A PRELIMINARY STUDY ON THE EFFECTS OF REPEATEDLY USED COOKING OIL ON HAEMATOLOGICAL PARAMETERS

by

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CERTIFICATE

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ABBREVIATION

1.	DNA	Deoxyribonucleic Acid
2.	FR	Free radicals
3.	HGB	Hemoglobin
4.	LDL	Low- Density Lipoprotein
5.	LPO	Lipid peroxidation
6.	MCH	Mean cell hemoglobin
7.	MCV	Mean cell volume
8.	MDA	Malondialdehyde
9.	NaOH	Sodium Hydroxide
10.	PPSP	School of Medical Science
11.	PUFAs	Polyunsaturated fatty acids
12.	R·	A free radical
13.	RBC	Red blood cell
14.	RNA	Ribonucleic acid
15.	ROOH	Hydroperoxide
16.	ROS	Reactive oxygen species
17.	TBA	Thiobarbituric acid
18.	TCA	Tricholoracetic Acid
19.	RH	Unsaturated fatty acid

ABSTRACT

This study was done to assess the effect of repeatedly used cooking oil on rats hematological parameter (HGB, RBC, MCV, MCH, and MCHC) and to investigate the morphological abnormalities of red blood cells on blood film of rats treated with repeatedly used cooking oil. This study will also focus on Lipid Peroxidation (LPO) product, malondialdehyde (MDA) in plasma samples of control rats and rats treated with fresh oil and with repeatedly used cooking oil. The repeatedly used cooking oil samples were obtained from stalls selling fried bananas. 5 ml of blood was withdrawn in each rat at 0 day, 20 day, 40 day and 60 day from retro orbital sinus/plexus.

Our study has shown that there are significant difference in the RBC parameter but other parameters studied did not show significant difference. The results showed that there was an increased in the malondialdehyde concentration in the plasma rats treated with repeatedly used cooking oil. The morphology of the red blood cells also showed a change in shape particularly in rats fed with repeatedly used cooking oil.

ABSTRAK

kajian ini dijalankan untuk mengkaji kesan minyak masak yang telah digunakan berulang kali ke atas parameter hematologi (HGB, RBC, MCV, MCH, danMCHC) dan menyiasat sebarang keabnormalan sel darah merah pada filem darah tikus yang telah diberi makan minyak masak yang telah digunakan berulang kali. Kajian ini juga difokuskan untuk mengkaji perbandingan produk pengoksidaan lemak iaitu malondialdehyde (MDA) dalam sample plasma kumpulan tikus kawalan, tikus yang diberi makan minyak masak yang segar dan tikus yang yang telah diberi makan minyak masak yang telah digunakan berulang kali. Minyak masak yang digunakan berulang kali diperoleh dari warung menjual goring pisang. Sebanyak 5 ml darah diambil adalah dengan menggunakan teknik retro orbital sinus iaitu dengan menembusi retro-orbital plexus/sinus pada hari pertama, ke- 20, ke- 40 dan ke- 60.

Kajian kami telah menunjukkan bahawa terdapat perbezaan yang signifikan dalam parameter sel darah merah dan parameter yang lain tidak memberikan perbezaan yang signifikan. Keputusan kajian juga menunjukkan terdapat peningkatan dalam kepekatan malondialdehyde dalam plasma tikus yang diberi makan minyak masak yang telah digunakan berulang kali. Morfologi sel darah merah tikus yang diberi makan minyak masak yang telah digunakan berulang kali juga menunjukkan perubahan.

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Repeatedly used cooking oil

Cooking oil is purified fat of plant or animal origin, which is liquid at room temperature. Some of the many different kinds of vegetable oils are grape seed oil, safflower oil, sunflower oil, olive oil, soybean oil, canola oil, peanut oil, cashew oil and sesame oil. Most animal fats are solid at room temperature and thus not considered as oils, but fish, whales and some other cold-climate animals have oils. Cooking oil is most often used to fry, deep-fry food, or in preserving food. Regardless of the type of cooking oil used, the key health factor is how much it consumed in the diet. While fats are a necessary part of a persons diet, they should not provide more than a third of the daily calories consumed and attention must be paid to the types of fat being used.

Among the oils, palm oil is widely used for cooking by most Malaysian because of its availability, great economic value, exceptional resistance to rancidity, contains high level of Vitamin A and E, low concentration of linolenic and linoleic acids, and does not produce unpleasant odor thus making it most suitable for use in frying and cooking. Hawkers food is famous among Malaysian. Most of these foods are either fry or deep fry. However, most of these hawkers prepare their food in the open environment especially by the road side. Aside from that, they also tend to repeatedly use this oil for a long period of time before changing the whole oil again for a new one. By the time

they change the oil, it has become rancid and tar like in appearance. The changes in the characteristics are due to the decomposing of the oil at very high cooking temperature.

A smoke point is the temperatures at which oil begins to decompose. The best oils for cooking and frying are those that have a high smoking point. Heating cooking oil to a temperature that is very high, will produces smoke fumes. The optimal temperature for frying foods at is 190°C (375°F). Cooking at higher temperatures will cause the food to burn on the outside and however at lower temperature, the food will absorb oil and tastes greasy.

The number of times cooking oils are repeatedly used can also have a negative health effect. The greatest hazard is allowing the fat to become rancid (spoiled) and deteriorated to the point it produces undesirable flavors and odors. Besides ruining what would have been a perfectly good meal, rancid oils also contain free radicals that are potentially carcinogenic (Alice, 2002). Frying foods at very high temperature while exposed to the open environment is unhealthy. Chemical in environment will easily absorb in the hot oil. Thus consuming food cooked with this oil is unhealthy for it contains free radical and also other carcinogenic compound.

A recent study by researchers in Spain has revealed a correlation between reusing cooking oil and high blood pressure (Federico Soriguer *et al.*, 2003). When the same port of oil is repeatedly reheated, the oil begins to degrade and results in release of substances known as polymers and polar

compounds. These substances are absorbed with the food. The researchers have concluded that the more polar compounds and polymer present in oil samples, the more likely an individual will have hypertension (Federico Soriguer *et al.*, 2003).

1.2 Lipid peroxidation

Lipids in biological systems can undergo oxidation, leading to deterioration. In oils, these reactions can lead to rancidity, loss of nutritional value from the destruction of vitamins (A, D, and E) and essential fatty acids, and the possible formation of toxic compounds and colored products. Lipid oxidation is a normal biological process by which we obtain energy from fat. Deleterious lipid oxidation occurring in the body generally is called peroxidation, however this oxidation process can be neutralize by the oxidase in the body.

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals `steal' electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylenes -CH2- groups that have especially reactive hydrogens.

The important lipids involved in oxidation are the unsaturated fatty acid moieties, oleic, linoleic, linolenic and arachidonic acid. The overall mechanism of lipid oxidation consists of three phases. As with any radical reaction the reaction consists of three steps that is initiation, propagation and termination.

Initiation is the step whereby a fatty acid radical is produced. The initiators in living cells are most notably reactive oxygen species (ROS), namely OH° which abstracts a hydrogen to make water and a fatty acid radical.

$$RH + O_2 \rightarrow R\cdot + \cdot OH$$

The fatty acid radical is not a very stable molecule and so it reacts readily with molecular oxygen thereby creating a peroxy-fatty acid radical. This too is an unstable species that reacts with another free fatty acid producing a different fatty acid radical and a hydrogen peroxide or cyclic peroxide if it had reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way.

$$R\cdot + O_2 \rightarrow \cdot + ROO\cdot$$

$$ROO\cdot + RH \rightarrow R\cdot + ROOH$$

$$ROOH \rightarrow RO\cdot + HO\cdot$$

Where RH is any unsaturated fatty acid; R· is a free radical formed by removing a labile hydrogen from a carbon atom adjacent to a double bond; and ROOH is a hydroperoxide, one of the major initial oxidation products that decompose to form compounds responsible for off-flavors and odors. Such secondary products include hexanal, pentanal, and malondialdehyde.

When a radical reacts it always produces another radical and that is why it is called the chain reaction mechanism. The only way a radical reaction can stop is for the two radicals to react and produce a non-radical species. This is

what happens when the concentration of radical species is high enough for there to be a high enough probability of two radicals to actually colliding. Living organisms have evolved different molecules that catch free radicals and protect the cell membrane, one of which is alpha-tocopherol also known as vitamin E.

$R\cdot + R\cdot \rightarrow RR$

$R\cdot + ROO\cdot \rightarrow ROOR$

$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

A free radical is a molecule that contains an unpaired electron in its outer orbit and that can exist independently. Molecular oxygen is a diradical, containing 2 unpaired electrons with parallel spin configurations. Because electrons must have opposite spin to occupy the same orbit, electrons added to molecular oxygen must be transferred one at a time during its reduction resulting in several highly reactive intermediates (Sen, 1995).

A free radical is a molecule that contains at least one unpaired electron. Free radicals (reactive oxygen species) are a byproduct of normal metabolism. They are highly reactive and bind with electrons from other molecules and potentially initiating chain reactions as successive molecules lose and gain electrons. The robbing of electrons by free radicals can disrupt normal cellular processes and cause cellular damage (oxidative stress). The most important reactants in free radical biochemistry in aerobic cells are oxygen and its radical derivatives (superoxide and hydroxyl radical), hydrogen peroxide and transition metals. Reactive free radicals formed within cells can oxidize biomolecules and lead to cell death and tissue injury. Establishing the involvement of free radicals

in the pathogenesis of a disease is extremely difficult due to the short lifetimes of these species (Cheeseman, 1993).

The complete reduction of oxygen to H_2O requires 4 steps and the generation of several free radicals and H_2O_2, which is not a free radical in itself because it contains no unpaired electrons. H_2O_2 is, however, considered a reactive oxygen species (ROS) because of its ability to generate highly reactive hydroxyl free radicals through interactions with reactive transition metals. The complete reduction of oxygen is summarized in the following equations:

$O_2 + e^- \rightarrow O_2^{-\bullet}$ superoxide radical (*1*)

$O_2^{-\bullet} + H_2O \rightarrow HO_2^{\bullet} + OH^-$ hydroperoxyl radical (*2*)

$HO_2^{\bullet} + e^- + H \rightarrow H_2O_2$ hydrogen peroxide (*3*)

$H_2O_2 + e^- \rightarrow {}^{\bullet}OH + OH^-$ hydroxyl radical (*4*)

Each of these oxygen-derived intermediates is considered highly reactive because its unstable electron configurations allow for the attraction of electrons from other molecules, resulting in another free radical that is capable of reacting with yet another molecule. This chain reaction is thought to contribute to lipid peroxidation, DNA damage (Kasai *et al.*, 1986), and protein degradation during oxidatively stressful events (Griffith *et al.*, 1988). Although all the intermediates are potentially reactive, the intermediates vary in their biological importance.

Although not as well described, free radicals can damage DNA, however, no direct measures are available to quantify such damage in vivo. Additionally, proteins appear to undergo modification when exposed to oxygen-derived free radicals. In summary, the univalent reduction of oxygen produces a series of free radicals and ROS that interact with lipids, DNA, and proteins. This interaction degrades proteins and promotes DNA-strand breakage and damage to other genomic structures. These reactive species affect lipids as well, compromising the integrity of polyunsaturated fatty acids, which in turn can affect the homeostatic environment of the cell.

Oxidation, particularly with its possible health and nutrition implications, is probably one of the most important areas of current lipid research. There are weighty problems, such as chronic diseases and the process of aging that have been found to be influenced by lipid oxidation (Haumann, 1993).

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems of plant and animal origin. The mechanism involves a process whereby unsaturated lipids are oxidized to form additional radical species as well as toxic by-products that can be harmful to the host system. Polyunsaturated lipids are especially susceptible to this type of damage when in an oxidizing environment and they can react to form lipid peroxides. Lipid peroxides are themselves unstable, and undergo additional decomposition to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides further react to form malonaldehyde (MDA). The extension of lipid peroxidation can be measured

based on MDA and thiobarbituric acid (TBA), generating a TBA-MDA complex which can be detected using a spectrophotometer at 530nm (Fatum and Haider, 2002).

The products of lipid peroxidation are malondialdehyde, ethane and penthane. MDA level which appear in blood and urine can be estimated. This estimation is used as a biomarker of free radical damage and lipid peroxidation (Marks *et al.*, 1996).

Malondialdehyde is a by product of lipid (fat) metabolism in the body an also a soluble compound that can be found in most biological samples including serum, plasma, tissues and urine as a result of lipid peroxidation, and has become one of the most widely reported analyses for the purpose of estimating oxidative stress effects on lipids. It is also found in many foods and can be present in high amounts in rancid food. MDA can modify proteins and, together with the changes in membrane lipids during lipid peroxidation, may be the main cause of damage to erythrocyte membranes and the subsequent hemolysis (Chiu *et al.*, 1989).

Measurement of malondialdehyde is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans and model systems. MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts, including cross-linked products. MDA also forms adducts with DNA bases that are mutagenic and possibly carcinogenic. DNA-protein cross-links are another result of the reaction between

DNA and MDA. The TBARS method is commonly used to measure MDA in biological samples.

1.3 Lipid peroxidation and its ill-health effects

As a result of singe electron rearrangement, the lipid undergoes degradation and malondialdehyde (MDA) is formed. The termination event can occur as the result of any reaction with another radical, protein, or compound that acts as a free radical trap to form stable oxidized compound. Escaped or release reactive oxygen metabolites into the microvascular environment can cause injury to tissue and connective tissue matrix. Reactive oxygen metabolites may lead to the peroxidation of lipids in the cell membranes resulting in the generation of fatty acid radicals in tissue. The overall effect may result in biochemical changes in the liver during the infection, including depletion of glycogen, lipid infiltration and decrease in nucleic acid content of both DNA and RNA (Al-Omar *et al.*, 2004).

Oxidative damage can result as a consequence of an unfavorable balance between free radical generation and antioxidant defenses. MDA is the most abundant individual aldehyde generated by free-radical attack on polyunsaturated fatty acids (PUFAs) of cell members. MDA also seems to be a promising noninvasive biomarker for free-radical damage. Identification of potentially modifiable protective factors for oxidative stress is an increasingly important task. Dietary intake represents one such set of factors that have

recently received a great deal of attention because of the ability to either reduce or promote oxidative stress. Therefore, a group of Spanish researchers assessed the association between dietary intake and lipid peroxidation via plasma MDA concentrations in elderly people (Lasheras *et al.*, 2003).

Oxidative damage to nucleic acids will occur in the presence of reactive oxygen species (ROS), radical mediated damage to DNA is complex and proceeds via peroxy radicals of the DNA bases and sugar, deoxyribose. DNA damage by ROS can lead to chromosome abnormalities. In proteins and amino acids, oxidative damaged caused by superoxide anions may prevent collagen gelation. Collagen gelation involves in the interaction of singe collagen peptide chain by hydrogen bonding to form triple peptide chain helices. Carbohydrates are also susceptible to oxidative damage by ROS. Hyaluronic acid is one of the main components of synovial fluid in joints that can be degraded by ROS (Al-Omar *et al.*, 2004).

Macrophages posses receptors that recognize and bind to modified low-density lipoprotein (LDL), called scavenger receptors. Modified LDL bound to these scavengers receptors are rapidly engulf by macrophages, so the intracellular cholesterol accumulates and may convert macrophage into a foam cell, which in turns involved in atherosclerosis development. ROS involvement in LDL peroxidation has been shown recently. It was also found that modified form of human LDL initiate the accumulation of cholesterol esters in macrophage as a result of oxidative stress. Vascular endothelial cells can also reuptake and destroy oxidized LDL. However, take up of oxidized LDL by

macrophage is more rapid and might be regarded as a defense mechanism to protect the vascular wall, but excess oxidized LDL can kill macrophages, either by initiating necrosis or by apoptosis. Macrophage death can release proteolytic enzymes and transition- metal ions, causing more oxidative stress to the surrounding cells that may leads to arthrosclerosis (Al-Omar *et al.*, 2004).

An increase in the lipid peroxidation in erythrocytes with age and during coronary heart disease makes red cell membranes more vulnerable to free radical damage via formation of reactive oxygen species. It is thus likely that peroxidative damage may be contributing to an increase in serum LDL-cholesterol, Apolipoprotein-B, probably after its oxidative modification, increase in ferritin and hyperuricemia in coronary heart disease patients. (Hameeda *et al.*, 2000).

The increased generation of reactive oxygen species that occurs in the condition of obesity may be responsible for oxidative injury to erythrocyte membranes, which could lead to a decrease in tissue oxygenation (Cazzola, 2004).

Reused cooking oil contains free radical and also possible carcinogenic compounds. The examples of carcinogenic compounds are dioxin, plumbum, and other heavy metal. Dioxin is the name generally given to a class of super-toxic chemicals, the chlorinated dioxins and furans, formed as a by-product of the manufacture, molding, or burning of organic chemicals and plastics that contain chlorine. It is the nastiest, most toxic man-made organic chemical; its toxicity is second only to radioactive waste. Dioxin exhibits serious health effects when it reaches as little as a few parts per *trillion* in our body fat.

Dioxin is a powerful hormone disrupting chemical. By binding to a cell's hormone receptor, it literally modifies the functioning and genetic mechanism of the cell, causing a wide range of effects, from cancer to reduced immunity to nervous system disorders to miscarriages and birth deformity. Because it literally changes the functioning of our cells, the effects can be very obvious or very subtle. Because it changes gene functions, it can cause so-called genetic diseases to appear, and can interfere with child development. There is no "threshold" dose - the tiniest amount can cause damage, and our bodies have no defense against it (Campbell, 2005).

Some of the diseases which are associated to free radical injury are ageing, acute renal failure, cervical cancer, cerebrovascular disorders, diabetes, Down's syndrome, ischemia reperfusion injury and Parkinson's disease (Marks *et al.,* 1996). Free radicals (FR) may injure erythrocyte membranes (Niki *et al.,* 1988). Similarly, free radicals damage to the erythrocyte membrane followed by hemolysis and anemia can be observed in hemodialyzed patients (Durak *et al.*, 1994).

1.4 Objectives

a) To determine probable change in haematological parameters of rats treated with repeatedly used cooking oil.

b) To investigate the morphological abnormalities of blood cells in rats treated with repeatedly used cooking oil.

c) To observe lipid peroxidation in rats by measuring concentration of malondialdehyde.

2.0 MATERIALS AND METHODS

2.1 Experimental animals

Eighteen male Sprague–Dawley male rats (Figure 1) weighing between 300-350 grams were used in the experiment. Only male rats will be included in this research to ensure homogenous representation of samples due to differences in body composition (fat and retention of water and electrolytes), hormonal factors and normal hematological data between the male and female. The local ethics committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institute of Health (NIH). The number of animals housed in a cage is 6. The rats were further divided into three groups; In each group, six (6) rats were used as control, six (6) were fed with 1.0 ml of fresh cooking oil and another six (6) were fed with 1.0 ml of repeatedly used cooking oil daily. Except for the control group, other rats were given food mixed with the oil daily for 60 days.

2.2 Blood collection

Whole blood preserved in EDTA was used for hematological procedures. Techniques that are used to obtain blood samples from rats depending on factors such as the species and the amount of blood required. Blood withdrawal from the retro orbital sinus has been used by penetrating the retro-orbital plexus/sinus with a glass capillary tube or Pasteur pipette. Retro-orbital sampling provides good sample quality. Concerns related to obtaining blood

samples relate primarily to the possibility that excessive frequency and volume of blood withdrawals could directly affect animal health and well being. The blood of each rats in each group were withdrawn on day 0, 20, 40 and 60. Noncoagulated blood were tested and analyzed immediately for red blood cell (RBC), hemoglobin (HGB), Mean cell volume (MCV) and Mean cell hemoglobin (MCH) in the laboratory, using Abott Cell Dyn model 4000.

Samples of peripheral blood were smeared and stained for peripheral blood film analysis. The well-prepared slides were analyzed under a light microscope and relevant features were photographed and recorded. EDTA was used as an anticoagulant and plasma samples were obtained by centrifugation for 5 min and stored at −60 °C. Stored plasma samples were analyzed for malondialdehyde concentration by thiobarbituric acid reaction method as described by Fatum and Haider (2002).

2.2.1 Rodent retro orbital bleeding

Animals were anesthetized with diethyl-ether placed voluntarily on a paper towel (Figure 2). The forefinger of the operator's nondominant hand is used to pull the facial skin taut and cause the eyes to protrude slightly while the skin at the back of the neck is grasped by the thumb and remaining fingers to restrain. Breathing and color are monitored throughout the procedure to ensure that the restraint does not compromise the airway.

Using the dominant hand, the tip of capillary tube or pipette was gently inserted below the eye at approximately 45 degree angle into the space between the globe and the lower eyelid (Figure 4). When the tip of the pipette contacts the boney floor of the orbit it was gently twisted between thumb and forefinger to rupture the capillary plexus/sinus. Blood was allowed to flow by capillary action into the pipette (Figure 5) Care is taken not to take any more blood than is needed. At the conclusion of the blood withdrawal tension on the animal is released and a gauze pad is gently pressed over the eye for a few seconds until the bleeding stopped. Normal color and respiration were reconfirmed and the animal was returned to its cage for recovery. The same eye was used for successive bleeds. A maximum of four bleeds per eye total were done.

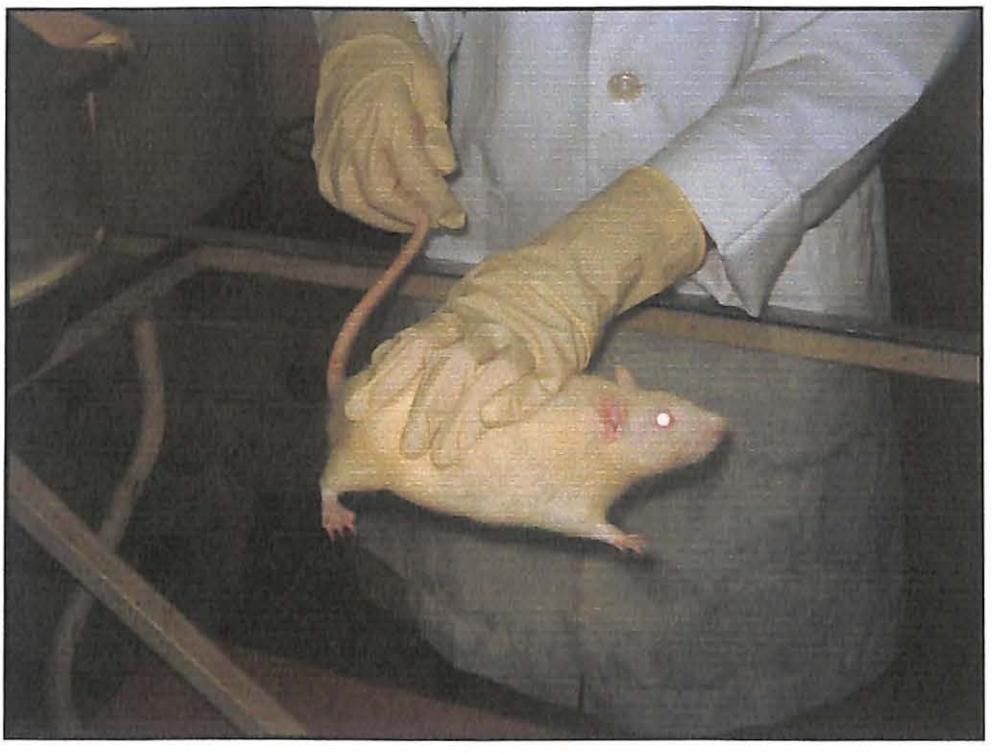

Figure 1: Sprague–Dawley male rat

Figure 2: Anesthetize rat with diethyl-ether

Figure 3: Rat after anesthetized

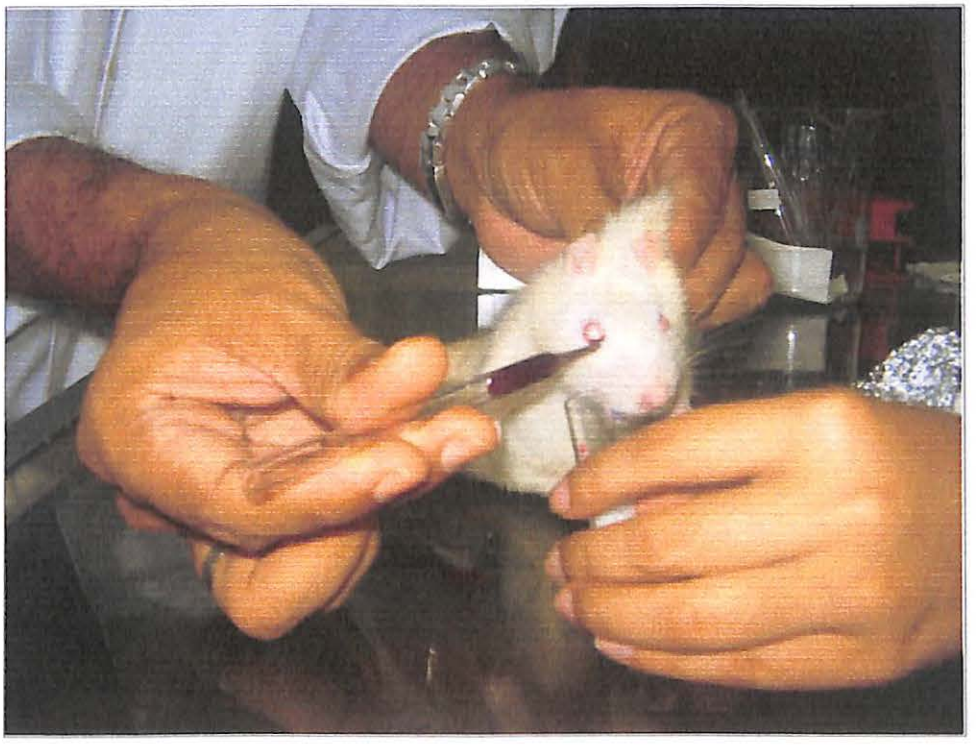

Figure 4: Rodent Retro orbital Bleeding

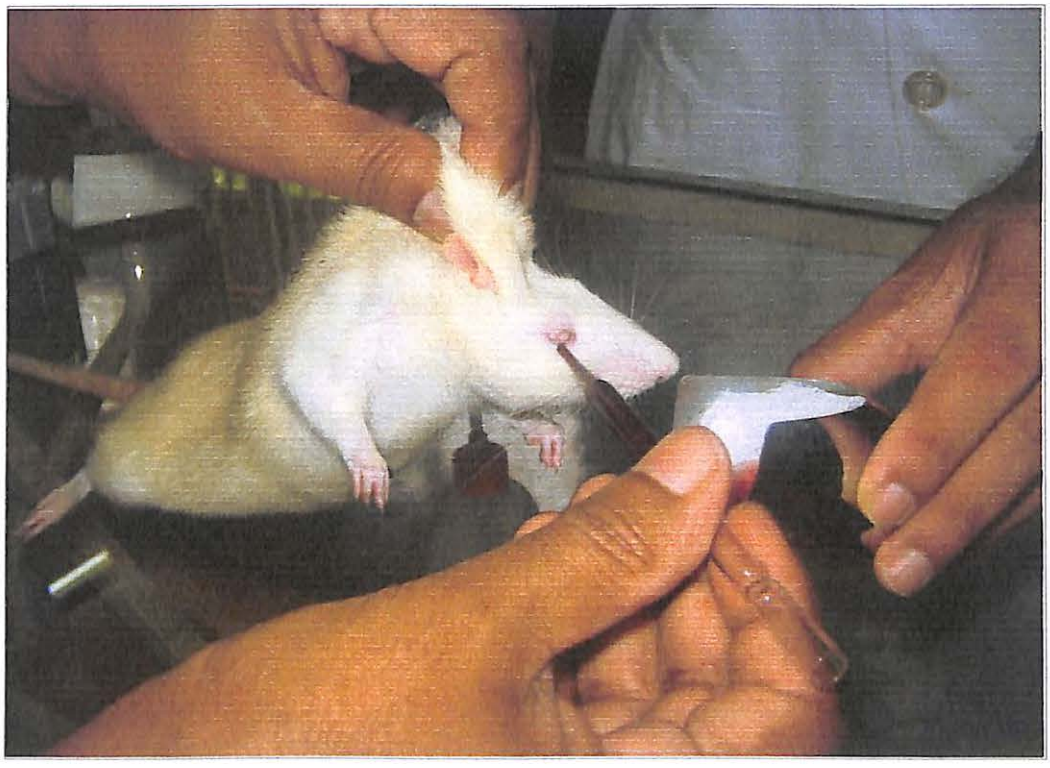

Figure 5: Blood flow by capillary action into the pipette

2.2.2 Haematology parameters

Abott Cell Dyn model 4000 haematology analyzer was used to determine the changes in blood parameters after following treatment with normal saline, fresh cooking oil and repeatedly used cooking oil. Before proceeding with the test, the calibration of haematology analyzer was first done to ensure the instrument was fully functioning and reliable. The blood parameters that were determined using haematology analyzer were:

1. Total Red Blood Cell (RBC)
2. Haemoglobin (HGB)
3. Hematocrit (HCT)
4. Mean cell volume (MCV)
5. Mean cell haemoglobin (MCH)
6. Mean cell haem. conc.(MCHC)
7. Red cell distribution width
8. Reticulocytes count
9. Immature reticulocytes fraction (RETC)
10. Nucleated RBC (NRBC)
11. Total Platelets count (PLT)
12. Mean platelet volume (MPV)
13. Platelet distribution width (PDW)
14. Plateletcrit (PCT)
15. Total White blood cell (WBC)
16. Neutrophils (NEU)

17. Lymphocytes (LYM)
18. Monocytes (MONO)
19. Eosinophil (EOS)
20. Basophil (BASO)

In this study, we will only analyzed, for red blood cell (RBC), hemoglobin (HGB), Mean cell volume (MCV) and Mean cell hemoglobin (MCH) using Abott Cell Dyn model 4000 haematology analyzer (Figure 6)

Figure 6: Abott Cell Dyn model 4000 haematology analyzer

2.2.3 Measurement of Malondialdehyde (MDA)

The reagents used were:

(i) Tricholoracetic Acid (TCA) (20%w/v)

This was prepared by dissolving 20 gram of TCA in 100ml of distilled water.

(ii) Thiobarbituric Acid (TBA) (0.8%w/v)

This was prepared by dissolving 0.8 gm of TBA in 100ml of distilled water with two pellets of Sodium Hydroxide (NaOH).

(iii) n-Butanol and Pyridine

This extraction solvent mixture was prepared by mixing 15.0ml of n-Butanol and 1.0 ml of Pyridine.

The assay for TBARS in the plasma sample was performed by thiobarbituric acid reaction method as described by Fatum and Haider (2002). The principle of the method was based on reaction of one molecule of malondialdehyde with two molecules of Thiobarbituric acid and the pink chromogen formed was measured at 532nm. The method consisted of the addition of the following reagents to tubes: one (1) ml of plasma sample, one (1) ml of distilled water, one (1) ml of 20% TCA solution and one (1) ml of 0.8% solution of TBA were pipetted into each tube by using automated pipette (1000µl). The blank was prepared by pipetting one (1) ml of distilled water. The total volume of the reaction mixture in the tubes was 4.0ml.

The tubes were covered with paraffin film and were mixed with vortex mixer. The tubes were then heated in water bath at 100^0C for 30 minutes. The tubes were next cooled with tap water, one (1) ml of distilled water and 5.0 ml of mixture of n-Butanol and Pyridine were added and shaken vigorously. The material in the tubes was centrifuged for 15 minutes at 4000 rpm. After centrifugation, the upper layer (organic layer) was transferred into the cuvette and absorbance was read against the blank at 532nm. The standard curve of the MDA concentration versus absorbtion were given as one unit of absorbtion is equivalent to MDA concentration of 92µg/ml (figure 7).

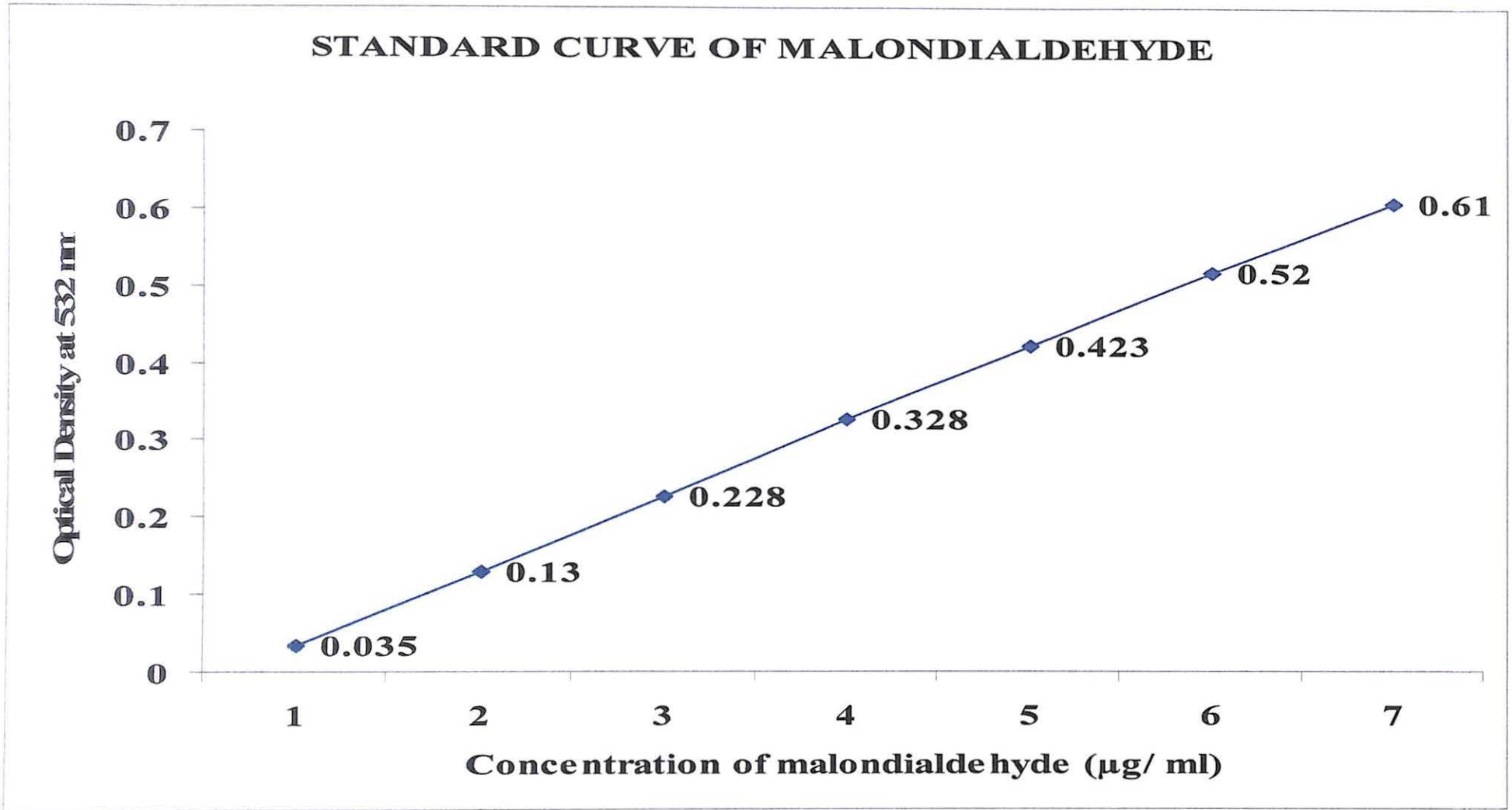

Figure 7: standard curve of malondialdehyde

2.3 Statistical analysis

The data obtained were subjected to Repeated Measure ANOVA (RM ANOVA) using SPSS 12.0 for Windows, with post hoc multiple comparisons for between group's treatment effect analysis based on day of the test. A repeated measure analysis of variance was applied when the same variables were measured on several occasions for each subject. Types of data required for this study were within subject variable and between subject variable The level of significance was evaluated at p= 0.005 by Banferroni Correction.

3.0 RESULTS

3.1 Red Blood Cell (RBC)

In this study, comparisons of hematological parameters for HGB, RBC, MCV and MCH within each treatment for each group based on day were done. The results were expressed as a mean of 95% confidence interval. Statistical analysis was determined by RM ANOVA with repeated measure. Statistical significance of the difference in value of control and treated animals was calculated at 5% significant level. There were 3 different times (day) for measurement within group analysis, which was on day 20, day 40 and day 60. The treatment that has been given to the rats were saline for control group, fresh cooking oil for fresh oil group and repeatedly used cooking for reused oil group. Except for the control group, other rats were given food mixed with the oil daily for 60 days.

The following treatment with reused cooking oil, that is between group analysis, there was a significant difference ($P<0.05$) in measurement of RBC parameters as compared to the control group and fresh oil group (table 1). Red cell count (RCC) is an estimation of the number of red blood cells per liter of blood. Figure 8 shows the comparisons of means Red Blood Cell (RBC) parameter among 3 different groups (control group, fresh oil group and reused oil group). This parameter was measured on day 20, day 40 and day 60. To summarize, table 1 compares the Mean (SD) and EMM (95% CI) of RBC parameter for each groups in which these studies were performed.

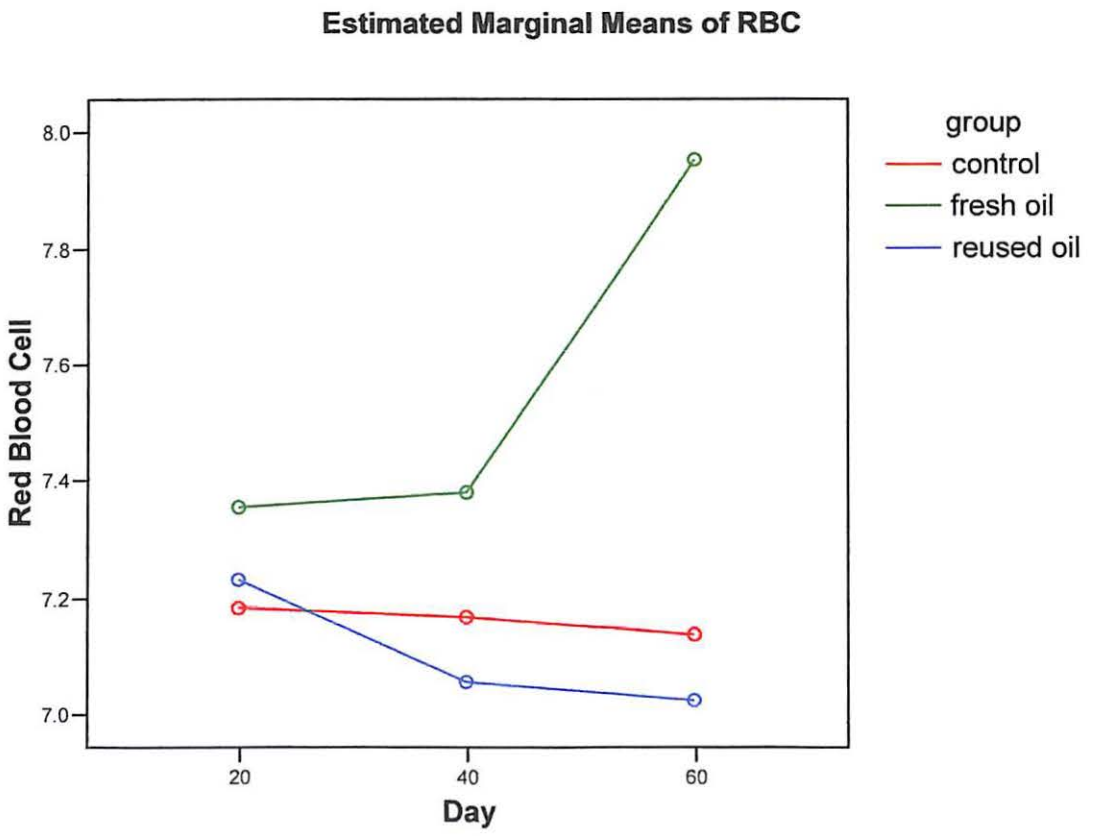

Figure 8: Comparisons of mean RBC parameter among 3 different groups based on day.

Figure 8 shows the comparisons of mean Total Red Blood Cell (RBC) parameter among 3 different groups (control group, fresh oil group and reused oil group). The parameter was determined on day 20, day 40 and day 60.

Scale	Group	Mean (SD)			EMM (95% CI)			F stat	P value
		Day 20	Day 40	Day 60	Day 20	Day 40	Day 60		
RBC	Control	7.186 (1727)	7.170 (0.1746)	7.140 (0.1373)	7.186 (6.944,7.428)	7.170 (6.941,7.399)	7.140 (6.622,7.658)	2.435	0.002
	Fresh oil	7.356 (0.3409)	7.380 (0.3436)	7.952 (0.8919)	7.356 (7.114,7.598)	7.380 (7.151,7.609)	7.952 (7.434,8.470)		
	Reused oil	7.234 (0.1989)	7.058 (0.1316)	7.026 (0.1845)	7.234 (6.992,7.476)	7.058 (6.829,7.287)	7.026 (6.508,7.544)		

Table 1: Comparison of mean RBC and estimated marginal means (EMM) among 3 different groups

P<0.05 are not significant
The percentage computed using spss
The results were significant at 5% leve

3.2 Haemoglobin (HGB)

The levels of means HGB parameters from day 20 to day 60 were found to have no significant difference (P >0.05) between 3 groups as shown in table 2. Hemoglobin is an iron-rich protein found inside red blood cells, which gives blood its red color. Oxygen travels through the bloodstream bound to hemoglobin. The amount of hemoglobin in the blood is an indicator of the amount of oxygen the blood can carry throughout the body. Figure 9 illustrated the changes in means of hemoglobin following treatment saline for control group, fresh cooking oil for fresh oil group and repeatedly used cooking for reused oil group. Hemoglobin parameters were decreased between day 0 and day 27 for groups treated with repeatedly used cooking compare to control group.

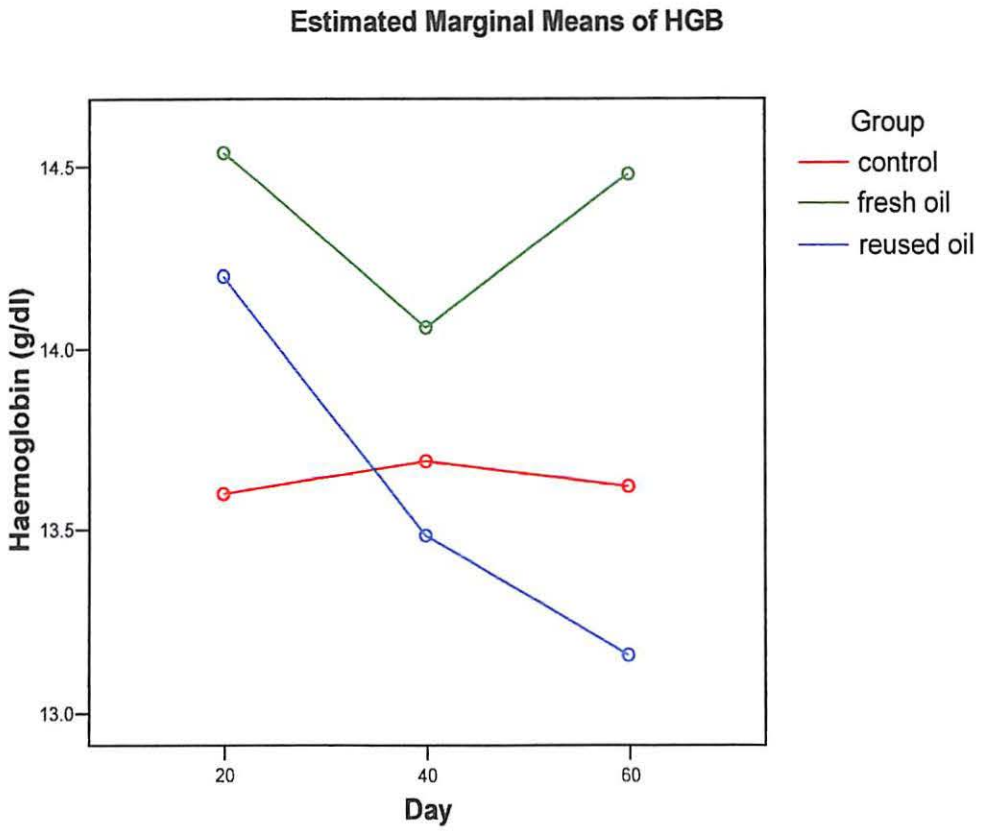

Figure 9: Comparisons of mean HGB parameter among 3 different groups based on day

Figure 9 shows the comparisons of mean Haemoglobin (HGB) parameter among 3 different groups (control group, fresh oil group and reused oil group). The parameter was determined on day 20, day 40 and day 60.

Scale	Group	Mean (SD)			EMM (95% CI)			F stat	P value
		Day 20	Day 40	Day 60	Day 20	Day 40	Day 60		
HGB	Control	13.600 (0.2449)	13.690 (0.2460)	13.620 (0.1789)	13.600 (13.065, 14.135)	13.690 (13.334, 14.046)	13.620 (13.289, 13.951)	4.479	.008
	Fresh oil	14.540 (0.5699)	14.060 (0.4921)	14.480 (0.4147)	14.540 (14.005, 15.075)	14.060 (13.704, 14.416)	14.480 (14.149, 14.811)		
	Reused oil	14.200 (0.7211)	13.484 (0.3139)	13.160 (0.3782)	14.200 (13.665, 14.735)	13.484 (13.128, 13.840)	13.160 (12.829, 13.491)		

Table 2: Comparison of mean HGB and estimated marginal means (EMM) among 3 different groups

P>0.05 are not significant
The percentage computed using spss
The results were significant at 5% level

3.3 Mean cell volume (MCV)

Besides, figure 10 illustrated the changes in means of MCV parameters from day 20 to day 60 for each groups. MCV parameters were decreased between day 0 and day 27 for groups treated with repeatedly used cooking compare to control group and fresh oil group. Results also showed that means of MCV parameters from day 20 to day 60 were found to have no significant difference (P >0.05) in parameter between 3 groups (table 3). Mean cell volume or mean corpuscular volume (MCV) is an estimate of the volume of red blood cells.

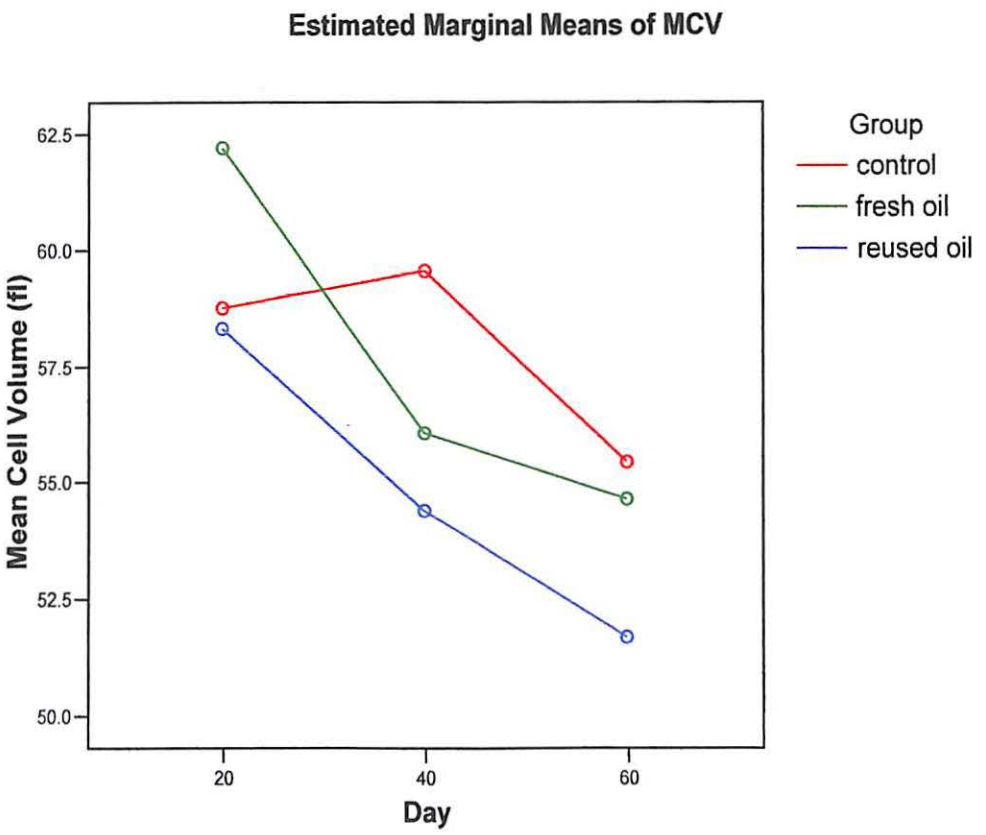

Figure 10: Comparisons of mean MCV parameter among 3 different groups based on day

Figure 10 shows the comparisons of mean cell volume (MCV) parameter among 3 different groups (control group, fresh oil group and reused oil group). The parameter was determined on day 20, day 40 and day 60.

Scale	Group	Mean (SD)			EMM (95% CI)			F stat	P value
		Day 20	Day 40	Day 60	Day 20	Day 40	Day 60		
MCV	Control	58.780 (1.2538)	59.560 (0.9555)	55.440 (2.5716)	58.780 (55.432, 62.128)	59.560 (57.400, 61.720)	55.440 (52.889, 57.991)	1.808	0.160
	Fresh oil	62.200 (5.5421)	56.060 (2.3017)	54.640 (3.4918)	62.200 (58.852, 65.548)	56.060 (53.900, 58.220)	54.640 (52.089, 57.191)		
	Reused oil	58.340 (1.7686)	54.380 (2.9218)	51.700 (1.3266)	58.340 (54.992, 61.688)	54.380 (52.220, 56.540)	51.700 (49.149, 54.251)		

Table 3: Comparison of mean MCV and estimated marginal means (EMM) among 3 different groups

P>0.05 are not significant
The percentage computed using spss
The results were significant at 5% level

3.4 Mean cell haemoglobin (MCH)

Data represented in table 4 also showed that treatment with reused cooking oil was found to have no significant difference ($P > 0.05$) of MCH parameters from day 20 to day 60 also between 3 groups. The MCH is the haemoglobin content of the average red cell. Figure 11 illustrated the changes in MCH following treatment saline for control group, fresh cooking oil for fresh oil group and repeatedly used cooking for reused oil group. Hemoglobin parameters were decreased between day 0 and day 27 for groups treated with repeatedly used cooking compare to control group.

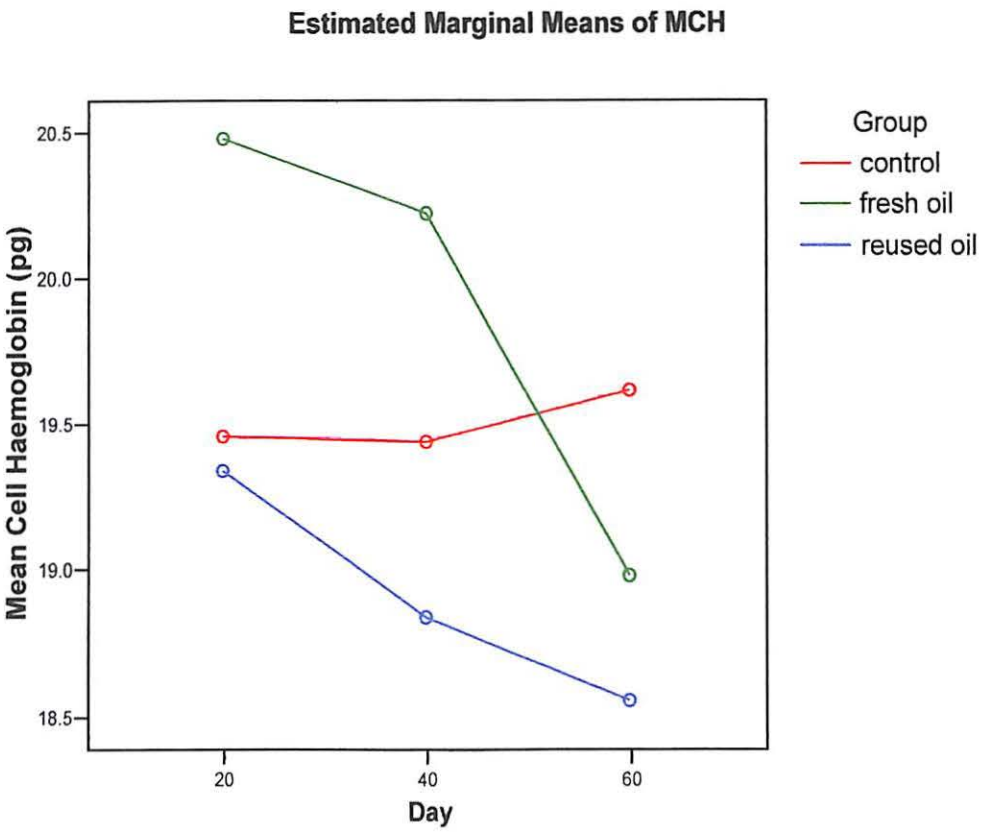

Figure 11: Comparisons of mean MCH parameter among 3 different groups based on day

Figure 11 shows the comparisons of mean Mean cell haemoglobin (MCH) parameter among 3 different groups (control group, fresh oil group and reused oil group). The parameter was determined on day 20, day 40 and day 60.

Scale	Group	Mean (SD)			EMM (95% CI)			F stat	P value
		Day 20	Day 40	Day 60	Day 20	Day 40	Day 60		
MCH	Control	19.460 (0.0894)	19.440 (0.0548)	19.620 (0.2490)	19.460 (18.237, 20.683)	19.440 (18.094, 20.786)	19.620 (19.071, 20.169)	1.398	0.281
	Fresh oil	20.480 (2.1394)	20.220 (2.3199)	18.980 (0.6181)	20.480 (19.257, 21.703)	20.220 (18.874, 21.566)	18.980 (18.431, 19.529)		
	Reused oil	19.340 (0.3715)	18.840 (0.5857)	18.560 (0.7127)	19.340 (18.117, 20.563)	18.840 (17.494, 20.186)	18.560 (18.011, 19.109)		

Table 4: Comparison of mean MCH and estimated marginal means (EMM) among 3 different groups

P>0.05 are not significant
The percentage computed using spss
The results were significant at 5% level

3.5 Concentration of Malondialdehyde

Plasma lipid peroxidation was determined by measuring the amount generated of thiobarbituric acid–reactive substances (TBARS). Results showed that lipid peroxidation in plasma rats treated with reused cooking oil were increase in TBARS formation in plasma (figure 12). The differences of malondialdehyde concentration between the 3 groups were observed clearly. The levels of malondialdehyde concentration in fresh oil and control group were nevertheless significantly lower and remain same along the treatment.

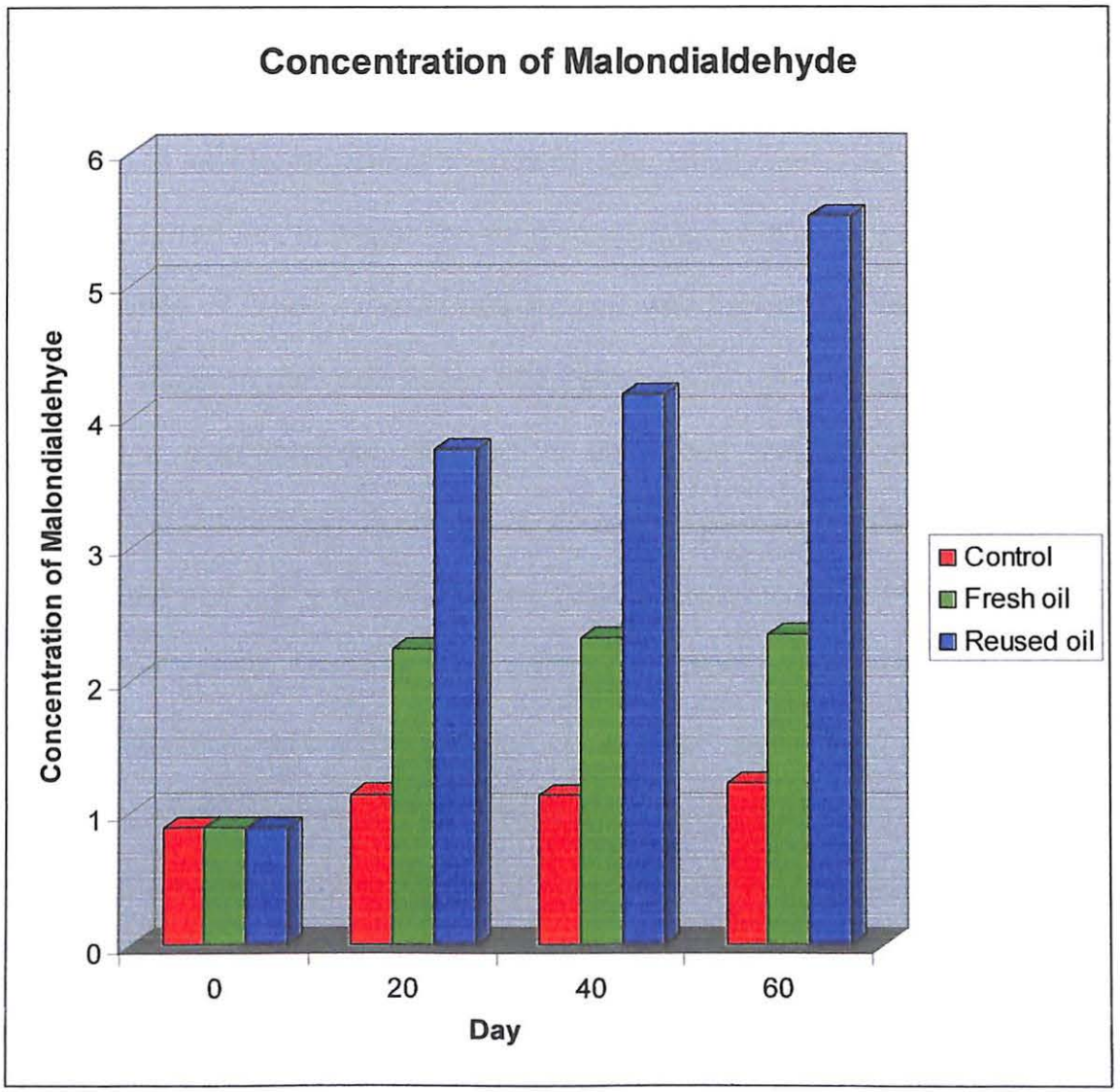

Figure 12: Comparison of malondialdehyde concentration in plasma samples of control rats with rats treated fresh oil and rats treated with repeatedly used cooking oil.

3.6 Blood morphology

Blood smear is a thin film of blood is examined under a microscope. This was used to look for abnormal shapes of cells which cannot be detected by the automated machine. In this study, we also want to investigate the morphological abnormalities of blood cells in rats treated with repeatedly used cooking oil compare to rats treated with saline and fresh cooking oil. Figure 16 showed that there were morphological changes in the blood film of rats treated with repeatedly used cooking oil on day 60. There were some teardrops cell (orange color arrow) and many acanthocytosis (yellow color arrow) in blood film. Many cells show marked crenation and contraction. The term acanthocytosis was used to determine an abnormality of red cell associated with abnormal phospholipid metabolism. Characteristically, the majority of the red cell was coarsely crenated, the size and number of the projections varying. Morphologically, rather similar irregularly crenated cells are to be seen in patients with anaemia (John, 1995).

It is almost too mundane to think of red cell morphology in only terms such as polychromasia, poikilocytosis, anisocytosis and occasional spherocytosis. There are many red cell shapes and sizes that are diagnostically useful. A few include codocytes and stomatocytes which also may be observed in immune-mediated hemolysis, burr cells in advanced glomerulonephritis, acanthocytes in some forms of cholestatic disease, schistocytes associated with disseminated intravascular coagulation, Heinz bodies when hemoglobin is oxidized, and blister cells observed in hypersplenic diseases. There are many more helpful red cell changes.

In health, the red blood cells vary relatively in size and shape. Photomicrograph of blood film as shown in figure 14 was blood smears of contol group rats on day 60. The blood film shows that the blood cell morphology is normal. The diameter of a typical erythrocyte disk is 6–8 μm and they are thus much smaller than most other human cells. The color of erythrocytes is due to the heme group of hemoglobin. Besides, blood film of blood smear rats treated with fresh cooking oil also show a normal morphology of red blood cells as shown in figure 15. This result showed that fresh cooking oil doesn't give any changes in red blood cell morphology.

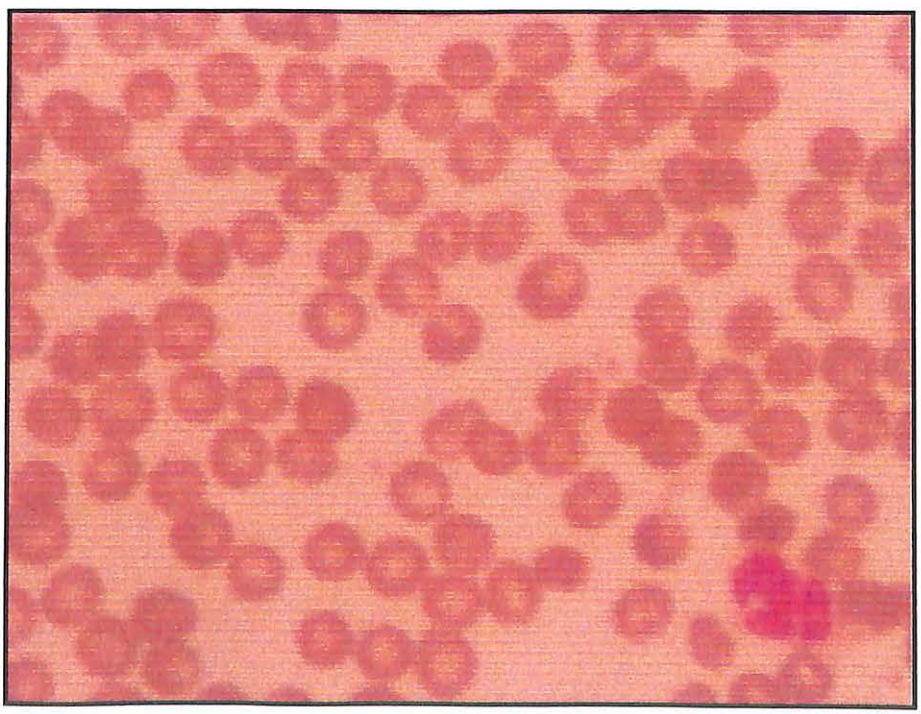

Figure 13: Photomicrograph of blood film on day 60 (control)

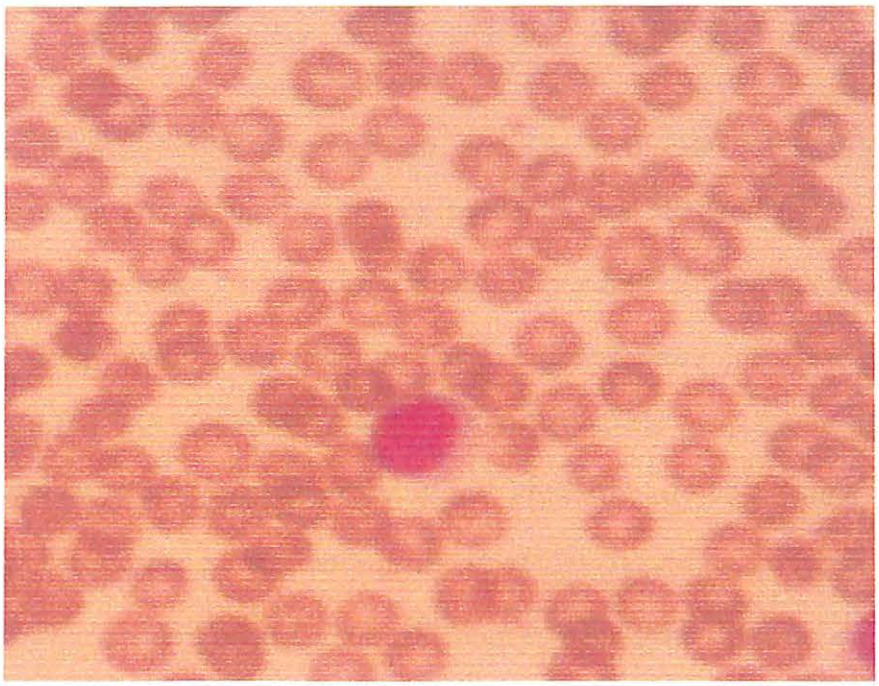

Figure 14: Photomicrograph of blood film on day 60 (fresh oil)

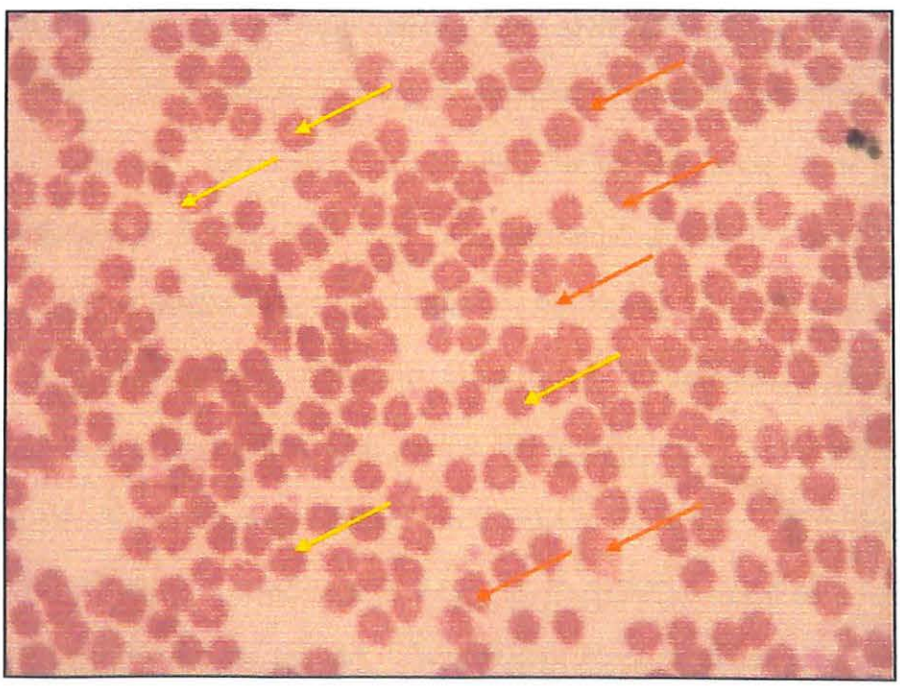

Figure 15: Photomicrograph of blood film on day 60 (reused oil)

4.0 DISCUSSION

During the process of frying, the oils and fats are heated to high temperatures and at the same time that they are exposed to the air, which results in a complex series of reactions that generate a wide spectrum of new components, both volatile and nonvolatile, that may have important physiologic effects. From the nutritional viewpoint, the nonvolatile products of degradation are more important, because they remain in the oil, are absorbed by the food, and are later consumed. Dobarganes *et al* (2000) have reported that among these nonvolatile products are the polymers and the polar compounds (PCs).

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, which is the most abundant malondialdehyde (MDA). This study was designed to determine whether there was a negative health effect relevant to reused cooking oil. We used an *in vivo* study in animal models following feeding of oil samples. Eighteen male Sprague–Dawley male rats were used in the experiment and the rats were then divided into 3 groups and different treatments were given. The sample size of experimental animal must obtained six or more for each group so that the statistical test would be applied. It was quite difficult in handling the animal so better handling technique can help this study work smoothly.

Jonathan Campbell (2005) has reported that reused cooking oil contains free radical and also possible carcinogenic compounds. The biological effects of fats submitted to various conditions of thermo-oxidation were studied in animals, and the results were contradictory (Marquez-Ruiz G *et el.,* 1990). Alice (2002) also reported that besides ruining what would have been a perfectly good meal, reused cooking oils also contain free radicals that are potentially carcinogenic

In this study, statistical analysis was determined by RM ANOVA with repeated measure. Hematology analyzer (abott cell dyn 4000) was used for measurement blood parameters. Our study has showed that only RBC parameter give a significant difference ($p<0.005$) between 3 groups based on day. Following treatment with reused cooking oil that is between group analyses there was a significant difference in measurement of RBC parameters as compared to the control group and fresh oil group (table 1). RBC parameter is slightly decreased in rat treated with reused cooking oil. The decrease in hematological this parameters indicative anaemia which means that have less red blood cells than normal and caused changes in the blood indices of rats. It was known that red blood cells have the capacity to change their deformability in response to various kinds of mechanical stress.

Our results have showed that reused cooking oil can give effect to RBC parameter. Free radicals (FR) may injure erythrocyte membranes (Niki *et al,* 1988). Similarly, free radicals damage to the erythrocyte membrane followed by hemolysis and anemia can be observed in hemodialyzed patients (Durak *et al,*

1994). Others parameters (HGB, MCV and MCH) were found to have no significant difference ($p>0.005$).

Malondialdehyde is the end-product of lipid peroxidation by reactive oxygen species. MDA level which appear in blood and urine can be estimated. This estimation is used as a biomarker of free radical damage and lipid peroxidation (Marks *et al*, 1996). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation. Our study also has showed that the levels of malondialdehyde concentration in plasma rats treated with reused cooking oil were increase by day (figure 13).

Increased of this product of lipid peroxidation has been associated with a variety of chronic diseases in both humans and model systems. MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts, including cross-linked products. MDA also forms adducts with DNA bases that are mutagenic and possibly carcinogenic. DNA-protein cross-links are another result of the reaction between DNA and MDA. Factors that may contribute to increase of malodialdehyde concentration in the reused cooking oil in could be air pollution, dirty utensils, and length of period that the oil has been reused and infrequent replacement of reused oil with fresh one. Air pollutants from the environment and motor vehicles that present in the air might contaminate the oil that is used for frying and may lead to the increased amount of malondialdehyde production.

Anane *et al* (2001) have reported that lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics. Also, oxidative damage to biomolecules, such as lipids, DNA, and proteins, has been implicated in many chronic diseases, in particular, cardiovascular disease, cancer, and cataract (Carr, 1999). Prakash (1995) found that lipid peroxidation induced by aluminium at sub-lethal levels, alter physiological and biochemical characteristics of biological systems. Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals `steal' electrons from the lipids in cell membranes, resulting in cell damage.

From this study, we also can investigate the morphological abnormalities of blood cells in rats treated with repeatedly used cooking oil. There were some teardrops cell (orange color arrow) and many acanthocytosis (yellow color arrow) in blood film (figure 16). Roberta Cazzola (2004) reported that The increased generation of reactive oxygen species that occurs in the condition of obesity may be responsible for oxidative injury to erythrocyte membranes, which could lead to a decrease in tissue oxygenation. Blood film of blood smear rats treated with fresh cooking oil also show a normal morphology of red blood cells as shown in figure 15. The normal red cell is normochromic, normocytic and has an area of central pallor. This result showed that fresh cooking oil doesn't give any changes in red blood cell morphology. Examination of a peripheral blood film is a simple haematological investigation, which can provide a significant amount of information.

The peripheral blood film shows the morphology of blood cells. There are many red cell shapes and sizes that are diagnostically useful. A few include codocytes and stomatocytes which also may be observed in immune-mediated hemolysis, burr cells in advanced glomerulonephritis, acanthocytes in some forms of cholestatic disease, schistocytes associated with disseminated intravascular coagulation, Heinz bodies when hemoglobin is oxidized, and blister cells observed in hypersplenic diseases. There are many more helpful red cell changes.

The red blood cell, shaped like a doughnut without a hole in the center, carries oxygen from the lungs to the rest of the body. The cell is remarkably flexible and can squeeze through the tiniest blood vessels. Three tests used that were the red blood cell count, hemoglobin, and mean cell volume look specifically at the red blood cells. The red blood cell count is a measure of the number of red blood cells in each cubic millimeter of blood, about 5 million. Hemoglobin is an iron-rich protein found inside red blood cells, which gives blood its red color. Oxygen travels through the bloodstream bound to hemoglobin. The amount of hemoglobin in the blood is an indicator of the amount of oxygen the blood can carry throughout the body.

5.0 CONCLUSION

The number of times cooking oils are reused can give negative health effect and the greatest hazard is allowing the fat to become rancid and deteriorated to the point it produces undesirable flavors and odors. Besides ruining what would have been a perfectly good meal, rancid oils also contain free radicals that are potentially carcinogenic. Frying foods at very high temperature while exposed to the open environment is also unhealthy. Chemical in environment will easily absorb in the oil. Thus consuming food cooked with this oil is unhealthy for it contains free radical and also carcinogenic compound.

From this study, there were several important findings that we have obtained. All these have been concluded in the form of results and the discussion. Our study has shown that the only RBC parameters have significant difference while others parameters has no significant difference. The blood morphology from each group has been estimated and the result shown that there were some morphological abnormalities in blood rats treated with reused cooking oil. Blood is an amazing and vitally important part of the body, because it contains many finely-tuned chemical systems that allow it to maintain the chemical environment needed for the body's metabolism. One of the most important functions of blood is delivering O_2 to all parts of the body by the hemoglobin protein. O_2 is carried in the hemoglobin protein by the heme group.

The MDA concentration was also has been measured. Malondialdehyde is the end-product of lipid peroxidation by reactive oxygen species and appear in blood. This estimation is used as a biomarker of free radical damage and lipid peroxidation. Our results have shown that there were increased levels of MDA in plasma rats treated with reused cooking oil.

Overall, reused cooking oil has potential to give negative health effect to human populations. For further study, the chronic study can be used to see the effect of reused cooking oil on various organs and long term effect of reused cooking oil in vivo.

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