CHAPTER 4

DISCUSSION AND CONCLUSION

A micronucleus assay using Feulgen-Fast Green stain was applied to study the genotoxic effect of embalming solution exposure occurred in the buccal cells of dentistry students. During and after the course of human gross anatomy practice, the dentistry students had an increased number of micronuclei in mononucleated buccal cells (MNBC) and the frequency of mononucleated buccal cells that had micronuclei (BCMN). Although, the average of the MNBC in male students was not statistically significant increased at 15 week-exposure, but even so still higher than 0 week-exposure. The initial high number of the baseline MNBC and BCMN was due to a large variation of 1,500 cells of MNBC and BCMN counted at 0-week exposure (0-5 micronuclei per 1,500 buccal cells). In this study, the baseline micronuclei in all subjects was 1.57 micronuclei per 1,500 buccal cells (0.11%) which higher than those of baseline micronuclei reported by Suruda *et al.* (1993), Titenko-Holland *et al.* (1996) and Ying *et al.* (1997). The baselines of those studies were 0.046, 0.6 and 0.568 micronuclei per 1,000 buccal cells, respectively. However, the average of micronucleus frequency in healthy population was reported as 1-3 micronuclei per 1,000 buccal cells (Fenech *et al.*, 1999). Thus, the baseline micronuclei of this study was below the average micronuclei found in normal population.

Suruda *et al.* (1993) reported that the number of initial low micronuclei in buccal cells at pre exposure was due to the large number of the subjects (27 of 29), who had no micronucleus in 1,500 cells. Suruda's result was similar to Sarto *et al.* (1990), who reported zero micronucleus per 1,500 buccal cells in 10 of 16 electroplating workers.

A significant inter-individual variability in the frequency of degenerated cells has been observed in buccal cells of healthy individuals, some of which may be due to inter-laboratory procedural differences. An important component in the interpretation of micronucleus assay

results is cell kinetics. Micronuclei observed in exfoliated buccal cells are not induced when the cells are at the epithelial surface, but they are when in the basal layer (Fenech *et al.*, 1999). In general, cells take 7-16 days to emerge to the surface and exfoliate (Stich and Rosin, 1984; Sarto *et al.*, 1990)

On the other hand, a great variation was detected in the individual of each exposed group. The micronucleus frequency variation observed within this study, characterized by the high values of the standard deviation (Table 2,3), must be due to the fact that the responses to a given genotoxic agent is different from person to person. This differential response may be the result of different factors, such as genetic constitution and life habits such as drinking and diet, etc. (Martino-Roth *et al.*, 2002). In addition to the standard deviation, the range of variation between the minimum and maximum values of the samples also reflects this variability, which goes from 0 to 5 micronuclei at 0 week-exposure, 0 to 16 micronuclei at 10 week-exposure and 0 to 9 micronuclei at 15 week-exposure. According to Dittberner *et al.* (1997), *in vivo* studies of micronucleus induction, like those performed on exfoliated buccal cells, showed frequently wide variations, and the micronuclei frequency was considered as a simple and trustworthy indicator of genotoxic damage in human tissue exposed to carcinogens. When spontaneous micronucleus frequency was compared to healthy individuals, up to a 17-fold difference was observed, which possibly reflected genetic and nonspecific exposure differences (Fenech *et al.*, 1999). The causes of this inter-individual variability are unclear at this time and deserve further investigation.

These data do not provide enough evidence for an exposure-response relationship between formaldehyde exposure to embalming solution and micronucleus formation, due to a lack of day-by-day measurements of exposure and the monitor of individual exposure. However, Norppa *et al.* (1993) failed to see a significant dose-response relationship between a rise in total micronucleus frequency and formaldehyde exposure, but did report a statistically significant increase in buccal cell micronuclei, similar to the result reported by Titenko-Holland *et al.* (1996). This appears disagree with the findings of Suruda *et al.* (1993), that found a dose-response relationship with formaldehyde exposure.

Suruda *et al.* (1993) also reported that females in their study tended to have a higher baseline micronucleus level than males. However, the baseline of micronuclei in the males and females in this study were not different. Twenty-five international laboratories participating in

The International Collaborative Project On Micronucleus Frequency in Human Populations (HUMN), reported an increase in micronucleus frequency in adult females compared to males, but not in the age range 0-19 years (Neri *et al.*, 2003). Age of the subjects in this study was 19 $\pm$ 0.6 years and there was no difference of micronucleus frequency by gender in which correlated to the finding of Barale *et al.* (1998), Ganguly (1993) and Neri *et al.* (2003). However, the MNBC and BCMN values at 10 and 15 week-exposure appeared to have higher micronucleus frequency in females than males. This effect could be attributed to the increase of aneuploidy in women, as suggested by Pastor *et al.* (2001). In fact, general high levels of micronuclei were observed in women rather than men and the gender effect was attributed to the high X-chromosome micronucleation (Barale *et al.*, 1998;Fenech, 1998). The X chromosome especially tends to lag behind in female lymphocyte anaphase, being micronucleated more efficiently than autosomes (Norppa and Falck, 2003).

There was no correlation between the confounding factors and MNBC and BCMN. There is no explanation for this non-correlation, since the positive and protective effects have been generally reported in a relation to DNA stability and food supplement. In this study, only a kind of nutritional food supplement information was collected from each student but had not determine food supplement level in blood.

In fifteen subjects who took vitamin C as a nutritional supplement, micronucleus frequency was still high. It is possibly because of the role of vitamin C in prevension of the DNA and lipid peroxidation (Fenech, 2002) which has different mechanism from those of formaldehyde and other compounds in this study to induce micronucleus formation. Although Piyathilake *et al.* (1995) reported that the presence of vitamin C in the plasma appeared to be marginally protective against chromosomal damage in buccal cells. But MacGregor *et al.* was reported in 1997 that ascorbic acid (vitamin C) and ∝-tocopherol (vitamin E) did not show any inverse correlation with the micronucleus frequency.

In five subjects who took vitamin B as a nutritional supplement, micronucleus frequency was still high. Piyathilake *et al.* (1995) reported that vitamin B_{12} appeared to marginally protect against chromosomal damage in buccal cells. Titenko-Holland *et al.* (1998) reported that the micronucleus frequency in buccal cells was decreased after dietary supplementation of 516 micrograms of folate per day. In addition Fenech *et al* (2002) found that humans who took folate

and vitamin B_{12} supplements in an intervention study showed lower chromosomal break and micronucleus formation when the plasma folate and vitamin B_{12} concentration were taken more than 34 and 300 nmol per liter, respectively. These concentrations are achievable at intake levels in excess of current recommended dietary allowance (RDA), which is more than 400 μg of folic acid daily and over 2 μg of vitamin B_{12} per day. In this study, subjects who had taken vitamin B did not show any correlation with MNBC and BCMN. Other nutrients that had been claimed such as vitamin E, A, Zinc and spirulina algae, also did not show any correlation with MNBC and BCMN. When the baseline of MNBC and BCMN were analyzed, no significant difference of micronuclei found in the case whether the subjects used dietary supplement food or not. It was suggested that vitamins or other supplements might not be taken high dosage enough to decrease or protect the micronucleus formation.

Several studies showed that X-ray exposure correlated with micronucleus induction (Thierens *et al.*, 2000; Muller *et al.*, 1993; Minodier *et al.*, 2002; Slonina *et al.*, 2003). However, this study showed no correlation between X-ray exposure and micronucleus formation. It is possible that radiation increases micronuclei and returns to the baseline within 1 month as proposed by Stich (1987).

There was also no correlation between spicy food consumption and the MNBC. This result was harmonious with that found by Picker and Fox (1986), when micronucleus frequency was enhanced in buccal cells by increasing consumption of curried food. However, the result was not statistically significant.

Dentistry students were also exposed to other chemicals such as, denture based resin during this study, which was a prominent material used in denture base. Denture based resin is usually composed of pre-polymerized poly-methyl methacrylate (MMA) powder particles, which is mixed with the monomers of MMA and a crosslinking agent (Ruyter, 1980). MMA is widely used in dentistry for the manufacture of dental prostheses and orthopedic surgery as bone cement to fill space in bone (Yang *et al.*, 2003). Despite the various methods were used to initiate the polymerization of denture based resin, the conversion of monomer to polymer was not completed, and some un-reacted monomer MMA, which was left in the denture base, can be leachable into saliva (Tsuchiya, *et al.*, 1994; Kedjarune, *et al.*, 1999; Huang *et al.*, 2000).

In 1984, Przybojewska *et al.* found a significantly induce chromosomal aberration (CA) resulting in micronuclei formation in mouse bone marrow polychromatic erythrocytes. In agreement with this study, MMA might induce micronuclei in the student's buccal cells.

Yang *et al.* (2003) found that MMA induced CA. It might induce several types of DNA lesions and the unrepaired or misrepaired lesions lean to CA. The CA induced by MMA was one of the chromosomal aberration types in the CHO cells. Moreover the chromosomal gap and break were the most common types of chromosomal aberration to be observed. The sister-chromatid exchange (SCE) frequency was also found to increase when the concentration of MMA increased. SCE is a cytological revelation of DNA breakage and misrepair, and represents as a method detect of genotoxicity of several substances. A dose-dependent increase in the number of SCE, observed after the treatment of MMA, suggests that it is a potent inducer of SCE. In this study, students were exposed to the resin for about 8 weeks before the second re-sampling of buccal cells. It could be increase of MNBC and BCMN found at 10 week-exposure that was higher than the micronucleus found at 15 week-exposure. The possible explanation might be due to the synergistic effect of formaldehyde and the resin at the 10 week-exposure.

Broken egg nucleus was also found in this study before and after exposure. Sarto *et al.* (1987) has described the broken egg nucleus as an abnormal nucleus with unknown cause. This phenomenon can best be described as a nucleus divided into two portions as if it had been "pinched", with one usually larger than the other had. Roberts (1997) reported that broken egg nucleus was in close proximity and connected by a thin band of material that was reported to be Feulgen negative. The result in that study was contradicted with the finding in our study, which found Feulgen positive. Cells that appeared to have an early stage of the broken egg formation were often noted by Roberts (1997). The current available data did not address the possibility of a relationship between embalming solution vapor and micronuclei. They were generally considered as separated phenomena. Other phenomenon was anucleated cell (ghost nucleus) and multinuclei cell. Anucleated cell is a barely visible remnant of the main nucleus with no staining characteristic and pale appearance (Roberts, 1997). Anyway, cells that appeared to contain multinuclei (tri- or tetra-micronuclei) have not been reported prior to our study. However, the mechanism of multinuclei formation remains to be further investigated.

Several studies have shown a relationship between embalming solution exposure, especially formaldehyde, and the presence of micronuclei (Ballarin *et al.*, 1992; Suruda *et al.*, 1993;Titenko-Holland *et al.*, 1996; Ying *et al.*, 1997). Suruda *et al.* (1993) and Titenko-Holland *et al.* (1996) found that the analysis of phenol and methanol in autopsy room revealed undetectable. The exposure level was below the OSHA permissible exposure limits (PEL). Air sampling measurements in that study indicated little or no exposure to other volatile substances in the laboratory than formaldehyde. Thus, they concluded that micronucleus formation was due to formaldehyde exposure. It is similar to our finding that the average of methanol and phenol concentrations in the autopsy room were lower than OSHA's PEL and National Institute of Occupational Safety and Health (NIOSH)'s recommended exposure limit (REL).

The average of exposed formaldehyde level received by the dentisty students was 0.19 $\pm$ 0.06 ppm per 4 hr, which was lower than the OSHA's PEL, 0.75 ppm for 8-hrTWA and 2 ppm for 15-min short term exposure limit (STEL). However, the formaldehyde level was higher than NIOSH's REL (0.016 ppm for 8 hr-TWA and 0.1 ppm for STEL). PEL expressed as a time-weighted average concentration of substance to which most human can be exposed without adverse health effects for a conventional 8-hr workday and 40-hr workweek, which it is believed that nearly all human may be repeatedly exposed, day after day. STEL is the concentration to which it is believed that human can be exposed continuously for a short period of time provided that the daily TWA is not exceeded. It is defined as a 15-min exposure and should not occur more than 4 times per day even if the TWA is not exceeded. There should be at least 60 minutes between successive STEL exposures. For 4-hr PEL which should be higher than 8-hr PEL but lower than 15-min STEL (Confer and Confer, 1999). In this study, 4 hr formaldehyde exposure was 0.19 $\pm$ 0.06 ppm, which was higher than 15 min STEL (0.1 ppm) and exceeded standard permissible exposure level. However, concentrations of those compounds after converting to the ppm in air might be some miscalculation due to the differences in temperature in autopsy room.

Formaldehyde is quickly evaporated at 20°C resulting in high concentration levels at the first week in which the cadavers were just picked up from embalming solution. Moreover, the fume of formaldehyde in the autopsy room appeared to be increased after the cadavers were being dissected. Formaldehyde levels also depend on the studied topics, e.g. when dissecting the pharynx and larynx, the vapor of formaldehyde came out from the cadaver would be higher than

the level when dissecting the mouth. It could be conceived that methanol that was used to dilute formaldehyde was high at the first air sampling and low at the last air sampling due to its evaporated so quickly at 20°C (NIOSH, 2000b).

The average concentration of phenol was 0.08 ppm per 4 hrs, which was lower than the OSHA's PEL (5 ppm as an 8-hr TWA). Even though, the cadavers were sprinkled with phenol to protect fungal growth in the cadaver resulting in an increase of the phenol concentration in each week through out this study.

A limitation of the present study was a lack of control groups who were not exposed to embalming solution vapors and has the same environment like the study group. However, each student in this study before embalming solution vapor exposure served as his or her own control. It was suitable for used as a control in this study. Furthermore, this study did not measure the background level of formaldehyde, methanol and phenol in the indoor air at some other rooms, which has no vapor of embalming solution such as a lecture room.

As the reason that the students were exposed to phenol and methanol as well as the formaldehyde. So, a potential interaction between these volatile substances was predicted to cause more mutagenity to the student's buccal cells.

Formaldehyde can cause DNA-crosslinks; point mutation; single and double strand DNA breaks, which result in cytogenetic damage, mainly in the form of chromosomal breakage conversion of the initial DNA damage into chromosomal aberrations and changes in cell proliferation (WHO, 2002). Titenko-Holland *et al.* (1996) reported that micronuclei containing only chromosome fragments were mostly induced from exposure to embalming solution vapor. They showed chromosome breakage rather than aneuploidy occurred after was inducing by exposure to embalming solution.

The structure of methanol does not indicate that it would be genotoxic (WHO, 1997) and there is no evidence from animal studies to suggest that methanol is a carcinogen. This was confirmed in the study of Campbell *et al.* (1991) in *in vivo* studies. There was no increased frequency of micronuclei in blood and lung cells of mice exposed by inhalation to 1,050 or 5,200 mg/m^3 (800 or 4,000 ppm) methanol for 5 days (Campbell *et al*, 1991). However, in 2001, Gattas *et al.* reported that frequency of micronucleus was increased in gas operators who exposed to a mixed fuel, which contains 33% methanol, 60% ethanol and 7% gasoline. They concluded that

the effect could be related to methanol, and possibly synergistic association with other components of gasoline.

Several studies have shown that phenol induced micronuclei. However, the relationship between inhalation of phenol and micronucleus induction has not been reported. The potential for phenol to induce micronuclei appears to be related to the route of administration, with generally positive results when it is administered intraperitoneally (Marrazzini *et al.*, 1994), but it had negative or equivocal results when administered orally (Ciranni *et al*, 1988). This is likely due to the potential first pass detoxification of phenol when it is administered via the oral route (EPA, 2002). Phenol was positive in an *in vitro* assay for the development of micronuclei in human lymphocytes (Yager *et al.*, 1990). Miller *et al.* (1995) also found that phenol was positive in an *in vitro* micronucleus test on CHO cells in the presence or absence of S9 from the livers of phenobarbital/betanapthoflavone induced rats, although a stronger response was observed in the presence of S9.

Thus, inhalation of formaldehyde, MMA, methanol and phenol has given positive results for micronucleus induction, and co-exposure of these compounds may cause an additive effect. However, the detailed mechanisms of micronucleus formation from those substances remain to be defined further.

In conclusion, this study suggests the use of buccal cells and the micronucleus assay, which are valuable to study the health effect of an exposure to embalming solution vapor. The finding is consistent with known clastogenic properties of formaldehyde, and the components in embalming solution and MMA are most likely responsible for micronucleus induction. These findings do not indicate a direct mechanism for carcinogenesis, but does indicate that DNA alteration has occurred after embalming solution exposure.