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RAT BLOOD PROFILE EVALUATION AFTER Fe_3O_4/CHITOSAN COLLOID INJECTION

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ABSTRACT

RAT BLOOD PROFILE EVALUATION AFTER Fe_3O_4/CHITOSAN COLLOID INJECTION. The application of iron oxide (Fe_3O_4) magnetic nanoparticles in the biomedical field is still being explored, mainly related to its toxicity and side effects. This article reported results of the study aimed at analyzing the effect of chitosan-coated magnetic nanoparticles (NPM-C) on rat blood profiles. Magnetic colloid as much as 1 mL (concentration of 5 mg NPM-C/mL aquabidest) for 1 kG rat body weight was injected through intravenous to the treated rat group (4 Wistar rats aged 6 months; weight ± 275 grams; male sex) while another four rats injected with sterile aquabidest used as a control group. The blood taking from each group of rats was carried out on 1 day before injection and several days after injection (days 1, 7, 14, 21, 28) through veins in the tail. To these blood samples, a series of blood profile analyzes is carried out including basic hematology, blood chemistry, and fragility of the erythrocyte membrane. The results of the analysis showed no significant differences between blood profiles after treatment and control, which indicated that chitosan-coated magnetic nanoparticles did not trigger cellular stress responses in the blood. The stability of blood magnetism analyzed by VSM (Vibrating Sample Magnetometer) also shows that magnetic nanoparticles are detected in the blood and tend to decrease in number with increasing time, so it is thought that these nanoparticles can be degraded or have been distributed into organs. These stable properties are analyzed due to an existence of chitosan coating around magnetic nanoparticles. Based on this study it can be concluded that up to the given concentration limit, iron oxide nanoparticles coated by chitosan are not toxic and have the potential to be used as drug carriers, MRI contrast agents, and other biomedical applications.

Keywords: Iron oxide, Magnetic nanoparticles, Blood profiles, Wistar rat, VSM

ABSTRAK

EVALUASI PROFIL DARAH TIKUS SETELAH INJEKSI KOLOID Fe_3O_4/CHITOSAN. Penerapan nanopartikel magnetik oksida besi (Fe_3O_4) di bidang biomedis masih dieksplorasi, terutama terkait dengan toksisitas dan efek sampingnya. Artikel ini melaporkan hasil penelitian yang bertujuan menganalisis efek nanopartikel magnetik berlapis kitosan (NPM-C) pada profil darah tikus. Koloid magnetik sebanyak 1 mL (konsentrasi 5 mg NPM-C/mL aquabidest) untuk 1 kG berat badan tikus disuntikkan melalui vena ke kelompok tikus yang dirawat (4 tikus Wistar berusia 6 bulan; berat ± 275 gram; jantan jenis kelamin) sementara empat tikus lainnya disuntik dengan aquabidest steril yang digunakan sebagai kelompok kontrol. Pengambilan darah dari masing-masing kelompok tikus dilakukan pada 1 hari sebelum injeksi dan beberapa hari setelah injeksi (hari 1, 7, 14, 21, 28) melalui vena di ekor. Untuk sampel darah ini, serangkaian analisis profil darah dilakukan termasuk hematologi dasar, kimia darah, dan kerapuhan membran eritrosit. Hasil analisis menunjukkan tidak ada perbedaan yang signifikan antara profil darah setelah perawatan dan kontrol, yang menunjukkan bahwa nanopartikel magnetik berlapis kitosan tidak memicu respons stres seluler dalam darah. Stabilitas magnet darah yang dianalisis oleh VSM (*Vibrating Sample Magnetometer*) juga menunjukkan bahwa nanopartikel magnetik terdeteksi dalam darah dan cenderung berkurang jumlahnya seiring bertambahnya waktu, sehingga diduga

bahwa nanopartikel ini dapat terdegradasi atau telah didistribusikan ke dalam organ. Sifat-sifat stabil ini dianalisis karena adanya lapisan kitosan di sekitar partikel nano magnetik. Berdasarkan penelitian ini dapat disimpulkan bahwa hingga batas konsentrasi yang diberikan, nanopartikel besi oksida yang dilapisi oleh kitosan tidak beracun dan berpotensi untuk digunakan sebagai pembawa obat, agen kontras MRI, dan aplikasi biomedis lainnya.

Kata kunci: Iron oxide, Nanopartikel magnetik, Profil darah, Tikus Wistar, VSM

INTRODUCTION

Nanomaterial applications in the field of biomedical have been researched and developed in this last decade. Nano-sized particles were being developed to be used as drug delivery systems [1]. Iron oxide magnetic nanoparticles (Fe3O4) are one of the nanomaterials that are approved in clinical use. These particles have also been used for protein immobilization and the separation of proteins or enzymes [2]. In addition, this nanomaterial was also developed as a contrast material in the diagnostic process by magnetic resonance imaging (MRI) [3].

The use of magnetic nanoparticles in the biomedical field still raises concerns about its toxicity and other side effects on health. Various studies have been carried out to determine the level of safety and possible biological impacts [4]. Various ways have been done to make magnetic nanoparticles that are safe to use for the body. One way is to coating nanoparticles with organic polymers or biodegradable polymers. Coating with biodegradable polymers is expected to produce a magnetic nanoparticle system that is not toxic and does not cause blood clots, or blockage of blood vessels.

Biodegradable polymers are chemical compounds that can be accepted by the body and do not cause negative effects. Some commonly known biodegradable polymers are poly lactic-co-glycolic acid (PLGA), Polylactid acid (PLA) and Poly(N-isopropylacrylamide) [5]. Another biodegradable polymer that has the potential to be used as a nanoparticle coating is chitosan. Chitosan is a natural biopolymer with reactive amino groups and functional hydroxyl groups. Chitosan can be synthesized from chitin, have biocompatibility properties, good adhesion properties, non-toxic, stable, mechanically strong and has high hydrophilicity [6]. Previous research has shown that insulin loaded chitosan nanoparticles system can be a promising alternative dosage form for oral protein delivery [7]. However, chitosan-coated magnetic nanoparticles have not been widely studied in vivo to determine their effect on blood profiles.

Blood is a body fluid consisting of the liquid component in the form of plasma and blood cells in the form of white blood cells (leukocytes), red blood cells (erythrocytes), and blood platelets (platelets), each of which has a specific function [8]. Blood plays a role in the regulation of body physiology, transportation, and the body's defense against foreign objects. Changes in blood profile can be used as an indicator of the body's response to foreign objects that enter the body. Therefore, a study on blood profile before and after nanoparticle injection could be used to determine toxicity of nanoparticle to the body.

In this study, magnetic nanoparticles of iron oxide (Fe3O4) dispersed in a water medium and formed stable colloids were injected intra-vein in test animal (Wistar rat) and their effects on several blood profile parameters were analyzed from rat blood samples taken as a function of time. Magnetic analysis of blood samples was also carried out to obtain profile changes of magnetic nanoparticles in the blood. The analysis was carried out using Vibrating Sample Magnetometer (VSM).

ANIMAL ETHIC STATEMENT

This study has followed a code of ethics approved by the Ethics Committee of the Faculty of Veterinary Medicine, Bogor Agricultural University (FKH-IPB) related to the use of animals as research subjects.

EXPERIMENTAL METHOD

Materials and Equipment

The magnetic colloid used was provided from the Advanced Materials and Technology Center Laboratory (PSTBM)-BATAN with 5 mg/mL magnetic nanoparticle concentration with water as a medium and chitosan as a coating [9]. The 8 rats (Wistar rats age 6 months; ± 275 grams weight; male sex) used were sterile mice from PT. IndoAnilab PSSP which has been given standard feed (Indonesia Formula Feed). Another reagent is ketamine, xylazine, hayem solution, turkish solution, methanol, giemsa dye, Buffer Neutral Formaline (BNF), saline and NaCl solution.

The tools used were rat cages (temperature 22-25 °C; humidity 55-63%; light control 12 hours bright and 12 hours dark), syringes, perfusion equipment, camera light microscopes and other equipment commonly used for profile analysis blood. Vibrating Sample Magnetometer (VSM) Oxford 1.2H was used for characterizing blood magnetic properties.

Animal Preparation and Treatment

This study used 8 male rats with 2 different treatments. Before being treated, rats were acclimatized for one week. After acclimatization, rats were divided into two groups randomly. One group becomes a control while the other group is treated. The control group consisted of four rats given sterile aqua-bidest injection, while the treatment group was given injection of magnetic nanoparticles with normal doses consisting of four rats following the method of Kim et al. [10] with time modification.

Magnetic colloids of 1 ml / Kg of body weight were intra-venously injected in rats of the treatment group while rats injected with sterile aqua-bidest were used as controls. The blood taking from each group of rats was carried out on 1 day before injection and several days after injection (day(s) 1, 7, 14, 21, 28) through veins in the tail.

Basic Hematology Observation

Blood-based hematology analysis was carried out by a standard method [11] which aims to determine blood conditions in general. Blood profiles observed were erythrocyte count, leukocyte count, hemoglobin value and hematocrit value. Basic hematological data were analyzed by Anova technique [12].

Fragility of Erythrocyte Membrane

Fragility of erythrocyte membranes was analyzed through the Osmotic Fragility Test (OFT) [13] by modifying the concentration of NaCl solution. The level of erythrocyte membrane strength can be known through the OFT by inserting erythrocytes into hypertonic, isotonic and hypotonic solutions in various concentrations. The solution used is NaCl with levels of 0.3% - 0.9%. Various concentrations of NaCl solution were added as much as 5 ml in a test tube serially. Each tube is added with 5 drops of rat blood. The solution is homogenized and allowed to stand for three hours. Erythrocyte damage was observed by looking at the formation of separate layers.

Blood Chemistry Analysis

Blood chemistry analysis is done by using blood analysis services from PSSP. The parameters observed were Blood Ureum Nitrogen (BUN), creatinine, total protein, albumin, globulin, total bilirubin, Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP).

Blood Magnetism Analysis

The magnetic properties of the blood were analyzed using the Vibrating Sample Magnetometer (VSM), in the PSTBM-BATAN laboratory [14]. A total of 10 µL of blood is inserted into the microcapillary tubes. The test is carried out at room temperature with an external magnet of 1 T and the speed of data acquisition is 0.25 T / minute.

RESULT AND DISCUSSION

This study used two groups of rats namely control and treatment. Each group consisted of four acclimatized rats. But on the 6th day after injection, one of the control rats was dead. Death is not caused by injection, but it is influenced by rat behavior. One rat from the control group showed aggressive symptoms from the first day of acclimatization. Aggressive behavior continues to increase during acclimatization and continues with activities of self-mutilation. Henceforth, this study still uses two test groups with a comparison of three control rats and four treatment rats to observe their blood profile.

Basic hematology

Figure 1 to Figure 4 show the data of erythrocytes count, leucocytes count, hematocrit value and hemoglobin level for each time of blood sample withdrawn respectively. All the graphical picture present of almost no significance difference between control and treated samples for each time point. The stable value of erythrocytes counts indicates that magnetic nanoparticles may not interfere with the number of red blood cells, so they do not interfere with the function of erythrocytes in the body. Previous studies have reported that the use of Fe_2O_3 nanoparticles (25 mg/L) does not have a significant effect on the number of red blood cells [15]. Medina-Ramirez et al. [16] also reported the use of TiO^2-Fe^{3+} nano-powder are not toxic to red blood cells.

The absence of differences in the number of leukocytes indicates that the use of nanoparticles may

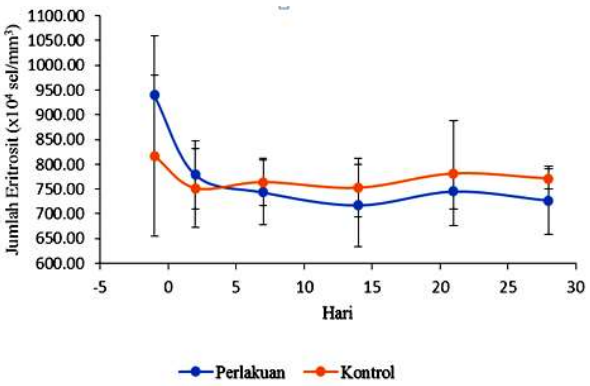

Figure 1. Changes in the number of rat erythrocytes before treatment and 4 weeks after injection of magnetic nanoparticles. The number of erythrocytes was not significantly different between treatment and control (Anova; P> 0.01).

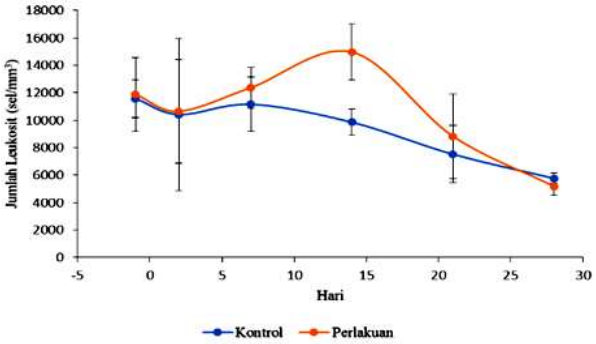

Figure 2. Changes in the number of rat leukocytes before treatment and 4 weeks after injection of magnetic nanoparticles. The number of erythrocytes was not significantly different between treatment and control (Anova; P> 0.01).

not interfere with the body's immune system. The main function of leukocytes is to recognize, digest cells or foreign proteins, kill pathogens and specific immune systems [17]. The absence of changes in leukocyte counts proves that there is no stimulation of specific immune responses due to injection of nanoparticles [18]. This result is also in line with the research of Javanovic et al. [19] who reported that the use of TiO_2 nanoparticles did not cause any changes in the number of neutrophils as one type of leucocytes.

The effect of the use of magnetic nanoparticles was also analyzed for hematocrit values which is define as the percentage of erythrocytes per 100 ml of blood. Besides being associated with the number of erythrocytes, hematocrit values are also closely related to the value of hemoglobin in the blood. Hematocrit value analysis was performed to confirm the effect of magnetic nanoparticles on the number of erythrocytes. The test results also showed that there were no significant differences between the treatment and control groups for each time point (Figure 3). The hematocrit value still shows that magnetic nanoparticles may not have a negative effect on the health of the body. Other studies have shown that changes in hematocrit values can occur due to dehydration, body activity and temperature, environmental conditions, and erythrocyte swelling [20].

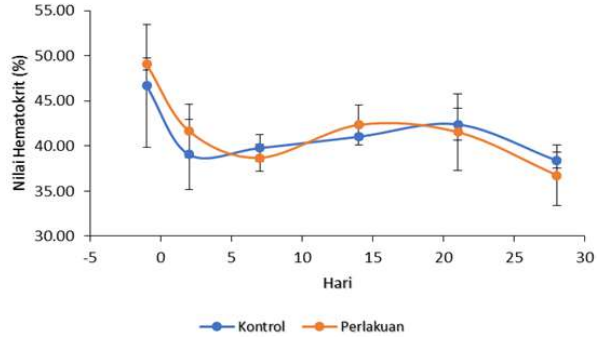

Figure 3. Changes in the hematocrit value of rats before treatment and 4 weeks after injection of magnetic nanoparticles. Hematocrit values were not significantly different between treatment and control (Anova; P> 0.01).

Based on these results, the hematocrit levels obtained are in the ranged of 40%, which level is normal in men [21] and support the data that the injected nanoparticles does not have a negative impact on the number of erythrocytes in the body and no effect on physiological processes.

Hemoglobin is a red pigment that carries oxygen in erythrocytes, which amount is closely related to the number of erythrocytes and blood hematocrit values (Swenson 1993). Hemoglobin levels in the body can be influenced by microminerals, vitamins, oxygen levels, gender and age (Patria et al. 2013). Hemoglobin levels in both treatment and control were relatively stable at around 15 g / dl (Figure 4), so the possibility of treatment did not affect hemoglobin levels. Normal hemoglobin in men ranges from 13-18 g/dl [21].

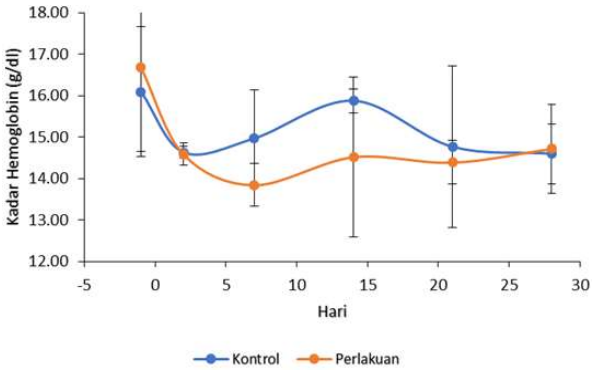

Figure 4. Changes in hemoglobin levels of rats before treatment and 4 weeks after injection of magnetic nanoparticles. Hemoglobin level was not significantly different between treatment and control (Anova; P> 0.01).

All this absence of significance difference in baseline hematology (erythrocyte count, leukocyte count, hematocrit value, and hemoglobin level) indicated that injection of chitosan-coated magnetic nanoparticles did not cause cellular stress. While the use of other nanoparticles such as Fe_2O_3 through veins in mice for 28 days gives rise to an inflammatory response that induces oxidative stress adversely affects cellular function [22]. Chen et al. [23] have also examined that administration of Fe_3O_4 magnetic nanoparticles without coating material via intravenously for 72 hours causes differences in immune responses (CD4 + and CD8 + T lymphocytes) which depend on the dose-dependent response. It is proved then, that the existence of chitosan coating around Fe_3O_4 nanoparticle will give more biocompatible surface, causing good recognition and interaction of the particles to the blood cells and environment.

Fragility of Erythrocyte Membrane

Erythrocyte membrane fragility describes the limits of the strength of erythrocyte membranes to resist pressure until hemolysis occurs. Fragility testing using

the OFT method is done by inserting red blood cells in a solution that is isotonic, hypertonic to hypotonic in various concentrations. The usual concentration range is 3-9%. If hemolysis occurs in a slightly hypotonic solution, there is an increase in erythrocyte fragility (decreased erythrocyte resistance), whereas if hemolysis occurs in very hypotonic solutions it shows a decrease in osmotic fragility (increased erythrocyte resistance).

The results of erythrocyte fragility testing at Table 1, showed that the initial hemolysis of erythrocytes in groups of rats given magnetic nanoparticle injection and control after four weeks ranged in the range of 5-6% NaCl concentration. The total hemolysis point of the control group was in the range of 4% NaCl concentration, while the value for treated rats was in the range of 4-5% NaCl concentration. This result show that injection of magnetic colloid did not give increasing pressure which will cause any preliminary hemolysis of erythrocyte membranes. Previous studies have reported that poly ethylene glycol (PEG) nanoparticles are also not harmful to the integrity of erythrocyte membranes [24]. Hou et al. [25] also succeeded in synthesizing polyethersulfone immobilized heparin nanoparticles (PES) and their use only caused less than 5% of the lysis of erythrocytes in the 9% NaCl concentration. Thus, these magnetic nanoparticles prepare in this research have the potential to be applied in the field of pharmacology.

Table 1. Fragility of rat erythrocyte membranes at 4 weeks after injection of magnetic nanoparticles (Anova; P> 0.01)

Groups	Initial Hemolysis (%)	Total Hemolysis (%)
Control	5-6	4
Treated	5-6	4-5

Blood Chemistry

Blood chemistry was tested on liver and kidney to identify effect of colloid injection to their function. Generally, kidney conditions are evaluated by looking at creatinine levels, Blood Ureum Nitrogen (BUN) and total protein in the blood. Liver conditions were evaluated from the conditions of albumin, globulin, total bilirubin, Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP) in the blood. The kidneys will secrete metabolic waste in the form of urea (from amino acid metabolism), creatinine (from muscle keratin), uric acid (from nucleic acid), the end product of hemoglobin (bilirubin), hormone metabolites, and toxins (drugs and pesticides) [26].

Creatinine and urea are indicators of renal excretion function and increases when there is impaired renal function [27]. The results of measurements in this study at Table 2 showed that there were no significant differences between treatment and control sample. The absence of significant changes in the value of BUN, creatinine and total protein is likely due to doses that can still be tolerated by the body. Ali Ahmad et al. [28] reported the use of Fe_3O_4 nanoparticles not coated with chitosan at concentrations of 10-100 ppm / day did not change BUN and creatinine levels in male Wistar rats, whereas at higher concentrations (1000 ppm) it could increase BUN and creatinine levels. This study proves once again that the use of chitosan as a coating on Fe_3O_4 surface will lower the toxicity and give higher concentrations of nanoparticles possibly used for in-vivo application hence increasing the strength of magnetic signal needed for drug delivery and MRI contrast agent application.

More detailed results showed that magnetic nanoparticles did not have a significant effect on liver function seen from albumin, globulin, bilirubin, ALT and ALP levels after four weeks of injection (Table 2). This reinforces the notion that injection of magnetic nanoparticles does not interfere with liver function which is indicated by the absence of changes in blood chemistry. Thus, these nanoparticles are not nephrotoxic. Kumari et al. [29] investigated that the application of Fe_2O_3 without coating on Wistar rats of females could increase ASP, ALP, and lactate dehydrogenase in serum and liver which indicated tissue necrosis in the liver due to an adaptive mechanism caused by stress by nanoparticles. This study proves that the use of magnetic nanoparticles (Fe_3O_4) coated with chitosan does not cause a response to such stress.

Table 2. The blood chemistry of mice at 4 weeks after injection of magnetic nanoparticles (Anova; P> 0.01).

Indicator	Unit	Control	Treated
		level ± SD	level ± SD
BUN	mg/dl	19.48 ± 4.76	24.50 ± 1.28
Creatinine	mg/dl	0.80 ± 0.13	0.77 ± 0.02
Protein Total	g/dl	5.13 ± 0.62	5.17 ± 1.00
Albumin	g/dl	2.68 ± 0.15	2.70 ± 0.10
Globulin	g/dl	2.45 ± 0.61	2.47 ± 1.03
A/G Ratio		1.13 ± 0.30	1.20 ± 0.50
Bilirubin Total	mg/dl	0.21 ± 0.18	0.06 ± 0.05
ALT/SGPT*	U/I	60.23 ± 13.35	68.43 ± 26.87
ALP**	U/I	391.50 ± 193.88	427.90 ± 197.25

*ALT/SGPT: Alanine Aminotransferase/ Serum Glutamic Piruvic Transaminase
**ALP: Alkaline Phosphatase

Blood Magnetism Analysis

The existence of magnetic particles can be detected by analyzing its magnetic properties. Magnetic properties of a material is formed from the arrangement / regularity of the magnetic moments of the constituent atoms in response to the signal of the external magnetic field to the particle in a particular direction. A material is said to have diamagnetic properties, if it gives a very weak response and forms the arrangement of moments in the opposite direction to the direction of the external magnetic field. Paramagnetic properties possessed by materials that provide a weak magnetic moment response but are in line with the external magnetic field. Strong

responses are given by ferromagnetic materials which have high magnetic moments per atom and interact to form a composition of moments in the direction of the external magnetic field with certain regularities. This material if it is very small to the nanoscale will decrease its interaction so that it has a weaker response and forms a paramagnetic response to a low magnetic field. Magnetic interactions get stronger as the external magnetic field increases and bring back the ferromagnetic response which gives overall superparamagnetic properties to magnetic nanoparticles.

The magnetism of this material can be analyzed from its magnetic hysteresis curve which is measured using magnetometer equipment. Magnetic nanoparticle of Fe_3O_4, especially in bulk particle size, has ferromagnetic magnetic properties with saturated magnetization values reaching 92 emu / gram. In this study, we used ~ 20 nm Fe_3O_4 particles coated with chitosan, which has superparamagetic properties with saturated magnetization, Ms = 74 emu / gram and 68 emu / gram for samples before and after chitosan coating respectively [9]. Chitosan coated nanoparticles were dispersed in water medium and formed stable magnetic colloid with magnetic nanoparticle concentration of 5 mg/ml.

Magnetic interactions between nanoparticles in the form of colloid will be weaker because they are blocked by water around the nanoparticles so that the magnetism decreases even though superparamagnetic properties are still reflected. Upon injecting this colloid into a blood vessel will cause further dilution by blood fluids so that the magnetic nanoparticle concentration decreases and the nanoparticles are more far apart from each other. Magnetic interactions between particles are increasingly blocked and reduce the strength of their interactions. Figure 5 shows the hysteresis curve of the blood sample for both the control (a), and those injected with magnetic colloid (b) after 8th day of injection. The hysteresis curve shows paramagnetic properties in both samples, which magnetic values (magnetic moments) is higher for treated samples, showing the existence of magnetic nanoparticles within the blood sample.

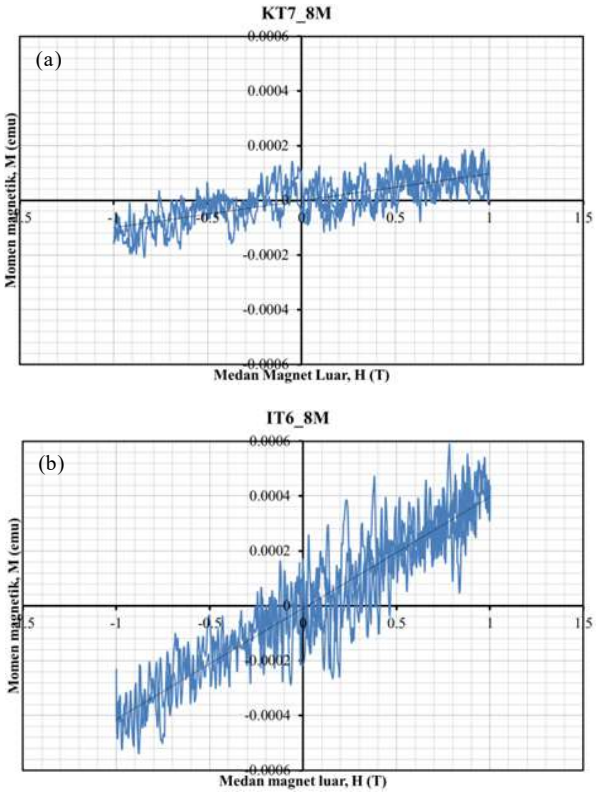

Figure 5. Magnetic hysteresis curve of blood control samples ((a), rats 7) and those injected with magnetic nanoparticles ((b), rats 6) on day 8 after injection.

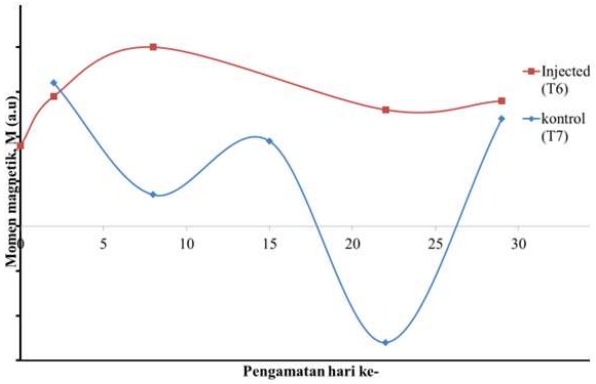

Figure 6. Maximum magnetic values of blood of control rat (a) and magnetic nanoparticles (b) treated rat after 29 days of observation. Treated rats tend to have higher and more stable magnetic values compared to lower and fluctuating values of controls samples.

Paramagnetic properties in control samples can be explained from Fe ions contained in blood. Injection of magnetic nanoparticles, adds the amount of magnetic moments in the blood, thereby increasing the total moment value as indicated by the increase in the blood paramagnetic curve. Figure 6 shows a graph of the maximum magnetic value of blood in treated and control rats after 29 days of treatment. The magnetic values in injected rat blood increased until the 7th day and then dropped. The decrease is probably caused by nanoparticles that have been degraded by the body or have been distributed to organs. On the other hand, control rats tend to have lower maximum magnetism values and fluctuate without a pattern. This graph has a tendency similar to the pattern of changes in leukocytes and hemoglobin shown in Figure 2 and Figure 4.

CONCLUSION

The injection of chitosan-coated magnetic nanoparticles to a concentration value of 1 ml / Kg body weight relatively did not give a significant change to the blood profile of all baseline hematology parameter, i.e the number of erythrocytes, the number of leukocytes, the percentage of hematocrit, and hemoglobin levels. The presence of magnetic nanoparticles in the blood also tends to be stable which is indicated by the maximum

magnetic value of the blood. In addition, magnetic nanoparticles up to these doses did not affect the condition of erythrocyte fragility and blood chemistry. Based on these characters, magnetic nanoparticles have no negative impact on blood profiles and have the potential to be applied as drug carriers and MRI contrast agents.

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