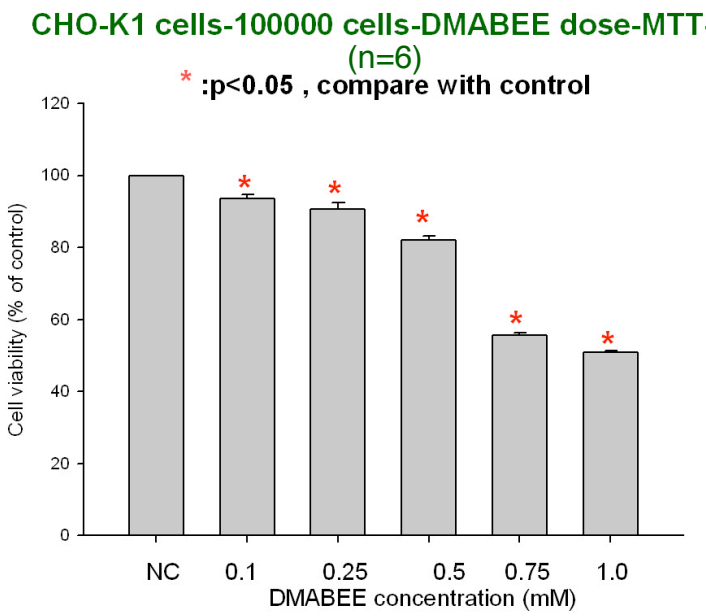


Fig. 1.2a & b Effect of DMAEMA on the growth of CHO-K1 cells.

(a) Cells (1.0×10^5 cells) in 6-well plate were exposed to DMAEMA for 24 hours and were measured by MTT assay. (b) Cells (200 cells) in 6-well plate were exposed to DMAEMA, then were cultured for 7 days and were calculated by CFC assay. Results were expressed as percentage of control (mean ± SE). *Denotes marked difference when compared with control.

(a)



(b)

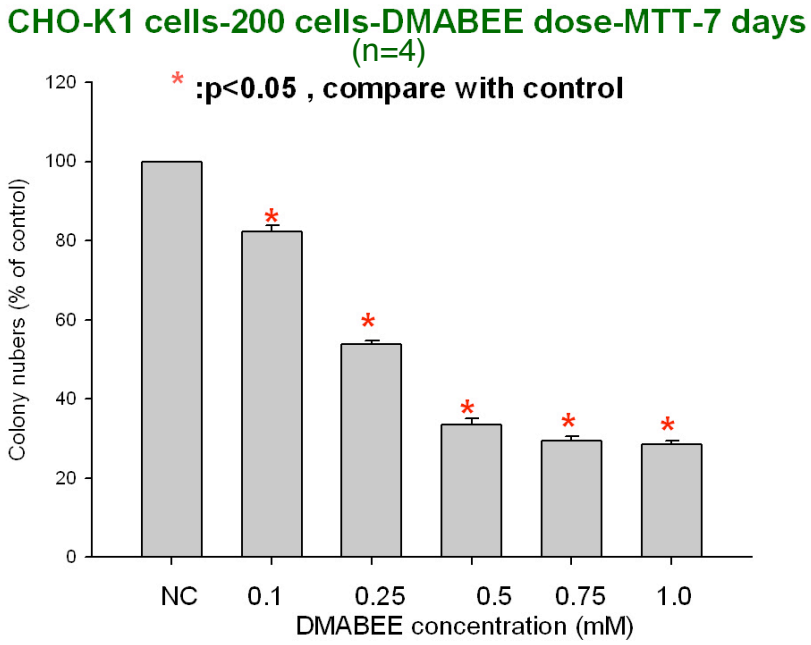


Fig. 1.3a & b Effect of DMABEE on the growth of CHO-K1 cells.

(a) Cells (1.0×10^5 cells) in 6-well plate were exposed to DMABEE for 24 hours and were measured by MTT assay. (b) Cells (200 cells) in 6-well plate were exposed to DMABEE, then were cultured for 7 days and were calculated by CFC assay. Results were expressed as percentage of control (mean ± SE). *Denotes marked difference when compared with control.

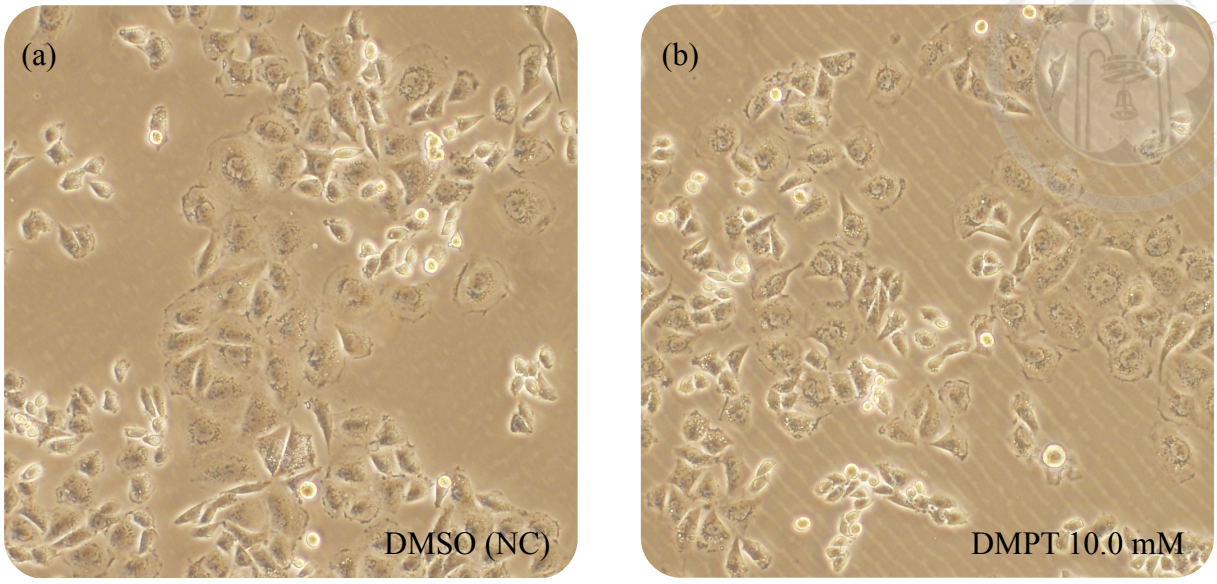


Fig. 2.1a & b Morphology alternation of CHO-K1 cells.

(a) Control CHO-K1 cells. (b) CHO-K1 exposed to 10.0 mM. DMPT for 24 hours. (100x, original magnification)

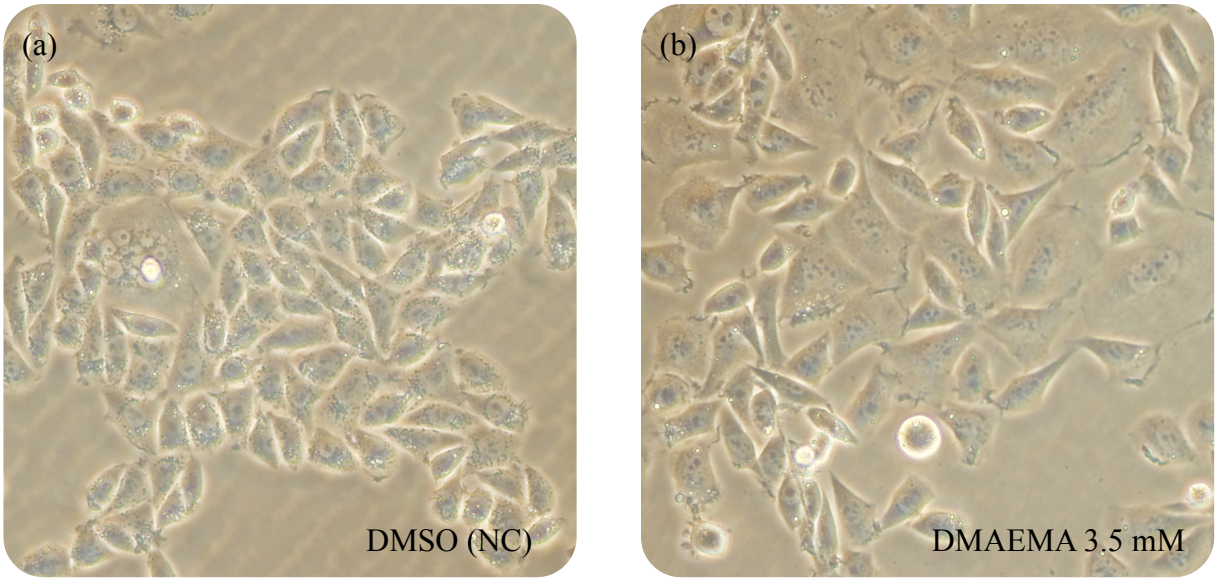


Fig. 2.2a & b Morphology alternation of CHO-K1 cells.

(a) Control CHO-K1 cells. (b) CHO-K1 exposed to 3.5 mM. DMAEMA for 24 hours. (50x, original magnification)

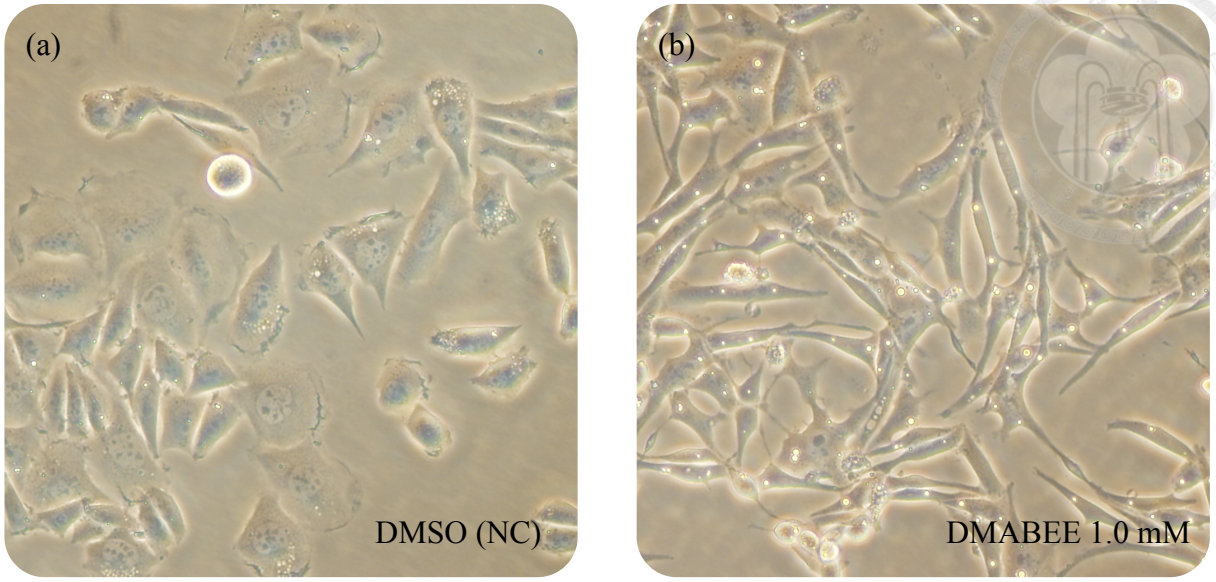


Fig. 2.3a & b Morphology alternation of CHO-K1 cells.

(a) Control CHO-K1 cells. (b) CHO-K1 exposed to 1.0 mM. DMABEE for 24 hours. (100x, original magnification)

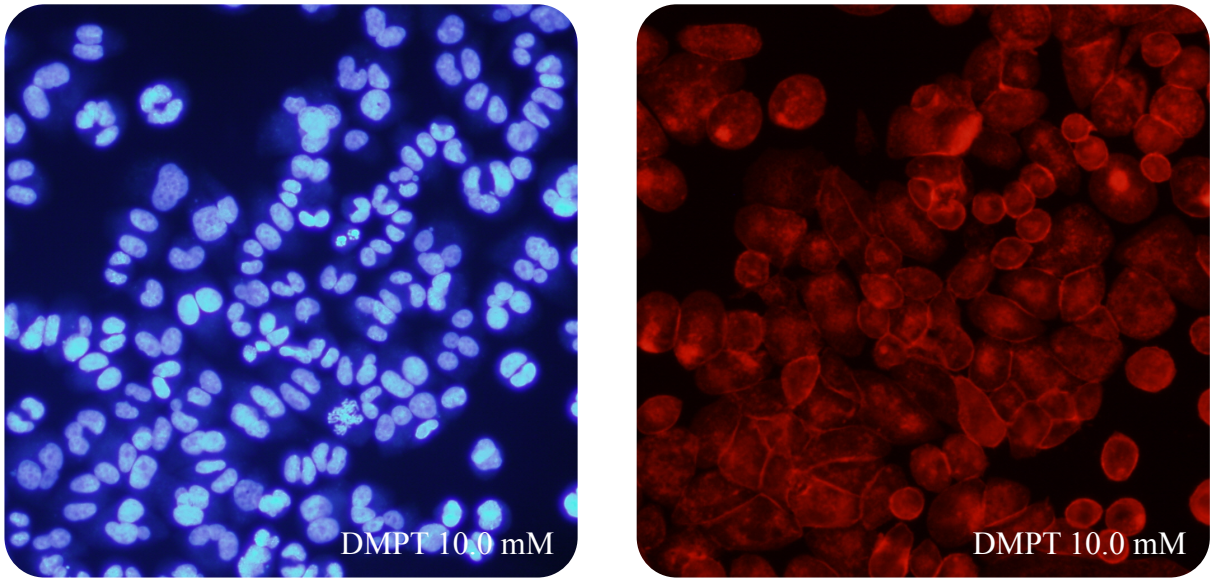
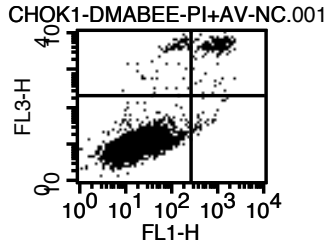


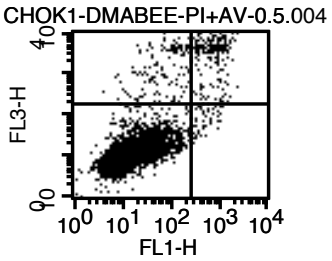
Fig. 2.4a & b Morphology alternation of CHO-K1 cells via immunofluorescence staining.

(a) Nucleus of CHO-K1 cells stained with DAPI (blue), and (b) actin stained with rhodamine phalloidium. (100x, original magnification)



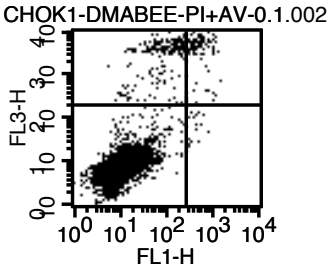
File: CHOK1-DMABEE-PI+A

Quad	% Gated	% Total
UL	0.90	0.90
UR	1.76	1.76
LL	97.00	97.00
LR	0.34	0.34



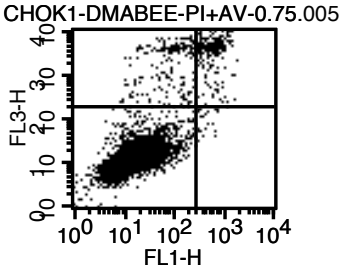
File: CHOK1-DMABEE-PI+AV-

Quad	% Gated	% Total
UL	1.44	1.44
UR	2.10	2.10
LL	95.93	95.93
LR	0.53	0.53



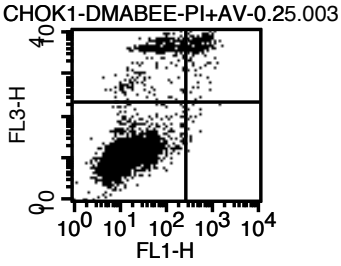
File: CHOK1-DMABEE-PI+A

Quad	% Gated	% Total
UL	1.99	1.99
UR	1.15	1.15
LL	96.70	96.70
LR	0.16	0.16



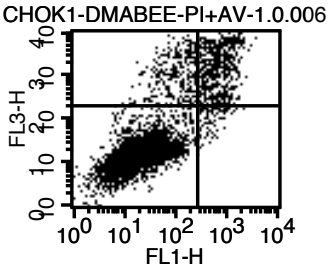
File: CHOK1-DMABEE-PI+A

Quad	% Gated	% Total
UL	2.08	2.08
UR	2.60	2.60
LL	95.06	95.06
LR	0.26	0.26



File: CHOK1-DMABEE-PI+AV

Quad	% Gated	% Total
UL	3.51	3.51
UR	2.18	2.18
LL	94.13	94.13
LR	0.18	0.18

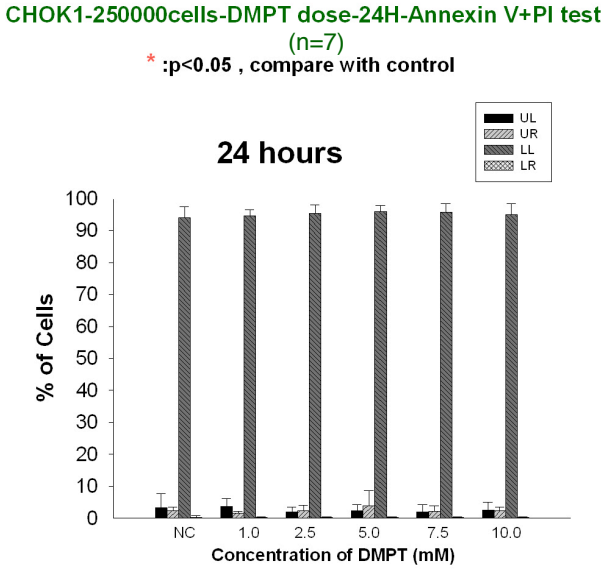


File: CHOK1-DMABEE-PI+A

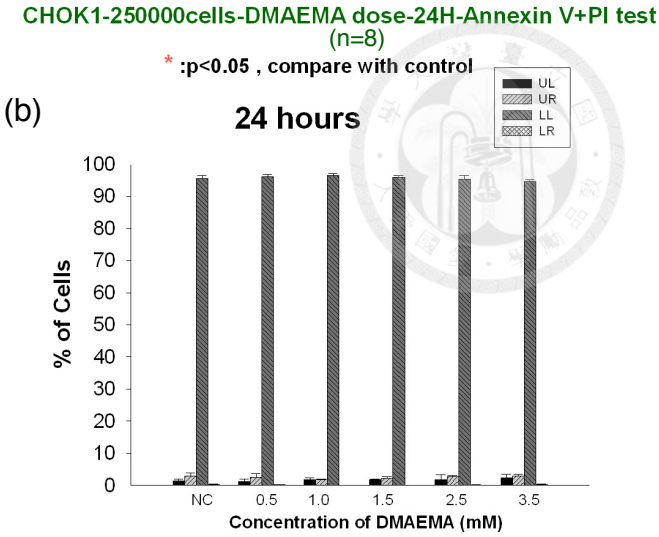
Quad	% Gated	% Total
UL	2.93	2.93
UR	5.39	5.39
LL	90.77	90.77
LR	0.91	0.91

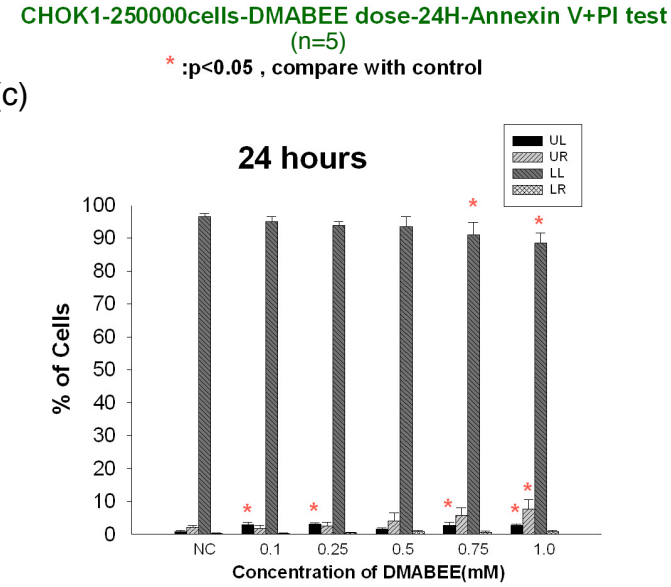
Induction of necrosis and apoptosis of CHO-K1 cells by DMABEE

Dot gram analysis by flow cytometry of CHO-K1 cells treated with DMSO (as NC) and DMABEE (0.1mM-1.0 mM). Result of one representative experiment from each group was shown.



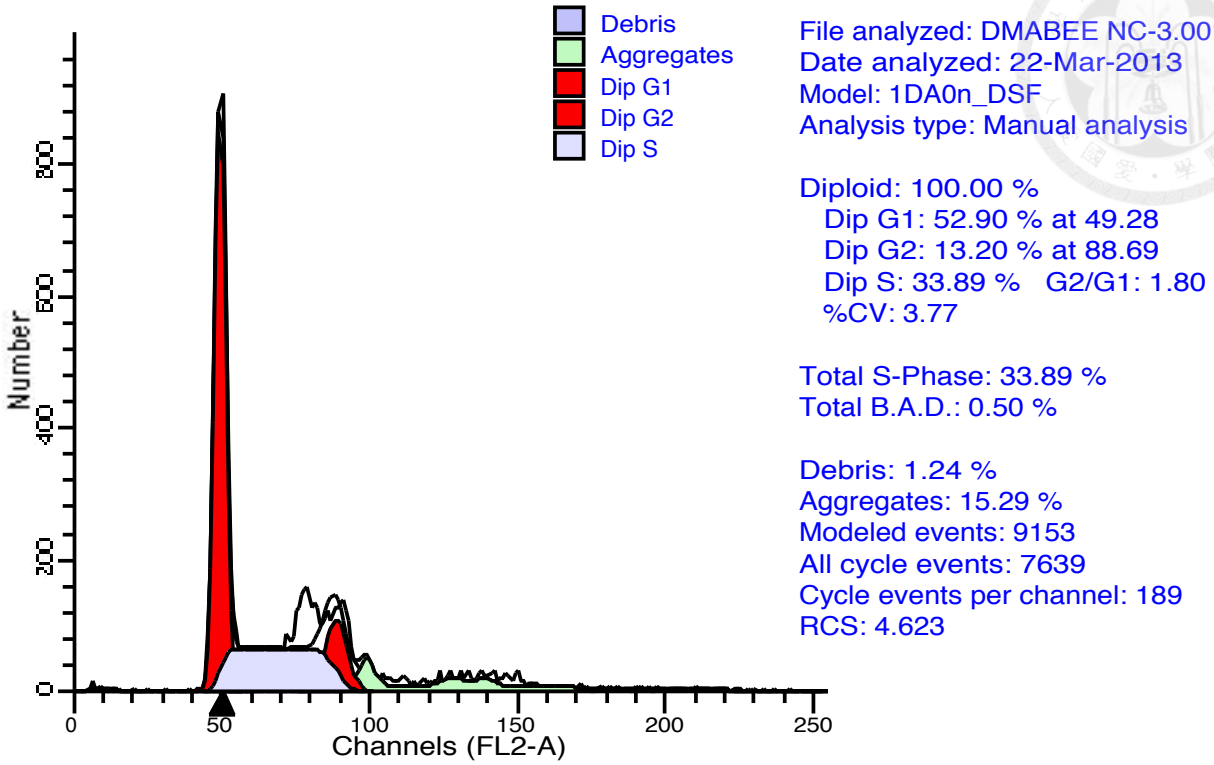
(b)





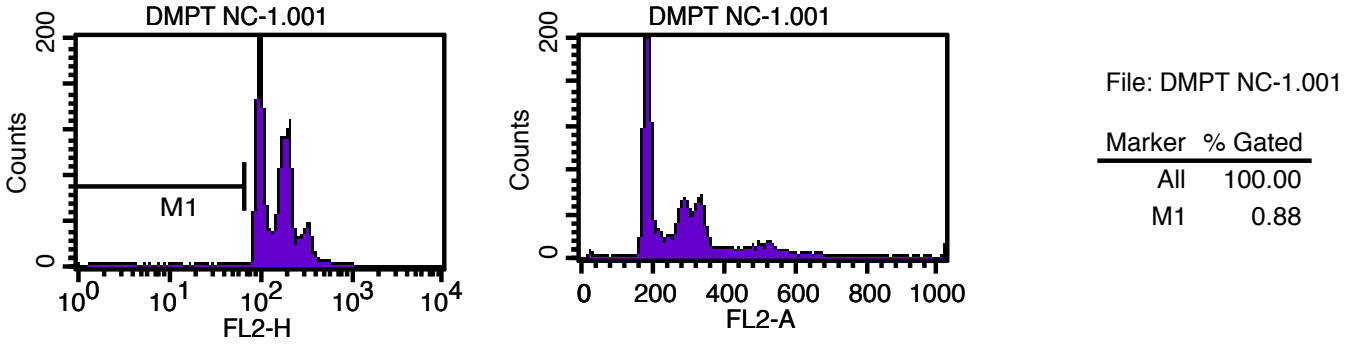
(c)

Fig. 3.1a, b & c Effect of three chemicals analysis by Annexin V-FITC / PI assay flow cytometry. Cells (2.5×10^5 cells) in 6-well plate. (a) Cells were exposed to DMPT for 24 hours and were measured by Annexin V-FITC / PI flow cytometry. (b) Cells were exposed to DMAEMA for 24 hours and were measured by Annexin V-FITC / PI flow cytometry. (c) Cells were exposed to DMABEE for 24 hours and were measured by Annexin V-FITC / PI flow cytometry.



Effects of DMSO on the cell cycle progression (G0/G1; S; G2/M phase) of CHO-K1 cells

Histogram analysis by flow cytometry of CHO-K1 cells treated with DMSO. Result of one representative experiment from control group.



Effects of DMSO on the cell cycle progression (Sub-G0/G1 phase) of CHO-K1 cells

Histogram analysis by flow cytometry of CHO-K1 cells treated with DMSO. Result of one representative experiment from control group.

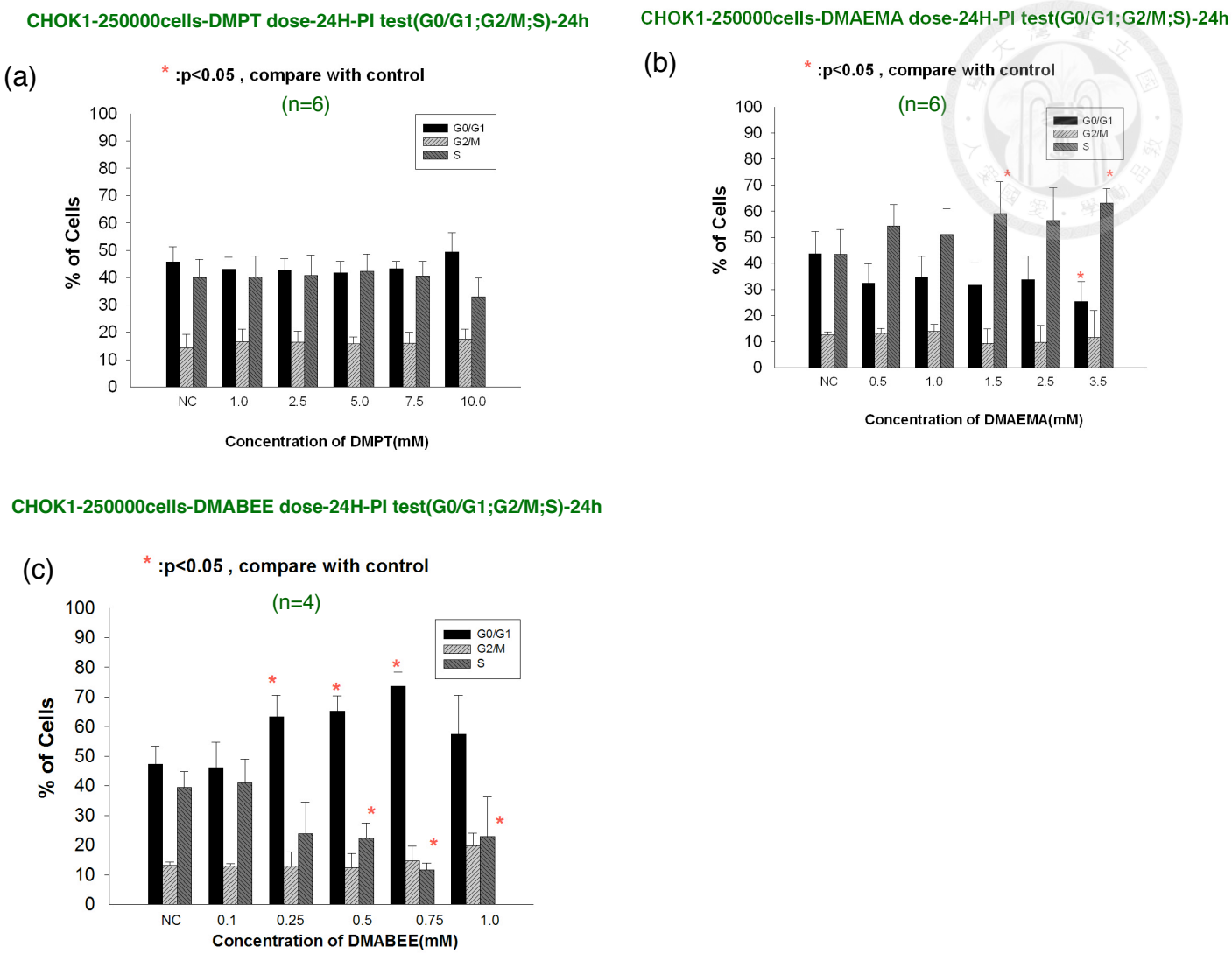


Fig. 4.1a, b & c Effect of three chemicals on the cell cycle of CHO-K1 cells (G0/G1, S, G2/M). Cells (2.5×10^5 cells) in 6-well plate. (a) Cells were exposed to DMPT for 24 hours and were measured by PI flow cytometry. (b) Cells were exposed to DMAEMA for 24 hours and were measured by PI flow cytometry. (c) Cells were exposed to DMABEE for 24 hours and were measured by PI flow cytometry.

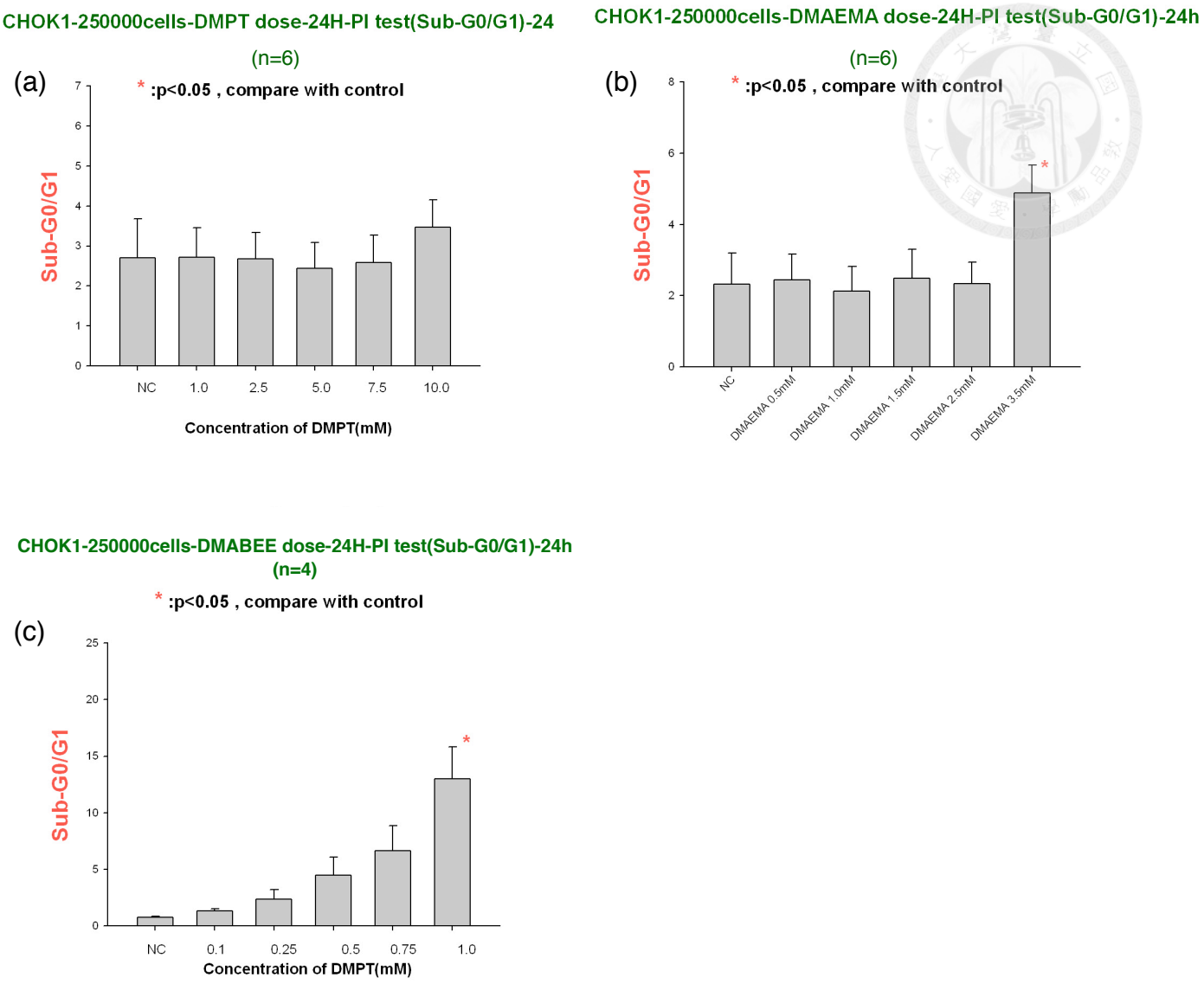
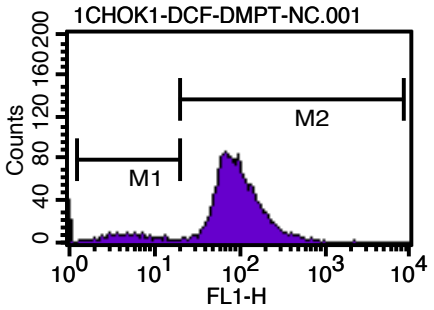


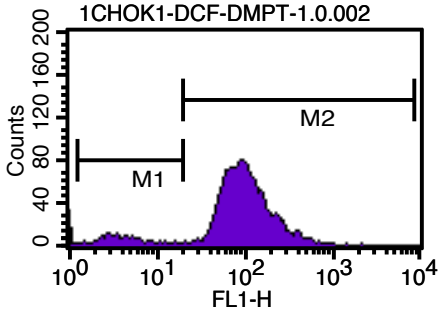
Fig. 4.2a, b & c Effect of three chemicals on the cell cycle of CHO-K1 cells (Sub-G0/G1).

Cells (2.5×10^5 cells) in 6-well plate. (a) Cells were exposed to DMPT for 24 hours and were measured by PI flow cytometry. (b) Cells were exposed to DMAEMA for 24 hours and were measured by PI flow cytometry. (c) Cells were exposed to DMABEE for 24 hours and were measured by PI flow cytometry.



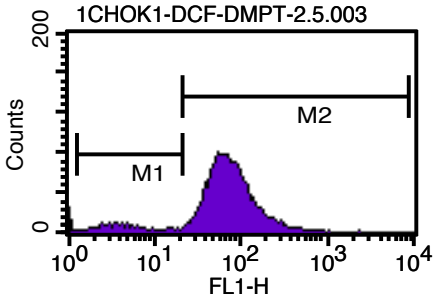
File: 1CHOK1-DCF-DMPT-NC.0(

Marker	Mean
All	99.39
M1	5.74
M2	106.36



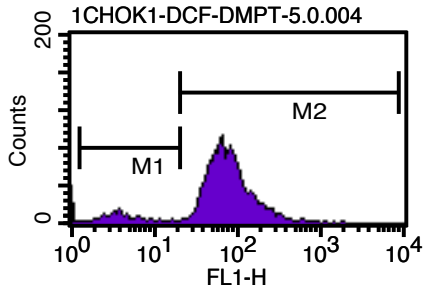
File: 1CHOK1-DCF-DMPT-1.0.00

Marker	Mean
All	106.84
M1	4.94
M2	115.32



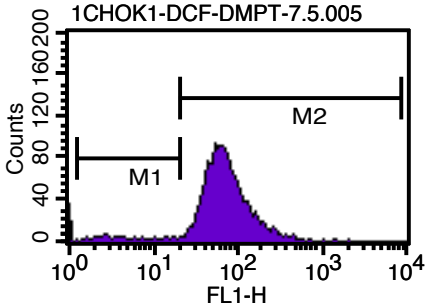
File: 1CHOK1-DCF-DMPT-2.5.003

Marker	Mean
All	77.84
M1	5.33
M2	83.26



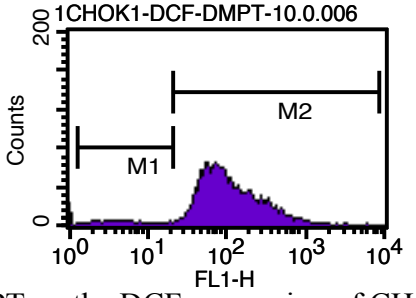
File: 1CHOK1-DCF-DMPT-5.0.004

Marker	Mean
All	80.45
M1	4.84
M2	87.07



File: 1CHOK1-DCF-DMPT-7.5.005

Marker	Mean
All	77.69
M1	6.30
M2	80.42



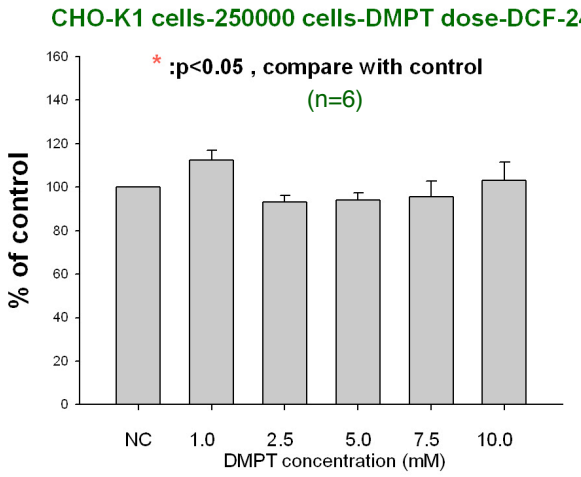
File: 1CHOK1-DCF-DMPT-10.0.006

Marker	Mean
All	137.22
M1	5.34
M2	142.56

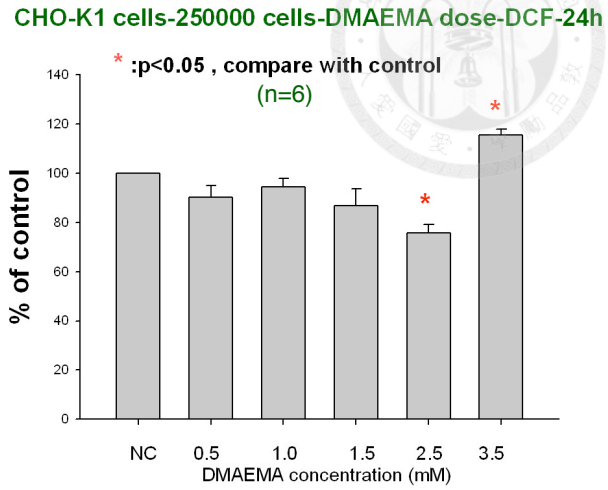
Effects of DMPT on the DCF expression of CHO-K1 cells

Histogram of DCF fluorescence of CHO-K1 cells treated with 0 to 10.0 mM DMPT. M2 population indicated ROS content were noted. Result of one representative experiment was shown

(a)



(b)



(c)

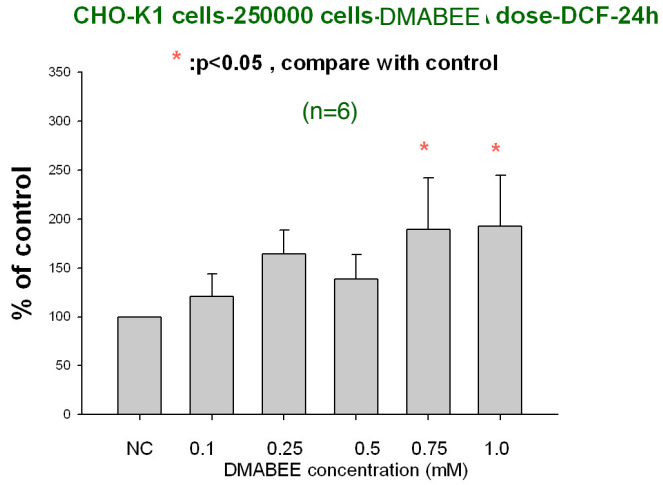
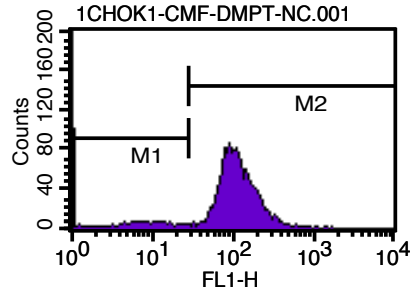


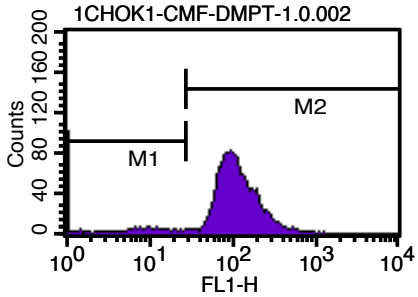
Fig. 5.1a, b & c Effect of three chemicals analysis by DCF flow cytometry

Cells (2.5×10^5 cells) in 6-well plate. (a) Cells were exposed to DMPT for 24 hours and were measured by DCF flow cytometry. (b) Cells were exposed to DMAEMA for 24 hours and were measured by DCF flow cytometry. (c) Cells were exposed to DMABEE for 24 hours and were measured by DCF flow cytometry.



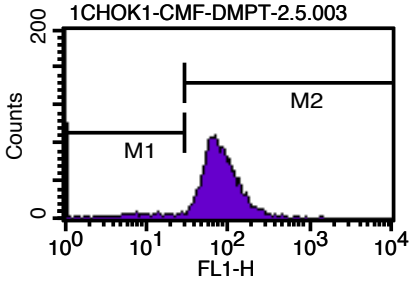
File: 1CHOK1-CMF-DMPT-NC.001

Marker	Mean
All	95.18
M1	3.20
M2	122.48



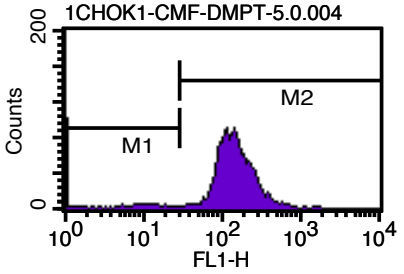
File: 1CHOK1-CMF-DMPT-1.0.002

Marker	Mean
All	100.25
M1	2.76
M2	123.15



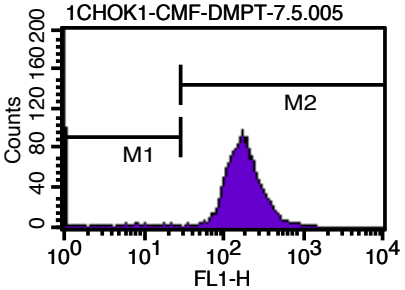
File: 1CHOK1-CMF-DMPT-2.5.00

Marker	Mean
All	67.41
M1	2.71
M2	85.59



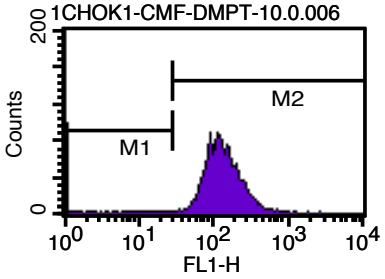
File: 1CHOK1-CMF-DMPT-5.0.004

Marker	Mean
All	128.95
M1	2.30
M2	152.35



File: 1CHOK1-CMF-DMPT-7.5.00

Marker	Mean
All	162.34
M1	2.00
M2	187.36



File: 1CHOK1-CMF-DMPT-10.0.006

Marker	Mean
All	115.41
M1	1.59
M2	140.12

Effects of DMPT on the CMF expression of CHO-K1 cells

Histogram of DCF fluorescence of CHO-K1 cells treated with 0 to 10.0 mM DMPT. M2 population indicated GSH depletion content were noted. Result of one representative experiment was shown

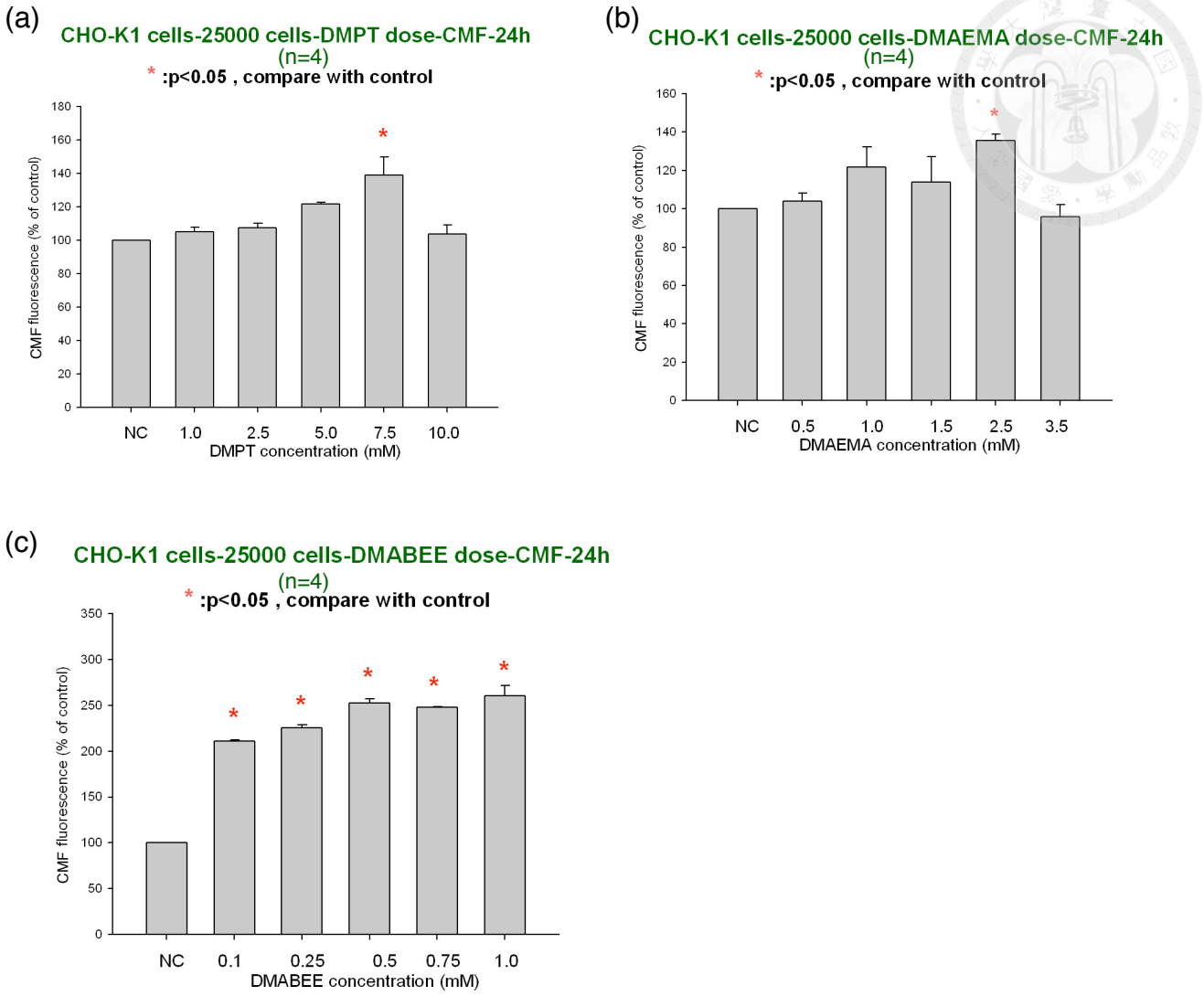
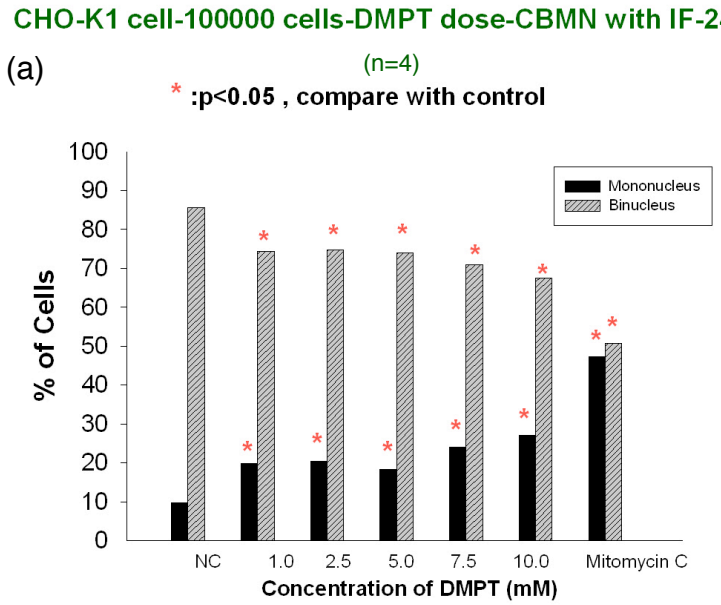


Fig. 5.2a, b & c Effect of three chemicals analysis by CMF flow cytometry

Cells (2.5×10^5 cells) in 6-well plate. (a) Cells were exposed to DMPT for 24 hours and were measured by CMF flow cytometry. (b) Cells were exposed to DMAEMA for 24 hours and were measured by CMF flow cytometry. (c) Cells were exposed to DMABEE for 24 hours and were measured by CMF flow cytometry.



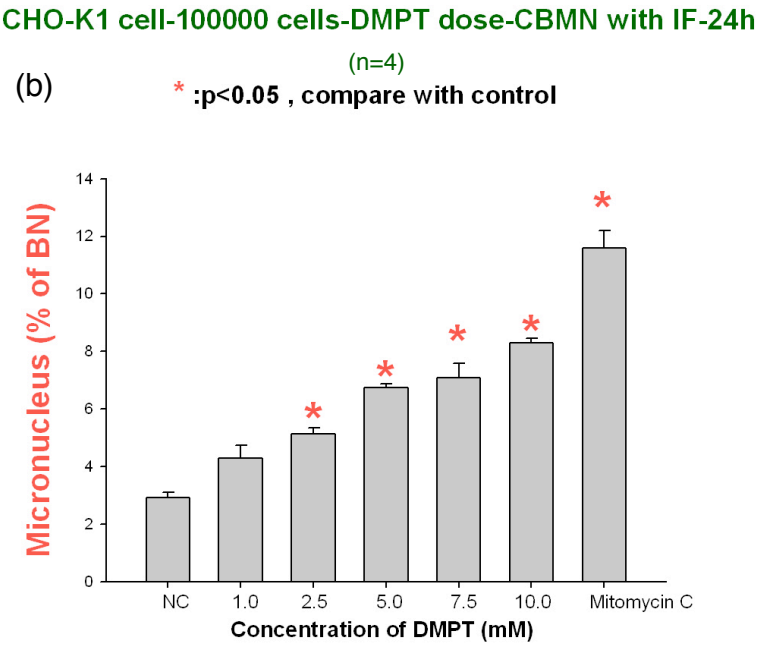


Fig. 6.1a & b CBMN assay of CHO-K1 cells.

Cells (1.0×10^5 cells) in 6-well plate on 18 × 18 mm slides were exposed to DMPT for 24 hours and the group of mitomycin c was regarded as positive control. (a) The ratio of mononuclear and binuclear cells were calculated by CBMN assay with IF staining. (b) MN / binuclear cells ratio was measure by CBMN assay (the number of binuclear cells > 500, at least)

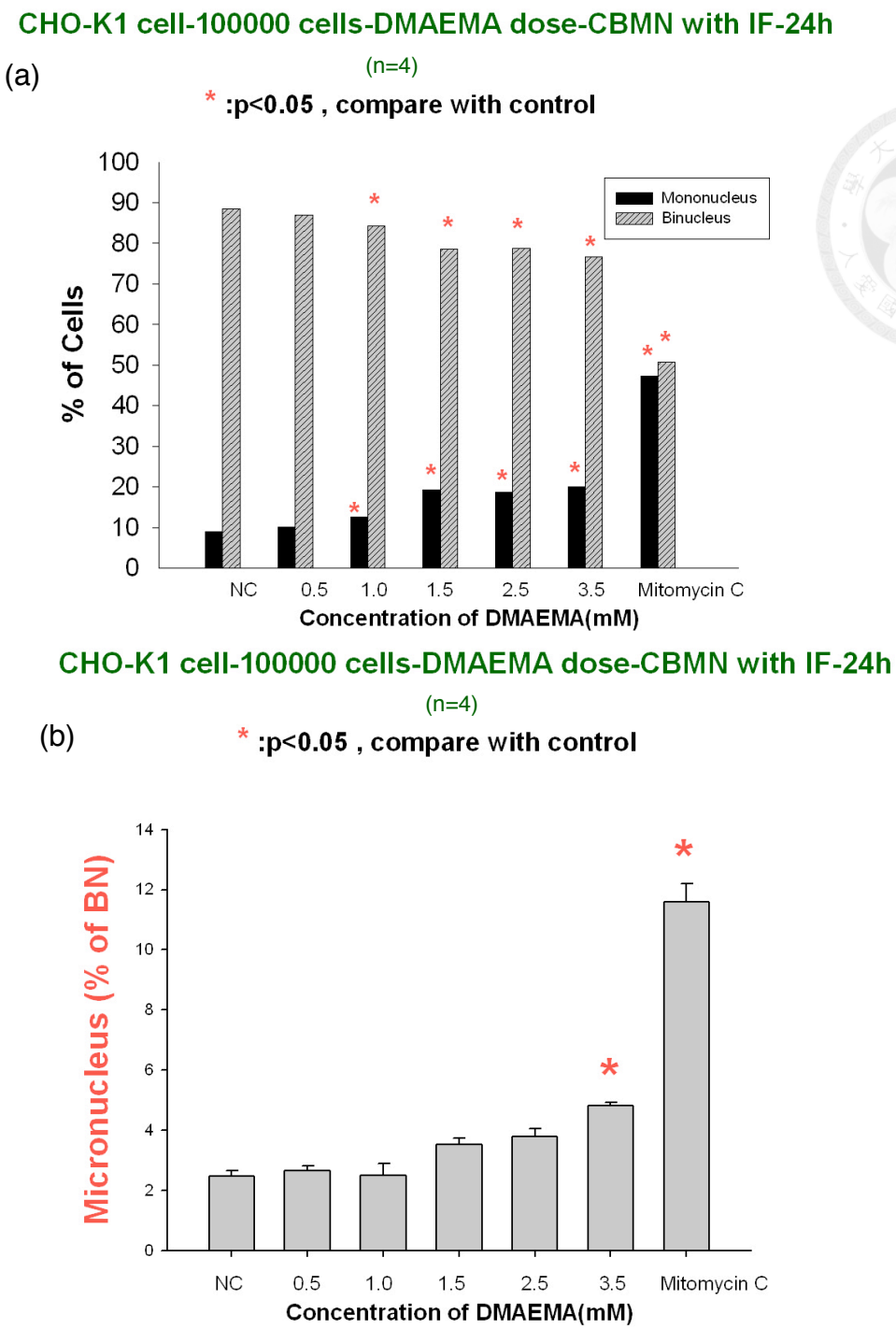
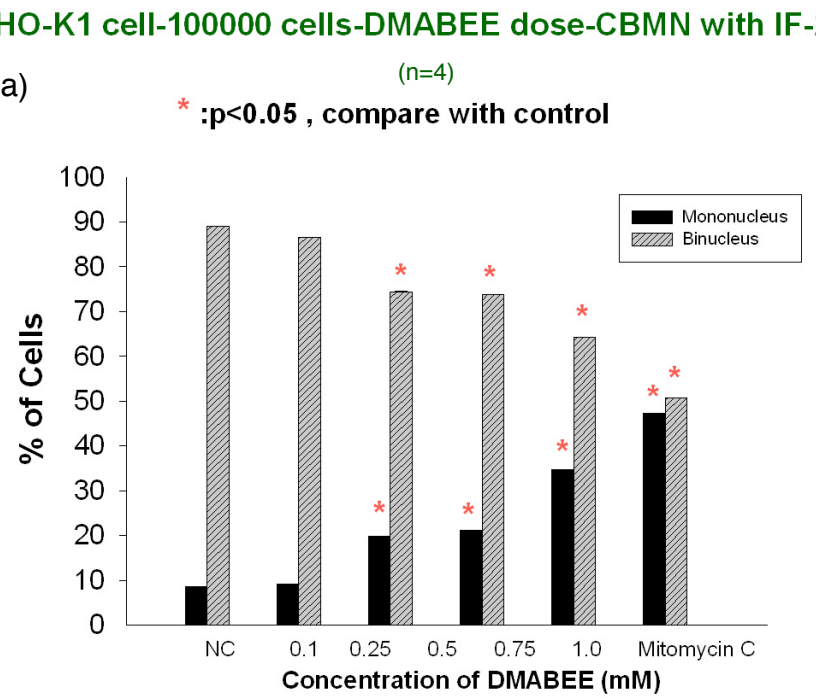


Fig. 6.2a & b CBMN assay of CHO-K1 cells.

Cells (1.0×10^5 cells) in 6-well plate on 18 × 18 mm slides were exposed to DMAEMA for 24 hours and the group of mitomycin c was regarded as positive control. (a) The ratio of mononuclear and binuclear cells were calculated by CBMN assay with IF staining. (b) MN / binuclear cells ratio was measure by CBMN assay (the number of binuclear cells > 500, at least)



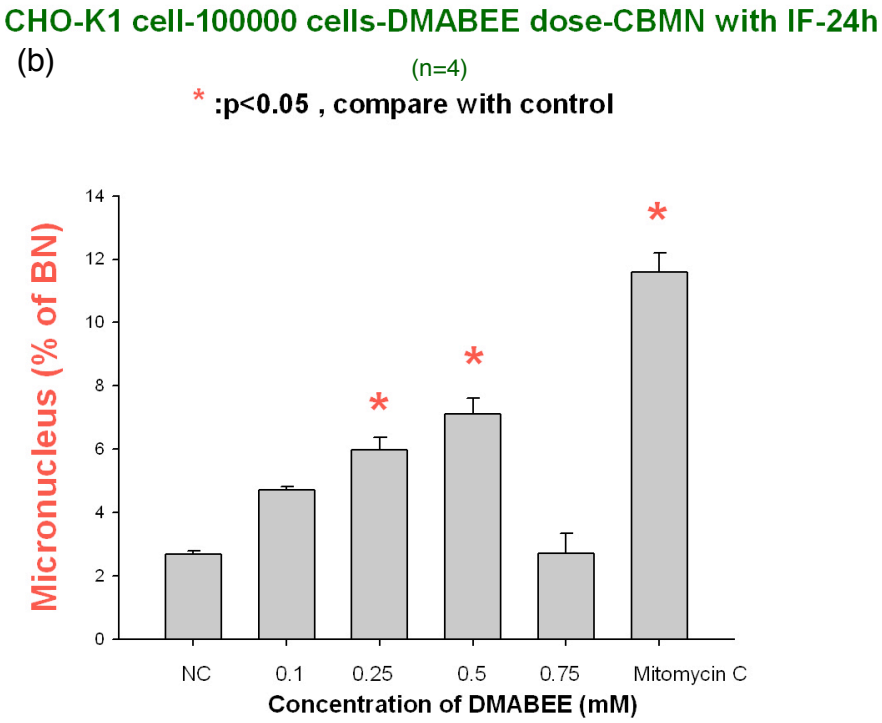
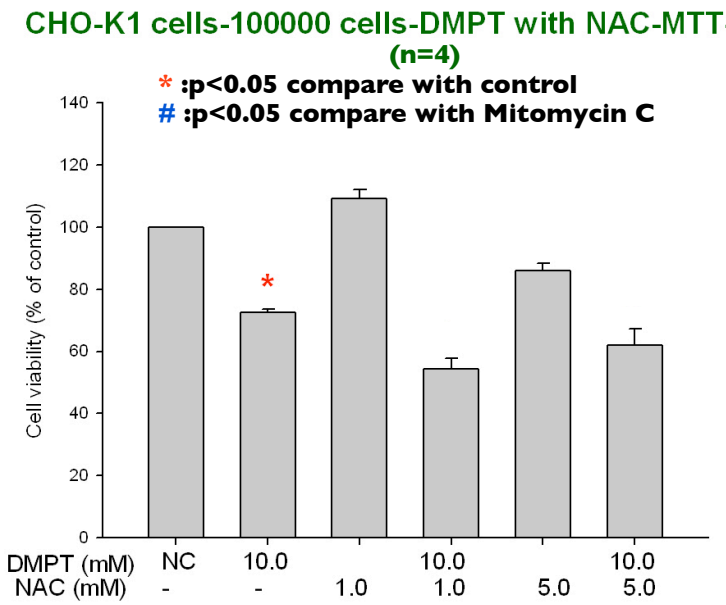


Fig. 6.3a & b CBMN assay of CHO-K1 cells.

Cells (1.0×10^5 cells) in 6-well plate on 18 × 18 mm slides were exposed to DMABEE for 24 hours and the group of mitomycin c was regarded as positive control. (a) The ratio of mononuclear and binuclear cells were calculated by CBMN assay with IF staining. (b) MN / binuclear cells ratio was measure by CBMN assay (the number of binuclear cells > 500, at least)

(a)



(b)

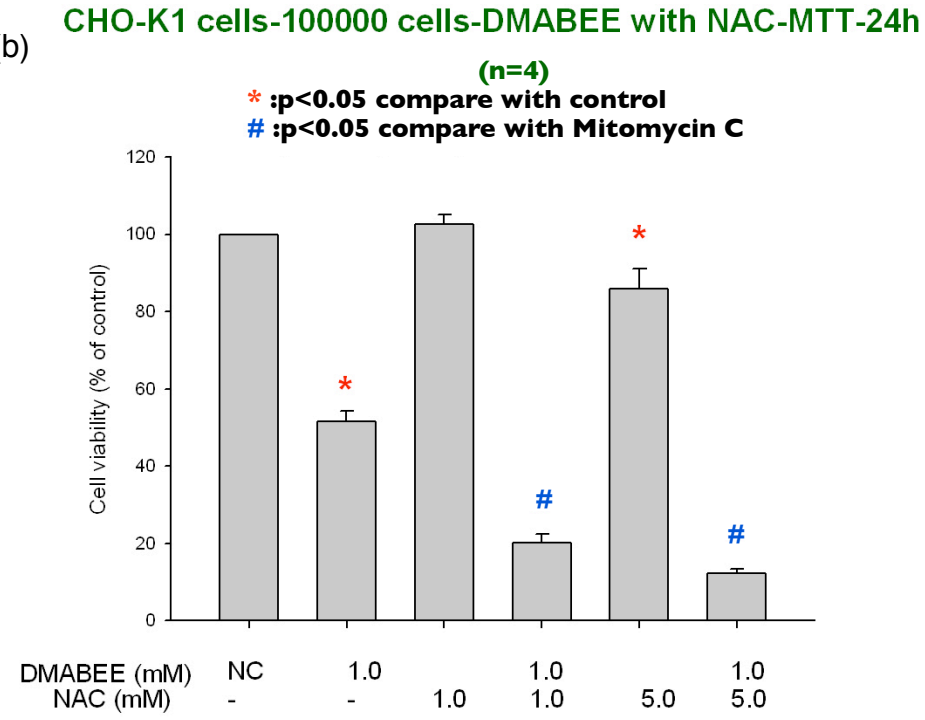
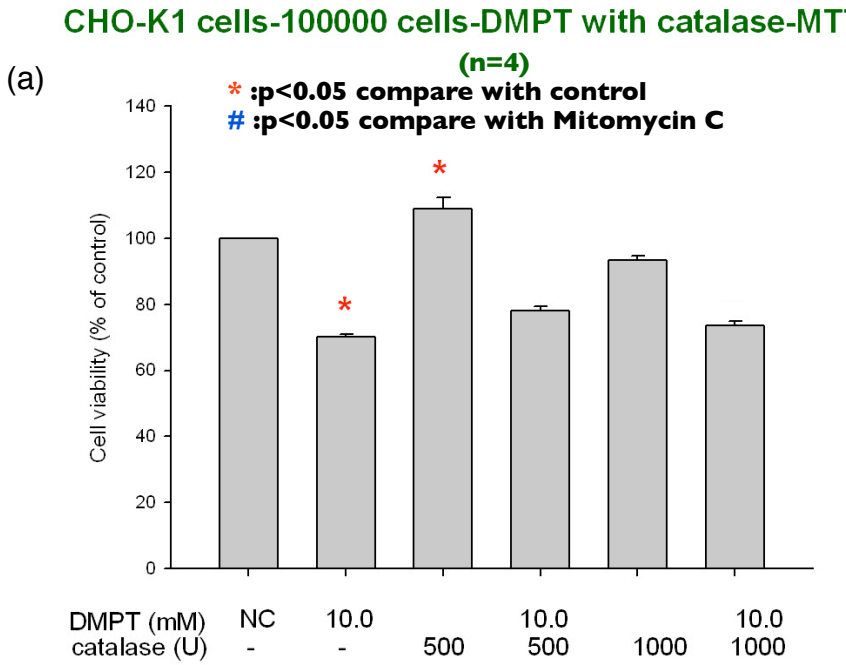


Fig. 7.1a & b Effect of CHO-K1 cells co-treating with NAC on the growth ability.

Cells (1.0×10^5 cells) in 6-well plate were incubated with NAC for an hour. (a) Cells were exposed to DMPT for 24 hours and were measured by MTT assay. (b) Cells were exposed to DMABEE for 24 hours and were measured by MTT assay.



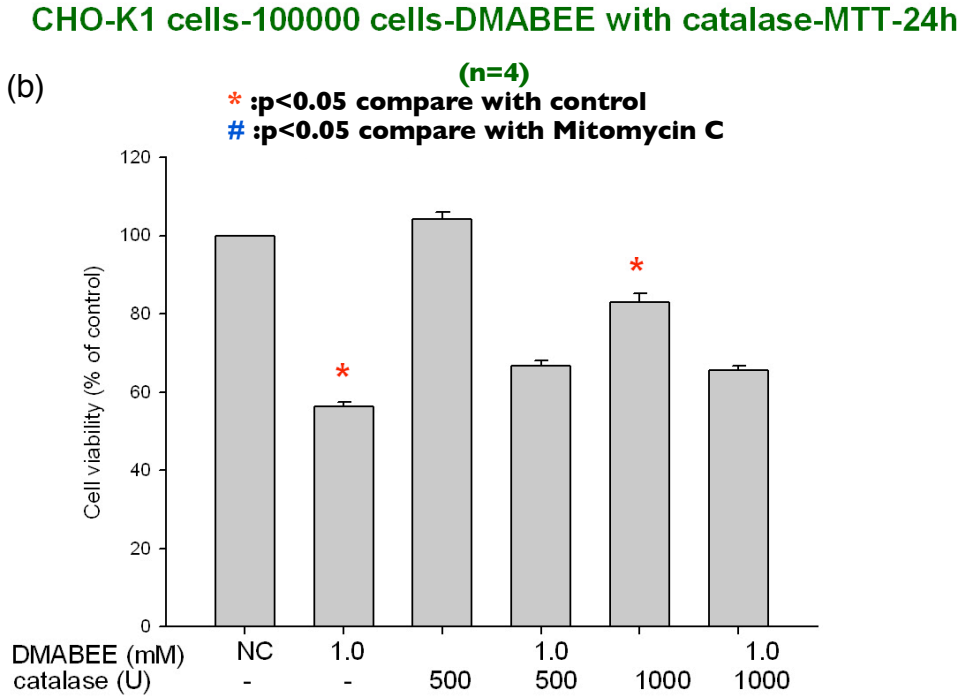
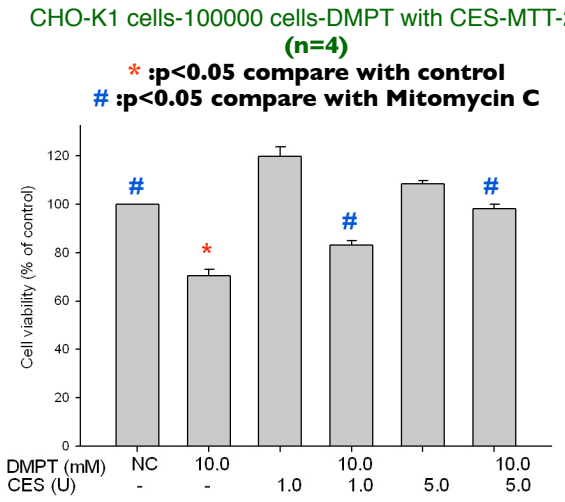


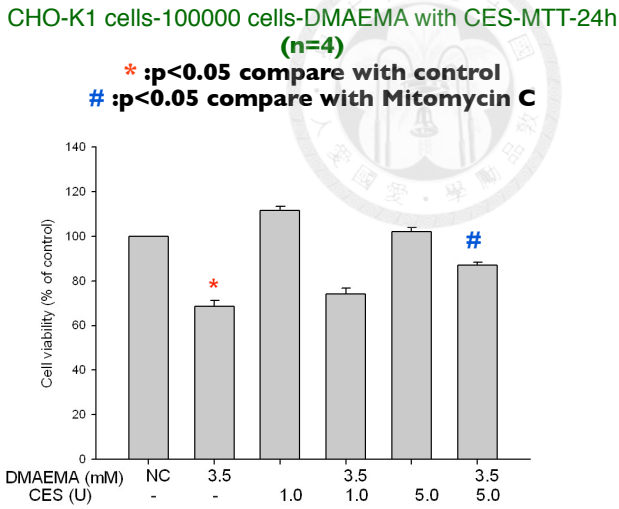
Fig. 7.2a & b Effect of CHO-K1 cells co-treating with catalase on the growth ability.

Cells (1.0×10^5 cells) in 6-well plate were incubated with NAC for an hour. (a) Cells were exposed to DMPT for 24 hours and were measured by MTT assay. (b) Cells were exposed to DMABEE for 24 hours and were measured by MTT assay.

(a)



(b)



(c)

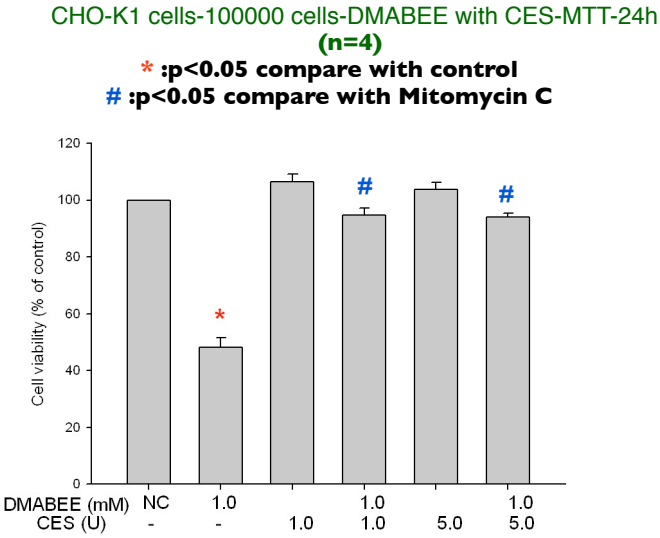


Fig. 7.3a, b & c Effect of CHO-K1 cells co-treating with carboxylesterase on the growth ability

Cells (1.0×10^5 cells) in 6-well plate were incubated with carboxylesterase for an hour. (a) Cells were exposed to DMPT for 24 hours and were measured by MTT assay. (b) Cells were exposed to DMAEMA for 24 hours and were measured by MTT assay. (c) Cells were exposed to DMABEE for 24 hours and were measured by MTT assay.