CHAPTER 6
DISCUSSION

Halothane is an inhalation anesthetic agent and has been used widely for the induction and maintenance of general anesthesia. The usual dosage of halothane is 0.5% to 1.5%. Halothane may cause dose-dependent respiratory depression resulting in hypoxia. Adverse effects on the liver range from liver dysfunction to hepatitis and necrosis, and are more frequent following repeated use. Defective proteolytic degradation of the immunogenic trifluoroacetylated protein caused hepatotoxicity.

Medical person especially anaesthetist, medical technician, and nurses need the best condition of operating room, i.e. there is adsorption tubes or diffusion dosimeters for personal halothane concentration. The total dosage of wasted halotane exposure depend on cumulative duration of the anesthet time. Therefore, such medical personnel have a risk of tissue damages; e.g. liver, kidney, cardiovascular system, pulmonary system, renal system, and skeletal muscle system.

This research aimed to identify and analyze the liver cells changes of halothane exposure to liver cells of Balb/C mice. We investigated the liver changes using HE staining and immunohistochemistry evaluation. The time of halothane exposure was modified as the independent variable. In HE staining evaluation, we examined the liver cells nucleus changes. Since halothane can
easily evaporate at room temperature, we only put it on a plate and keep inside the cages and let the mice inhale it for 3 hours each day.

EB Kurutas, *et al.* used 30 male, non-inbred *Mus musculus* albino mice, 6-10 weeks old to investigate the effect of halothane anesthesia in different doses and a period of two weeks on glucose-6-phosphate dehydrogenase (G6PDH) activity of mouse liver.\textsuperscript{42} The mice were anesthetized with halothane using the same method with this research. Liver tissue after being processed routinely, were underwent 5µm sections and stained with Harris hematoxyline-eosin then examined under light microscope. The liver tissues showed eosinophil leukocyte and mononuclear inflammatory cell infiltration on portal tracts. Cloudy degenerations, congestions, micro- and macrovesicular fatty degeneration, spotty necrosis and centrilobuler necrosis of the parenchymal cells were observed. They did not statistically test those findings. In this research, we counted the abnormal nucleus cells which could reflect cell damage.

From counting the abnormal cells through HE staining examination, we found that control group has the smallest percentage of nucleus changes (mean: 17.65\%) and group 3 has the highest percentage of nucleus changes (mean: 61.22\%). Total abnormal cells were positively correlated with the time of exposure. Interestingly, in the group 4, after six weeks halothane exposure followed by two weeks of free halothane, the mean of abnormal cells were less than group 3 which exposed by halothane for six weeks. However, overall abnormal cells were higher in group 4 which suggest the cells damage still continued regardless the cessation
of halothane exposure. The comparison between these two groups was not statistically significant. Thus, I suggest that the liver damage may be irreversible.

Mechanisms of halothane toxicity are idiosyncratic reaction, immunoallergic type, and the protein adducts formed in the initial toxic reaction provide the hapten for the formation of antibodies which augments damage on re-exposure. Up to one-third of inhaled halothane may be oxidatively metabolized by cytochrome P450 2E1 and 2A6. Trifluoroacetyl chloride, which has a strong ability to acetylate liver proteins, is formed during the synthesis of trifluoroacetic acid (TFA). Only a small amount (~1%) of halothane is metabolized through the reductive pathway by cytochrome P450 2A6 and 3A4. This pathway is favored under hypoxic conditions, resulting in the release of bromide and fluoride ions and in the formation of other volatile organic metabolites.\textsuperscript{22} The release of these ions damage membrane integrity of cells and results in fatty change, acute yellow atrophy and widespread centrilobular hepatocellular necrosis that is indistinguishable from fulminant viral hepatitis.

Two types of halothane-mediated hepatotoxicity have been defined. The first type, type I, is a mild, self-limited postoperative hepatotoxicity, with a mild form of hepatocellular injury that can be observed in about 20% of halothane–treated patients. The mild hepatic injury is assumed to result from the direct action of halothane on the liver cells. Two clinically detectable factors appear to contribute to the mild form of hepatic injury. The first is a transient elevation of liver enzymes and the second is alteration of cellular integrity, which can be detected by electron microscopy. Lesions result from intracellular degradation of
halothane via its anaerobic and aerobic pathways in combination with local hypoxia caused by an alteration of the hepatic oxygen demand and supply relationship. The second type of halothane-mediated hepatotoxicity is type II-halothane hepatitis. The incidence of this type of hepatotoxicity after halothane administration is one case per 10000-30000 adult patients. The probable mechanism is most likely an immune-mediated hepatotoxicity; antibodies are against modified liver microsomal proteins on hepatocyte surfaces.6

Sufficient tissue perfusion and oxygenation are vital for all metabolic processes in cells and the major influencing factor of tissue repair and resistance to infectious organisms. A concept covering both oxygen deliveries, tissue oxygen transport and oxygen consumption of the cells could be named tissue oxygen perfusion. In hepatic hypoxia, reductive pathway may metabolize halothane. Thus, halothane has reputation as liver toxin. In other hand, some believe that hepatic dysfunction after the administration of halothane and other anesthetics is related to global or surgical effects on liver perfusion rather than to the agent itself.43

The phenotype can be distinguished by the actual activity or the amount of the expressed CYP enzyme. The genotype, however, is determined by the individual DNA sequence. Since human has two sets of chromosomes, that mean the same genotype enables different phenotypes. Depending on the metabolic activity (phenotype), three major categories of metabolizers are separated: extensive metabolizer (normal), poor metabolizer, and ultra-rapid metabolizer (increased metabolism of xenobiotics). However, until recently there is no
published evidence of CYP2E1, CYP2A6, and CYP3A4 genetic polymorphism that correlate with halothane metabolism.  

Spracklin, et al. conducted a research to test the hypotheses that disulfiram prevents halothane-dependent protein trifluoroacetylation in vivo, and that TFA represents a biomarker for hepatic protein trifluoroacetylation. Using rats pretreated with isoniazid (CYP2E1 induction), isoniazid followed by disulfiram (CYP2E1 inhibition), or nothing (controls), then anesthetized with halothane or nothing (controls), plasma and urine TFA were quantified by ion HPLC and hepatic microsomal TFA-proteins were analyzed by Western blot. He revealed that CYP2E1 induction increased both TFA and TFA-protein formation compared with uninduced halothane-treated rats.

Prokes B, et al. tried to determine concentrations of wasted halothane in operating rooms and investigated the effect of halothane pollution on liver functions of exposed personnel. They examined liver function test of 191 operating room personnel. Average concentration of waste halothane detected in examined surgical theaters has remained under TLV for halothane of 40 mg/m$^3$ (4.9 ppm). Analyses showing the status of hepatic cell membrane (AST, ALT, LDH) revealed that average concentrations of all three parameters were significantly higher in the exposed medical personnel than in the control group.

In this study, we examined the cytochrome P450 2E1 expression in mice liver cells after halothane exposure using immunohistochemistry. We did not directly count the amount of TFA since Cytochrome P450 2E1 (CYP2E1) is a major catalyst in the formation of trifluoroacetylated proteins; however, our result
was not statistically significant. There are some factors that can influence the result of immunohistochemistry examination. These factors can be divided into pre-analytical, analytical and postanalytical factors.

The pre-analytical factors include optimal preservation of the antigenic epitope. IHC can be affected by the duration of anoxia at surgery, time gap between resection to fixation, the type of fixative, the duration in the fixative, the size of the tissue, the thickness, and whether freezing was done. Finally, the quality of reagents such as the company, the batch, and the shelf-life of antibodies can affect the kind of result obtained. Of these, optimal fixation is of special interest because it is a critical yet manageable step.

The analytical factors include the various techniques used for antigen retrieval namely heat, microwaving, pressure cooking, trypsin digestion, autoclaving with different buffers etc. Proper endogenous peroxidase blocking is vital to prevent background staining. Further, whether an autostainer is being used or the staining is done by hand will influence the end result. The biochemical process involved is important, for example the relatively recent “catalyzed signal amplification” method is considered more sensitive than avidin-biotin or extraavidin methods.

The postanalytical (interpretative) factor is the actual interpretation of the IHC result by the surgical pathologist. Several large studies have addressed the issue of inter and intra-observer errors. The positive or the negative interpretation has several subtle nuances which only a busy practitioner of IHC will realize. Suffice it to say a combination of several observations such as intensity, quantity
and localisation of the IHC reaction and visualization of the immunostain in the lesional cells - as opposed to the immuno reaction seen in normal tissues, reactive tissues and other ‘bystanders’ (often referred to as “background” staining) – are features vital to the the final interpretation. Nevertheless the lack of a prescribed threshold level for interpreting a reaction as positive leaves immense scope for inter and intra observer errors. Finally, whatever the interpretation – it is the integration of the information obtained from the IHC test in the histopathologic picture is what matters most in the final interpretation.43

Regardless of those factors, I propose that this non-significant difference of the cytochrome P450 (2E1) expression of the experiment groups compared with the control group was because it was analyzed in the liver tissue. It is possible when it analyzed in the serum/plasma, the result would be different. Furthermore, each mice probably has different metabolic activity (phenotype), those are: extensive metabolizer (normal), poor metabolizer, and ultra-rapid metabolizer (increased metabolism of xenobiotics), that in turn influence the expression of cytochrome P450.

This study has several limitations. First, it was difficult to design cages size that resembled the proportion of operating room and the halothane wasted. It was also difficult to make sure that every mouse got the same dose one and another since I did not control the air circulation. Finally, the acute effect of halothane administration intravenously when terminated the mice did not taken into account whether it would caused acute effect on the liver cells or not. It may explain why enlarged nucleus in the control group was found.