Original article

Safety evaluation of human peripheral blood mononuclear cells in naive rats:a chronic toxicitystudy

Basuki Supartono[1], Siti Farida[2], Sony Suhandono[3], Ahmad Aulia Yusuf[4],

Abstract

Human peripheral blood mononuclear cells (PBMC) are widely used within tissue engineering therapy, thus, the evaluation of their safety is mandatory. The present study aims to assess the safety of human PBMC transplantations in naive rats in terms of chronic toxicity. Rats received intravenous injections of human PBMC with doses of 10^5, 10^6, or 10^7 cells (n = 5/group/sex) every month for three months without administration of any immunosuppressant drugs. We evaluated clinical, physical, laboratory, and pathology parameters. No morbidity, mortality, or significant differences occurred within all of the tested parameters between control and experimental groups until the finalization of the study. In conclusion, the current work indicates that human PBMC transplantation in naive rats did not induce chronic toxicity reaction.

Keywords: Human peripheral blood mononuclear cells, Chronic toxicity, Naive rat, Tissue engineering, Stemcell transplantation

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1. Introduction

Recently tissue engineering techniques are widely employed within therapies for several diseases. The primary component of this technique involves mononuclear cells (MC); MC transplantation is increasingly being used in tissue engineering therapy [1]. MC includes rounded nucleated cells, which consists of various cell types, including monocytes and stem cells [2,3]the main players are cells of the epithelial lining and the immune system. Human peripheral blood mononuclear cells (PBMCs. MC transplantations have been reported in several clinical studies including those of liver cirrhosis [4], ischemic limb [5], ischemic stroke [6], bedsores [7]this study examines the use of autologous stem cells from bone marrow to promote the healing of pressure ulcers in patients with SCI. Objective: To obtain preliminary data on the use of bone marrow mononuclear cells (BM-MNCs, burger disease [1], patellar tendinopathy [8], hematologic disease, oncologic disease, immunodeficient disorders, acute refractory autoimmune rheumatologic diseases [9], erectile dysfunction [10], acute respiratory distress syndrome [11], osteoarthritis [12], diabetic ulcer [13]. MC sources include both solid and liquid tissues, such as bone marrow (BM) and peripheral blood (PB). Compared with BM aspiration, PB isolation is easy and more accessible [2]. PBMC could potentially revolutionize regenerative medicine [14], however, use of human PBMC has raised concerns regarding safety due to growing demand in clinical use. Thus, assessment of toxicity is essential. In this study, naive rats were deliberately used, and not genetically engineered animals as stem cell researchers have done in the past [15], due to several reasons. Firstly, the aforementioned engineered animals were not available in our country, secondly, these animals are

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costly and difficult to maintain, and thirdly, the aim herein was to test the highest safety of human PBMC. Previously we have performed acute and sub chronic toxicity tests of human PBMC in naive Sprague Dawley (SD) rats without immunosuppressant drugs [16], however there is no data available on chronic toxicity. Therefore, our current study aims to evaluate the safety of human PBMC transplantations in naive rats during chronic toxicity.

2. Materials and methods

2.1. Ethical and regulations statement

The study was performed according to National Regulation of Ethical Research, and the principles and procedures of the Organization for Economic Cooperation and Development (OECD) Guidance No. 453, Section 4: Health Effects with Appropriate Modifications [17,18]. All procedures were approved by the Ethical Committee of Health Research of UPN Veteran Jakarta, Indonesia, (No: B/2159/VIII/2019/KEPK).

2.2. Donors, isolation, and sample preparation

Donors were healthy young adult males. The procedures were explained to the participants, and they provided informed consent. The health status of each donor was analyzed. There was no history of hepatitis, BM disorders, or other severe diseases within any of the participants. Donor blood samples underwent laboratory examination including that of HBsAg, anti-HCV, anti-HIV, prostate serum antigen, carcinoembryonic antigen, and hematology parameters. PB was collected, diluted with NaCl, filtered with pancoll solution, and centrifuged. We isolated the buffy-coat layer, washed the cells, collected the MC, and checked for cell viability. We prepared four suspensions, i.e., a control suspension and three intervention suspensions containing three different amounts of cells: 10^5, 10^6, and 10^7 cells. All of the suspensions were diluted with 500 uL NaCl solution and loaded into a syringe [19].

2.3. Animals

We used SD naive rats. Forty rats (20 male and 20 female) at 11 weeks of age were bought from the Faculty of Husbandry of IPB University. All rats were healthy and had never previously undergone any experimental procedures. the weight of the male rats varied between 157–165 g with an average weight of 162 $\pm$ 9 g, while the weight of the female rats varied between 139–142

g with an average of 141 $\pm$ 6 g. Each rat obtained an identification number. Animals were acclimated for one week and maintained following the principles of animal welfare. Rats were randomly assigned to the groups. After randomization, the mean body weight of the groups did not exhibit significant differences. We housed rats of the same sex in small groups within cleaned cages in a semi-sterile room. Room conditions were as follows: 25°C ± 30°C, 50% humidity, and a 12-hour light cycle. All rats were given a diet according to standard nutritional requirements and sterile water for drinking. Male and female rats were grouped into control and experimental groups (n = 5/sex/group). All animals received serial intravenous injections via tail vein every month for three months. The control group received a NaCl solution. Three experimental groups received human PBMC suspensions consisting of either 10^5 cells, 10^6 cells, or 10^7 cells. We evaluated several parameters, including those of clinical, physical, laboratory, and histopathological. We conducted these experiments in compliance with the OECD TG 453 protocol for chronic toxicity studies [17]. We anesthetized rats via intraperitoneal injection of ketamine and xylazine solution (60 and 6 mg/kg) [20]. The body and internal organs of all the animals were examined upon sacrifice. The livers and kidneys were sent to the laboratory for histopathology examination. Heart puncture was performed to harvest blood, which was subsequently sent for laboratory examinations.

2.4. Transplantations of human PBMC

Shortly before transplantation, we examined the PBMCs under an inverted microscope. The number and viability of MC were counted prior to intravenous injection.

2.5. Drugs

Ketamine HCl (KTM, 100 mg/10 ml, Guardian Pharmatama), heparin sodium (5000 IU/ml, Fahrenheit), and xylazine 2 % (Xyla, Holland). Pancoll (Biotech GmbH) for MC isolation.

2.6. Observation of chronic toxicity

2.6.1. Clinical signs

Observations were performed once a day for three days following cell transplantations, and then twice a week until the end of the study. The animals are strictly monitored for morbidity, mortality, and other clinical signs [21].

2.6.2. Physical observations

Rats were weighed at the beginning of the study and again every week until the conclusion of the study. We also recorded the rats' food consumption every

week [21].

2.6.3. Hematology and biochemical examinations

We transferred a fraction of the blood samples into EDTA tubes, and the remaining samples were centrifuged at 3000 rpm for 15 minutes to collect the serum. EDTA tubes were inserted into an analyzer (Mindray BC-30s) for examining hematology parameters, including: hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell

(RBC) count, white blood cell (WBC) count and platelet (Plt) count. Non-heparinized tubes were inserted into the photometer machine (Rayto 194c Semi-auto Chemistry Analyzer) for measurement of biochemical parameters, namely: alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), and creatinine (Cr).

The examination was performed by comparing rat enzyme levels (DiaSys, Indonesia) with control (HumaTrol N, Germany). Analysis of hematology and blood chemistry was conducted according to the reference and values of control and experimental rats for both sexes [19].

2.6.4. Macroscopic examinatio

Macroscopic examinations of the liver and were performed, including such parameters as relative weight and color.

2.6.5. Histopathological analysis

Slides were prepared using kidney and liver tissue sections and histopathological analysis was conducted by performing hematoxylin and eosin staining. We fixed tissues with 70% alcohol and 4% paraformaldehyde solution for 20 hours at 4°C. Specimens were encased in paraffin in 5 µm slices and dissolved in xylol. Humidity was restored by submersion into alcohol with a graded concentration. Samples were washed with Aqua Dest laboratory water and stained with H&E. Dehydration and clearing were performed at the end of this stage, followed by mounting. We examined slides using a light microscope and microphotography tools [19].

2.7. Statistical analysis

Statistical analyses were performed to determine significant differences using one-way ANOVA. A p-value < 0.05 was considered significant, with a 95% confidence limit. Measurement values are denoted as mean ± SD.

3. Results

3.1. Blood collection and PBMC isolation

The blood test results indicated that all human donors were healthy and free from hepatitis B, hepatitis E, HIV, as well as prostate or digestive tract malignancies. The volume of blood collected varied between 100–120 (113 + 11.5) ml. The numbers of MC per sample varied between 310–339 × 10^6 cells (326.3 + 14.7 × 10^6 cells). PBMC viability was 99% for all samples.

Table 1 Donor laboratory parameters and PBMC viability

Characteristics	Donor 1	Donor 2	Donor 3
Age (year)	25	26	25
S ex	Male	Male	Male
HIV	Neg	Neg	Neg
Hbs Ag	Neg	Neg	Neg
Hb e Ag	Neg	Neg	Neg
PSA (ng/mL)	2.15	1.87	2.15
CEA (ng/mL)	1.00	0.94	1.00
HGB(g/dL)	16,1	16,2	16,1
HCT (%)	46,2	49,0	46,2
RBC (10^6/ul)	5.12	5.28	5.12
WBC (10^3/ul)	9.3	9.2	9.3
Platelet(10^3/ul)	355	275	355
Blood (ml)	120	120	100
PBMC()	339.4	310.3	329.3
Viability (%)	99	99	99
Month	1	2	3

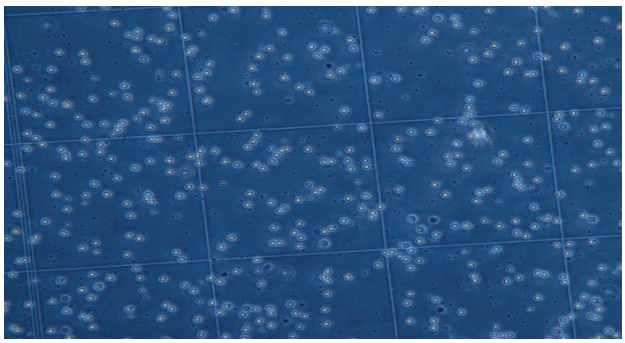

Fig. 1. Microscopic image of human PBMC, viability: 99% (Inverted microscope, 100 ^{x} magnification))

3.2. Transplantations of human PBMC

Human PBMC were successfully injected via tail

vein once a month for three months without any injection complications in all naive SD rats.

3.3. Clinical signs

Throughout the duration of the study there were no observations of morbidity, mortality, toxicity symptoms, and any other abnormalities within the experimental animals. There were also no notable changes in daily behavior. There were no signs of tremor, diarrhea, hyperactivity, convulsion, change of sound, or change in skin color.

3.4. Body weight

Transplantations of human PBMC did not interrupt weight gain within rats of either sex. Throughout the study, the body weight of the rats showed a gradual and predictable increase (Fig. 2).

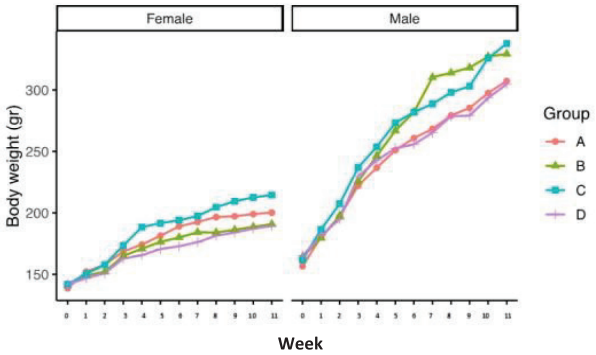

Fig. 2. Rat body weight gain after administration of repeated intravenous injection of human PBMCs once a month for three months. A) Control group, B) 10^5 cells, C) 10^6 cells, D) 10^7 cells.

3.5. Food and water consumption

Human PBMC transplantations did not affect the consumption of food and water of the control and treated rats of either sex (Fig. 3.).

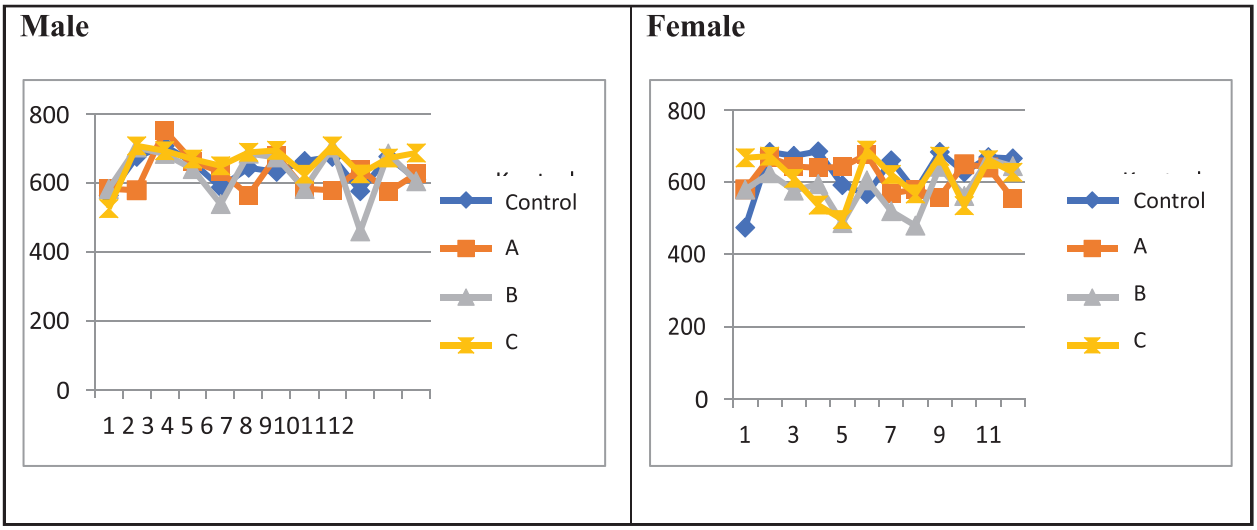

Fig 3. Effect on food consumption of rats administered with repeated intravenous injection of human PBMC once a month for three months. A) Control group, B) 10^5 cells, C) 10^6 cells, D) 10^7 cells.

3.6. Necropsy

All of the rats were normal; there were no evidence of pathological conditions.

3.7. Hematological parameters

Human PBMC transplantations up to doses of 10^7 cells had a nonsignificant effect on hematologicalpar ameters(Table2).

3.8. Biochemical parameters

Human PBMC transplantations up to doses of 10^7 cells did not affect the biochemical parameters of naive rats. We did not detect any significant differences within liver and kidney enzymes between the control and experimental groups (Table 3).

Table 2. Effect on hematological parameters of naive SD rats administered with repeated intravenous injection of human PBMC once a month for three months.

Parameters	Groups(n = 5)							
	Control M	10^5 M	10^6 M	10^7 M	Control F	10^5 F	10^6 F	10^7 F
Hemoglobin(g/dL)	14.1 ± 0.6	14.2 ± 1.4	14.4 ± 0.6	14.7 ± 0.9	13.1 ± 0.5	14.1 ± 1.1	13.5 ± 0.4	14.1 ± 0.5
Hematocrit(%)	37.3 ± 1.2	37.6 ± 3.2	37.5 ± 1.7	38.3 ± 2.3	34.2 ± 1.4	35.8 ± 3.3	34.5 ± 1.1	36.1 ± 1.4
MCV (µm3)	56.02 + 2.5	55.86 + 2.6	55.66 + 2.2	56.68 + 3.1	59.66 + 4.2	56.58 + 1.6	59.8 + 3.3	55.4 + 2.7
MCH (pg)	21.16 + 1.1	21.18 + 1.0	21.3 + 0.7	21.66 + 1.1	22.8 + 0.7	22.26 + 0.4	23.32 + 1.1	21.6 + 0.6
MCHC (g/dL)	37.8 + 0.7	37.92 + 0.6	38.28 + 0.5	38.16 + 0.2	38.34 + 1.8	39.36 + 1.0	39.02 + 0.8	39.06 + 1.2
RBC(x106 µL)	6.7 ± 0.4	6,7 ± 0.6	6.7 ± 0.5	6.9 ± 0.5	5.7 ± 0.4	6.3 ± 0.5	5.7 ± 0.4*	6.5 ± 0.3
WBC(x10^3µL)	8.1 ± 1.4	8.3 ± 1.1	9.8 ± 1.4	5.2 ± 0.7	6.0 ± 1,7	6.3 ± 0.5	5.2 ± 0.3	5.7 ± 3.3
Platelets(x10^3µL)	740 ± 49	659 ± 253	756 ± 71	770 ± 96	744 ± 298	885 ± 177	765 ± 30	817 ± 100

Table 3. Effect on clinical and biochemical parameters of naive SD rats administered with repeated intravenous injection of human PBMC once a month for three months.

Parameters	Groups(n = 5)							
	Control M	10^5 M	10^6 M	10^7 M	Control F	10^5 F	10^6 F	10^7 F
AST (IU/l) ALT	216 ± 86	270 ± 136	205 ± 57	z179 ± 98 76	256 ± 128	224 ± 118	181 ± 51	299 ± 88
(IU/l)	76 ± 71	75 ± 21	70 ± 9	± 12	59 ± 11	49 ± 7	53 ± 5	82 ± 22
BUN (mg/dL)	47 ± 6	43 ± 8	47 ± 5	43 ± 9	51 ± 7	45 ± 7	46 ± 5	47 ± 8
Cr(mg/dL)	0.61 + 0.05	0.66 + 0.06	0.64 + 0.06	0.64 + 0.10	0.61 + 0.10	0.63 + 0.05	0.61 + 0.10	0.66 + 0.06

3.9. Macroscopic examination of liver and kidney

Human PBMC transplantations at different doses did not affect the color and relative organ weight. We did not find any significant differences between the control group and the experimental groups within either sex (Table 4).

Table 4. Relative weight and color of naive rat liver and kidney administered with repeated intravenous injection of human PBMC once a month for three months.

Organ	Groups (n=5)							
	Control M	10^5 M	10^6 M	10^7 M	Control F	10^5 F	10^6 F	10^7 F
Liver (%)	2,95 ± 0,11	2,83 ± 0,25	2,70 ± 0,24	2,69 ± 0,35	3,08 ± 0,44	2,99 ± 0,53	2,94 ± 0,23	3,13 ± 0,19
Kidney (%)	0,37 ± 0,03	0,33 ± 0,05	0,30 ± 0,03	0,32 ± 0,03	0,37 ± 0,04	0,34 ± 0,03	0,34 ± 0,01	0,38 ± 0,05
Liver (OD)	0,7 ± 0,1	0,7 ± 0,1	0,6 ± 0,0	0,7 ± 0,0	0,8 ± 0,3	0,6 ± 0,1	0,7 ± 0,1	0,7 ± 0,1
Kidney(OD)	1,2 ± 0,1	1,2 ± 0,1	1,1 ± 0,1	1,1 ± 0,1	1,0 ± 0,1	1,3 ± 0,1	1,2 ± 0,1	1,2 ± 0,1

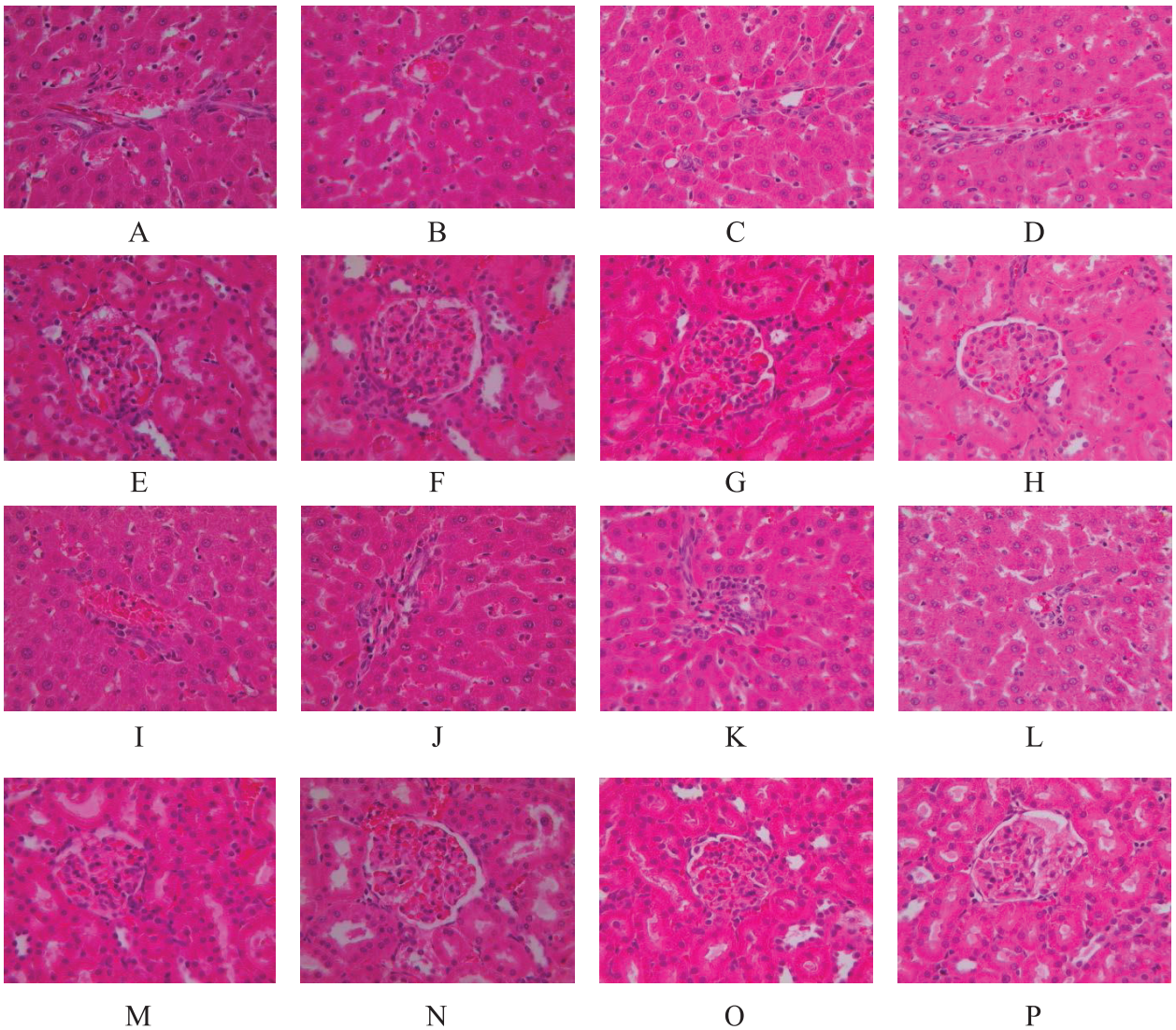

Fig. 4. Microscopic images of liver and kidney sections stained with hematoxylin and eosin (200× magnification) of naive rats after repeated intravenous injection of human PBMC once a month for three months. Microscopic images of liver and kidney were normal in terms of structure and architecture. There were no significant differences in the microscopic images of the liver and kidneys between the control and treatment groups for both sexes. A–D) male rat livers:

3.10. Microscopic of liver and kidney

Transplantations of human PBMC did not appear to impact the microscopic structure of either the liver or kidney organs within rats (Fig. 4-5). Histopathological results for liver and kidney showed no differences between the control and treated rats. There was no liver cell damage and inflammatory process in either the control group or the treated groups. Nevertheless, there was appearing of increasing the number of oval cells in all of the treated groups (Fig. 6).

A) control, B) 10^5 cells, C) 10^6 cells, D) 10^7 cells. E–H) male rat kidneys: E) control, F) 10^5 cells, G) 10^6 cells, H) 10^7 cells. I–L) female rat livers: I) control, J) 10^5 cells, K) 10^6 cells, L) 10^7 cells. M–P) female rat kidneys: M) control, N) 10^5 cells, O) 10^6 cells, P) 10^7 cells.

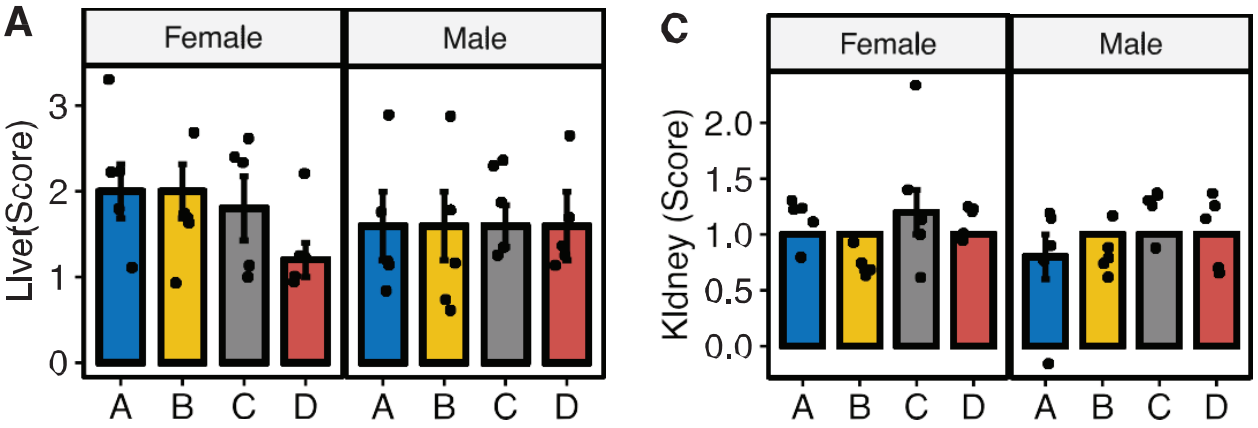

Fig. 5. Microscopic scoring of liver and kidney within naive rats after repeated intravenous injection of HPBMNCs once a month for three months. A) control, B) 10^5 cells, C) 10^6 cells, D) 10^7 cells. Scores; 0: normal, 1: mild degeneration, 2: mild degeneration with inflammation, 3: moderate degeneration with inflammation.

4. Discussion

PBMC can be used both as a diagnostic tool as well as disease therapy. PBMC have been used in several diagnostic studies, including genetic study and others. PBMC can be used to test the capabilities of the cell to proliferate. For example, Ikhsan et al. used PBMC SLE patients to test the mesenchymal cell's capabilities to increase the number of T - reg cells [22]. PBMC is also used by Achter et al. in a genetic study to describe the susceptibility of certain alleles among Bangladeshi Hepatitis infected patients [23]. Whereas, Tungjai used PBMC as a normal cell model to determine intracellular organelles characteristic such as lysosomes in response to low-dose medical diagnostic X-rays [24]. Although PBMC has been widely used as a diagnostic tool, it is predicted that these cells may provide additional benefits to cure diseases, especially in tissue engineering therapy. Human PBMC plays an important role in tissue engineering therapy. However, the use of human PBMC has raised concerns regarding their safety due to the growing demand for clinical use. It is necessary to determine the safe dose and the maximum number of injections that can be administered for effective therapy, as well as to assess toxicity [25]. We have performed both acute and subchronic study of human PBMC in naive rats without immunosuppressant drugs. In our previous study, we reported that administration of a relatively high number of cells was safe (10^7 cells/200 g). There were no negative findings observed in any of the animals following a single intraarticular injection of human PBMC into the knee. We conducted the study using an intraarticular route; however, there was no data available on the intravenous route in terms of chronic toxicity [16]. Thus, in the present study, we conducted a chronic toxicity study of human PBMC with repeated intravenous injection of differing cell doses.

We found that human PBMC transplantations were tolerated well in naive rats, and thereby demonstrated that human PBMC administration was safe for these animals. This is the first study to our knowledge to report the absence of chronic toxic reactions in naive rats were administered human PBMC without immunosuppressant drugs. We did not find any significant changes within any of the evaluation parameters. Since the wellness status of animals reflected good conditions, this indicates that human PBMC was not toxic [20,26]. The administration of certain compounds may cause some interaction in the hematological and biochemistry system of animals

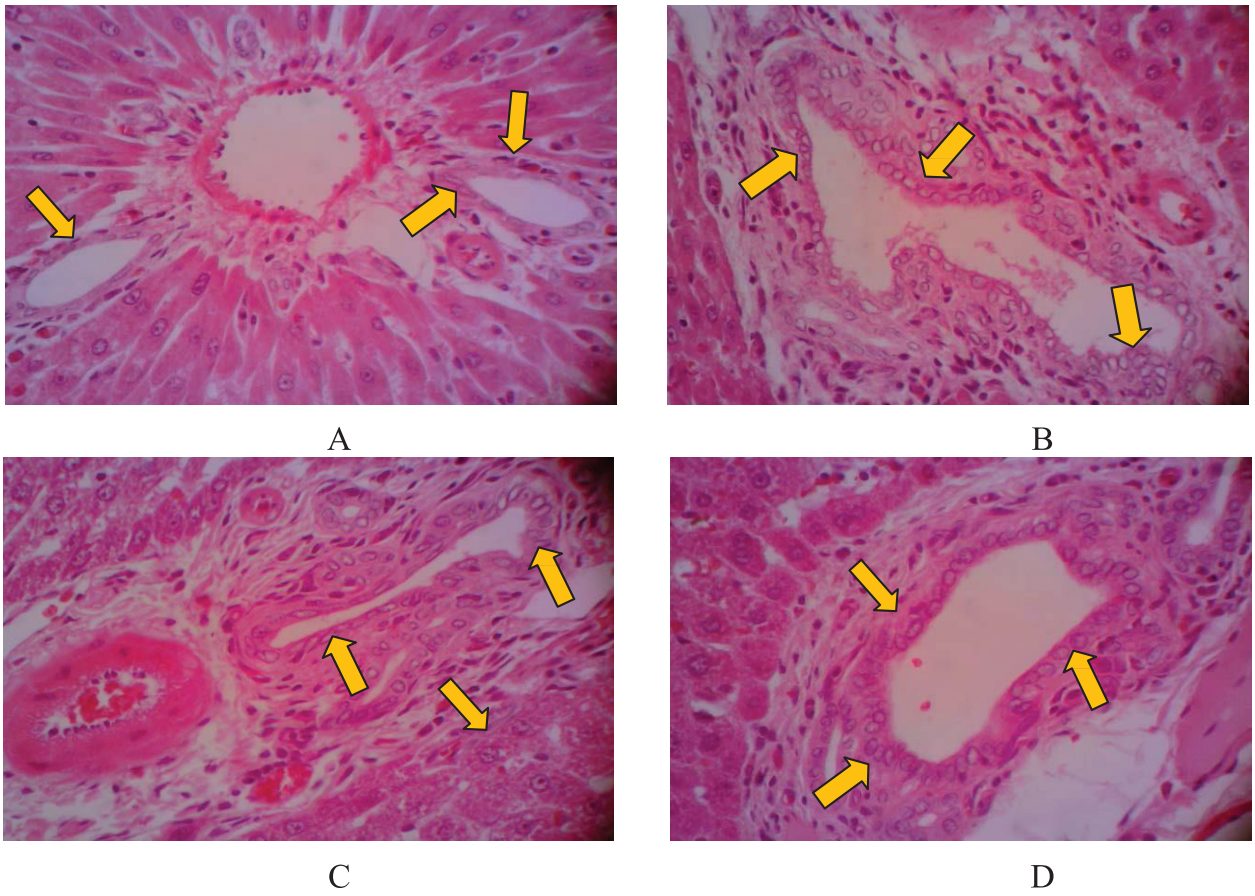

Fig. 6. Microscopic images of liver sections stained with hematoxylin and eosin (400× magnification) of naive rats after repeated intravenous injection of human PBMC once a month for three months. The number of cells in the bile duct has increased in all of the treated groups compared to the control group, as shown by yellow arrows. The shape of the cells in the treated groups looked more oval than the control group. A) control, B) 10^5 cells, C) 10^6 cells, D) 10^7 cells.

[25], and hematological parameters are a sensitive indicator of animal health status [20,27].

In three months of study, we did not observe significant changes in most of the hematological parameters examined. We found that in male rats, there was a significant difference in WBC between the experimental and control groups; however, this remained in normal limits. We did not find a decrease in hemoglobin value as a response to toxic compounds [27]. The result showed that repeated injections of human PBMC up to 10^7 cells did not result in toxicity for the hematological system of naive rats.

Biochemical parameters are very important to assess for toxicity reactions to certain drugs. Transaminases enzymes can be used to determine the effect of drugs on liver cells and have been used for predicting liver toxicity [28]. Human PBMC transplantations produced slight changes in liver enzyme levels in all rats; however, these increases were not statistically significant between the control and experimental groups for either sex. Insignificant changes in the levels of liver enzymes after transplantation are a clear indication that repeated intravenous injections of human PBMC without immunosuppressant drugs up to the amount of 10^7 cells did not cause liver damage. Blood urea and creatinine are important biomarkers of renal toxicity [29]. The results showed that human PBMC transplantations did not significantly affect blood urea and creatinine values in all naive rat groups. The absence of significant changes in liver enzymes and renal indicators indicated that the transplantation of human PBMNC neither changed the metabolism nor the function of the liver and kidney tissues. Thus, this supports that repeated intravenous injections of human PBMC without immunosuppressant drugs were safe for the health of these organs.

Weight of the liver and kidney are indicating signs of toxicity [26]. We did not find any significant differences between all groups in terms of relative weight or color of organs. We therefore concluded that the human PBMC transplantations did not affect the

macroscopic parameters of these organs; histopathology results further confirmed these results. There were no significant microscopic changes observed between the groups. Inflammation was noted in some slides, however, this could be due to certain intervening factors, such as difficulty in intravenous injection or handling the animals. In general, histopathology analysis showed that the cellular structure and tissue architecture of the organs were normal. Nevertheless, there was an increase in the oval cell in all treated rat's liver than the control group. The proliferation and differentiation of these cells were most likely facilitated by growth factors and other proliferation and differentiation-inducing factors released by human PBMC. The increasing of these specific cell progenitors showed the effect of human PBMC transplantations [30]specific liver progenitors, are activated in response to injury. The human umbilical cord blood (hUCB.

All of these results indicate that human PBMC transplantations did not cause chronic toxicity in naive rats. Moreover, no lethality and nor significant adverse changes in all parameters during the 90 days of treatment can be used as support to justify the safety of human PBMC usage. The current research is consistent with our previously conducted toxicity study [16].

Transplantation of human cells into animals can result in toxic reactions, such as when donor cells are recognized as foreign antigens by host T cells. This can trigger the proliferation of host cytotoxic T cells, macrophages, and neutrophils to attack the donor cells, and thereby result in tissue damage. All of the cells in this study were washed before injection, which may have contributed to the lack of any toxic reactions following transplantation. The results also indicated that increasing the numbers of cells injected did not cause a toxic reaction, and a dose of 10^7 cells was still considered safe for rats weighing ~ 200 g. This result could also be attributed to the nature of immature MC cells harboring antigen has not yet fully developed [10].

These results provide evidence for the use of naive rats in tissue engineering research and may make it easier for researchers because this eliminates the requirement to use genetically engineered animals, which can be costly and difficult to maintain. Based on the data shown here, researchers performing tissue engineering studies could safely use human PBMC with a dose of at least 10^7 cells 200 g animal weight. Repeated administration of high doses of PBMC to experimental animals was performed here in an attempt to simulate daily clinical situations, since a single dose of PBMC is often not sufficient to treat disease. Typically, repetitive dosing is necessary to treat several diseases, such as ischemic stroke [6] and ulcers [7]this study examines the use of autologous stem cells from bone marrow to promote the healing of pressure ulcers in patients with SCI. Objective: To obtain preliminary data on the use of bone marrow mononuclear cells (BM-MNCs. The intravenous (IV) route of transplantation of this study was selected due to its suspension characteristics and this being the predominant route of transplantation in humans. IV administration is easily performed, and this route is not known to be inferior to other routes, such as intraarterial (IA) [31].

We administered MC, namely those that are the source of stem cells for tissue engineering therapy. These cells have biological properties that allow them to adapt, integrate, and differentiate to form target tissues. Our future research is expected to present better recommendations regarding the safety of PBMC applications.

Of note, a limitation to our study was that we did not perform a labeling procedure to trace the PBMC, therefore we could not ultimately trace the fate of these cells. Our main research objective was initially to investigate the effect of administering human cells to naive rats. Similarly, other researchers investigating stem cells and tissue engineering did not carry out a labeling procedure. However, we would deem this procedure necessary to include in further research.

5. Conclusion

In summary, chronic transplantation of human PBMC in naive rats was safe up to 10^7 cells for 12 weeks. The results of the study support the use of non-engineered rats in stem cell research. These data could also open up the possibility of allotransplantation research of human PBMC in future clinical studies. Considering that this study only consisted of a three month duration, it would be necessary to conduct further research with longer observation periods to address other potential chronic toxicity reactions.

Nevertheless, the aforementioned prior research provides us with insight to conduct additional studies by including the long-term effect variable.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors Contribution

Data gathering and idea owner of this study: Basuki Supartono, Soni Suhandono, Ahmad A. Yusuf

Study design: Basuki Supartono

Data gathering: Basuki Supartono, Ahmad A. Yusuf

Writing and submitting manuscript: Basuki Supartono, Siti Farida, Sony Suhandono

Editing and approval of final draft: Basuki Supartono, Soni Suhandono, Ahmad A. Yusuf, Siti Farida

Source of Fund: UPN Veteran Jakarta, Indonesia

Ethical Clearance: All procedures were approved by the Ethical Committee of Health Research of UPN Veteran Jakarta, Indonesia, (No: B/2159/VIII/2019/KEPK).

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