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Toxicity Assessment of Gas Phase in Cigarette Smoke Using Cell-free Assay

Chul-Hoon Park*, Hyung-Ok Sohn, Han-Jae Shin, Hyeong-Seok Lee,
Young-Keun Min and Hak-Chul Hyun
KT&G Central Research Institute
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ABSTRACT : ***In vitro*** **toxicity tests such as cytotoxicity, mutagenicity and genotoxicity assay are useful for evaluating the relative toxicity of smoke or smoke condensates obtained from different cigarette configurations. A major disadvantage of these tests is relatively time-consuming, complicated and expensive. Recently, a cell-free glutathione consumption assay (GCA) as a rapid and simple screening method for the toxicity assessment of smoke has been reported by Cahours *et al.* (CORESTA, 2006). This study was carried out to assess the GCA application capable of predicting the toxicity of gas/vapor phase (GVP) of cigarette smoke and to identify individual compounds responsible for the glutathione (GSH) consumption in smoke. Each GVPs from 2R4F, standard cigarette, carbon filter cigarette (ExC) and new carbon filter cigarette (ExN), test cigarettes were collected by automatic smoking machine and evaluated the relative toxicity by GCA and neutral red uptake (NRU) assay. Toxic compounds existed in smoke were also chosen, relative toxicities of these compounds were screened by using two methods and compared individually. The overall order of toxicity by GCA was 2R4F > ExC > ExN, which was consistent with the result of Neutral Red Uptake assay. The levels of carbonyl compounds of ExN were lower than those of 2R4F and ExC, indicating that GSH consumption was associated with carbonyl compound yields. A major toxicant under current study is acrolein, which contributed to more than half of the GSH consumption. Collectively, the toxicity of GVP determined by GCA method may be mainly attributed to acrolein.**

Key words **: Glutathione consumption assay, cytotoxicity, gas/vapor phase**

GVP of mainstream smoke contributes greatly to the cytotoxicity of cigarette smoke, because it contains a myriad of reactive components, including 10^{14}~10^{16}alkyl, alkoxyl and peroxyl radicals per puff, up to 1000 mg/g of the free radical nitric oxide and high levels of reactive aldehydes (Reddy *et al.*, 2002). Previous reports also demonstrate that GVP represents a stronger oxidizing species than whole smoke (Pouli *et al.*, 2003). Therefore, there has been a growing interest in the *in vitro* cytotoxicity of smoke GVP. Cytotoxicity of GVP can be assessed by NRU assay, as is the cytotoxicity of total particulate matter (TPM). NRU assay has been proposed as an easy and sensitive assay for assessing the cytotoxic potential of cigarette smoke and validated by the international regulatory agencies (Andreoli *et al.*, 2003; Putnam

*연락저자 : 305-805, 대전광역시 유성구 신성동 302 번지, KT&G 중앙연구원
*Corresponding author : *KT&G Central Research Institute, 302 Shinseong-dong, Yuseong-gu, Daejeon 305-805, Korea(phone: 82-42-866-5598; fax: 82-42-866-5544; e-mail: chpark@ktng.com)*

et al., 2002). However, the disadvantages of this test for screening the toxicity of new tobacco product are relatively time-consuming and expensive. Recently, GCA reported by Cahours X. *et al.* (2006), has been known as a rapid and simple screening method showing good correlation with cytotoxicity assay. GCA was carried out in a cell free system and the cytotoxicity of GVP could be speculated from GSH consumption caused by oxidants in GVP.

GSH is a tripeptide antioxidant containing a sulfhydryl group. GSH plays a protective role against oxidants, electrophilic comounds and xenobiotics in cells. Previous studies showed that GSH is critical to lung cellular antioxidant defenses in *in vitro* and *in vivo* assay (Rahman and MacNee, 1999) and this depletion often parallels cell activation or toxicity (Reddy *et al.*, 2002). The direct attack by oxidants can deplete GSH, which may be at least in part responsible for the cytotoxicity caused by GVP. In addition, some acute cytotoxic carbonyl compounds such as α, β-unsaturated aldehydes spontaneously react with GSH (Reddy *et al.*, 2002). These suggest that common determinants of GCA and in vitro cytotoxicity assay may exist in GVP.

Present study was carried out to evaluate the suitablility of GCA for predicting cytotoxic potential of GVP from new carbon filter cigarettes and identify individual compounds responsible for the GSH consumption.

MATERIALS AND METHODS

Experimental cigarettes

2R4F cigarettes, reference cigarette, were purchased from the University of Kentucky, Lexington, KY, USA. Carbon filtered (ExC) and new carbon filtered cigarettes (ExN), test cigarette, were designed from KT&G central research institute. A brief designation for each cigarette is given in Table 1.

Sample preparation

Experimental cigarettes were conditioned and smoked according to ISO Standard 3308 (2000). GVP was freshly prepared by bubbling through 20 mL of PBS from mainstream smoke which was passed through a Cambridge filter disc to get rid of TPM. GVP was diluted with culture media immediately before use for NRU assay, and intactly used for GCA.

Cell culture for the Neutral red assay

NRU assay was performed using the BALB/c 3T3 clone A31 cell line (ATCC CCL 163, Manassas, VA) according to the National Institute of Health (NIH, 2001) and INVITTOX protocol 1 (1990). Briefly, 3T3 cells were cultured in DMEM medium, supplemented with 2 % FBS

Table 1. Description of experimental cigarettes including ISO analyses of TPM

	Description	TPM (mg/cig)
Ky2R4F	Reference Cigarette	
ExC	Carbon filter with low carbon content	7.15 ± 0.33
ExC1	Carbon filter	7.10 ± 0.23
ExN1	New carbon experimental filter, Type 1	6.88 ± 0.49
ExN2	New carbon experimental filter, Type 2	6.66 ± 0.36

All experimental cigarettes used in this study were commercial blend except for 2R4F. Data represent mean ± SD of 3 individual experiments.

(JRH), 10 % Serum Plus Medium Supplement and Antibiotic-Antimycotic 1X. Cells were seeded into 96-well tissue cultured plate at a density of 10,000 cells (in 100 μL of medium) per well and incubated at 37 ℃ and 5 % CO_2. When cells were confluent, cells were treated for 24 hr with at least 8 dilutions of GVP in culture medium. After 24 hr, the culture medium was aspirated from the wells and cells were washed with PBS. Immediately, 200 μL of a neutral red solution (50 μg/mL) was added to each well and the cells were incubated at 37 ℃ for 3 hr. The neutral red solution was aspirated and 200 mL of 1 % formalin solution was added to each well. The formalin solution was replaced with 50 % ethanol solution containing 10 mL glacial acetic acid/liter. The plates containing neutral red solution was placed on a microplate shaker for 10 min and the absorbance of each well was measured at 540 nm on a microplate reader (Bio-Tek, USA). Relative cell viability was calculated and expressed as percent of untreated vehicle control. The concentration of GVP reflecting a 50 % inhibition of cell viability is determined from the concentration-response.

Selection of carbonyl compound for GSH consumption assay

Acrolein, formaldehyde, acetaldehyde, propionaldehyde and acetone were chosen for cytotoxic potential and abundance in cigarette smoke. All these compounds were purchased from Sigma Chemical Co (St. Louis, MO). For GCA, stock solutions were prepared by dissolving them in PBS.

Chemical analyses of GVP

In order to identify the contents of carbonyl compounds in GVP from 2R4F, GVP was dissolved in acetonitrile with 2,4-dinitrophenylhydrazine (DNPH) derivatives and carbonyl compounds were determined by liquid chromatography with photometric detection of the hydrazones.

Glutathione consumption assay

Glutathione consumption was measured by colorimetric method with DTNB (5,5'-Dithiobis (2-nitrobenzoic acid)). In brief, 500 μL of GSH solution (0.8 mM) prepared in PBS was aliquoted to 1.5 mL disposable tube. We mixed 500 μL of GSH solution with 500 μL of GVP or selected carbonyl compound solution. Additionally, two vials of 500 μL PBS were included in each assay, one with 500 μL of GSH solution for the negative control, and the other for the blank. All vials were capped, mixed by gently vortex and left to stand for 1 hr. 100 μL of GSH mixtures were added to the microtiter plate wells containing 100 μL of DTNB (1 mM). Absorbance was measured at 405 nm on a microplate reader (Bio-Tek, USA)

Statistics

Values are expressed as mean ± SD. Statistical analysis was performed using one way analysis of variance (ANOVA) and the Duncan's test. P values of <0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

NRU cytotoxicity assay has been described as a suitable and sensitive method for the assessment of cytotoxic potential of GVP as well as TPM (Tewes *et al.*, 2003). Our results show that NRU assay can differentiate ExC from the ExNs and furthermore discriminate among ExNs. Cytotoxicity determined by the NRU assay of the GVP is shown in Fig. 1. The GVP dose-dependently induced cytotoxicity and reduced cell viability. The GVPs from all the test cigarettes had significantly lower cytotoxicity compared to the 2R4F. The overall order of cytotoxicity was

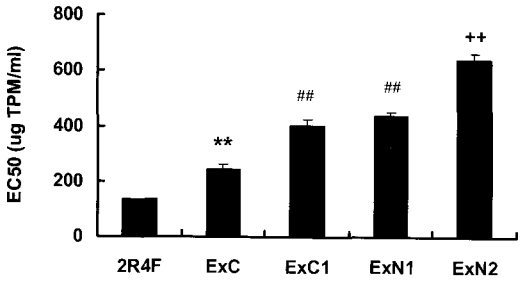

Fig. 1. Cytotoxicity of GVP from 2R4F, ExCs and ExNs. Data represent mean ± SD of 3 individual experiments. ANOVA followed by Duncan's test: ** P<0.01vs. 2R4F; ## P<0.01 vs. 2R4F and ExC; ++ P<0.01 vs. 2R4F, ExCs and ExN1

ExC > ExC1 ≥ ExN1 > ExN2. Cytotoxicity of GVP from ExC1 and ExN1 was 40-45 % lower than that of ExC. ExN2 was 62 % less cytotoxic than ExC, 35 % and 31 % less cytotoxic than ExC1 and ExN1, respectively.

Since some of carbonyl compounds found in GVP are extremely cytotoxic (Bombick *et al.*, 1995; Grafstrom *et al.*, 1986; Harris *et al.*, 1985), reductions of carbonyl compounds by new carbon filter could have significant effect on reducing the cytotoxicity. For each filter type, the levels of five carbonyl compounds such as acrolein, formaldehyde, propionaldehyde, acetaldehyde and acetone, were investigate in GVP by HPLC. The cigarettes containing new carbon filter significantly reduced the carbonyl compounds (Table 2). For the ExN2, about 70 % of acrolein and propionaldehyde and 40~60 % of acetaldehyde, acetone and formaldehyde were reduced in comparison with ExC.

The order of GSH consumption was ExC > ExC1> ExN1> ExN2 (Fig. 2) indicating that the data obtained GSH consumption assay were correlated well with the results of NRU assay (Fig. 3). These results are consistent with those of a previous study which showed a good correlation between GSH consumption and cytotoxicity (Cahours *et al.*, 2006; Heinz-Verner *et al.*, 2004). In abiotic condition, since many reactive GVP components can react directly with GSH, exposure of solutions of GSH to GVP also resulted in rapid GSH consumption (Fig. 2).

In living cells, GSH depletion is considered as a major mechanism of cytotoxicity (Kelly, 1999). Acute exposure of whole smoke or its condensate

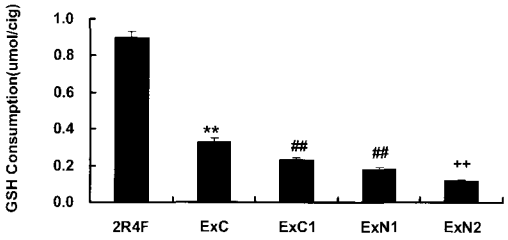

Fig. 2. GSH consumption caused by GVP from 2R4F, ExCs and ExNs. Data represent mean ±SD of 3 individual experiments. ANOVA followed by Duncan's test: ** P<0.01 vs. 2R4F; ## P<0.01 vs. 2R4F and ExC; ++ P<0.01 vs. 2R4F, ExCs and ExN1

Table 2. Yields of selected carbonyl compounds in GVP from experimental cigarettes

	Formaldehyde (μg/cig)	Acetaldehyde (μg/cig)	Acetone (μg/cig)	acrolein (μg/cig)	propionaldehyde (μg/cig)
ExC	5.17 ± 0.42	181.5 ± 10.9	104.2 ± 5.1	12.2 ± 1.0	9.5 ± 0.4
ExC1	4.58 ± 0.41	146.7 ± 1.3	69.1 ± 1.5	7.5 ± 0.5	6.8 ± 0.4
ExN1	3.43 ± 0.37	130.4 ± 10.6	59.5 ± 5.2	6.4 ± 0.9	5.9 ± 0.7
ExN2	3.19 ± 0.63	103.7 ± 4.4	39.6 ± 2.5	3.7 ± 0.2	3.0 ± 0.2

Data represent mean ± SD of 3 individual experiments.

Table 3. The estimated contribution rate (%) of each carbonyl compound to the GSH comsumption by Ky2R4F

Smoke constituents	Yield (μg/cig)	GSH consumption (nmol/cig)	Contribution rate (%)
	Ky2R4F	904.8 ± 12.6	100
Acetaldehyde	398.2 ± 3.5	0	0
Acetone	231.0 ± 2.7	0	0
Acrolein	34.3 ± 1.3	572.6 ± 14.8	63
Formaldehyde	5.7 ± 0.9	15.4 ± 8.6	2
Propionaldehyde	27.5 ± 0.9	0	0

Data represent mean ± SD of three individual experiments.

induced the depletion of intracellular GSH (Aufderheide *et al.*, 2003; Joshi *et al.*, 1988; Kode *et al.*, 2006; Li *et al.*, 1994; Rahman *et al.*, 1995; Ritter *et al.*, 2004), which can give rise to cytotoxic effects such as lipid peroxidation, protein oxidation and DNA modification, if the glutathione pool and other antioxidative cellular mechanisms are not sufficient to balance the redox situation (Pryor, 1997; Ritter *et al.*, 2004). These indicate that reactive oxygen species and aldehydes which can be easily react with GSH are play a key role in the cytotoxicity and futhermore, might also be highly responsible for GSH consumption in GCA.

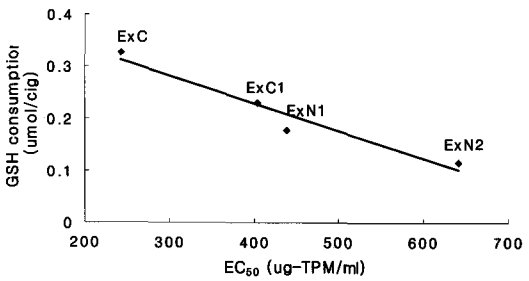

Fig. 3. The correlation between NRU cytotoxicity test and GCA (R^2=0.9437). Relationship between $EC_{50}$and GSH consumption caused by GVP of experimental cigarettes were evaluated. The line was obtained by linear regression analysis.

The data in table 3 show the contribution rate (%) of each carbonyl compound to the GSH consumption by 2R4F. 2 % of the GSH consumption by GVP from 2R4F was attributed to formaldehyde and 63 % to acrolein. However, acetaldehyde, propionaldehyde and acetone had almost no effects on the GSH consumption in the dose range of 1~66 mM. According to the previous study, n-alkanals such as formaldehyde, acetaldehyde and propionaldehyde, were 1000 times less effective in depleting GSH than 2-alkenals, of which acrolein was the most potent in the adult rat lung type II alveolar cells (Meacher and Menzel, 1999). It was also reported that nearly 50 % of the GSH depletion by GVP is due to reaction with acrolein (Reddy *et al.*, 2002) and that 40~60 % of the cytotoxicity by GVP can be attributed to acrolein (Tewes *et al.*, 2003; Shin *et al.*, 2007). Acrolein can bind rapidly (both enzymatically and non-enzymatically) with cellular components. Many of the toxicological effects of acrolein may occur due to the saturation of protective cellular mechanisms (most notably glutathione) and subsequent reaction with critical sulfydryl groups in proteins and piptides (Gurtoo *et al.*, 1981; Marinello *et al.*, 1984). Therefore, acrolein is considered as a key determinant of cytotoxicity and GSH consumption induced by GVP.

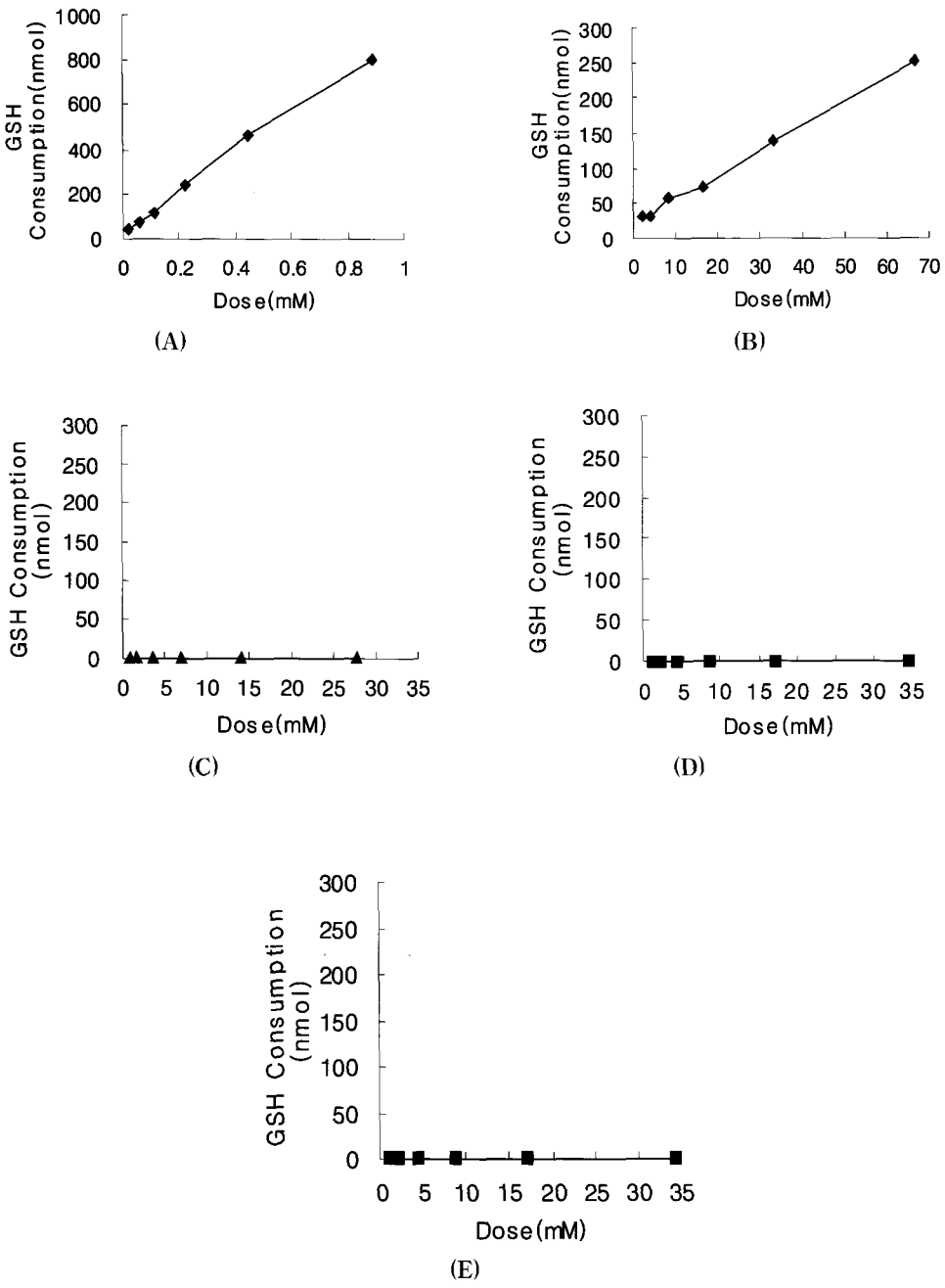

Fig. 4. Dose-dependent effect of selected carbonyl compounds on GSH consumption. The effect of selected carbonyl compound on GSH consumption was tested in the dose range of 0.02~66 mM. (A) Acrolein, (B) Formaldehyde, (C) Acetaldehyde, (D) Propionaldehyde, (E) Acetone

CONCLUSIONS

We confirmed the compatibility of in-house GCA with the prediction model for in vitro cytotoxicity test using the new carbon filter cigarettes. There was a statistically significant correlation (R^2=0.9437) between cytotoxicity and GSH consumption. Furthermore, the GSH consumption of each carbonyl compound was investigated to identify what are responsible for GCA. Our data shows that more than 60 % of the GSH consumption by GVP from 2R4F was attributed to acrolein. Taken together, although not responding to all toxicants and/or combinations of toxicants in GVP, GCA is a useful method for predicting the toxicity of gas phase cigarette smoke.

REFERENCES

Andreoli, C., Gigante, D. and Nunziata, A. (2003) A review of *in vitro* methods to assess the biological activity of tobacco smoke with the aim of reducing the toxicity of smoke. *Toxicol. In vitro* 17: 587-594.

Aufderheide, M., Knebel, J.W. and Ritter, D. (2003) An improved *in vitro* model for testing the pulmonary toxicity of complex mixture such as cigarette smoke. *Exp. Toxic. Pathol.* 55: 51-57.

Bombick, D. W. and Doolittle, D. J. (1995) The role of chemical structure and cell type in the cytotoxicity of low molecular weight aldehydes and pyridine. *In Vitro Toxicol.* 8: 349-356.

Cahours, X., Blanchet, M, Haond, C., Marchand V. and Dumery, B. (2006) A cell-free method to assess the gaseous phase cytotoxicity of cigarette smoke. Coresta Conference Paris, France 4.

Grafstrom, R. C., Willey, J. C., Sundqvist, K. and Harris, C. C. (1986) Pathobiological effects of tobacco smoke-related aldehydes in cultured humn bronchial epithelial cells. In Banury Report 23: Mechanisms in Tobacco Carcinogenesis (D. Hoffmann and C. C. Harris, Eds.), p273-285 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gurtoo, H. L., Marinello, A. J., Struck, R. F., Paul, B. and Dahms, R. P. (1981) Studies on the mechanism of denaturation of cytochrome P-450 by cyclophosphamide and its metabolites. *J. Biol. Chem.* 256: 11691-11701.

Harris, C. C., Willey, J. C., Saladino, A. J., and Grafstrom, R. C. (1985) Effects of tumor promoters, aldehyde, peroxides and tobacco smoke condensate on growth and differentiation of cultured normal and transformed human bronchial cells. In Cancer of the Respiratory Tract: Predisposing Factors P. 159. Mass, et al., ds., Plenum Press, New York.

Heinz-Verner, H., Gerhard, G., Dinamis, J. and Gerhard, S. (2004) Mechanistically-based Tests for toxicity of tobacco smoke. Coresta Conference Kyoto, Japan 1-8.

INVITTOX (1990) The FRAME modified neutral red uptake cytotoxicity test. The ERGATT/ FRAME data bank of in vitro techniques in toxicology, Protocol No. 3a, INVITTOX, Nottingham.

ISO Standard 3308 (2000) International Organization for Standardization. Routine analytical cigarette-smoking machine-Definitions and standard conditions. 4th ed.

Joshi, U. M., Kodavanti, P. R., and Mehendale, H. M. (1988) Glutathione metabolism and utilization of external thiols by cigarette smoke-challenged, isolated rat and rabbit lungs. *Toxicol. Appl. Pharmacol.* 96: 324-335.

Kelly, F. J. (1999) Glutathione: in defence of lung. *Food and chemical Toxicol.* 37: 963-966.

Kode, A., Yang, S. R. and Rahman, I. (2006) Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine

release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. *Respir. Res.* 7: 132.

Li, X. Y., Donaldson, K., Rahman, I., and MacNee, W. (1994) An investigation of the role of glutathione in the increased permeability induced by cigarette smoke *in vivo* and *in vitro*. *Am. J. Respir. Crit. Care Med.* 149: 1518-1525.

Marinello, A. J., Bansal, S. K., Paul, B., Koser, P. L., Love, J., Struck, R. F. and Gurtoo, H. L. (1984) Metabolism and binding of cyclophosphamide and its metabolite acrolein in rat hepatic microsomal cytochrome P450. *Cancer Res.* 44: 4615-4621.

Meacher, D. M. and Menzel, D. B. (1999) Glutathione depletion in lung cells by low molecular weight aldehydes. *Cell Boil. & Toxicol.* 15: 163-171.

NIH-National Institutes of Health (2001). Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity. Report of the Interagency Coordinating Committee on theValidation of Alternative Methods (ICCVAM) and the National Toxicology Program. Department of Health and Human Services, US Public Health Service, National Institutes of Health, Research Triangle Park, NC, NIH Publication NO. 01-4500.

Pouli, A. E., Hatzinikolaou, D. G., Piperi, C., Stavridou, A., Psallidopoulos, M. C. and Stavrides, J. C. (2003) The cytotoxic effect of volatile organic compounds of the gas phase of cigarette smoke on lung epithelial cells. *Free Radic. Biol. Med.* 34(3): 345-355.

Pryor, W. A. (1997) Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ. Health Perspect.* 105: 875-882.

Putnam, K. P., Bombick, D. W. and Doolittle, D. J. (2002) Evaluation of eight *in vitro* assays for assessing the cytotoxicity of cigarette smoke condensate. *Toxicol. In Vitro* 16: 599-607.

Rahman, I., Li, X. Y., Donaldson, K., Harrison, D.J. and MacNee, W. (1995) Glutathione homeostasis in alveolar epithelial cells *in vitro* and lung *in vivo* under oxidative stress. *Am. J. Physiol.* 269: L285-L292.

Rahman, I. and MacNee, W. (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* 277: L1067-L1088.

Ritter, D., Knebel, J. and Aufderheide, M. (2004) Comparative assessment of toxicities of mainstream smoke from commercial cigarettes. *Inhal. Toxicol.* 16: 1-10.

Reddy, S., Finkelstein, E. I., Wong, P. S., Phung, A., Cross, C. E. and van der Vliet, A. (2002) Identification of glutathione modifications by cigarette smoke. *Free Radical Biol. Med.* 33: 1490-1498.

Shin, H. J., Sohn, H. O., Park, C. H., Lee, H. S., Min, Y. K. and Hyun, H. C. (2007) Evaluation of the *in vitro* biological activity of selected 35 chemicals. *J. Kor. Soc. Tob. Sci.* 29: 30-40.

Tewes F. J., Meisgen, T. J., Velter, D. J., Roemer, E. and Patskan, G. (2003) Toxicological evaluation of an electrically heated cigarette. Part 3: Genotoxicity and cytotoxicity of mainstream smoke. *J. Appl. Toxicol.* 23: 341-348.