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Investigation of the myocardial damage due to acute anemia in a rabbit model of acute normovolemic anemia

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Abstract: Myocardial damage and increased circulating cardiac troponin I (cTnI) concentrations have been shown in various diseases that cause anemia in animals. Whether the acute anemia that occurs during these diseases directly contributes to myocardial damage and to the release of cTnI to the circulation still remains unclear. The objective of this study was to investigate the effect of acute anemia on myocardial damage and serum cTnI concentration using experimentally-induced acute normovolemic anemia. New Zealand rabbits were randomly separated into 2 groups: an acute anemia group (AG, n = 8) and a control group (CG, n = 6). To induce acute anemia, repeated jugular phlebotomy was performed. Blood samples were collected before the experiment (0) and at 24, 48, 72, 96, and 120 h. Serum cTnI concentrations were measured using a human specific cTnI assay. After the last blood sample was taken, euthanasia was performed to evaluate the histopathological changes and the cTnI immunolabelling in cardiomyocytes. In the AG, the mean packed cell volume (PCV) was 13.1 ± 1.4% at 120 h. The median serum cTnI concentrations in the AG and the CG were 0.075 (IQR; 0.017–0.077) and 0.015 ng/mL (IQR; 0.01–0.02) at the 120-h mark, respectively (P < 0.01). There was a negative correlation between PCV and serum cTnI concentrations (r = –0.52, P < 0.001). In addition, serum cTnI concentrations were significantly correlated with both the histopathological score (r = 0.75, P < 0.001) and cTnI immunoreactivity (r= –0.79, P < 0.001). The results of this study showed that acute anemia caused histopathological lesions and loss of cTnI immunolabelling in cardiomyocytes, along with an increase in serum cTnI concentrations.

Key words: Acute normovolemic anemia, cardiac troponin I, myocardial damage, rabbit

1. Introduction

Acute anemia is a frequently encountered problem in veterinary medicine, and it is characterized by a reduction of circulating red blood cells and a decrease in oxygen carrying capacity [1,2]. It may result from different causes, such as loss of blood from circulation, intravascular or extravascular hemolysis, and lack of production from bone marrow [3].

The heart plays a central role in maintaining tissue oxygenation during anemia. The continuation of tissue oxygenation during acute anemia is provided by compensatory mechanisms, such as an increase in both cardiac output and tissue oxygen extraction [4]. Decreased blood viscosity and increased nitric oxide activity play a key role in increased cardiac output [5,6]. Additionally, the activation of aortic chemoreceptors results in an increase in myocardial contractility [7]. All of these changes increase the oxygen requirement of the cardiac muscle during anemia by elevating cardiac muscle workload [8]; therefore, it is hypothesized in this study that anemia alone could cause cardiac muscle damage.

Cardiac muscle damage has been demonstrated by detecting elevated serum or plasma cardiac troponin I (cTnI) concentrations in various diseases that cause anemia, including babesiosis, theileriosis, ehrlichiosis, tick infestation, and primary immune mediated hemolytic anemia (IMHA) [9–14]. As shown in previous experimental sepsis studies [15,16], researchers have argued that the increase in circulating cTnI concentration is caused by systemic inflammatory reactions. Additionally, these studies have stated that acute anemia may also contribute to myocardial damage and increased circulating cTnI concentration, but the direct effect of anemia on myocardial damage and the release of cTnI to the circulation are still controversial. Some researchers argue that because of the simultaneous presence of possible causes of cardiac muscle damage, such as acute anemia, infectious agents, and systemic inflammatory reactions during diseases

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causing anemia, it is not possible to establish a causative relationship between acute anemia and cardiac muscle damage [11,17,18]. The aim of this study is to demonstrate whether anemia alone could cause cardiac muscle damage without any systemic inflammatory reactions by using an experimentally induced acute normovolemic anemia model in rabbits. Measuring serum cTnI concentrations and detecting the histopathological changes and cTnI immunolabelling in cardiomyocytes comprise the basic steps of the methodology followed in the present study.

2. Materials and methods

2.1. Animals

This study was performed with the approval of the Fırat University Local Ethics Committee of Experimental Animals (13.01.2016, 2015/117). The 14 New Zealand rabbits, at 3–6 months old, were supplied by the Fırat University Experimental Researching Centre (Elazığ, Turkey). The weight of the rabbits ranged from 2.73 to 3.63 kg. The experimental groups of this study consisted of an acute anemia group (AG, n = 8) and a control group (CG, n = 6). The rabbits were maintained individually in stainless steel cages (750 × 750 × 650 mm, length × width × height) with sawdust bedding at room temperature (20–22 °C), 60% relative humidity, and 12 h light/12 h dark photoperiods.

2.2. Induction of acute normovolemic anemia

The focus of this study was to test whether acute anemia could cause myocardial damage. In accordance with this aim, acute normovolemic anemia was induced using a modification of the previously described repeated phlebotomy technique [19]. As previously stated [20], 10 mL/kg of blood was safely removed from circulation with each jugular phlebotomy. Only one jugular phlebotomy was performed daily for 5 days to obtain a PCV value between 10%–15%. The PCV of each rabbit was determined daily before jugular phlebotomy. The blood was collected into 4.5 mL Lithium Heparin PST II Plus Blood Collection Tubes (BD, Plymouth, UK) for induction of anemia.

Immediately after phlebotomy, lactated Ringer's solution, the volume of which was equal to the removed PCV, was infused via ear vein catheter to maintain normovolemia. The volume of the removed packed cells was calculated by multiplying the collected blood volume by the PCV value that was measured before jugular phlebotomy. Subsequently, the collected blood to the lithium heparin tubes were centrifuged at 4 °C and 3000 × g for 10 min. After centrifugation, the plasma was immediately infused into the same rabbit via ear vein catheter.

2.3. Blood sampling and measurement of hematological and biochemical parameters

Blood samples were taken from the rabbits' jugular veins using a 21 G Safety-Lok blood collection set (BD Vacutainer, Plymouth, UK) for hematological and biochemical analysis. Blood sampling times occurred at 0, 24, 48, 72, 96, and 120 h in both groups. In the AG, blood samples were collected before daily jugular phlebotomy. While serum cTnI concentrations were measured to determine myocardial damage, PCV measurements were performed to determine the anemia. In addition, total white blood cell (WBC) counts and serum C-reactive protein (CRP) concentrations were measured to determine whether a systemic inflammatory reaction occurred during the experiment. Following blood collection, the serum samples were centrifuged at 3000 × g for 10 min. Serum cTnI concentration measurements were performed on the same day. In addition, the serum samples were stored at –20 °C for 2 months before CRP analysis. The PCVs were determined with a heparinised capillary tube by centrifuging for 5 min at 15000 × g (Sigma 1-16 Laboratory Centrifuge, Osterode, Germany). A rabbit specific ELISA kit (Fine Test, Wuhan Fine Biological Technology, Wuhan, China) was used to measure serum CRP concentrations. In order to measure serum cTnI concentrations, a human specific cTnI assay (Advia Centaur TnI-Ultra, Siemens Healthcare Diagnostics, Eschborn, Germany) was used. The Advia Centaur TnI-Ultra is a second-generation, 3-site sandwich immunoassay with chemiluminescence detection. The measurement range of this assay is 6–50.000 ng/L, as specified by the manufacturer. This human-based analyzer has been previously used and validated in rabbits [21].

2.4. Histopathological examination

The rabbits were euthanized by intravenous injection of ketamine hydrochloride (Ketasol, Richter Pharma AG, Vienna, Austria) at a dose of 120 mg/kg immediately after the last blood sampling. Initially, the hearts were examined macroscopically. Afterwards, tissue samples were collected from different parts of the hearts (interventricular septum, atriums, and ventricles) for microscopic examination. The samples were fixed in 10% neutral formalin solution. Selected heart tissues were processed routinely and embedded in paraffin. Subsequently, the heart tissue specimens were cut into 3-µm thick sections and stained with hematoxylin and eosin to examine inflammatory changes. The tissue sections were assessed for the presence and severity of both degeneration/necrosis and inflammatory cell infiltration. Histopathological changes were graded semiquantitatively as follows: 0 = no change, 1 = mild, 2 = moderate, and 3 = severe.

2.5. Immunohistochemical evaluation

An immunohistochemical examination was also performed on the tissue sections to evaluate cytoplasmic cTnI immunoreactivity by using a commercially available immunohistochemistry kit (ab64259, Abcam, Cambridge, UK). Initially, the formalin-fixed, paraffin-embedded

tissue sections were deparaffinized and rehydrated using xylene and graded alcohols, respectively. Afterward, endogen peroxidase activity was inhibited using 3% hydrogen peroxide for 10 min; then, the sections were placed in citrate-buffered saline for 5 min, and washed 2 times with phosphate buffered saline (PBS). Subsequently, the sections were incubated with primary antibody (ab10231, Abcam) for 1 h at room temperature. At the end of incubation with primary antibody, tissue sections were washed 4 times with PBS and incubated with biotinylated goat antimouse immunoglobulin G (ab64259, Abcam) for 10 min at room temperature. Having been washed 4 times, tissue sections were exposed to 3, 3′ -diaminobenzidine and counterstained with Mayer's hematoxylin. Tissue sections were examined with a fluorescence attachment on a triocular microscope (BX43; Olympus, Tokyo, Japan) and visualized with an image analysis system (DP72, Olympus, Tokyo, Japan). The immunolabeling score was assessed semiquantitatively by determining the percentage of cTnI-positive cells in 10 different fields of each section.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 21 (IBM Corp., Armonk, NY, USA). To test the normality of variables, the Shapiro–Wilk test was administered. The statistical significance value was set at $P < 0.05$. The data was reported as the mean ± standard deviation of the mean or median with interquartile range (IQR; 25th–75th percentile). The statistical difference between groups at different time points was analyzed with an independent sample t-test or Mann–Whitney U test. The correlation between variables was assessed using the Spearman rank correlation method.

3. Results

All of the rabbits used in this experimental study were clinically healthy before the experiment. In the AG, no changes were detected in appetite, water consumption, or attention to external stimuli until 96 h into the experiment. After this time point, 6 of the 8 rabbits in the AG were observed to have clinically apparent depression, inappetence, a reluctance to move, and a tachypneic breathing pattern.

3.1. Hematological and biochemical changes

The mean serum CRP concentrations and total WBC counts did not change in the AG and the CG throughout the experiment (Figures 1a and 1b). The baseline PCV means were 35.8 ± 0.9% and 38.0 ± 1.9% in the AG and the CG, respectively. The PCV means gradually decreased, and the targeted PCV was reached at the 96-h point of the experiments in the AG. Before the termination of the experiment (120 h), PCV means were 13.1 ± 1.4% and 30.5 ± 1.3% in the AG and the CG, respectively (Figure 1c). In the CG, PCV gradually decreased during the experiment in comparison to the baseline value, depending on the repeated blood sampling for hematological and biochemical analysis.

The baseline serum cTnI concentrations were 0.025 ng/mL (IQR; 0.01–0.03) and 0.011 ng/mL (IQR; 0.01–0.015) in the AG and the CG, respectively (Figure 1d). The serum cTnI concentration significantly increased ($P < 0.05$) to 0.050 ng/mL (IQR; 0.017 –0.077) at 72 h in the AG. The highest serum cTnI concentration was 0.075 ng/mL (IQR; 0.025–0.11) at 120 h. A statistically significant negative correlation ($r = -0.52$, $P < 0.001$) was detected between serum cTnI concentration and PCV.

3.2. Histopathological and immunohistochemical changes

There were no pathological lesions in the macroscopic examination of the hearts. Microscopically, histopathological lesions were not observed in the CG (Figure 2a), whereas focal myocardial degeneration and mononuclear cell infiltrations were evident in the AG (Figure 2b). Additionally, the nuclei of cardiomyocytes were pictonic, and muscle striations were invisible in the affected parts of the heart. The most affected parts were the interventricular septum and the ventricular endocardium. The mean of the histopathological grade was 2.50 ± 0.53 and 0.66 ± 0.51 in the AG and the CG, respectively. Analyzing the mean of the histopathological score revealed a significant difference between the AG and the CG ($P < 0.01$). In addition to the histopathological evidence, the loss of cTnI immunolabelling was prominent ($P < 0.01$) in the AG compared to the CG (Figures 2c and 2d). The positive cTnI immunolabeling score was significantly different between the AG (61.66 ± 9.83%) and the CG (95.00 ± 5.47%) Serum cTnI concentrations were also significantly correlated in both the histopathological score ($r = 0.75$, $P < 0.01$) and cytoplasmic cTnI immunoreactivity ($r = -0.79$, $P < 0.001$).

4. Discussion

The main result of this study is that acute anemia causes cardiac muscle damage and an elevation of serum cTnI concentrations. Previous studies have shown increased circulating cTnI concentration in various diseases accompanied by anemia in both companion and farm animals [9,11,12,14,17]. In a study performed in dogs with babesiosis, serum cTnI concentrations were 9.9 ± 5.76 ng/mL and 6.53 ± 4.32 ng/mL in complicated and concurrent IMHA groups, respectively [9]. Lalor et al. [17] demonstrated that anemic cats had higher serum cTnI concentrations than nonanemic sick cats. Increased serum cTnI concentrations were also reported for dogs with primary IMHA [11]. Serum cTnI concentrations were measured at 0.028 ± 0.008 ng/mL and 0.012 ± 0.009 ng/

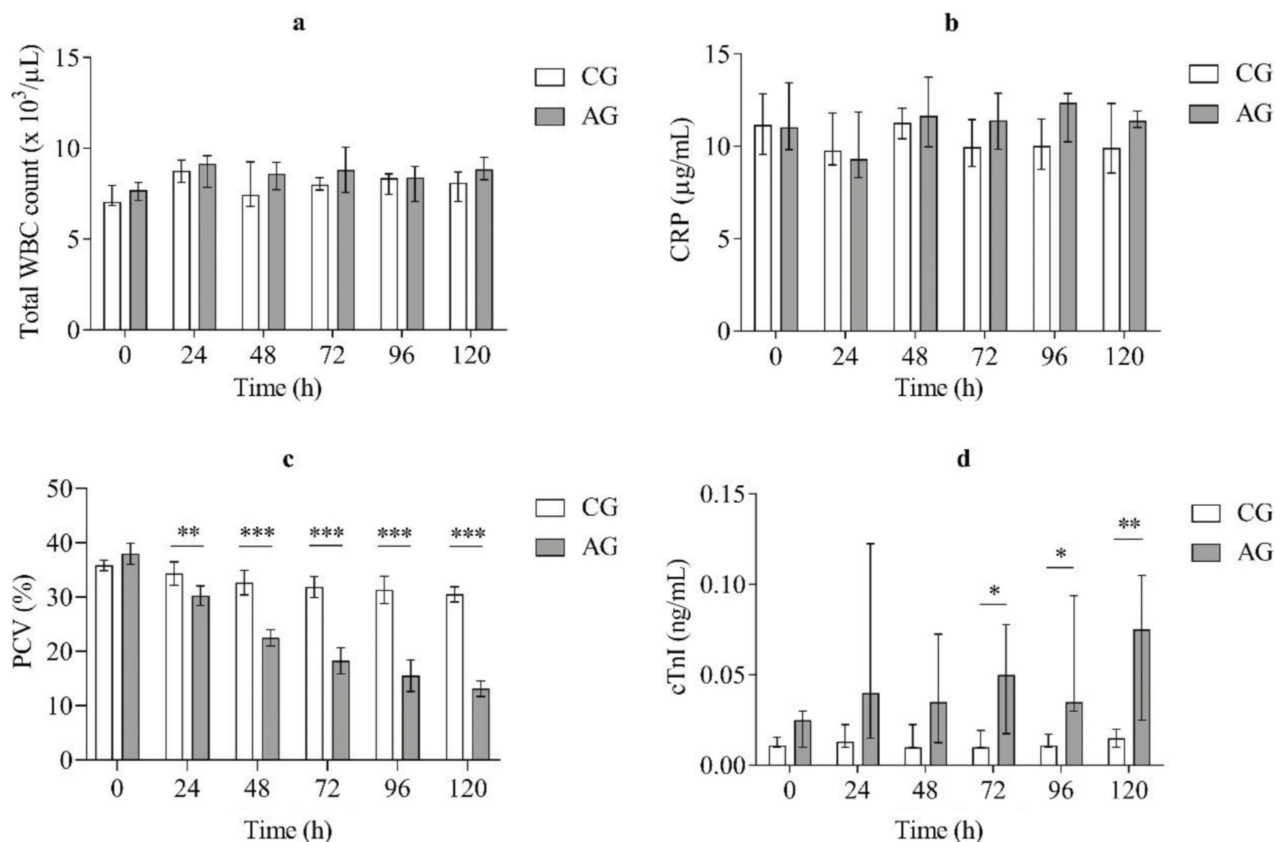

Figure 1. Changes and statistical differences in total WBC count (a); serum CRP concentration (b); PCV % (c); and serum cTnI concentration (d) at different time points between groups. CG: control group (n = 6); AG: anemia group (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001.

mL in cattle with tropical theileriosis and healthy control cattle, respectively [12]. Further evidence regarding increased serum cTnI concentration comes from a study conducted on sheep with babesiosis [14].

The myocardium is particularly susceptible to a decreased supply of oxygen due to its restricted anaerobic capacity [22]. It is thought that anemia contributes to myocardial damage by causing oxygen supply/demand mismatching [23]. Although the above-mentioned studies have shown myocardial damage with increased circulating cTnI concentrations in various diseases that cause acute anemia, whether acute anemia that occurs during these diseases directly contributes to myocardial damage and the release of cTnI to the circulation remains to be addressed. In these studies, the authors state that the simultaneous presence of different factors such as anemia, infectious agents, neoplasia, and systemic inflammatory reactions in these diseases may directly cause cardiac muscle damage; thus, it is not possible to establish a causative relationship between anemia and cardiac muscle damage [11,17,18]. Therefore, an experimental study in which only the effect of anemia on cardiac muscle damage is examined without any other cardiac muscle damage inducer was considered necessary.

An acute normovolemic anemia model, previously described in dogs, was used in the present study to examine the effect of acute anemia on histopathological change and cTnI immunoreactivity in cardiomyocytes and serum cTnI concentrations [19]. This acute normovolemic anemia model consists of the removal of blood from circulation and the simultaneous infusion of a crystalloid or colloidal solution to maintain normovolemia [24]. This approach has been used for many years in human medicine to decrease homologous blood transfusion during surgery [25]. Furthermore, an acute normovolemic anemia model was previously used to investigate the role of anemic hypoxia in the development of canine babesial nephropathy [26]. An acute normovolemic anemia model was also used to evaluate the effects of acute anemia on echocardiographic and hemodynamic changes in dogs, but serum cTnI concentrations were not evaluated in these studies [27,28]. The results of our experimental study clearly showed that acute anemia induced cardiomyocyte damage and caused the release of cTnI to the circulation. When damage occurs

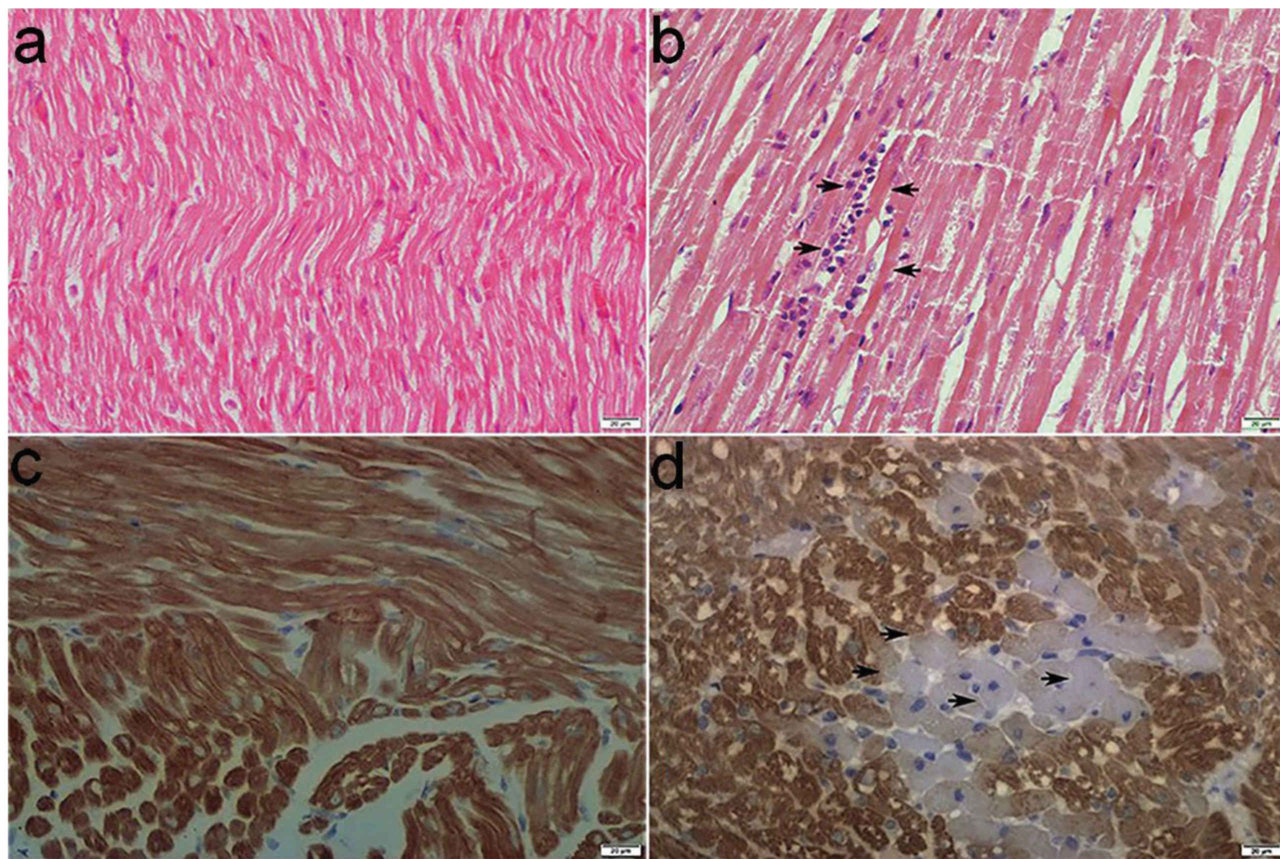

Figure 2. (a) section of left ventricle from a control group rabbit (H&E; × 40); (b) focal myocardial degeneration and inflammatory cell infiltrations (black arrow) in a left ventricle section of a rabbit in the anemia group (H&E; × 40); (c) cTnI immunoreactivity in a left ventricle section of a rabbit in the control group (ABC; × 40); (d) focal loss of cTnI immunoreactivity (black arrows) in a left ventricle section of a rabbit in the anemia group (ABC; × 40).

that disrupts cardiac myocyte membrane integrity, cTn is released into the circulation [29]. The investigators have shown that circulating cTnI concentrations reflected the magnitude of the histopathological changes and that increased serum cTnI concentrations were negatively correlated with cTnI immunoreactivity in cardiomyocytes [30,31]. Similar to previous reports, serum cTnI concentrations were positively correlated with the histopathological lesions score in this study. In addition,, a negative correlation was detected between serum cTnI concentration and cytoplasmic cTnI immunoreactivity in cardiomyocytes.

The exact mechanism of myocardial damage during acute anemia has not yet been completely understood. Several theories have been proposed to explain these underlying mechanisms, some focusing on hypoxia and others on the hyperdynamic ventricular state [18,27]. Numerous studies have investigated the correlation between the severity of anemia and serum cTnI concentrations [9,10,12]. Serum cTnI concentrations were correlated with PCV in cattle with tropical theileriosis [12]. In another study, a significant correlation was reported between serum cTnI concentrations and the severity of anemia in dogs infected with *Ehrlichia canis* [10]. A negative correlation was also detected between hemoglobin levels and serum cTnI concentrations in canine babesiosis [9]. In addition to the above-mentioned studies, Cartwright et al. [18] reported that serum cTnI concentrations decreased following the treatment of dogs with IMHA and improvement in hematocrit. Supporting the results in the relevant literature, as outlined above, the present study found a significant negative correlation between PCV and serum cTnI concentrations.

In conclusion, the results of this study showed that acute anemia caused histopathological lesions and loss of cTnI immunoreactivity in cardiomyocytes and an increase in serum cTnI concentrations. Further investigations are needed to establish a diagnostic cut-off point for both PCV and serum cTnI concentrations that can predict histopathological changes during acute anemia.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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