Toxicity Effects of Caffeine in ICR Mice Prior to Anti-malarial Study

Ahmad Zakwan Noriman[1], Wan Nurhayati Wan Hanafi[1,2], Rusliza Basir[3], Hani Kartini Agustar[4], Mohd Ridzuan Mohd Abd Razak[5] and Wan Rozianoor Mohd Hassan[1,2*]

[1]School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
[2]Genetic and Biochemistry, Research Nexus of UiTM (ReNeU), Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
[3]Pharmacology Unit, Department of Human Anatomy, Faculty of Medicine and Health Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[4]School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[5]Bioassay Unit, Herbal Medicine Research Center, Institute for Medical Research, National Institute of Health, 40170 Shah Alam, Selangor, Malaysia.
rozianoor@uitm.edu.my

Abstract. Caffeine is a stimulant that increase alertness in the body. However, little is known on the toxicity effects of caffeine in ICR mice. This study aimed to identify the toxicity effects of caffeine in ICR mice for further anti-malarial investigation. Adult ICR mice were injected with caffeine (5, 10, 20 mg/kg) for four days via intraperitoneal injection (i.p.) and were monitored for 30 days. All doses exhibited no changes in body temperature, locomotor activity and urine colour when compared to control mice. Besides that, there were no signs of piloerection and lethargic whereas the mice body weight kept increasing in 30 days of monitoring. Therefore, the treatment of caffeine with 5, 10 and 20 mg/kg b.w. in ICR mice exhibited no toxicity signs and can be used for further anti-malarial study.

Keywords: Anti-malarial study, caffeine, ICR mice, toxicity effects.

1 Introduction

Caffeine or the scientific name 1,3,7-trimethylxanthine is a plant alkaloid with a molecular weight of 194.19 [1]. Caffeine can be found in daily food intake such as tea and coffee [2]. This compound has a bitter taste which function as a stimulant to the central nervous system that increase the body alertness and energy level [3]. Caffeine has also the effects such as decrease reaction time and as psychostimulant [4]. The caffeine can be extracted from *Theobroma cacao* and *Paullina cupana* [5, 6]. Caffeine is consumed by human in the average of 1.5 cups per day which is equivalent of 150 mg. After consumption, the absorption is within 45 minutes and the peak concentration

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in the blood level is within 15 to 2 hours [1]. The half-life of caffeine in a healthy individual is 5 hours but it can be influenced by other conditions such as physiological and environmental changes [1]. Caffeine can be broken down depending on various factor in the liver. The factor includes pregnancy and oral contraceptive which delay the breakdown up to 15 hours while smoking cigarette can speed up the breakdown of caffeine [7]. When taken regularly, a person can develop caffeine tolerance which reduces its stimulant function. Stopping the consumption of caffeine in an instant will cause withdrawal symptoms such as irritability and followed with headache, agitation, fatigue and depression of mood. Reduction of caffeine consumption gradually will help to reduce the side effects [7].

Research has been done scientifically on caffeine to identify their beneficiary effects such as protection against oxidative stress in Alzheimer's disease and Parkinson disease. A report by reference [8] stated that caffeine based chalcones and pyrazolines show high inhibition percentage against *P. falciparum*. The study also stated that that caffeine-based, pyrazoline possess greater inhibition effect against the parasite compared to chloroquine (anti-malarial drug). The alkaloid also has shown anti-inflammatory and neuroprotective ability [9, 10]. Besides that, caffeine has been identified in having protective effects against Alzheimer's and Parkinson's disease by preventing and stabilizing the blood brain barrier so that the barrier remains intact [11]. Caffeine also has been used in medical application such as a catalyst that can be used as a drug delivery material [12]. Caffeine also can increase in efficacy of drugs when consumed together with over-the-counter medicine compared to analgesic alone based on clinical trial report.

Caffeine mechanism of action is on the adenosine receptor in the brain [13]. Due to caffeine being water and fat-soluble, it can cross the blood brain barrier which result in antagonism towards adenosine receptor [14]. Specifically, the receptor of A2A receptor which function for the brain alertness can be affected by caffeine. The receptor is not limited in the central nervous system but also present in the body such that antagonism of A1 receptor causes the cardiac muscle inotropic effect [15]. The adenosine receptor also caused release of catecholamines contributing the systemic stimulatory effect of caffeine and further stimulating chronotropy. The adenosine receptor also causes vasodilation of vascular and stimulate the release of nitric oxide from the endothelial cells [16]. This causes the muscle relaxation. The receptor inhibition stimulates respiratory drive which cause increase of ventilator response to carbon dioxide and stimulate central respiratory drive and improving diaphragm contractility [17]. Caffeine also causes the level of hormones such as dopamine, serotonin, norepinephrine and melatonin to be altered as the hormones can affect the promotion of sleeping [18].

The toxicity study of caffeine in an adult human body also has been conducted in identifying the minimum starting point of negative side effects. The threshold of caffeine is at 400 mg/day in a healthy adult, 100 mg/day in an adolescent and 2.5 mg/kg/day in a healthy child [18]. High consumption of caffeine (>500 mg) has shown to increase tension, nervous, irritability, nausea, tremor, and restlessness. The consumption of caffeine at the sub lethal dose (7-10 mg/kg per body weight) in a normal adult causes symptom such as flushing, nausea, headache and tremor [19]. The human life-threatening dose is estimated at 10 to 14 grams, but the side effect has been seen in

1.2 grams [20]. The impact on adolescence and children of caffeine toxicity is related towards stress, tiredness, anxiety and depression [18]. Caffeine intoxication clinical features have also been reported to include cardiovascular symptoms such as hypertension, hypotension, bradycardia, tachycardia, blockage of artery and vein, ventricular fibrillation, myocardial ischemia and myocardial infarction. Gastrointestinal symptom such as nausea, vomiting, abdominal pain and diarrhea are among the clinical features from caffeine intoxication. The neurological symptoms that can occur are delusion, hallucination, anxiety, agitation, seizures, headache, cerebra1 edema and coma. The musculoskeletal symptoms and pulmonary symptoms include weakness, rigidity, tremor, rhabdomyolysis, hyperventilation and respiratory failure [19].

There have been studies in identifying the lethal dose (LD) of caffeine which include minimum lethal dose, median lethal dose or the lethal dose ranges between 100-360 mg/kg in laboratory animals via oral intake. Various studies have been conducted in determination of lethal dose of caffeine in animal studies. The study conducted by reference [21] identified LD_{50} intravenous and oral gavage of caffeine was at 62 mg/kg body weight (b.w) and 340 mg/kg b.w. respectively in CD2F1/Cr1BR mice. The oral median lethal dose of caffeine was at 192 mg/kg b.w. in albino rats [22]. Another study conducted and identifies the LD_{50} of oral mice was at 185 mg/kg b.w and the level for no observed adverse effect was at 22 mg/kg b.w. These indicated that the mice require less than 22 mg/kg b.w. in order for all the mice to survive any toxicity effects of caffeine. The question remains whether or not the dose of 20 mg/kg b.w. and below, given for four consecutive days via intraperitoneal injection (i.p.) will shows any side effects in ICR mice. Thus, the current study aimed to identify the toxicity effects of caffeine at 5 mg/kg, 10 mg/kg and 20 mg/kg in ICR mice. The concentration that shows no toxicity changes will be used for further anti-malarial study of caffeine in *P. berghei*-infected ICR mice via i.p. injection.

2 Materials and Methods

A total of 28 ICR mice with the average weight of 30-40 grams were used in this study. The mice were divided into 4 groups which were saline (0.85%) and 3 different dosages of caffeine respectively (5 mg/kg, 10 mg/kg, 20 mg/kg body weight). The mice were then injected with the treatment group via intraperitoneal injection for four consecutive days. The treatment given for four days were used to mimic the treatment given to infect ICR mice in malarial study [23]. The experimental mice were then observed for 30 days for any physiological changes such as their behaviour, visual, axillary temperature, body weight and urine colour. After 30 days, the mice were then sacrificed via CO_2 inhalation. The mice were then wrapped in tissue and aluminium foil and placed into a waste bag and tied. The bag is then placed into a freezer for disposal collection by the waste management. The dosage use in this experiment were below than the *LD*50 of caffeine administered in male albino rat which is 20 mg/kg based on *in vitro* studies [8].

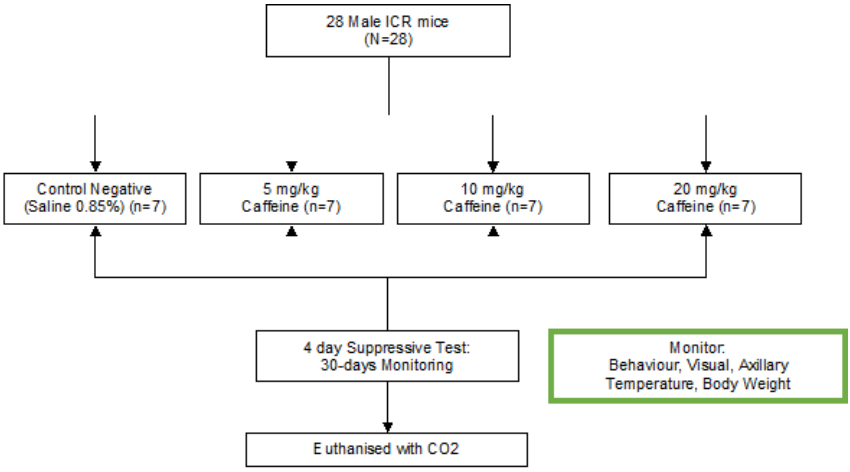

Fig. 1. Flowchart of toxicity test of caffeine

Throughout the study, the basic parameter such as body weight, body temperature, and physical changes were measured and recorded every two days. The mice body weight was measured using a weighing balance (Camry, Hong Kong). A body thermometer (medACCU, China) was used to measure the axillary temperature of mice. Throughout the experiment, all control and malaria-infected mice were visually observed for behavioural changes and signs of illness such as diarrhea, lethargy, piloerection, and decreased locomotor activity. Any indications of illness were categorized as absent (-), mild (+), moderate (++), or severe (+++) using an arbitrary scale. The categorization is based on a study conducted according to reference [23]. Throughout the experiment, the deaths of mice were recorded.

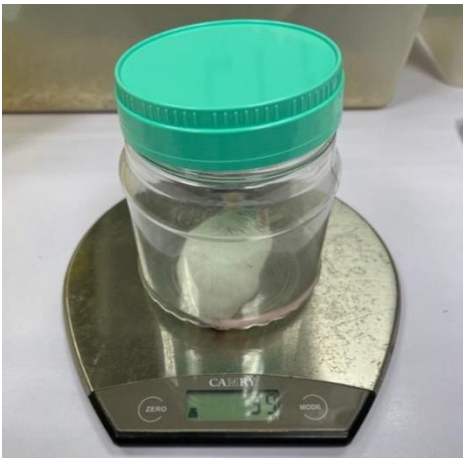

Fig. 2. Measurement of mice body weight using a weighing balance

Fig. 3. Measurement of the axillary temperature using a thermometer

3 Results

3.1 Body weight

The data collected from all four groups showed that the body weight kept increasing after treatment of caffeine for four consecutive days throughout the 30 days monitoring. When comparing the means using statistical analysis software, the results showed that the p-value was more than 0.05 which meant the difference between the body weight of each group was not significant. This indicated that the treatment given to the mice did not reduce the body weight of the mice when compared to the normal control group. Figure 4 shows the means value of body weight of the mice throughout 30 days monitoring.

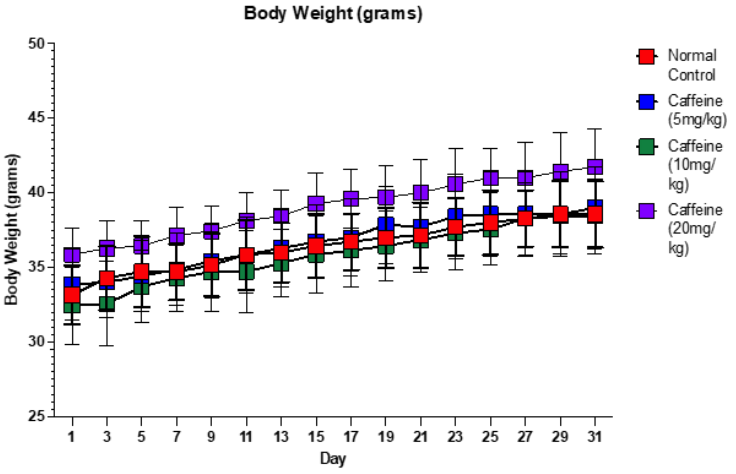

Fig. 4. Body weight of the mice throughout 30 days of monitoring

3.2 Body temperature

The data collected from all four groups show that the average body temperature maintained within the range of 37.0 °C to 37.5 °C after given treatment for four consecutive days throughout the 30 days of monitoring. The results showed that the p-value > 0.05 indicated no significant difference between the body temperature of each group of the mice. The treatment given to the mice exhibited no significant fluctuation of the mice body temperature when compared to the normal control group. Figure 5 shows the means value of body temperature of the mice throughout 30 days of monitoring.

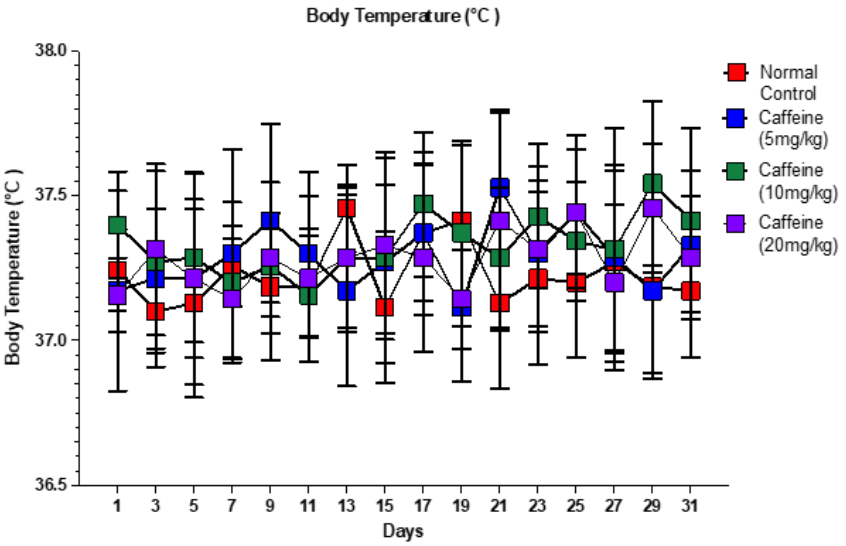

Fig. 5. Body temperature of the mice throughout 30 days of monitoring

3.3 Lethargic

The data collected from all four groups showed that the lethargic sign was absent after given treatment for four consecutive days throughout the 30 days of monitoring. This indicated that the treatment given to the mice did not cause the mice to become lethargic as compared to the normal control group. Figure 6 shows the means of lethargic activity of the mice throughout 30 days of monitoring.

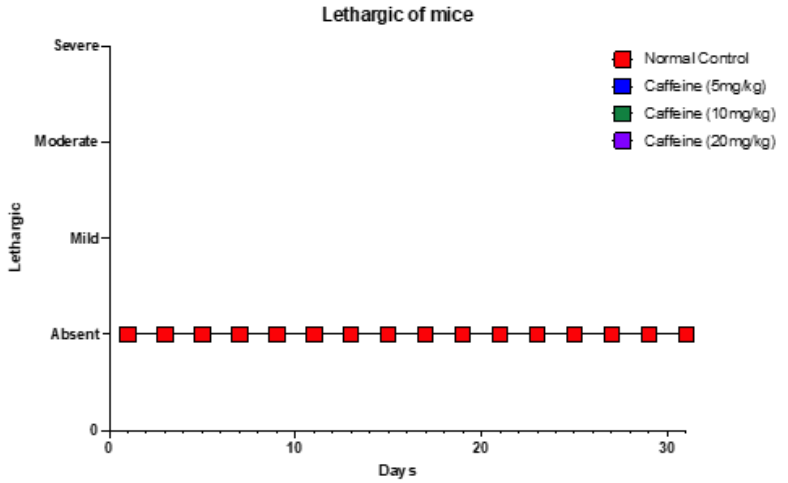

Fig. 6. Lethargic sign of the mice throughout 30 days of monitoring

3.4 Reduced locomotor activity

The data collected from all four groups showed that there was no reduction of locomotor activity after given treatment of caffeine for four consecutive days throughout the 30 days of monitoring. This indicated that the treatment given to the mice did not cause the locomotor activity to be reduced as compared to the normal control group. Figure 7 shows the group means of reduced locomotor activity of the mice throughout 30 days of monitoring.

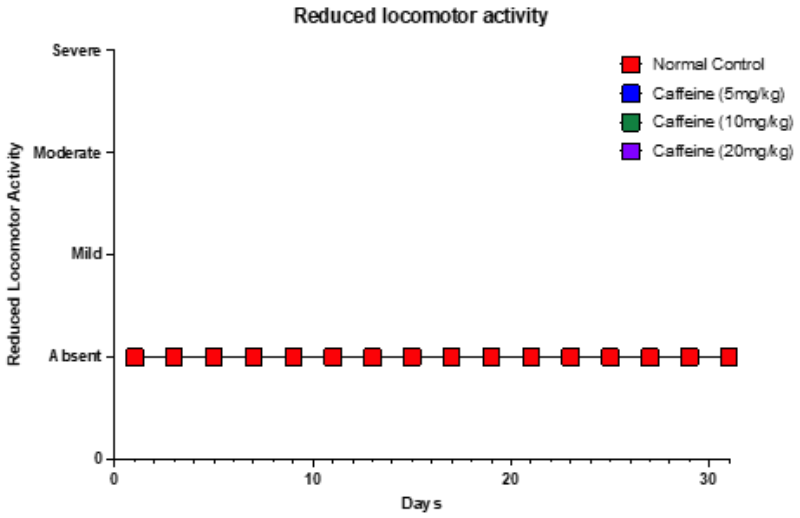

Fig. 7. Reduced locomotor activity of the mice throughout 30 days of monitoring

3.5 Piloerection

The data collected from all four groups showed that the piloerection was absent after given treatment for four consecutive days throughout the 30 days of monitoring. Figure 8 shows no presence of piloerection in ICR mice. This indicated that caffeine did not cause any piloerection in ICR mice after treated with caffeine for four consecutive days. Figure 8 shows the group means of piloerection of the mice throughout 30 days of monitoring. Figure 9 shows the physical appearance of normal, 5 mg/kg caffeine, 10 mg/kg caffeine and 20 mg/kg caffeine on Day 29 of the study.

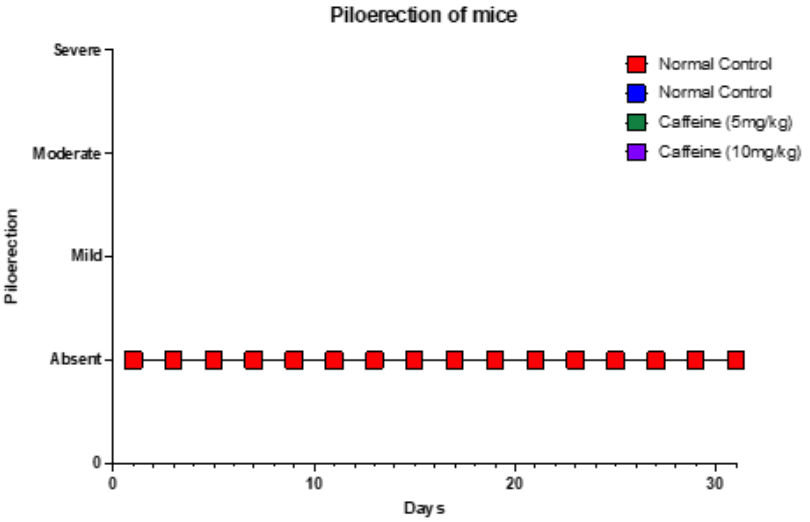

Fig. 8. Piloerection of the mice throughout the 30 days of monitoring

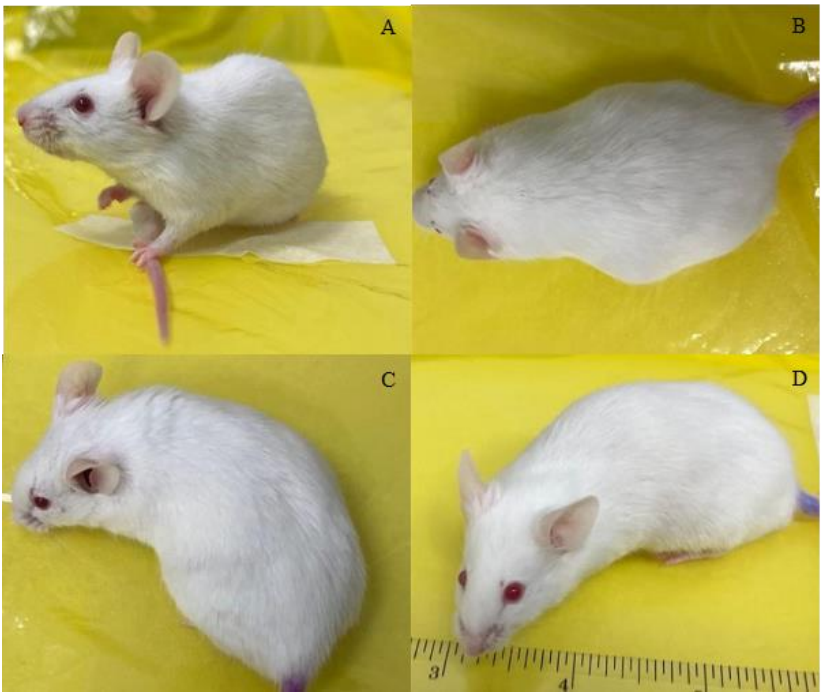

Fig. 9. Physical features of mice on Day 29. A: Normal Group, B: mg/kg b.w. Caffeine, C: 10 mg/kg b.w. Caffeine, D: 20 mg/kg b.w. Caffeine

3.6 Urine colour

The data collected from all four groups throughout the 30 monitoring days showed that the urine colour change of the mice was absent after treated with caffeine for four consecutive days. This indicated that the treatment given to the mice did not cause the urine colour to change from light yellow to black when compared to the normal control group. Figure 10 shows the group means of urine colour of the mice throughout 30 days of monitoring.

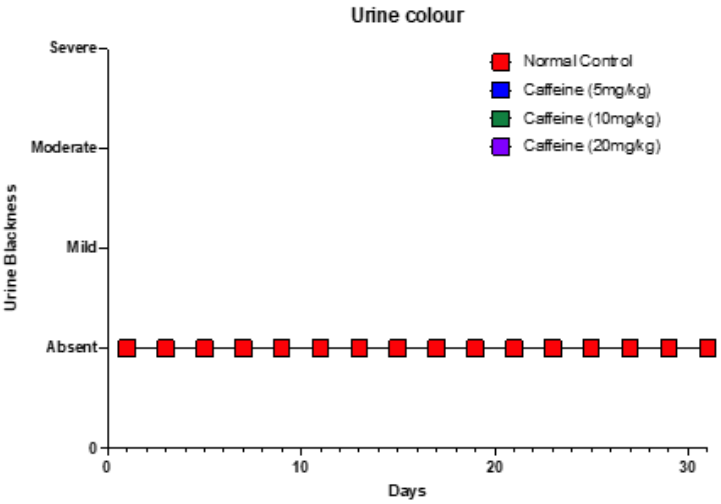

Fig. 10. Urine colour of the mice throughout 30 days of monitoring

4 Discussion

The average consumption of caffeine is approximately 4 mg/kg b.w. per day [24]. The high intake of caffeine is considered as one of the four that was associated with daily cigarette use, aggressive behavior and social problem in adolescence [25]. Despite the fact that caffeine is consumed by adolescence and children, the majority of pre-clinical study is performed in adult animals. The dosage use in this experiment is below than the LD_{50} of caffeine administered in male albino rat which is 20 mg/kg based on previous *in vitro* studies [8]. The concentration of 20 mg/kg b.w. is chosen as the high dose, 10 mg/kg b.w. as the moderate dose and 5 mg/kg b.w. as the low dose. This is because of the previous study that reported that treatment with 10 mg/kg of caffeine via i.p. injection showed neuroprotective effect which cause the blockage of adenosine receptor [11]. In addition, low concentration of caffeine can block adenosine receptor [11]. Furthermore, caffeine at 6.25-25 mg/kg showed stimulation of locomotion while concentration at 100 mg/kg showed lack of locomotion. A 5 mg/kg of caffeine in rodents is equivalent to one cup of caffeine in human, approximately 100 mg of caffeine exposure. Thus, the dosage is chosen at 5, 10 and 20 mg/kg which mimicking human consumption as one, two and four cups. Dosage at 30 mg/kg is not chosen due to the fact that there was a report on excessive systemic toxicity by reference [11].

From the results obtained, the concentration at 5,10 and 20 mg/kg of caffeine showed no changes in body temperature, locomotor activity and urine color when compared to control mice. There were no signs of piloerection and lethargic were detected and the mice body weight kept increasing in 30 days of monitoring. Therefore, the treatment of caffeine with 5, 10, 20 mg/kg b.w. in ICR mice exhibited no toxicity signs.

The result shows increased of body weight for all three doses, but the increment was not significant. This indicated that caffeine did not cause the mice body weight to

decrease compared to normal controlled mice. This is proven by the previous study conducted by reference [24], stated that caffeine below 100 mg/kg b.w. did not affect the growth rate of the albino rats within 28 days of the study which relate to the body weight that increased the same as the normal control mice. The study also stated that as the daily dose of caffeine given through the range of lethal dose (LD_{50}) and the minimal LD (LD_{100}), clinical signs were observed such as anorexia, loss of body weight, hypothermia, blepharitis, gastroenteritis, pulmonary edema and nephritis. Other signs such as dehydration and diuresis are among those that survive acute lethal dose [24]. From the observation of the physical features, the mice did not show any clinical signs for anorexia or dehydration. In addition, the food pellet and water supply were given ad libitum.

The results showed that the body temperature of the mice was within the range of normal temperature throughout the experiment (36.7-37.5 °C). Fluctuation of body temperature indicated that it is either hypothermia or fever. When comparing the result of treatment group and normal controlled mice, the caffeine treatment did not cause the average body temperature to fluctuate over the normal range. This indicated that the caffeine did not cause any toxicity effects in ICR mice. Furthermore, the increase of body temperature above the range of normal temperature indicated presence of infection while hypothermia was caused by loss of body heat [23].

The locomotor activity of the mice showed no reduction for all caffeine treatment when comparing with normal controlled mice which indicated that caffeine did not cause the reduction of locomotor activity. This is further proven by a study that showed 30 and 60 mg/kg b.w of caffeine exhibited increment in locomotor activity while 100 mg/kg b.w. showed reduced locomotor activity [25]. This supported that at the concentration of 5, 10 and 20 mg/kg b.w. of caffeine, ICR mice exhibited reduce locomotor activity. This might be because caffeine has biphasic effect where small or moderate dose of caffeine increased locomotor activity while higher dose reduced locomotor activity [25].

No presence of piloerection was shown in caffeine-treated mice throughout the experiment when compared to normal controlled mice. All mice also showed no presence of lethargic as the activity of the mice remain the same when compared to normal controlled mice. This indicated that the mice have no lethargic effect when given treatment of caffeine at 5, 10 and 20 mg/kg b.w. This is proven by a study in identifying anti-inflammatory activity of caffeine with virulent *Listeria monocytogenes* in Swiss mice stated that the toxicity study of caffeine showed no presence of piloerection or decrease in physical activity [26].

The urine color of caffeine-treated mice remains the same as light yellow when compared to the normal controlled mice. In addition, there is no presence of diarrhea that have been identified. This result is further proven by a study conducted by reference [24], where in chronic oral toxicity study of 100 mg/kg b.w. of caffeine, the albino rats did not show any diarrhea or urine color changes. The concentration of caffeine given to the rats was a higher concentration as compared to the current study. The normal color of urine varies from colorless to yellowish white to light brown. The color can change to dark brown and black due to the myoglobin [27]. Thus, the color that change from light yellow to dark brown indicates sign of toxicity.

The experiment showed that the concentrations used are suitable for anti-malarial study of caffeine in *P. berghei* ANKA-infected ICR mice. Results showed that no physical changes affecting the clinical symptoms of *P. berghei* ANKA-infected ICR mice. Among the reported physical signs of malarial infection in ICR mice were loss of body weight, reduced temperature, piloerection, reduced locomotor activity, lethargic and black urine which appeared 3 days after infection [23].

5 Conclusion

The study has shown that all three concentrations (5, 10 and 20 mg/kg b.w.) exhibited no toxicity effects as the physical signs showed no changes to the mice. The concentrations also are suitable to be used for anti-malarial study in *P. berghei* ANKA-infected ICR mice as all of the physical appearance were not affected by the caffeine.

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Novelty Statement. Caffeine has been an interesting compound in drug discovery due to their ability to treat and prevent diseases which have potent biological activity. The compound ability in anti-parasitic and anti-inflammation treatment helps in the discovering of new caffeine-based drugs. In addition, previous studies have identified caffeine toxicity in human and animals. However, no study has yet to be done on the toxicity effects of caffeine in ICR mice via intraperitoneal injection for four consecutive days prior to anti-malarial study. The work presented in this article deemed to be useful prior to murine malarial study.

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