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TRIPOLAR ELECTRICAL STIMULATION AS A POTENTIAL MODULATOR OF METABOLIC AND HEMATOLOGICAL PROFILES IN MICE

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ABSTRACT

Elevated blood cholesterol and glucose levels are common conditions among the elderly, often requiring continuous pharmacological intervention. To explore alternative approaches, the present study investigated the effects of alternating electrical simulation on these parameters, as well as on hemoglobin concentration and blood viscosity, using mice as experimental subjects. Treatment were applied at voltages ranging from 0 to 2.0 V, with a frequency of 500 kHz, for durations of 3 or 5 min day^{-1} over a 5-day period. The results revealed that electric stimulation at 2.0 V for 5 min reduced cholesterol levels by 7.53-9.0% and glucose levels by 28.41%, while hemoglobin levels tended to increase without a consistent pattern. Blood viscosity decreased under stimulation at 1.0 and 1.5 V but increased at 2.0 V, indicating a voltage-dependent response. In conclusion, electrical stimulation effectively lowered cholesterol and glucose levels in mice; however, its effects on hemoglobin and blood viscosity were non-linear, thus suggesting the need for further studies to optimize treatment parameters.

Keywords: Frequency, glucose, mice, tripolar, voltage

INTRODUCTION

Elevated blood cholesterol and glucose levels are common conditions, frequently experienced by elderly people. Reportedly elevated total cholesterol ($\geq$ 5.0 mmol L^{-1}) globally affects approximately 39% of adults (37% of men and 40% of women) (Ray *et al.*, 2022). High cholesterol levels can clog blood vessels, which may put patients at risk of high blood pressure, heart disease, and stroke (Yusuf *at al.*, 2016; Saputra *at al.*, 2019). High glucose levels can damage blood vessels and nerves around the heart, potentially causing cardiovascular disease, particularly heart disease (Petrofsky, 2011; Rask-madsen and King, 2014). Total cholesterol reflects the accumulation of cholesterol transported by various lipoprotein fractions in circulation, mainly low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and to a lesser extent very low-density lipoprotein (VLDL). For adults in good health, the recommended total cholesterol level is < 200 mg dL^{-1}, with LDL levels < 100 mg dL^{-1} and HDL < 60 mg dL^{-1} (Setyawati and Lasroha, 2021). Fasting blood glucose levels in healthy adults typically range from 70 to 100 mg dL^{-1}, while preprandial glucose level is typically between 70-110 mg dL^{-1} (Chamhuri *et al.*, 2022).

Several efforts have been made by individuals with high cholesterol and blood glucose levels to restore these levels to normal. Common approaches include taking pharmaceutical drugs to lower cholesterol and glucose levels, using herbal medicines, and regulating diet. Statins have been used for more than 20 years as safe and effective cholesterol-lowering drugs (Armitage, 2007). However,

taking statins in large amounts at once may potentially cause kidney or liver disease and, in some cases, may lead to a small body posture (Navaneethan *et al.,* 2006). Metformin is an antidiabetic drug that works by reducing glucose production in the liver and increasing insulin sensitivity in peripheral tissues (Rena *et al.,* 2017; Taylor *et al.,* 2021). However, prolonged use may cause some side-effects, such as gastrointestinal problems and diarrhea (Alibrahim *et al.,* 2023). Herbal medicines have also proved useful in lowering cholesterol levels (Saad and Al-Shawk, 2023) or blood glucose levels (Verma *et al.,* 2018). However, herbal medicines tend to act more slowly in lowering cholesterol and blood glucose levels (Zaidi *et al.,* 2024**).** In addition, dietary adjustment has proved effective in lowering cholesterol and blood glucose levels, but not everyone is able to adhere to such changes. Recently, various electrical devices have been used in health and beauty therapy. Stein *et al.* (2013) reported that transcutaneous electrical nerve stimulation improved pain relief in patients with diabetic neuropathy. Four weeks of pulsed electrical stimulation resulted in significant improvement in glucose tolerance, as measured by the oral glucose tolerance test (Galvan *et al.,* 2022). Providing electrical pulse stimulation for 40 min day^{-1} over two weeks resulted in a greater reduction in blood glucose levels in the experimental group (12.71%) compared to the control group (4.06%) [Sharma *et al.,* 2010]. In previous studies, electrical stimulation using pulsed voltage was commonly used, thus requiring a fairly high voltage and long treatment time. Meanwhile, it has been reported that cell membranes have capacitive properties that inhibit the flow of direct current (Shigimaga, 2014). The skin and fat are poor electrical conductors, whereas water is a good conductor of electricity (Miklavčič *et al.,* 2006). Therefore, this study employed tripolar alternating electrical stimulation with low voltage and high frequency, so as to achieve more optimal results within a shorter treatment time. The present study was aimed to reduce cholesterol and blood glucose levels using low-voltage alternating electrical stimulation and to assess its impact on hemoglobin levels and blood viscosity in mice.

MATERIALS AND METHODS

Experimental set up

This research was conducted at the Biophysics Laboratory, Maulana Malik Ibrahim State Islamic University (MMISIU) of Malang from April to September 2024. The study was approved by the Ethics Commission of the Faculty of Science and Technology, MMISIU [Approval No. 01/EC/ KEP-FST/2024 dated January 19, 2024]. The study involved 35 male mice (*Mus muculus*) with an average age of 8 weeks. The mice were acclimatized for 7 days, with one mouse housed per cage. The cages were lined with rice husks, which were replaced daily. Each mouse was fed commercial BR-1 feed produced by ZZN Pelhřimov a.s. (Czech Republic), which is a complete feed with balanced nutritional composition. Feeding was done once daily, while drinking water (100 mL) was provided *ad libitum* to meet the animal's fluid requirements. After acclimatization, the fur of mice's backs was shaved to optimize the therapy process.

The alternating voltage used for treatment was generated by an HTP-brand cavitation ultrasound device (model HPTB0105), supplemented with a radio frequency signal. The voltage was generated from one ground electrode and two alternating channels, one of which produced a specific signal waveform (Fig. 1). The treatment given to the mice used effective voltages of 0, 1.0, 1.5 and 2.0 V at a measured frequency of 500 kHz. Treatment was given to the backs of mice whose fur had been shaved.

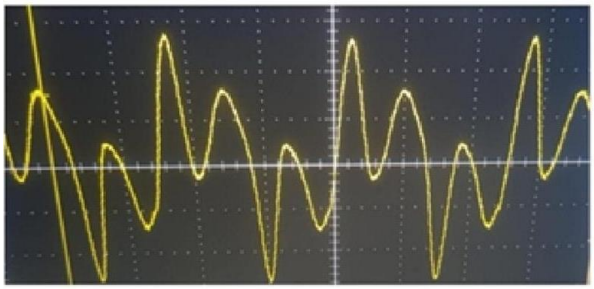

Fig. 1: Waveform of the electrical stimulation output voltage used for treatment

Treatment and blood collection process

Acclimatized mice were randomly divided into seven groups of five each. These groups comprised one control group and six treatment groups, each receiving varying stimulation duration (3 and 5 min) and voltages (1.0, 1.5 and 2.0 V). Treatments were administered once daily for five consecutive days. One day after final treatment, the mice were fasted for about 6 h prior to blood sampling. Blood samples were collected via tail vein. Prior to sampling, the mice's tails were immersed in warm water at 40-42°C for 1-2 min to promote vasodilation. The tails were then cleaned with cotton wool soaked in 70% alcohol and allowed to dry. The blood sampling was performed by making a small incision in the distal part of tail using sterile instruments. The first drop of blood was discarded, and subsequent drops were collected using a fine-tipped micropipette for further analysis.

Cholesterol level measurement

Total cholesterol levels were measured using the enzymatic colorimetric cholesterol oxidase-phenol aminophenazone (CHOD-PAP) method (Thorat and Phalak, 2023). The principle of this method is the oxidation of cholesterol by enzyme cholesterol oxidase, which produces hydrogen peroxide and subsequently reacts with 4-aminoantipyrine and phenol. This reaction, catalyzed by enzyme peroxidase, forms a red complex that can be measured spectrophotometrically. Blood samples (0.5 mL) were collected from the tail vein of mice using a tail vein puncture technique with a sterile 25-27G needle after the animals were restrained. The blood collection procedure was performed aseptically in accordance with animal laboratory standards (Parasuraman *et al.,* 2010). The blood was transferred into tubes without anticoagulant and left at room temperature for 30 min to allow clotting. The samples were then centrifuged at 4000 rpm for 10 min to separate the serum from the cellular components. The resulting serum was carefully collected using a micropipette and used as the test sample. A 10 µL sample serum was placed in a test tube, followed by the addition of 1000 µL cholesterol working reagent (CHOD-PAP). This reagent typically contains cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine, phenol, and phosphate buffer, consistent with formulations described in modern enzymatic cholesterol assays and commercial diagnostic kits (Nakamura *et al*., 2014). The mixture was homogenized and incubated at 37°C for 10 min as per the kit's instructions. After incubation, the absorbance was measured spectrophotometerically at 500 nm. The sample absorbance was compared with a standard cholesterol solution to determine total cholesterol levels. Total cholesterol levels were calculated using the equation given by Tietz (2018):

$$\text{Total cholesterol (mg dL}^{-1}) = \frac{A_{sample} \times C_{standard}}{A_{standard}}$$

Where A_{sample} is the absorbance of the sample, $A_{standard}$ is the absorbance of the standard solution, and $C_{standard}$ is the concentration of the standard cholesterol solution.

Glucose level measurement

Blood glucose levels were measured using an Accu-Chek® Active digital glucometer (Roche Diagnostics GmbH, Mannheim, Germany), which operates using an electrochemical method using the enzyme glucose dehydrogenase or glucose oxidase (Baumstark *et al.,* 2018). Before sampling, the rat's tails were cleaned and, if necessary, shaved distally to facilitate visualization of blood vessels. The area was then disinfected with 70% alcohol and allowed to dry. Blood was collected using the tail-tip incision technique, which involves a minimal incision (±2 mm) of tail tip using sterile scissors or a scalpel to obtain capillary blood (Parasuraman *et al.,* 2010). The first drop of blood was wiped away to avoid tissue fluid contamination, while subsequent drops were used as samples. The blood was then dripped directly onto a test strip compatible with Accu-Chek® Active glucometer. The test strip was inserted into the device, which automatically activated and performed the measurement. Blood glucose results were displayed within ±5-10 sec. After measurement, the wound on rat's tail was pressed with sterile cotton until the bleeding stopped (King *et al.,* 2012).

Hemoglobin level measurement

Prior to blood collection, the test animals were fasted for about 6 h with *ad libitum* access to drinking

water to minimize metabolic variations (Parasuraman *et al.,* 2010). Blood samples were collected from the tail vein using the tail tip incision technique under aseptic conditions (Parasuraman *et al.,* 2010) and transferred into tubes containing ethylenediamine tetra-acetic acid (EDTA) to prevent clotting. The samples were centrifuged at 3000 rpm for 10 min. However, whole EDTA-treated blood was used for hemoglobin analysis since hemoglobin is contained within erythrocytes (Bain *et al.,* 2017). Hemoglobin concentration was determined using the cyanmethemoglobin (hemiglobincyanide) method (WHO, 2011; Bain *et al.,* 2017). In this assay, blood reacts with Drabkin's reagent (potassium ferricyanide and potassium cyanide) where hemoglobin is oxidized to methemoglobin and subsequently converted to stable cyanmethemoglobin. For analysis, 20 µL EDTA-treated blood was mixed with 5 mL Drabkin's reagent, homogenized and incubated at room temperature for 10 min. The resulting colour intensity was measured using at 540 nm using a UV-Vis spectrophotometer. Hemoglobin concentration was calculated by comparing sample absorbance with a standard cyanmethemoglobin solution and expressed in g dL^{-1}.

Blood viscosity measurement

Blood samples were obtained from mice via tail tip amputation using microhematocrit capillary tubes. To minimize stress and facilitate access, the test mice were restrained (NRC, 2011). The tail tip was either cut with sterile surgical scissors or incised with a sterile lancet and blood flow was enhanced by immersing the tail in warm water (45-50°C) for several minutes to induce venous vasodilation (Fox *et al.,* 2015). The blood samples were collected directly into capillary tubes by capillary action (NRC, 2011) and transferred into microhematocrit tubes containing anticoagulant. Heparin was primarily used as it prevents clotting without altering erythrocyte volume (Keohane, 2020), while EDTA served as an alternative, providing improved plasma separation after centrifugation (Keohane, 2020). The capillary tubes were sealed at one with clay and centrifuged at 12,000 rpm for 5 min to achieve complete separation of erythrocytes, buffy coat, and plasma, in accpordance with the hematocrit protocols for laboratory animals (Dallarosa *et al.,* 2023). Hematocrit (packed cell volume) values was calculated by comparing the height of the erythrocyte column with the total height of blood column in the capillary tube using a microhematocrit reader. It was expressed as a percentage (%), using the following equation (Harmening, 2012):

$$\% \text{ Hematocrit } = \frac{h_{erythrocyte}}{h_{totlal}} \text{ x } 100$$

where $h_{erythrocyte}$ is the height of the erythrocyte column and h_{total} is the total height of the blood column.

Hematocrit values reflect the volume fraction of red blood cells in blood and is a key determinant of blood viscosity (Alexy, 2022). As hematocrit rises, the erythrocyte interactions increase, producing a non-linear elevation in blood viscosity. This relationship can be estimated empirically using the following polynomial equation of Trejo-Soto and Machado (2022):

$$y = 1.5 + 0.0708x – 0.0019x^2 + (4 \times 10^{-5}) x^3$$

where *y* represents the relative blood viscosity and *x* represents the hematocrit value (%).

This equation indicates that an increase in hematocrit leads to a non-linear increase in blood viscosity due to enhanced interactions between erythrocytes and increased resistance to blood flow (Trejo-Soto and Machado, 2022).

Erythrocyte staining

Blood samples were collected as per the procedure of Ratnaningsih *et al.* (2006). For this, 1-3 mL blood samples were drawn into EDTA-containing vacutainer tubes to prevent clotting and ensure plasma separation. In total, 45 blood preparations were made. The samples were fixed using a 96% fixative solution with fixation times varied at 3min (standard), 5 min, 10 min, and 15 min to assess preservation quality. Giemsa staining was performed using a 1: 9 dilution (1 mL Giemsa mixed with 9 mL distilled water). The slides were stained for 25 min, rinsed with running water, and air-dried in a vertical position. Once dry, the slides were examined under a microscope at 400 x magnification using immersion oil. Images were captured with an Optilab Advance camera mounted on microscope.

Statistical analysis

This study employed a true experimental design with a post-test-only control group design. The test animals were divided into several treatment groups, each consisting of five animals. Statistical analysis began with a test of data normality. For normally distributed data one-way ANOVA was performed, followed by Tukey post hoc test to determine differences between groups. The significance level was set at $p \leq 0.05$. All data analyses were performed using SPSS software.

RESULTS AND DISCUSSION

Cholesterol levels

High frequency alternating current electrical stimulation affected blood cholesterol levels. Electrical stimulation at 1.0 and 1.5 V for 3 min day^{-1} resulted in an insignificant increase in cholesterol levels (Fig. 2). Cholesterol levels in control (without stimulation) were 110.67 ± 1.53 mg dL^{-1}, while stimulation at 1.0 and 1.5 V for 3 min increased the levels slightly to 111.33 ± 0.58 and 111.33 ± 1.53 mg dL^{-1}, respectively. In contrast, stimulation at 2 V caused a significant decrease in cholesterol levels ($p \leq 0.05$), reaching 105.67 ± 1.15 mg dL^{-1}. Extending the stimulation duration for 5 min potentially reduced cholesterol level. A significant decrease was observed at 2 V, with levels decreasing to 102.33 ± 4.04 mg dL^{-1}, representing a decrease of 7.53%.

The decrease in cholesterol levels may be attributed to the ability of electrical stimulation to enhance the permeability of cell membranes, thereby facilitating the penetration of ions, molecules and macromolecules into cells (Sweeney *et al.*, 2016). Electrical stimulation has also been reported to enhance the transfer of nutrients into cells (Albin *et al.*, 2024). Increased nutrient uptake may increase the lipase enzyme (Alkaade, 2024), which can lower cholesterol levels. Lipase plays a vital role in the breakdown of triglycerides, which are lipid compounds stored in adipose tissue, into fatty acids and cholesterol (Al-asadi, 2020).

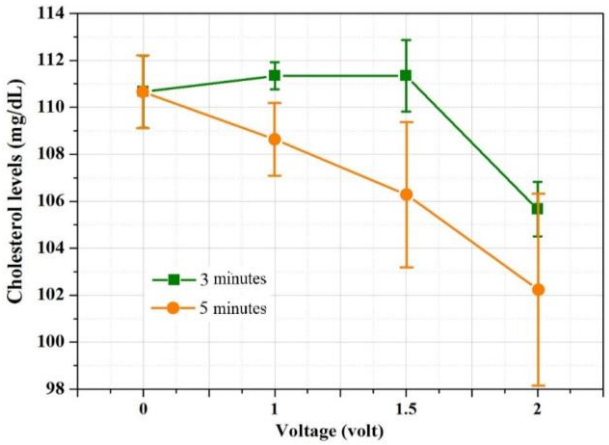

Fig. 2: Changes in blood cholesterol levels after electrical stimulation treatment

Glucose levels

Electrical stimulation for 3 min reduced blood glucose levels; however, stimulation at 1.0 and 1.5 V did not cause any significant reduction (Fig. 3). In contrast, stimulation at 2 V for 3 min significantly reduced glucose levels from 154 ± 5.29 mg dL^{-1} to 138.33 ± 5.03 mg dL^{-1}, thus showing 10.17% decrease. Electrical stimulation for 5 min at voltages ranging from 1.0 to 2.0 V a significantly reduced glucose levels. Stimulation at 2 V for 5 min reduced glucose levels from 154 ± 5.29 mg dL^{-1} to 113.33 ± 2.08 mg dL^{-1}, revealing 28.41% decrease.

The decrease in glucose levels may be attributed to radiofrequency electrical stimulation, which induces muscle contractions and stimulates nerves. Muscle contractions increase glucose uptake by muscle cells through an insulin-independent pathway, specifically through the activation of adenosine monophosphate-activated protein kinase (AMPK) enzyme (Jensen *et al.,* 2014). This mechanism directly reduces monophosphate-activated protein kinase blood glucose levels. Stimulation of vagus nerve may also increase insulin secretion from the pancreas by activating the parasympathetic response (Magnone *et al.,* 2020). In addition, vagus nerve stimulation can increase the sensitivity of peripheral tissues to insulin. Therefore, enhancing the electrical voltage and stimulation duration lead

to greater reductions in blood glucose levels. Stimulation at 2.0 V for 5 min reduced blood glucose levels by 28.41%. Earlier studies have reported that low-frequency electrical stimulation applied for a short duration suppresses hepatic glucose production (Catalogna *et al.*, 2016). After 2 weeks of electrical stimulation, blood glucose levels decreased in the experimental group, indicating that electrical stimulation can be used to help control blood glucose levels in type 2 diabetes patients (Sharma *et al.*, 2010).

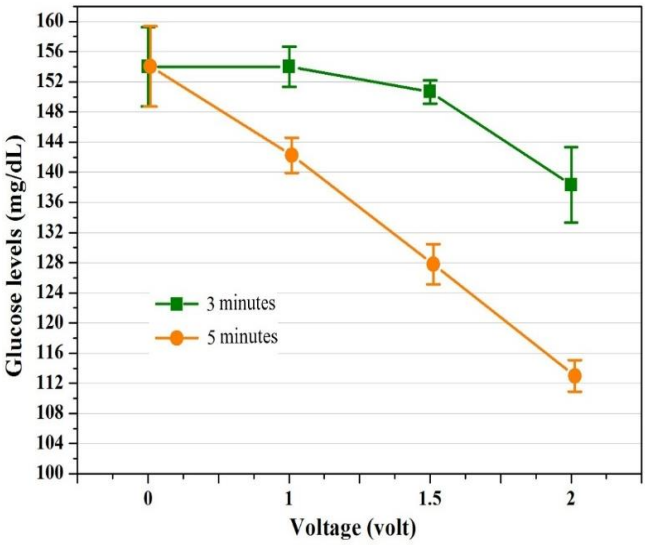

Fig. 3: Blood glucose level changes after electrical stimulation treatment

Hemoglobin levels

The use of high-frequency electrical stimulation not only affected blood cholesterol and glucose levels, but also affected hemoglobin levels. Electrical stimulation at 1.0-2.0 V for 3 min day^{-1} showed potential to increase hemoglobin levels. A significant increase ($p \leq 0.05$) of 11.11% was observed at 2 V, wherein hemoglobin levels increased from 17.4 ± 0.62 gr dL^{-1} to 19.33 ± 0.40 gr dL^{-1} in comparison to the non-simulated control group. Electrical stimulation for 5 min at 1.0 and 1.5 V significantly increased hemoglobin levels, with highest increase of 44.06% observed at 1.0 V. This resulted in an increase from 17.4 ± 0.62 gr dL^{-1} to 25.07 ± 0.40 gr dL^{-1} compared to the control group (no electrical stimulation). However, electrical stimulation for 5 min at 2.0 V significantly decreased hemoglobin level to 15.83 ± 0.31 gr dL^{-1}, showing a 9% decrease.

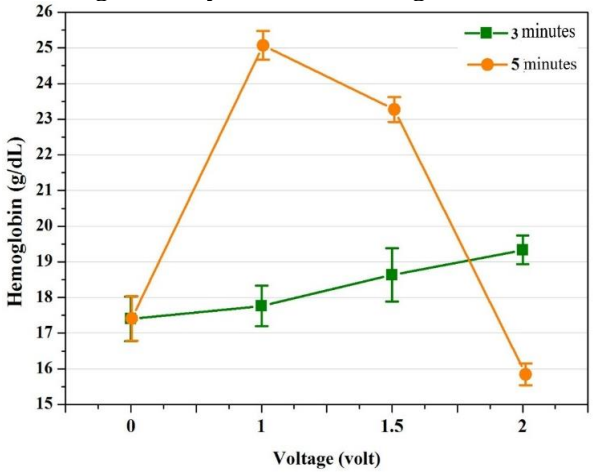

Fig. 4: Changes in blood hemoglobin levels treated using electrical stimulation

Muscle contraction induced by electrical stimulation increases local oxygen demand, thereby triggering a hypoxic response that activates HIF-1α. This factor stimulates the production of erythropoietin (EPO) in kidneys, which increases hemoglobin synthesis through increased red blood cell production (Hidmark e*t al.*, 2017). Sympathetic nerve stimulation via transcutaneous electrical nerve stimulation can increase the release of catecholamines (e.g. adrenaline), which stimulate the bone marrow to accelerate the production of RBC and hemoglobin (DeSantana *et al.*, 2009). In addition, low-intensity electrical stimulation can stimulate bone marrow stromal cells to release growth factors [e.g., stem cell factor (SCF) and interleukin-3 (IL-3)], which support the differentiation of hematopoietic stem cells into RBC (Xueling *et al.*, 2019). Therefore, electrical stimulation for 3 min resulted in a gradual increase in hemoglobin levels up to 2.0 V. In contrast, with a treatment duration of 5 min, hemoglobin levels increased at 1.0 and 1.5 V but decreased at 2.0 V. The low-frequency electrical stimulation can cause a significant decrease in hemoglobin concentration, glycated hemoglobin, total cholesterol, and systolic and diastolic blood pressure (Rubinowicz-Zasada *et al.*, 2021). Furthermore, electrical stimulation applied for 6 weeks reportedly improves muscle function and blood circulation (Dobsák *et al.*, 2016).

Blood viscosity

Blood viscosity is closely related to the proportion of erythrocytes in blood. Higher the erythrocyte content, greater is the viscosity. High-frequency alternating electrical stimulation at 1.0-2.0 V affects blood viscosity. Stimulation at 1.0 and 1.5 V for 3 and 5 min day^{-1} resulted in a slight non-significant decrease in blood viscosity. However, electrical stimulation at 2.0 V for 3 and 5 min significantly ($p \leq 0.05$) increased blood viscosity. Stimulation at 2.0 for 3 min increased blood viscosity from 5.13 ± 0.64 mPa.s to 6.74 ± 0.85 mPa.s, depicting 31.47% increase. Similarly, stimulation for 5 min increased blood viscosity to 5.97±0.10 mPa.s, corresponding to a 16.38% increase.

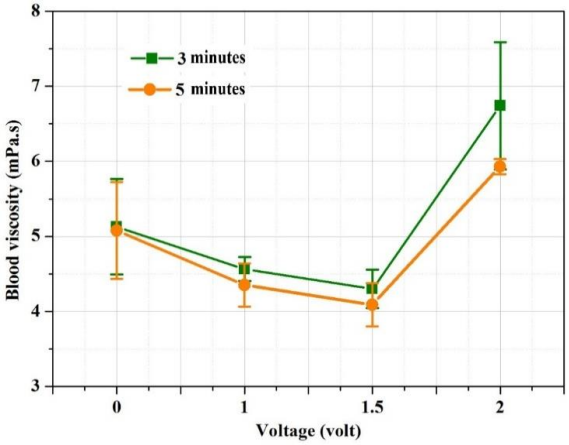

Fig. 5: Changes in blood viscosity after electrical stimulation

Electrical stimulation increases peripheral blood flow and reduces shear stress in blood vessels, thereby increases erythrocyte deformability and inhibits platelet aggregation (Beana *et al.*, 2023). Electrical stimulation also reduces fibrinogen (a blood-thickening protein) levels through anti-inflammatory effects and regulation of the sympathetic nervous system (Reeve *et al*., 2009). Furthermore, electrical stimulation-induced muscle contractions increase circulatory efficiency, reduce the need for high hematocrit, and help in the optimization of blood viscosity (Ando *et al.*, 2021). These mechanisms may explain why blood viscosity decreased at stimulation voltages of 1.0 and 1.5 V but increased at 2.0 V.

Blood histology

The microscopic analysis of blood histological sections demonstrated progressive alterations in erythrocyte morphology following five consecutive days of electrical stimulation (3 min day^{-1}) (Fig. 6A-D). In control the erythrocytes were sparsely distributed with characteristic biconcave shape without aggregation, reflecting a healthy physiological state (Fig. 6A). The electrical simulation at 1.0 V caused increase in cell numbers with a more uniform distribution, accompanied by mild anisocytosis (variation in cell size) (Fig. 6B). The electrical simulation at 1.5 V caused a significant increase in erythrocyte density, with cells tightly packed and relatively uniform, suggesting increased blood viscosity or relative polycythemia (Fig. 6C). The electrical simulation at 2.0 V, in addition to the increased density, showed irregular morphological

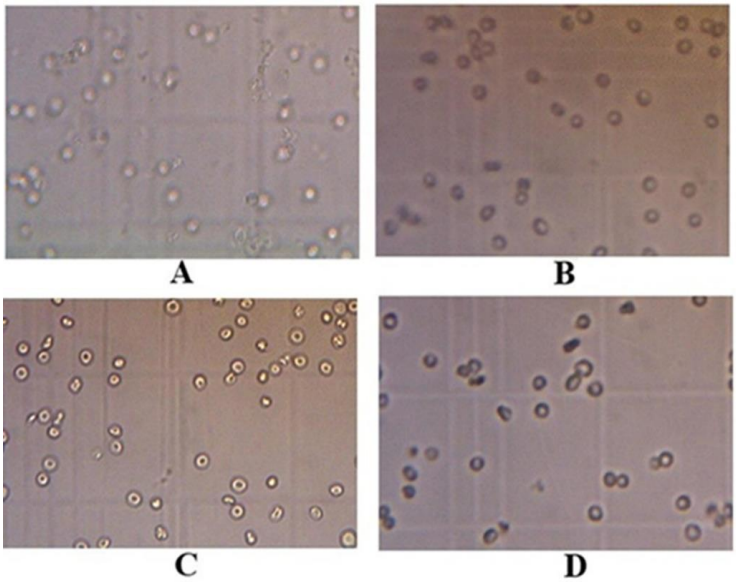

Fig. 6: Blood histological sections demonstrating the progressive alterations in erythrocyte morphology following 5 consecutive days of electrical stimulation at 3 min day^{-1}; A) control; B) electrical stimulation at 1.0 V; C) electrical stimulation at 1.5 V; and D) electrical stimulation at 2.0 V. Images were taken using a microscope equipped with an Optilab Advance camera at 400x magnification.

changes, including aggregation in the form of rouleaux formation (Fig. 6D). Collectively, these findings indicated a shift from normal morphology towards the states characterized by increased erythrocyte density and abnormal aggregation, reflecting both the biophysical impact of electrical simulation and the potential onset of pathological-like conditions.

Alternating current (AC) exerted notable biophysical effects on erythrocytes, particularly influencing cell aggregation and shape. These effects are dualistic, dependent on intensity, frequency, and duration of stimulation. Under normal physiological conditions (Fig. 6A), erythrocytes were discrete with characteristic biconcave shape. At low frequency and controlled intensity AC stimulation can polarize the cell membrane, inducing dipole interactions that align erythrocytes to chain-like rouleaux structures resembling stacks of coins (Jahangiri *et al.*, 2020; Trevino *et al.*, 2023). Importantly, this process is reversible; once the stimulus is removed, the aggregates disperse (Laha, 2023).

Conclusion: Tripolar low-voltage electrical stimulation affected cholesterol, glucose, hemoglobin, and blood viscosity levels in mice. Stimulation at low voltages tended to decrease cholesterol and blood glucose levels, while hemoglobin levels tended to increase. Electrical stimulation at voltages at 1.0 and 1.5 V decreased blood viscosity, whereas stimulation at 2.0 V increased viscosity. Electrical stimulation therapy has demonstrated potential in modulating and improving hematological parameters.

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Ethical statement: The research was approved by the Ethics Commission of the Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University of Malang (Approval No. 01/EC/KEP-FST/2024), dated 19 January, 2024.

Conflicts of interest: The authors declare no conflicts of interest.

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