Role of antibody-dependent enhancement in DENV-infected Wistar rats as a dengue murine model

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ABSTRACT

Preclinical studies for discovering and developing a drug for a disease involve utilizing animals as experimental subjects. The search for an effective and efficient murine model of dengue infection is ongoing to support further scientific updates. This study aimed to explore the suitability of Wistar rats as a murine model for dengue infection. Twenty-four Wistar rats (male sex, 2-3 months old, 200-300 grams weight) were randomly divided into four groups (n=6 per group): control group (no infection), SC-Group (DENV-2 s.c.), IV-Group (DENV-2 i.v.), and ADE-Group (DENV-3 i.p. twice and DENV-2 i.v. once). Inactive 0.2 mL of 10^{11} ffu/mL DENV-3 were injected on days -14 and -5. Active 0.2 mL of 5 x 10^{8} ffu/mL DENV-2 were injected on day 0. Rectal temperature was measured on day 0 until 6. NS1 antigen tests were carried out from the viral medium on days -14, -5, and 0 and from the blood serum samples on day 4. Hematological parameters (leukocytes, hemoglobin, hematocrits, and platelets) were analyzed on days 0, 4, and 6. Biochemical parameters (albumin, ALT, and AST) were analyzed on day 6. SC-Group showed significant increases in the temperature from day 0 to day 1 (p=0.028). IV-Group showed significant increases in the temperature from day 0 to day 1 (p=0.007), day 2 (p=0.002), and day 3 (p=0.006). There were significant temperature increases on day 1 (p=0.047), day 2 (p=0.009), and day 3 (p=0.001) compared to the normal group. ADE-Group had a mortality rate of 33.3%, lusterless and ruffled hair coat, and several hemorrhagic manifestations. ADE-Group also showed significant increases in the temperature from day 0 to day 2 (p=0.043) and day 3 (p=0.038). There were significant temperature increases on day 1 (p=0.048), day 2 (p=0.002), day 3 (p=0.000), and day 4 (p=0.004) compared to the normal group. Leukocytes in the ADE-Group showed significant decreases from day 0 to day 6 (p=0.021). ALT (p=0.033) and AST (p=0.011) of the ADE-Group also showed significant increases compared to the normal group. Dengue infection through an induction method adapted from the antibody-dependent enhancement phenomenon shows the most severe clinical spectrum and laboratory manifestations compared to other induction methods in Wistar rats.

Keywords: Dengue virus, dengue infection, Wistar rat, antibody-dependent enhancement, murine model

INTRODUCTION

Dengue infection is a mosquito-borne disease rapidly spreading among five dengue virus (DENV) serotypes [1]. This positive-strand ribonucleic acid virus from the family of Flaviviridae and genus Flavivirus is transmitted to humans through female Aedes aegypti and Aedes albopictus bites as their vectors [2,3]. DENV causes a wide range of clinical spectrum in humans, manifested as asymptomatic, mild fever as in dengue fever, and even life-threatening as in dengue hemorrhagic fever or dengue shock syndrome [4,5]. The DENV-2 serotype has been reported to increase the presence and severity of plasma leakage in severe dengue, in addition to the host's vascular integrity and the antibody-dependent enhancement (ADE) phenomenon. ADE is a phenomenon where antibodies will support the entry of the virus thereby increasing its binding into the host cell and its replication. This phenomenon is described in severe dengue due to secondary dengue infection.

The incidence of dengue infection has a high prevalence worldwide, especially in tropical and subtropical regions. World Health Organization (WHO) reported that from the beginning until the end of 2023, there have been around 5,000 deaths per over 5 million dengue infection cases in more than 80 countries [6].

Providing the dengue vaccine and vector controls are preventive strategies to reduce the incidence of dengue infection [7]. Current therapy only relies on supportive treatment, with a relatively high risk of mortality if it is not sufficiently provided [8]. The various clinical manifestations and complications due to severe dengue infection give rise to the urgency of searching for anti-DENV agents as definite therapy to reduce mortality rates.

One of the preclinical stages in discovering and developing a drug for a disease is using animals as experimental subjects. The murine models must be able to represent conditions that mimic, are similar to, or are precisely the same as those experienced in humans. Mice (*Mus musculus*) in different strains, including AG129, BALB/c, and C57BL/6, are explored to be effective as murine models for experimental dengue infection [9-11]. Several limitations related to the availability of

strains in some regions and the maximum blood volume produced by a mouse initiate the need to search for other more effective and efficient murine models of dengue infection.

The laboratory rat (*Rattus norvegicus*) is an animal often utilized in scientific studies due to its straightforward care requirements, widespread availability, and rapid metabolic rates. They produced quite a large maximum blood volume, making it more efficient to conduct research with various examination parameters. The most commonly used strain is Wistar, a sub-strain derivative of the Norway rat. These albino rats have a long-life expectancy and a high resistance to disease other than the interventions.

This study aimed to explore the suitability of Wistar rats as a murine model for dengue infection by examining the impact of DENV infection from several administration routes and induction methods on its clinical spectrum and laboratory findings. Several parameters are assessed, such as temperature, leukocytes, hemoglobin, hematocrits, platelets, albumin, and transaminases.

MATERIALS AND METHODS

Ethics Statement

The animal protocols followed the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines 2.0 and ethical principles outlined by the Council of International Organizations of Medical Sciences (CIOMS) [12]. This study was reviewed by the Health Research Ethics Committee (HREC), Faculty of Medicine, Universitas Katolik Widya Mandala Surabaya, Surabaya, Indonesia, under approval reference number 0005/WM12/KEPK/DSN/T/2024.

DENV-2 and DENV-3 Preparation

Vero cell medium was prepared by mixing Dulbecco's modified eagle medium (Gibco®, Thermo Fisher Scientific Inc., USA) and fetal bovine serum (Sigma-Aldrich®, Merck Group, Darmstadt, Germany). Cell passage was carried out by mixing Vero cells with its medium, then centrifugated for 5 minutes using a laboratory centrifuge (Hettich®, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Centrifugation yielded two distinct layers: the media on top and the Vero cells at the bottom. The top layer was removed, and Vero cells were added to their new medium

and mixed until homogeneous. A small amount of the mixture was stained with trypan blue stain 0.4% (Gibco®, Thermo Fisher Scientific Inc., USA) for observation and enumeration under a light microscope.

A confluent monolayer of Vero cells was infected with 0.5 ffu/cell DENV-2 and then incubated at 37°C with 5% CO2 for seven days. The supernatant was collected and centrifuged for 5 minutes. The cell culture supernatant was kept at -80°C, and then the titer of DENV-2 and DENV-3 was determined using a focus assay [13,14]. The method was done multiple times until the desired titer was achieved for the dose required in the study. DENV-3 was treated by heat inactivation of 56°C for 30 minutes.

Animal Preparation

Twenty-four male Wistar rats (2-3 months old, 200-250 grams weight) were randomly assigned to four groups (*n*=6 per group) and then divided equally into their wire-covered sterilized cage (*n*=3 per cage) with paddy husk inside as the bedding. All rats were declared healthy by the certified veterinarian and were acclimated (22±4°C room temperature, 45-55% relative humidity, 12-hour light/12-hour dark cycle) for seven days before treatment.

Animal Experimental Design and Protocol

The experimental groups consisted of the uninfected (control or normal group) and the infected group. Rats in the normal group were not treated with any chemical intervention. Rats in the infected group consisted of three treatment groups with different virus administration routes: SC-Group (0.2 mL subcutaneous injection of 5 x 10^8 ffu/mL activated DENV-2 on day 0), IV-Group (0.2 mL intravenous injection of 5 x 10^8 ffu/mL activated DENV-2 on day 0), and ADE-Group (0.2 mL intraperitoneal injection of 10^{11} ffu/mL inactivated DENV-3 on day -14 and -5, then 0.2 mL intravenous injection of 5 x 10^8 ffu/mL activated DENV-2 on day 0). All experimental animals were given ad libitum access to nutritious standard chow and distilled water.

Validation of Dengue Viability

A non-structural protein 1 (NS1) dengue antigen test was performed using a standard dengue NS1 antigen kit (StandaReagen®, Nantong Egens Biotechnology Co., Ltd., China). The test was

carried out four times on two different samples: virus with their media and animal blood serum. The validation of DENV-3 viability was carried out on days -14 and -5, right before the virus was inactivated. The validation of DENV-2 viability was carried out on day 0, right before the injection into rats. The NS1 dengue antigen test of the animal blood serum was carried out on day 4.

Assessment of Body Temperature

The body temperature of each rat in the experimental groups was rectally examined from day 0 until 6 using a digital rectal thermometer (OMRON®, OMRON Corp., Japan).

Animal Blood Collection

All rats were anesthetized with a mixture of ketamine 100 mg/kg BW and xylazine 10 mg/kg BW in a ratio of 1:1 by intramuscular injection in the caudal thigh muscle [15]. A 0.5 mL of blood samples were collected by cardiac puncture without surgery on days 0 and 4 to perform the hematological analysis. On day 6, surgery was conducted to collect all the intracardiac blood samples to perform the hematological and biochemical analysis.

Hematological Parameters Analysis

Hematological parameters were analyzed on days 0, 4, and 6. Leukocytes, hemoglobin, hematocrits, and platelets were estimated using an automatic hematology analyzer (ABX Micros 60®, HORIBA, Ltd., Japan).

Biochemical Parameters Analysis

Biochemical parameters were analyzed on day 6. Blood serum samples were obtained from the whole blood samples following centrifugation at 3000 rpm for 5 minutes. Albumin and transaminases were estimated using an automatic clinical chemistry analyzer (Pentra C200®, HORIBA, Ltd., Japan).

Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical differences between experimental groups were analyzed using one-way analysis of variance (ANOVA) and Fisher's least significant difference tests for the parametric analysis. Kruskal-Wallis and Mann-

Whitney tests were used for the non-parametric analysis. Statistical analysis was performed using GraphPad Prism software version 9 (GraphPad Software Inc., San Diego, California, USA). A *p*-value of less than 0.05 ($p<0.05$) indicates a statistically significant.

RESULTS

Mortality Rate and Clinical Spectrums

No deaths occurred in Wistar rats treated with subcutaneous (SC-Group) and intravenous (IV-Group) injection routes of DENV-2. Mortality in 33.3% (two out of six) of Wistar rats was reported in the ADE-Group, in which rats were injected with DENV in different serotypes at different time intervals. These rats were observed to have limping behavior and died on day 4. Wistar rat (n=1) representatives from each experimental group showed different clinical spectrums of their hair coats. The normal group was seen in a healthy physical condition, indicated by the neat-shiny hair coat and agile behavior (Fig 1a). There were only slight changes in the SC-Group and IV-Group (Figs 1b and 1c). Lusterless and ruffled hair coat were found on the ADE-Group (Fig 1d).

Several clinical hemorrhagic manifestations were found in ADE-Group, including hematuria and hematochezia on day 3 and ear bleeding on day 4. Chromodacryorrhea and reddish flaky skin followed by minimal hair loss were also observed on day 4, as seen in Fig 2. These results were only found in the ADE-Group.

Fig 1. Infection of DENV in Wistar rats with different administration routes and injection methods showed a different clinical spectrum of hair coat in (a) Normal Group, (b) SC-Group, (c) IV-Group, (d) ADE-Group.

Fig 2. Antibody-dependent enhancement injection method of DENV showed several clinical hemorrhagic manifestations in Wistar rats, including hematuria (red arrow), ear bleeding (green arrow), and hematochezia (yellow arrow). Other findings, including chromodacryorrhea (blue arrows) and reddish flaky skin with minimal hair loss around the nose (black arrows).

Non-Structural Protein 1 Dengue Antigen Test

Table 1. Summary of non-structural protein 1 antigen test in Wistar rat treated with DENV in subcutaneous injection (SC-Group), intravenous injection (IV-Group), and the injection method adapted from the antibody-dependent enhancement phenomenon (ADE-Group)

Group Name	Day -14[a]	Day -5[a]	Day 0[b]	Day 4[c]
SC-Group (*n*=6)	-	-	Positive	Negative
IV-Group (*n*=6)	-	-	Positive	Negative
ADE-Group (*n*=4)	Positive	Positive	Positive	Negative

SC-Group: Subcutaneous Group; IV-Group: Intravenous Group; ADE-Group: Antibody-dependent Enhancement Group
Samples were obtained from:
[a]DENV-3 with their medium
[b]DENV-2 with their medium
[c]Animal blood serum

Positive NS1 dengue antigen test results of DENV-3 in their medium were seen on days -14 and -5. The results were also positive on day 0, with DENV-2 in their medium as the samples. In the animal blood serum on day 4, the tests revealed negative results in all rats in the SC-Group, IV-Group, and ADE-Group (Table 1).

Fever Pattern of Dengue Infection

The rectal temperature of the rats from days 0 to 6 in Fig 3 were described as follows: the normal group were 35.73±0.07°C, 35.7±0.16°C, 35.78±0.17°C, 35.63±0.26°C, 35.53±0.17°C, 36±0.16°C, 35.83±0.22°C, respectively. There was no significant change from day to day (p=0.656); The SC-Group were 35.63±0.37°C, 36.52±0.35°C, 36±0.33°C, 35.88±0.16°C, 35.78±0.16°C, 35.82±0.23°C, 35.87±0.21°C, respectively. There was a significant increase from day 0 to 1 (p=0.028); The IV-Group were 35.58±0.25°C, 36.6±0.39°C, 36.8±0.14°C, 36.62±0.16°C, 36±0.22°C, 35.67±0.3°C, 35.73±0.21°C, respectively. There were significant increases on day 1 (p=0.007), day 2 (p=0.002), and day 3 (p=0.006) compared to day 0; and the ADE-Group were 35.68±0.5°C, 36.7±0.24°C, 37.23±0.36°C, 37.18±0.05°C, 36.68±0.42°C, 36±0.26°C,

35.53±0.84°C, respectively. Significant increases from day 0 were seen on day 2 (p=0.043) and day 3 (p=0.038).

If the rectal temperature in the dengue experimental group was compared with the normal group, then on day 0, there was no significant difference (p=0.986). On day 1, there was a significant increase in the IV-Group (p=0.047) and ADE-Group (p=0.048), while in the SC-Group was not (p=0.069). On day 2, the IV-Group (p=0.009) and ADE-Group (p=0.002) were found to be significantly increased, while there was no significant difference in the SC-Group (p=0.602). On day 3, there were also significant differences in the IV-Group (p=0.001) and the ADE-Group (p=0.000) when the SC-Group still did not show a significant result (p=0.343). Surprisingly, on day 4, there was a significantly higher temperature that was only found in the ADE-Group (p=0.004) when there were no significant differences in the IV-Group (p=0.146) and the SC-Group (p=0.426). There were no significant differences between the dengue experimental group and the normal group on day 5 (p=0.718) and day 6 (p=0.992).

Fig 3. Line graph showing the rectal temperature from day 0 to day 6 of Wistar rats untreated with DENV (normal group, red); treated with DENV in subcutaneous injection (SC-Group, orange), intravenous injection (IV-Group, green), and the injection method adapted from the antibody-dependent enhancement phenomenon (ADE-Group, blue). *: p<0.05 vs day 0. #: p<0.05 vs normal group.

Hematological Indices

Hematological indices of Wistar rats in all experimental groups were illustrated in Fig 4. Leukocytes (10^9/L) in the normal group were 16.9±1.59 (day 0), 16.5±1.06 (day 4), and 17.92±4.62 (day 6); The SC-Group were 11.42±0.82 (day 0), 16±1.82 (day 4), and 14.53±0.74 (day 6); The IV-Group were 17.25±2.98 (day 0), 12.77±1.09 (day 4), and 13.15±1.47 (day 6); and the ADE-Group were 23.35±1.16 (day 0), 19.7±1.68 (day 4), and 11.25±3.48 (day 6). There were no significant differences between days 0 to 4 and 6 in the normal group (p=0.676), SC-Group (p=0.051), and IV-Group (p=0.459). In the ADE-Group, compared to day 0, was found to have no significant change on day 4 (p=0.083), but a significantly lower change was seen on day 6 (p=0.021).

Hematocrits (%) in the normal group were 40.5±1.4 (day 0), 40.88±0.33 (day 4), and 38.9±1.3 (day 6); The SC-Group were 39.43±0.61 (day 0), 39.33±0.92 (day 4), and 36.88±1.11 (day 6); The IV-Group were 38.52±0.81 (day 0), 39.1±0.72 (day 4), and 37.4±0.67 (day 6); and the ADE-Group were 39.5±2.7 (day 0), 37.45±1.41 (day 4), and 32.75±4.88 (day 6). There were no significant differences between days 0 to 4 and 6 in the normal group (p=0.433), SC-Group (p=0.110), IV-Group (p=0.281), and ADE-Group (p=0.378).

Platelets (10^9/L) in the normal group were 783.5±89.67 (day 0), 873.33±83.09 (day 4), and 726.5±54.46 (day 6); The SC-Group were 995.5±106.49 (day 0), 907.5±80.39 (day 4), and 812.67±71.72 (day 6); The IV-Group were 882.17±91.57 (day 0), 866.33±66.61 (day 4), and 680.67±82.16 (day 6); and the ADE-Group were 842.5±72.22 (day 0), 926.25±69.06 (day 4), and 866±40.36 (day 6). There were no significant differences between days 0 to 4 and 6 in the normal group (p=0.421), SC-Group (p=0.360), IV-Group (p=0.180), and ADE-Group (p=0.633).

Hemoglobin (g/dL) in the normal group were 14.4±0.49 (day 0), 14.37±0.16 (day 4), and 13.62±0.5 (day 6); The SC-Group were 14.38±0.23 (day 0), 14.03±0.34 (day 4), and 13.28±0.43 (day 6); The IV-Group were 13.97±0.37 (day 0), 14.17±0.2 (day 4), and 13.62±0.26 (day 6); and the ADE-Group were 13.55±0.82 (day 0), 12.9±0.58 (day 4), and 11.33±1.72 (day 6). There were no significant differences between days 0 to 4 and 6 in the normal group (p=0.369), SC-Group (p=0.102), IV-Group (p=0.411), and ADE-Group (p=0.409).

Fig 4. Bar diagrams of hematological parameters on day 0, day 4, and day 6 of Wistar rats untreated with DENV (normal group); treated with DENV in subcutaneous injection (SC-Group), intravenous injection (IV-Group), and the injection method adapted from the antibody-dependent enhancement phenomenon (ADE-Group). *: p<0.05 vs day 0.

Biochemical Indices

Table 2. Summary of biochemical indices of Wistar rat untreated with DENV (normal group); treated with DENV in subcutaneous injection (SC-Group), intravenous injection

(IV-Group), and the injection method adapted from the antibody-dependent enhancement phenomenon (ADE-Group)

Group Name	Albumin (g/dL)	ALT (IU/L)	AST (IU/L)
Normal Group (n=6)	3.46±0.11	51.62±3.17	190.67±12.83
SC-Group (n=6)	3.43±0.14	56.3±6.67	236.15±28.71
IV-Group (n=6)	3.5±0.13	42.57±2.66	174.37±9.46
ADE-Group (n=4)	3.49±0.17	58.38±5.56*	274.83±19.3*

SC-Group: Subcutaneous Group; IV-Group: Intravenous Group; ADE-Group: Antibody-dependent Enhancement Group
*$p<0.05$ vs normal group

The albumin value of the normal group was 3.46±0.11 g/dL, the SC-Group was 3.43±0.14 g/dL, the IV-Group was 3.5±0.13 g/dL, and the ADE-Group was 3.49±0.17 g/dL (Table 2). There was no significant result between all groups (p=0.984).

The ALT value of the normal group was 51.62±3.17 IU/L, the SC-Group was 56.3±6.67 IU/L, the IV-Group was 42.57±2.66 IU/L, and the ADE-Group was 58.38±5.56 IU/L. The ADE-Group was found to have a significantly greater ALT value than the normal group (p=0.033). No significant differences between the SC-Group (p=0.749) and IV-Group (p=0.078) compared to the normal group (Table 2).

The AST value of the normal group was 190.67±12.83 IU/L, the SC-Group was 236.15±28.71 IU/L, the IV-Group was 174.37±9.46 IU/L, and the ADE-Group was 274.83±19.3 IU/L. There was a significant difference between the normal group and the ADE-Group (p=0.011). There were no significant differences between the SC-Group (p=0.200) and IV-Group (p=0.337) compared to the normal group, as seen in Table 2.

DISCUSSION

The dengue NS1 antigen test is detectable during the acute phase, usually the first week of symptoms [16]. The examination of the dengue NS1 antigen test in Wistar rats revealed negative results in this study. As a clarification, it was confirmed that the injected virus was viable based on the positive results shown in the virus medium samples before it was injected into the rats. These results are also in line with another study, which stated that the results of the NS1

examination are not correlated with the disease severity of dengue fever in mouse model [17]. Thereby, this result can be deemed to be a false negative.

There were three phases in dengue infection: febrile, critical, and convalescence [18]. The febrile phase occurs on days 0 to 7, characterized by increasing temperature that reaches its peak on days 1 to 3, then gradually decreasing on days 4 to 7 [19]. The rectal temperature results in this study revealed that the IV-Group and ADE-Group showed a fever pattern consistent with dengue infection. The fever pattern was shown to be more severe and statistically more significant in the ADE-Group than in the IV-Group.

The critical phase occurs on days 4 to 6 [18]. Plasma leakage, an increase in hematocrit levels of more than 20% of the basal hematocrit, might occur in this phase [19,20]. In this study, hematocrit did not show any significant results in all groups. Other complete blood parameters, such as hemoglobin and platelets, also did not show any significant change in all groups. Only leukocytes showed a statistically significant decrease from day 0 to 6 in the ADE-Group, in which viral infections are usually characterized by leukopenia [21,22]. Laboratory manifestations that can also be found in dengue infection are hypoalbuminemia and transaminitis [23,24]. Surprisingly, the albumin also did not change significantly in all groups. Significant increases in ALT and AST were shown in the ADE-Group compared to the control or the normal group. Secondary dengue infection causes more severe infection reactions due to ADE [25]. The ADE phenomenon has two different pathways, namely extrinsic and intrinsic [26]. Extrinsic ADE occurs due to enhanced rates of receptor interaction and internalization of virus-immune complexes, while intrinsic ADE involves the modulation of innate immune effectors by internalized virus-immune complexes to favor increased viral replication and release [27]. The intrinsic ADE also promotes the expression of pro-inflammatory cytokines, leading to increases in the production of nitric oxide, which is a radical and ultimately leads to organ damage [28,29]. The clinical spectrum, mortality rate, fever pattern, leukocytes, and transaminases were found to be the most severe in Wistar rats using the dengue infection method that utilized the ADE phenomenon compared to other injection methods. The subcutaneous injection route does not provide results that are consistent with the dengue infection because viruses that enter

subcutaneously may require a longer research observation time, considering the DENV incubation period [30]. The intravenous injection route provides minimal insight into the disease. Further studies should be carried out in order to support these findings, such as examining the viral titers, the specific antibodies in the ADE pathway, and the pro-inflammatory cytokine or free radical parameters that also play a role in the mechanism of dengue infection.

CONCLUSION

Dengue infection through an induction method adapted from the antibody-dependent enhancement phenomenon shows the most severe clinical spectrum and laboratory manifestations compared to other induction methods in Wistar rats, making it a promising dengue murine model. Future studies of possible mechanisms of infection and different parameters of other disease severity pathway are warranted to confirm our findings.

CONFLICT OF INTEREST

The authors report no conflict of interest in this study.

AUTHOR CONTRIBUTIONS

LW, CPT, NS: experimental design, conceptualization, data analysis, manuscript writing. SS, THS: methodology, research supervision, manuscript editing.

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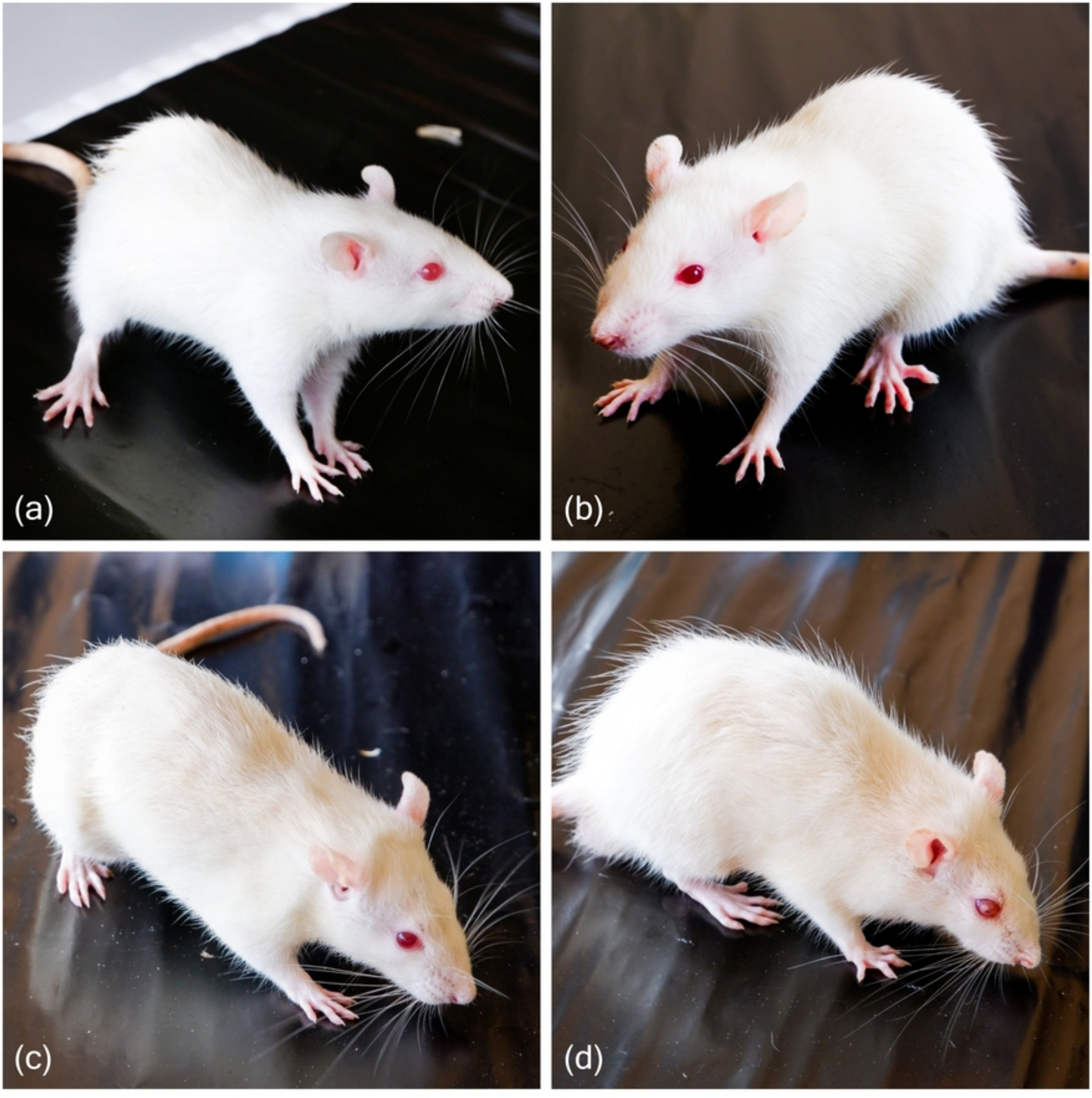
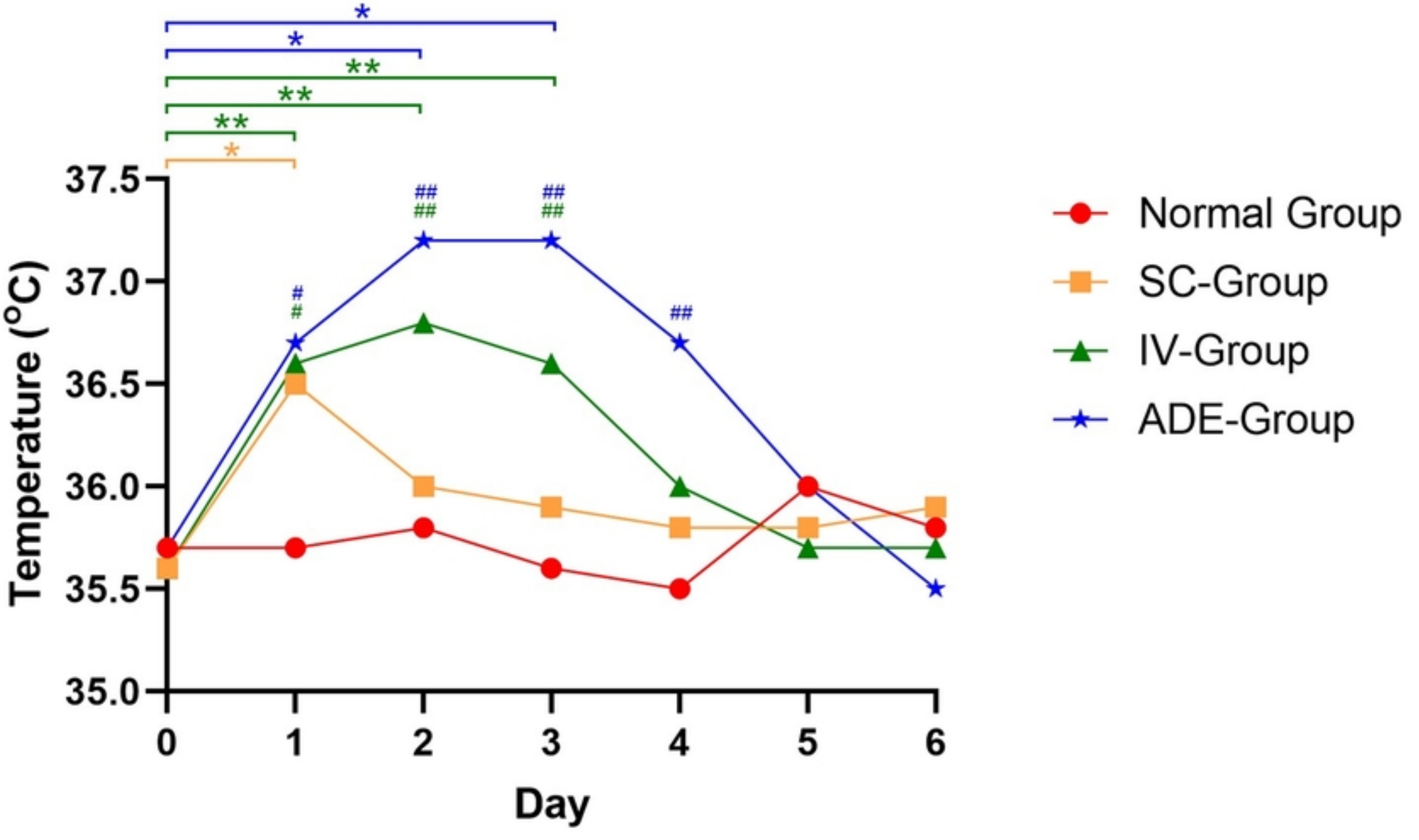


Figure 1

Figure 2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*
37.5
37.0
36.5
36.0
35.5
35.0
Temperature (°C)
0
1
2
3
4
5
6
Day
Normal Group
SC-Group
IV-Group
ADE-Group

Figure 3

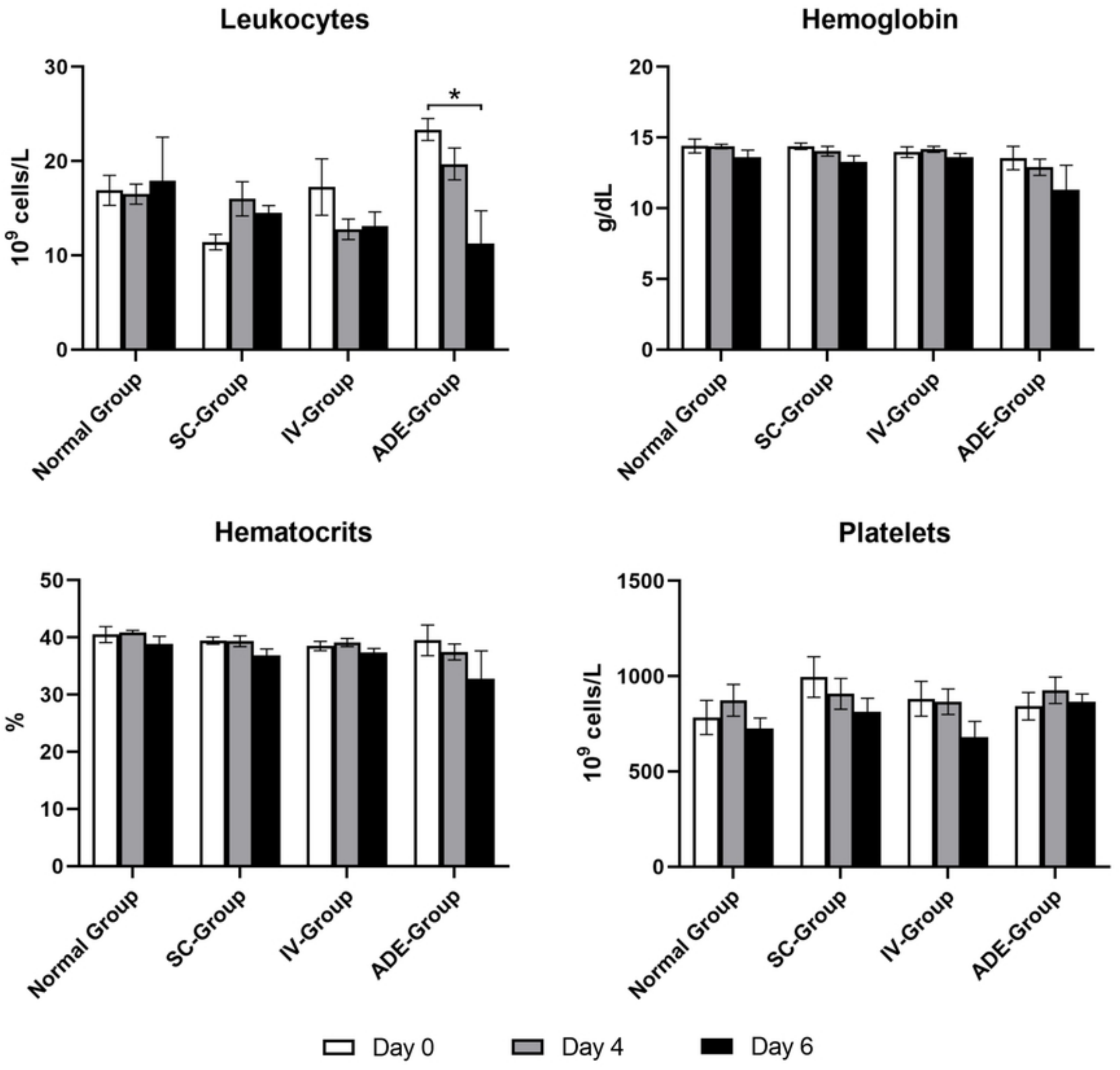
Leukocytes
10^9 cells/L
30
20
10
0
*
Normal Group
SC-Group
IV-Group
ADE-Group
Hemoglobin
g/dL
20
15
10
5
0
Normal Group
SC-Group
IV-Group
ADE-Group
Hematocrits
%
50
40
30
20
10
0
Normal Group
SC-Group
IV-Group
ADE-Group
Platelets
10^9 cells/L
1500
1000
500
0
Normal Group
SC-Group
IV-Group
ADE-Group
Day 0
Day 4
Day 6

Figure 4