

Effects of Thymoquinone On Erythrocyte Aggregation Kinetics: An In Vitro Study

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ABSTRACT

Erythrocyte aggregation is associated with a series of physio-pathological processes in body and according to this, current study was designed to examine how the thymoquinone (TQ) and aggregation kinetics relation changes when the aggregation properties of erythrocyte suspensions are intervened using different approaches, such as changing the suspension environment or erythrocyte surface properties.

Blood samples from 40 male *Wistar albino* rats (three months old weighing 250-300 g) were randomly divided into four groups, as 1) whole blood (control), 2) 1/2 dilution (plasma was diluted with PBS), 3) dextran 500 (1% dextran 500 was dissolved in plasma) and 4) glutaraldehyde. Blood samples were taken from abdominal aorta under anesthesia. The erythrocyte suspensions were incubated at 37°C for one hour in the absence or presence of TQ, with a final concentration of 1 mg/ml. Then, the electrical properties of erythrocyte suspensions were recorded, and the electrical capacitance was monitored using an inductance, capacitance, and resistance meter (LCR meter) at 100 kHz.

It was found that TQ incubation decreased the aggregation index in control and dextran groups while increased it in 1/2 dilution and GA groups. It was also found that TQ incubation increased the time constant in whole blood and dextran while decreased it in 1/2 dilution and GA.

The TQ can normalize the altered erythrocyte aggregation responses in both ways that is, it decreases the increased aggregation or increases the decreased aggregation. Therefore, it may have very critical role in rouleaux formation-induced circulatory disorders.

Keywords: Thymoquinone, *Nigella Sativa*, Ranunculaceae, Erythrocyte aggregation kinetics, Capacitance

Introduction

Erythrocyte aggregation, clustering of erythrocytes by forming parallel surfaces to each other, is affected by the changes in the property of both the plasma and the cellular structure of erythrocytes. These changes can be associated with a series of pathophysiological processes in body (1-6). Therefore, measurement of erythrocyte aggregation has a clinical importance. Various approaches and methods are used to determine erythrocyte aggregation *in vitro*. Photometric measurements are widely used in researching aggregation in erythrocyte suspensions (2, 7, 8). Besides, electrical properties of erythrocyte suspensions can also be used to monitor aggregation. It has been reported that, like photometric measurements, monitoring of the electrical properties of erythrocyte suspensions might inform us about the process running during aggregation (9-11).

Like all cells, erythrocytes are also exposed continuously to the excess amount of oxidants

produced by endogenous and exogenous sources. When the concentration of reactive oxygen species exceeds the antioxidants, oxidative stress occurs in tissues or cells (12). Erythrocyte oxidation produces osmotic fragility, methemoglobin formation, membrane injury, and thus the destruction of the cell (13). In addition, oxygen free radicals may damage red blood cells (RBCs) by elevating the strength of their aggregates (14), thus leading to an elevation in vascular resistance against to blood flow, especially at the precapillary level (15-17). Therefore, an alteration in RBC rheology may play an important role in the development of various acute or chronic complication (18).

Thymoquinone (TQ), the active ingredient of *Nigella sativa* plant, has antioxidant, anti-cancer (19), analgesic (20), and anticonvulsive (21), effects. It has also protective effects against cardiac, liver and kidney damages (22). It has been reported that *Nigella sativa* makes the cell skeleton mechanically more resistant, and that it protects erythrocytes against oxidative stress and fragility

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due to its antioxidant action (23, 24). It has been suggested that the *Nigella sativa* protects human erythrocytes against H₂O₂-induced protein degradation and loss of deformability (24). However, the evidence about the possible effect of *Nigella sativa* and TQ on erythrocyte rheology is still not enough. Assuming that TQ has a very strong antioxidant effect and protects human erythrocytes against degradation and loss of deformability, this study was designed to examine how the TQ and aggregation kinetics relation changed when the aggregation properties of erythrocyte suspensions were intervened using different approaches, such as changing the suspension environment or erythrocyte surface properties. Because we planned to investigate the effect of TQ on hemorheological parameters by using different experimental rat models in the future, rats were preferred as experimental animal in this *in vitro* study to obtain premise data. We could have used the healthy human blood for this study. However, it would be very difficult for us to continue to investigate the TQ's effects on cases such as sepsis (1), diabetes (25), hypertension (26), nephrectomy (27), pancreatitis (28), etc. models where rouleaux formation is affected.

Material and methods

Animals: In this study, three ages old, 40 male Wistar albino rats weighing between 250-300 g were used. Animals were obtained and kept in the Bezmialem Vakif University Experimental Animal Centre under standard laboratory conditions. The ethical approval was obtained from the Laboratory Animals Ethical Committee of Bezmialem Vakif University (No: 2014/42).

Blood sampling, preparation of erythrocyte suspension and study design: Blood samples were taken from abdominal aorta under ketamine 100 mg/kg and xylazine 10 mg/kg anesthesia from 4 animals (approximately 10 ml blood was obtained from one rat) into sodium heparin (15 IU/ml) containing injectors and mixed to obtain 40 ml blood in each study day. The same procedure was repeated for 10 days. Thus, 10 measurements (means 10 animals) were performed in each group. Obtained 40 ml blood was divide into four equal study groups as; 1) whole blood (control), 2) 1/2 dilution (plasma was diluted with PBS at a rate of 1/2 for decreasing aggregation degree by lowering macromolecule concentration in the plasma), 3) dextran 500 (1% dextran 500 was dissolved in plasma for increasing aggregation degree by

increasing the macromolecule concentration in the plasma, MW = 500 kDa, D-5251, Sigma Chemical Company, St. Louis, MO) and 4) glutaraldehyde (GA was fixed with RBCs for decreasing aggregation degree, GA, Merck, Darmstadt, Germany). Some of the blood samples were centrifuged to separate plasma and erythrocytes. Remaining blood was kept as whole blood sample. In addition, erythrocyte samples prepared in phosphate-buffered saline (PBS) were also used to control the electrical properties of erythrocyte suspensions (a validation group "5th group). The PBS solution was prepared by dissolving; 8.5 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ (anhydrous), 0,2 g KH₂PO₄ in 1000 ml of distilled water. The pH of the solution was adjusted to approximately 7.4. The erythrocyte suspensions were incubated at 37 °C for one hour in the absence or presence of TQ (Sigma Chemical Company, St. Louis, MO), with a final concentration of 1 mg/ml. The sample studies were completed in four hours after the blood drawing. The plasma and erythrocytes were separated by centrifuging approximately 30 ml of the obtained blood for 5 min at 1.400 g. Remaining blood (about 10 ml) was kept as whole blood sample. Whole blood was turned into suspension with a hematocrit value of 0.4 l/l by using unchanged autologous plasma, and obtained erythrocytes were turned into suspension with a hematocrit value of 0.4 l/l by using plasma diluted with PBS at a rate of 1/2 (for decreasing aggregation degree), and 1 % dextran 500-added (500 kDa) plasma (for increasing aggregation degree). In this way, aggregation change was done by changing only suspension environment, without any changes to the cellular properties of erythrocytes. On the other hand, aggregation change was also done by changing the cellular properties of erythrocytes. In this aim, erythrocyte deformability was decreased (for decreasing aggregation degree) by incubating them with GA for 30 minutes at low concentration (0.003 %). Then, they washed with PBS following the incubation, and the RBC packed was adjusted to a hematocrit value of 0.4 l/l in an autologous plasma.

TQ incubation: In order to achieve an adequate TQ solution, it was initially dissolved in ethanol. Then, it was diluted with PBS at the rate of 1:100. The prepared erythrocyte suspensions of each group were incubated with or without TQ (final concentration was 1 mg/ml and ratio of ethanol was 0.1%) at 37 °C for one hour (18, 24). After one hour of incubation period, the blood samples

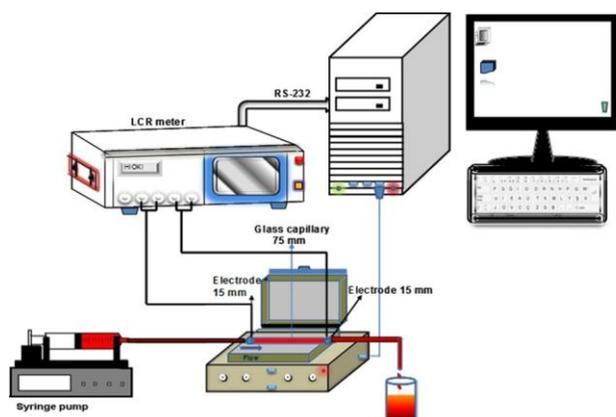


Fig. 1. The Measurement System

were placed in the system to measure the electrical properties in an order.

The electrical properties of erythrocyte suspensions: The electrical properties of erythrocyte suspensions were recorded in a horizontal glass capillary (diameter: 0.8 mm; length: 75 mm), during and after the stoppage of flow induced by a syringe pump (World Precision Instruments, Aladdin-4000, Florida, ABD). The glass capillaries and electrodes were placed in a heat-controlled box, and measurements were done at 37°C. After the flow rate of the pump was adjusted to 42 ml/min, the blood flow in capillary system was obtained as 5.5 m/s according to the diameter of capillary. Using a formula of wall shear stress [viscosity x (8 velocity / diameter)], this flow velocity corresponds to approximately 28 Pascal in blood samples with 0.4 l/l hematocrit (human blood viscosity is about 4-5 cP); and it is a value that exceeds the threshold value of disaggregation in glass capillaries. Electrical capacitance was monitored using an LCR meter (Hioki, 3532-50 LCR HiTester, Nagano, Japan) at 100 kHz that was also controlled by the same computer during and after the stoppage of flow for 2 minutes at 1 second intervals. The measurement system was schematized in Figure 1. The recorded capacitance curve was analyzed mathematically and erythrocyte aggregation index (AI) and, aggregation half time (T_{1/2}) were determined.

Statistical analysis: The time constants representing a similar exponential change were calculated using the course of time of the electrical parameters that were obtained (Figure 2). The results were expressed as mean ± standard error (SE). Statistical comparisons between binary groups were done by Student's t test whereas multiple comparisons were done using one way ANOVA with Bonferroni post hoc test. P < 0.05 was considered as statistically significant.

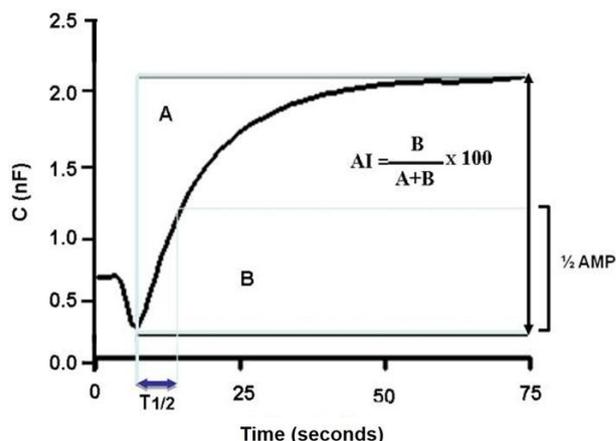


Fig. 2. Calculation graphic of erythrocyte aggregation index (AI) and erythrocyte aggregation half time (T_{1/2})

Results

Capacitance (C; nF) records of erythrocyte suspension obtained from the whole blood, 1/2 dilution, dextran, GA, and PBS groups in the absence of TQ incubation are shown in Figure 3A. As expected, the highest change in the data was observed in dextran, the lowest change was observed in 1/2 dilution, and no change was observed in PBS. Thus, the reliability of our system was confirmed. In addition, the C records of erythrocyte suspensions of whole blood, 1/2 dilution, dextran, GA and PBS groups incubated with TQ are shown in Figure 3B. The changes in the data were observed to be the highest in dextran, the lowest in whole blood; and no change was observed in PBS.

The AI calculated from the C records of whole blood, 1/2 dilution, dextran, and GA groups in the absence or presence of TQ incubation is shown in Figure 4A. In the absence of TQ incubation, compared to whole blood, the values of AI were found the highest in dextran (P < 0.01), and the lowest in 1/2 dilution (P < 0.001). However, in the presence of TQ incubation, compared to whole blood, the AI was found to be high in dextran (P < 0.001) and GA (P < 0.01) while no change (P > 0.05) was found in 1/2 dilution. While TQ incubation decreased the AI in whole blood (P < 0.001) and dextran (P < 0.001), it increased it in 1/2 dilution (P < 0.001) and GA (P < 0.001).

The T_{1/2} calculated from the C records of whole blood, 1/2 dilution, dextran, and GA groups in the absence or presence of TQ incubation is shown in Figure 4B. In the absence of TQ incubation, the T_{1/2} values were found the

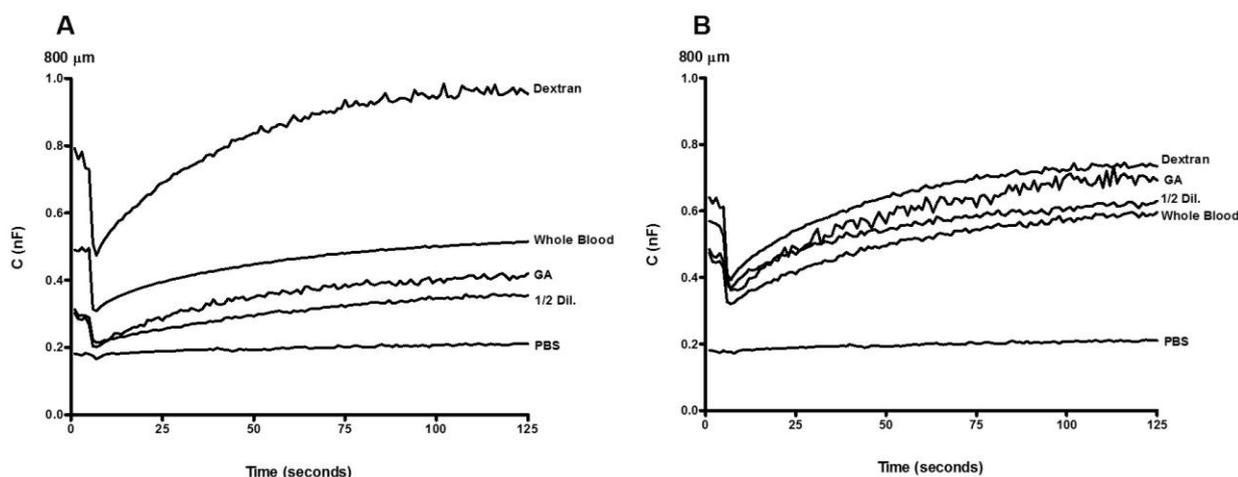


Fig. 3. The C records of erythrocyte suspensions obtained from the whole blood, 1/2 dilution, dextran, GA, and PBS groups in the absence (A) or presence (B) of TQ incubation. The C measurements were done at a frequency of 100 KHz.

lowest in dextran ($P < 0.05$) and highest in 1/2 dilution ($P < 0.001$). However, in the presence of TQ incubation, T 1/2 was low in dextran ($P < 0.001$) and GA ($P < 0.05$) while no difference ($P > 0.05$) was found in 1/2 dilution. While TQ incubation increased the T 1/2 in whole blood ($P < 0.001$) and dextran ($P < 0.001$), it decreased it in 1/2 dilution ($P < 0.01$) and GA ($P < 0.001$).

Discussion

This study was aimed to examine how the TQ and aggregation kinetics relation changes when aggregation properties of erythrocyte suspensions are intervened with different approaches such as changing the suspension environment or erythrocyte surface properties; and, thus, how TQ affects the erythrocyte aggregation kinetics. It was found that while TQ incubation decreased the AI in whole blood and dextran, it increased it in 1/2 dilution and GA. TQ incubation was also found to increase T 1/2 in whole blood and dextran whereas it decreased it in 1/2 dilution and GA. This study is the first one indicated that TQ can normalize the erythrocyte aggregation in both ways that is, it decreases the increased aggregation or increases the decreased aggregation serving as a regulator molecule.

Dextran, complex branched glucan, has been used to induce RBC aggregation in many previous *in vivo* or *in vitro* studies (29-30,35). Because it is a molecule itself, its application increases the erythrocyte aggregation rate by increasing the macromolecule concentration in the plasma. However, there are numerous molecular weight dextrans. Because dextran 70 and dextran 500,

which are the high molecular weight polymers might induce RBC aggregation, they are widely used to adjust the degree of aggregation. However, dextran 10 and dextran 40, which are the low molecular weight polymers have an inhibitory effect on RBC aggregation (29). Furthermore, it has been demonstrated that optimal or maximum erythrocyte aggregation occurs with dextran 500 (30). When normal erythrocytes are suspended in dextran 500, their aggregation is largely increased because it creates an effect similar to the fibrinogen and causes erythrocytes to form a rouleaux formation (3-6, 31-34). Dextran infusion also increases plasma viscosity and produces yield stress. The yield stress of blood is highly correlated with the level of RBC aggregation (35). It has been reported that the cross-bridging and depletion models are the main models for erythrocyte aggregation (30, 36). Because conclusive evidence has yet to appear, none of these approaches has been universally accepted. In bridging model, erythrocyte aggregation occurs when the bridging forces exceeds the disaggregation forces. However, erythrocyte aggregation in depletion model occurs due to a lower concentration of protein or polymer near the cell surface comparing to the suspending medium (36). Dextran infusion can also increase plasma viscosity and yield stress, thus leading to red blood cell aggregation (35). In this study, it was determined that the application of TQ decreased the erythrocyte aggregation rate that was increased by the application of dextran 500. The application TQ might have caused one or some of the followings while decreasing the erythrocyte aggregation rate that was increased by

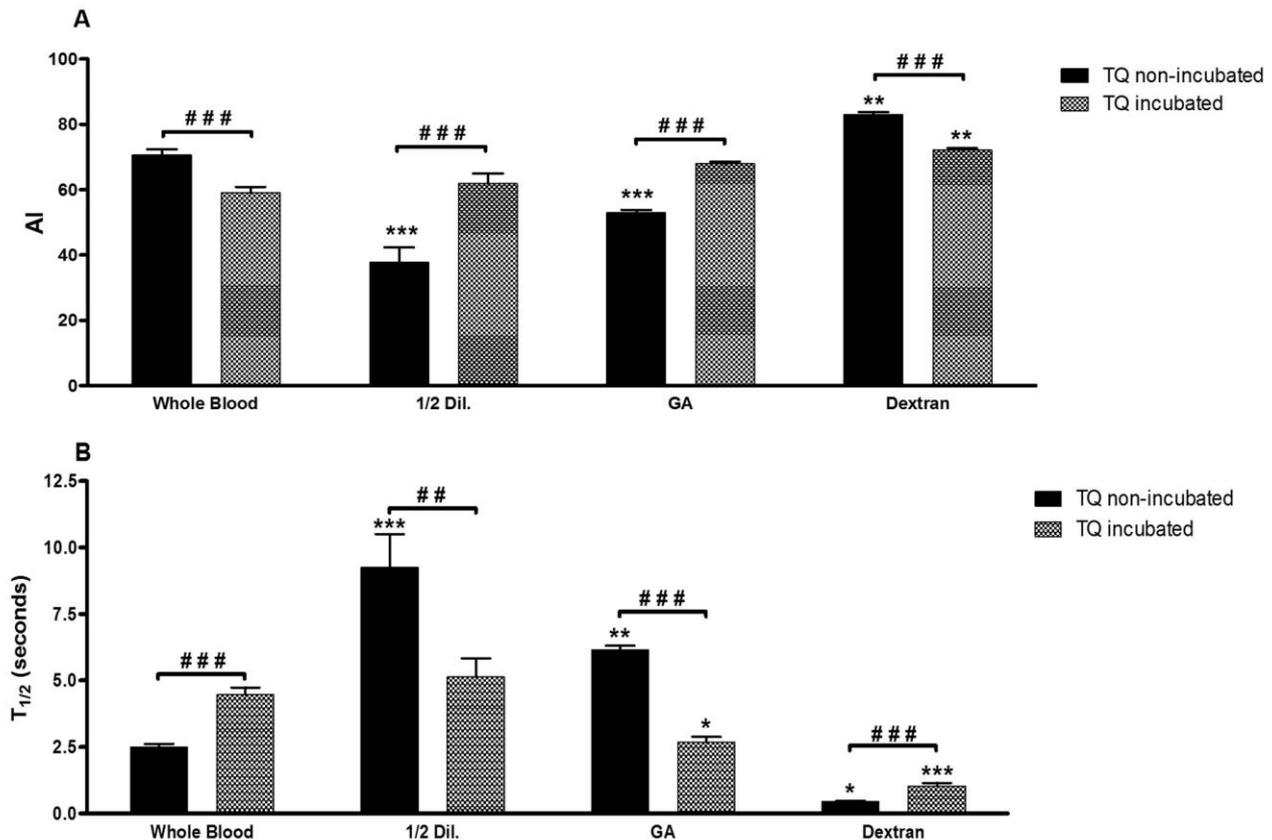


Fig. 4. The AI (4A) and T_{1/2} (4B) calculated from the C records of whole blood, 1/2 dilution, dextran, and GA groups in the absence or presence of TQ incubation. Results are expressed as mean ± standard error (n=10). Compared to whole blood; *: P < 0.05; **: P < 0.01; ***: P < 0.001, Compared to TQ non-incubated blood; ##: P < 0.01; ###: P < 0.001

the application of dextran 500: 1) It might have caused dextran 500, which was added into the suspension environment from the outside, to break down, 2) it might have caused the fibrinogen in the plasma to break down (fibrinolytic effect), 3) it might have caused the negative charge of the erythrocyte membrane to change, 4) it might have inhibited the potential of fibrinogen or/and dextran 500 to form aggregation. Since the fibrinolytic effect of TQ was not examined in the current study, further studies are needed. It has been demonstrated that *Nigella sativa* at 1.0 mg/ml produced an important protection against the loss of deformability of H₂O₂ treated erythrocytes and has been suggested that the protective effects of *Nigella sativa* on erythrocyte deformability could be due to its inhibitory activity on protein degradation. Since lower protein concentration near the cell surface versus the suspending medium cause erythrocyte aggregation (depletion model), it might be said that TQ decreased the elevated dextran 500-induced erythrocyte aggregation by inhibiting protein degradation (24).

Application of TQ increased the erythrocyte aggregation rate, which was decreased by GA. The GA, as a cross-linker is a small molecule with an uncomplicated structure and lowers the erythrocyte aggregation by decreasing the RBC deformability. It cross-links the entire cell and increases the cytoplasmic and lipid membrane viscosity with a reduction in cell deformability (37). A decrease in RBC deformability inhibits the formation of close contacts between adjacent cells. It has been reported that the maintenance of the normal shape and deformability of RBCs requires preservation of cellular ATP and low calcium ion content (38). An alteration in calcium metabolism causes a membrane stiffening, and thus reduced deformability of RBCs. The calcium antagonist activities of *Nigella sativa* has been shown in previous studies (39, 40). Therefore, increased-erythrocyte aggregation rate induced by the application of TQ in GA-treated RBCs could be due to decreased intracellular calcium accumulation due to the calcium antagonist activities of TQ.

It was also found that the TQ incubation increased the erythrocyte aggregation rate which

was decreased by plasma dilution in this study. Dilution of the plasma by a rate of 1/2 decreases erythrocyte aggregation rate by causing a decrease in the macromolecule concentration (generally fibrinogen) in plasma (3-6, 31-34). While it is not known how the TQ increased the erythrocyte aggregation in this suspension whose macromolecule concentration was decreased by half, it can be assumed that TQ itself acted like a macromolecule. In this case, however, it would have been expected to have the same effect on dextran suspensions, too; but it was found to have an opposite effect. In the laboratory stage of the study, TQ was observed not to dissolve in pure PBS. On the other hand, the dissolved TQ in ethanol turned into particles after being mixed with PBS at the rate of less than 1/100. That was the reason why we first dissolved the TQ in ethanol, then diluted with PBS at the rate of 1/100. Therefore, it might be said that the TQ turned into particles after being encountered with extra PBS in the 1/2 dilution suspension, and increased erythrocyte aggregation. However, TQ did not precipitate out of solution, given the solution electrochemical properties (and thus conductance data) were influenced by particle geometry and size. More studies are needed.

As a conclusion, TQ can normalize the altered erythrocyte aggregation responses in both ways that is, it decreases the increased aggregation or increases the decreased aggregation. In pathophysiological processes, increased fibrinogen level which leads to the erythrocyte aggregation causes the circulatory system, especially microcirculation disorders. Multiple organ failures and circulatory system disorders are among the most important causes of death. TQ might bring increased erythrocyte aggregation closer to normal levels, and this might have critical importance in the cases where circulatory disorders or increased rouleaux formation are observed in human. However, in this study, all experimental stages were performed *in vitro*. In further studies, possible changes in erythrocyte aggregation in pathological cases can be researched through the *in vitro* application of TQ. In addition, this is a rat model, therefore extrapolation of the results to any human relation is limited.

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