

## Original Article

# The effect of ozone on hypoxia, hemolysis and morphological change of blood from patients with aortic dissection (AD): a preliminary *in vitro* experiment of ozonated autohemotherapy for treating AD

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**Abstract:** This study aimed to investigate the effect of ozone on hypoxia, hemolysis and morphological change of blood from aortic dissection (AD) patients for providing preliminary evidence of application of ozonated autohemotherapy (Ozone-AHT) in AD patients. 20 AD patients and 20 healthy volunteers were consecutively included, and blood samples were collected from all participants and ozonized *in vitro*. PO<sub>2</sub>, SO<sub>2</sub>, malondialdehyde (MDA), superoxide dismutase (SOD), malformation percentage, morphology change and spatial distribution of filamentous actin (F-actin) in erythrocytes at different ozone concentrations were evaluated. After ozonation of whole blood, the median levels of PO<sub>2</sub> and SO<sub>2</sub> increased under Ozone concentrations at 40 µg/mL, 80 µg/mL and 160 µg/mL compared with samples exposed to 0 µg/mL in both AD group and control group. The MDA level was similar in samples exposed to 0 µg/mL, 40 µg/mL and 80 µg/mL ozone, while the levels of SOD increased in samples exposed to 40 µg/mL and 80 µg/mL in both AD group and control group. Compared with the samples exposed to 0 µg/mL ozone, Fhb level only increased in samples exposed to 80 µg/mL and 160 µg/mL Ozone in both AD group and control group. In addition, overdosed ozone (160 µg/mL) but not therapeutic ozone concentrations (0 µg/mL, 40 µg/mL and 80 µg/mL) increased malformation percentage and morphology change of erythrocytes in both AD group and control group. In conclusion, Ozone improves oxygen content and reduces oxidative damage in blood from AD patients, and therapeutic dose ozone do not induce hemolysis and morphology change of erythrocytes.

**Keywords:** Ozonated autohemotherapy (Ozone-AHT), aortic dissection, oxygenation, antioxidant, morphological change, erythrocytes

## Introduction

Aortic dissection (AD), caused by the tear of intima of the aorta, is a fatal disease with high mortality in patients both before and after hospital admission [1]. AD patients always suffer from unbearable chest or back pain, ischemia and systemic symptoms. The treatment options mainly include open surgery and endovascular management, between which the open surgery might be the most efficient treatment for patients, however, it is also one of the most complicated operations in cardiac surgery, with extremely high fatality rate and severe postoperative complications, such as hypoxemia and

neurological complications (delirium, confusion, et al) [2].

Ozone (O<sub>3</sub>) therapy has been applied and extensively researched for more than a century, despite of a few severe side effects, it is believed that the O<sub>3</sub> therapy is effective in treating multiple diseases such as heart failure and cervical spondylodiscitis [3-5]. O<sub>3</sub>, a strong oxidant that is 10-fold more soluble than oxygen, is capable of providing sufficient energy and oxygen supply to the damaged tissues. Accumulating studies illustrate that O<sub>3</sub> might improve the ischemia of injured tissue via delivering the oxygen to tissues, activating the pen-

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tose phosphate pathway, elevating 2,3-DPG content in erythrocyte and stimulating the erythrocyte oxygen metabolism [6]. Furthermore,  $O_3$  also can protect the blood cell from oxidative stress by inducing the expression of antioxidant enzymes [7]. Ozonated autohemotherapy (Ozone-AHT) is firstly applied in clinical practice in 1968, and now is widely used in various clinical disciplines, specifically in systemic disease and ischemic disease [8, 9]. In light of the benefit in other systemic and ischemic diseases, we assumed that Ozone-AHT could change the hypoxemia of AD, however there is no report about Ozone-AHT on treating AD patients.

Thus, our study aimed to investigate the effect of ozone on hypoxia, hemolysis and morphological changes of blood from AD patients for providing preliminary evidence of the application of Ozone-AHT in AD patients.

### Materials and methods

#### *Collection*

Twenty patients with AD (AD group) and 20 healthy volunteers (control group) with no smoking and treatment histories were consecutively included in the First Affiliated Hospital of Harbin Medical University from March 2016 to Aug 2016. Blood samples of 20 ml were drawn from vein from AD patients and healthy volunteers, and subsequently stored in the polyvinyl chloride (PVC) bags (20 U/mL blood). In addition, the blood samples from AD patients were collected right after the arrival in the emergency department, and AD patients did not receive any medication, including oxygen administration at the time of the sample collection. Approval from the Ethics Committee of the First Affiliated Hospital of Harbin Medical University was obtained and informed consents were collected from all participants.

#### *Ozone generation and measurement*

Ozone was generated from medical-grade oxygen ( $O_2$ ) through electrical corona arc discharge using the  $O_3$  generator (Model Ozonosan PM 100K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and  $O_3$  concentration to be controlled in real time. The procedure was conducted under standard condition

according to the Standardisation Committee of the International  $O_3$  Association (IOA). The ozone flow-rate was kept constant at 3 L/min in all machines. Tygon polymer tubes and single-use silicon treated syringes (ozone-resistant) were used throughout the procedure to ensure containment and concentrations.

#### *Gas delivery to blood samples*

According to the method described by Valter Travagli et al [10], gas delivery was carried out with a single dose of ozone (concentration per volume) as follows: a predetermined volume of gas mixture composed of  $O_2$  (95%) and  $O_3$  (5%) with ozone concentrations at 0, 40, 80, 160  $\mu\text{g/mL}$  was collected by a second syringe and immediately introduced into the blood samples. The optimal blood sample/gas ratio was 1:1. The syringes were in a monodirectional oscillator (60 cycles/min) for 20 min until the foaming became minimal, which indicated the liquid and gas were mixed completely.

#### *Erythrocyte membrane preparation*

Erythrocyte membranes were obtained according to the method modified by Dodge et al [11]. Erythrocytes were lysed using 4°C hypotonic Tris-HCL (PH=8.0, 10 mM) solution with 0.02 mM phenylmethanesulfonyl fluoride (PMSF) (PMSF was dissolved with glycerol) at 4°C for 12 h with the volume ratio 1:5. And then, the erythrocyte membranes were centrifuged at 15,000×g for 20 min, after the supernatant was extracted, the remaining part was washed three times with the same above-mentioned hemolysis buffer until the purified white membranes (ghost protein) were obtained, which only contains the erythrocyte membranes. The final membrane was resuspended in 250  $\mu\text{L}$  of cold Tris-HCL (PH=8.0, 10 mM) buffer and stored. The protein concentration at the membranes was determined by BCA Protein Assay Kit (Beyotime Biotechnology Co., Guangzhou, China).

#### *Physicochemical determinations*

The effect of ozone on improving the hypoxia of blood obtained from AD patients and controls were determined by evaluating  $PO_2$ ,  $SO_2$ , which were examined by an ABL800 blood gas analyzer (Radiometer, Danmark).

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**Table 1.** Characteristics of all participants

Parameter	AD group (N=20)	Control group (N=20)	P value
Age (years)	50.10±10.74	41.35±7.65	0.005
Gender (male/female)	16/4	13/7	0.288
Weight (kg)	66.10±9.50	70.90±10.76	0.143
History of hypertension (n/%)	12 (60.0)	0 (0.0)	<0.001
History of coronary heart disease (n/%)	6 (30.0)	0 (0.0)	0.007
History of drink (n/%)	9 (45.0)	4 (20.0)	0.176
HCT (%)	33.50 (30.75-35.00)	36.50 (35.00-44.00)	<0.001
PO <sub>2</sub> (mmHg)	49.40 (45.13-53.30)	49.98 (46.75-55.05)	0.499
SO <sub>2</sub> (%)	72.95 (69.33-77.38)	69.41 (64.64-74.16)	0.088
FHb (µg/mL)	111.59 (107.27-113.44)	23.89 (22.27-25.00)	<0.001
MDA (nmol/mg prot <sup>-1</sup> )	7.53 (6.68-8.11)	6.71 (5.13-7.87)	0.028
SOD (U/mg prot <sup>-1</sup> )	12.50 (11.83-13.33)	13.10 (12.48-14.35)	0.110
Malformation percentages of erythrocyte (%)	3.78 (2.87-5.21)	5.23 (2.72-6.45)	0.425
Erythrocyte membrane surface parameters			
D (µm)	7.76 (7.72-7.79)	7.67 (7.50-7.79)	0.078
H (µm)	1.57 (1.50-1.64)	1.64 (1.54-1.73)	0.208
Depth (µm)	1.31 (1.29-1.33)	1.33 (1.25-1.45)	0.378
Rq (nm)	17.15 (12.55-19.53)	8.45 (7.73-10.60)	<0.001
Ra (nm)	9.80 (9.30-14.48)	5.58 (3.63-7.87)	<0.001

Data was presented as mean ± standard deviation, median (25<sup>th</sup>-75<sup>th</sup>) or count (%). Comparison between two groups was determined by t test, Chi-Square test or Wilcoxon rank sum test. *P*<0.05 was considered significant. AD, aortic dissection; HCT, hematocrit; PO<sub>2</sub>, partial pressure O<sub>2</sub>; SO<sub>2</sub>, oxygen saturation; FHb, free hemoglobin; MDA, malondialdehyde; SOD, superoxide dismutase; D, diameter; H, height; Depth, concave depth; Rq, root-mean-square roughness; Ra, roughness.

### Enzyme-linked immuno sorbent assay (ELISA)

Free hemoglobin (FHb) concentration, malondialdehyde (MDA) and superoxide dismutase (SOD) of erythrocyte membrane were evaluated by ELISA kits (YuanMu Biological technology Co., Shanghai, China) following the manufacturer's protocol.

### Morphological determinations

**Scanning electron microscopy (SEM) sample preparation:** To begin with, the ozone preconditioned samples were centrifuged at 3000 rpm for 10 mins, after which the supernatant (plasma, white blood cells and platelets) was discarded. Then one drop of each sample was added into EP tubes containing 500 µL 2.5% glutaraldehyde DPBS with pH of 7.4 and stand for 120 mins. This was followed by rinsing twice for 3 mins before being fixed for 15 min with 1% osmium tetroxide (OsO<sub>4</sub>). The samples were rinsed thrice with dulbecco's phosphate buffered saline (DPBS) for 2 mins and were dehydrated serially 3 times with 100% ethanol, about 30 µL of each sample were placed on

glass cover slips, air-dried at room temperature, gold coated for 3 min at 13.3 Pa in a sputter device (Edwards S 150, Sussex, England) and examined in a scanning electron microscope (JEOL JSM-6380LV, Japan).

**Atomic force microscope (AFM) sample preparation:** All the ozone preconditioned samples were centrifuged (3000 rpm×5 min), after which the supernatant was discarded and the erythrocytes was suspended in 2.5% glutaraldehyde for 60 mins, and rinsed with DPBS and post-fixed with OsO<sub>4</sub> to protect membrane phospholipids. The samples were then dehydrated with ethanol and dried on a glass cover slip.

Cell morphology of erythrocyte were obtained from AFM (Dimension Edge, Bruker, Germany) tapping mode. 0.01-0.025 Ohm-cm Antimony (n) doped Si tips (RTESP, Bruker, Germany) with a spring constant of 40 N/m, a resonant frequency of 300 kHz and a nominal tip radius of 8 nm were employed. Six cells were scanned randomly in the air using tapping mode of each sample at the following fields: 20×20 µm<sup>2</sup>, 10

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**Table 2.** Levels of PO<sub>2</sub> and SO<sub>2</sub> in human blood samples after exposure to different Ozone concentrations

Concentrations	0 µg/mL	40 µg/mL	80 µg/mL	160 µg/mL	P value
PO <sub>2</sub> (mmHg)					
AD group	49.40 (45.13-53.30)	426.80 (395.60-474.93) <sup>a</sup>	576.51 (517.28-664.95) <sup>a,b</sup>	602.45 (538.69-654.53) <sup>a,b</sup>	<0.001
Control group	49.98 (46.75-55.05)	405.37 (361.24-526.32) <sup>a</sup>	584.54 (535.39-640.37) <sup>a,b</sup>	608.68 (534.10-658.76) <sup>a,b</sup>	<0.001
P value	0.499	0.589	0.871	0.935	
SO <sub>2</sub> (%)					
AD group	72.95 (69.33-77.38)	99.58 (99.23-99.78) <sup>a</sup>	99.90 (99.80-100.00) <sup>a</sup>	100.16 (99.82-100.77) <sup>a,b</sup>	<0.001
Control group	69.41 (64.64-74.16)	99.95 (99.44-100.08) <sup>a</sup>	99.70 (99.28-99.95) <sup>a</sup>	99.76 (99.33-100.31) <sup>a</sup>	<0.001
P value	0.088	0.013	0.046	0.042	

Data was presented as median (25<sup>th</sup>-75<sup>th</sup>). Comparison among different concentrations was determined by Kruskal-Wallis H rank sum test, and a Dunn-Bonferroni test for post hoc comparisons. Comparison between AD groups and C group was determined by Wilcoxon rank sum test. *P*<0.05 was considered significant. <sup>a</sup>Ozone concentrations 0 µg/mL vs 40 µg/mL, 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05), <sup>b</sup>Ozone concentrations 40 µg/mL vs 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05). AD, aortic dissection; PO<sub>2</sub>, partial pressure O<sub>2</sub>; SO<sub>2</sub>, oxygen saturation.

×10 µm<sup>2</sup>, 1×1 µm<sup>2</sup>. NanoScope Analysis (Bruker, Germany) was used to filter out any noise, subtract the plane of average inclination and perform all measurements. The following parameters were measured: the diameter, height and concave depth of the erythrocyte. The parameters for evaluating cell surface roughness are arithmetic average roughness (Ra) and root-mean-square roughness (Rq). Membrane roughness was measured by 1×1 µm<sup>2</sup> erythrocyte edge bulge.

**Confocal sample preparation:** Ozone preconditioned samples were centrifuged and supernatant was extracted, afterwards 100 µL of pellets of each sample were transferred to EP tubes containing 5 ml of 2.5% glutaraldehyde DPBS with pH of 7.4 and stand at room temperature for 12 h. Then 1 ml of cell suspension was taken after vortex and centrifuged at 3000 rpm for 5 mins, after which around 7×10<sup>6</sup> cells were collected, which were rinsed twice with DPBS (3000 rpm×5 min), and then the supernatant was discarded. The cells were suspended in 200 µL DPBS containing 0.1% TritonX-100 and subsequently incubated at room temperature for 5 mins, rinsed twice with DPBS (3000 rpm×5 min), and then extracted the supernatant. Subsequently, the cells were suspended in 200 µL DPBS containing 1% BSA, incubated at room temperature for 30 min, rinsed twice with DPBS (3000 rpm×5 min), then the supernatants was discarded. And then the cells were suspended in 200 µL DPBS containing 10 µL phalloidin-rhodamine (Sigma, St. Louis, MO) (500 µL/mL, dissolved in methanol); incubation for 60 min at 37°C in darkness, rinsed thrice with DPBS (3000 rpm×5 min), and then the

supernatants was discarded. Finally, the cells were suspended in 4 ml of DPBS, preserved at 4°C in darkness. Cell suspension of 100 µL was used to observe cytoskeleton of erythrocytes under laser confocal microscope.

### Results

#### Characteristics of AD patients and controls

As listed in **Table 1**, the mean age of patients in AD group was older than that in the control group (*P*=0.005). No difference of gender and weight was found between two groups (*P*=0.288 and *P*=0.143, respectively). The number of patients with history of hypertension and coronary heart disease were 12 (60.0%) and 6 (30.0%), and no patient had those histories in control group (*P*<0.001 and *P*=0.007, respectively). In addition, patients in the AD group had reduced median level of hematocrit (HCT) compared with control group (*P*<0.001). In the meantime, the median levels of Fhb and MDA in AD group were increased than that in the control group (*P*<0.001 and *P*=0.028, respectively). Additionally the median value of two erythrocyte membrane surface parameters (Rq and Ra) were notably greater in the AD group compared with control group (all *P*<0.001). No difference of other characteristics was found between two groups.

#### The effect of ozone on oxygenation

After ozonation of whole blood, the median levels of PO<sub>2</sub> and SO<sub>2</sub> were increased in samples with ozone concentrations at 40 µg/mL, 80 µg/mL and 160 µg/mL in both AD group and control group (all *P*<0.05) compared with samples

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**Table 3.** Levels of MDA and SOD in human blood samples after exposure to different Ozone concentrations

Concentrations	0 µg/mL	40 µg/mL	80 µg/mL	160 µg/mL	P value
MDA (nmol/mg prot <sup>-1</sup> )					
AD group	7.53 (6.68-8.11)	7.10 (6.09-8.02)	7.36 (6.38-7.96)	9.37 (8.51-10.01) <sup>a,b,c</sup>	<0.001
Control group	6.71 (5.13-7.87)	6.46 (5.42-7.46)	6.08 (5.39-7.45)	8.93 (8.23-9.55) <sup>a,b,c</sup>	<0.001
P value	0.028	0.152	0.012	0.310	
SOD (U/mg prot <sup>-1</sup> )					
AD group	12.50 (11.83-13.33)	14.45 (13.45-15.71) <sup>a</sup>	15.33 (13.71-16.36) <sup>a</sup>	10.85 (10.25-11.96) <sup>b,c</sup>	<0.001
Control group	13.10 (12.48-14.35)	16.38 (15.38-17.67) <sup>a</sup>	16.88 (15.88-18.49) <sup>a</sup>	12.13 (10.67-12.67) <sup>b,c</sup>	<0.001
P value	0.03e.110	0.006	0.012	0.055	

Data was presented as median (25<sup>th</sup>-75<sup>th</sup>). Comparison among different concentrations was determined by Kruskal-Wallis H rank sum test, and a Dunn-Bonferroni test for post hoc comparisons. Comparison between AD group and C group was determined by Wilcoxon rank sum test. *P*<0.05 was considered significant. <sup>a</sup>Ozone concentrations 0 µg/mL vs 40 µg/mL, 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05), <sup>b</sup>Ozone concentrations 40 µg/mL vs 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05), <sup>c</sup>Ozone concentrations 80 µg/mL vs 160 µg/mL are significantly different (*P*<0.05). AD, aortic dissection; MDA, malondialdehyde; SOD, superoxide dismutase.

**Table 4.** Levels of Fhb in human blood samples after exposure to different Ozone concentrations

Concentrations	0 µg/mL	40 µg/mL	80 µg/mL	160 µg/mL	P value
Fhb (µg/mL)					
AD group	111.59 (107.27-113.44)	115.81 (113.03-118.22)	119.89 (118.21-125.62) <sup>a</sup>	186.98 (183.80-189.58) <sup>a,b,c</sup>	<0.001
Control group	23.89 (22.27-25.00)	23.89 (22.27-25.00)	28.48 (24.51-30.47) <sup>a</sup>	30.99 (27.71-35.00) <sup>a,b</sup>	<0.001
P value	<0.001	<0.001	<0.001	<0.001	

Data was presented as median (25<sup>th</sup>-75<sup>th</sup>). Comparison among different concentrations was determined by Kruskal-Wallis H rank sum test, and a Dunn-Bonferroni test for post hoc comparisons. Comparison between AD groups and C group was determined by Wilcoxon rank sum test. *P*<0.05 was considered significant. <sup>a</sup>Ozone concentrations 0 µg/mL vs 40 µg/mL, 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05), <sup>b</sup>Ozone concentrations 40 µg/mL vs 80 µg/mL or 160 µg/mL are significantly different (*P*<0.05), <sup>c</sup>Ozone concentrations 80 µg/mL vs 160 µg/mL are significantly different (*P*<0.05). AD, aortic dissection; Fhb, free hemoglobin.

exposed to 0 µg/mL ozone (Table 2). In addition, the median PO<sub>2</sub> levels increased in samples exposed to 80 µg/mL and 160 µg/mL compared with that of samples exposed to 40 µg/mL ozone in both AD group and control group (all *P*<0.05). No difference of PO<sub>2</sub> median levels in samples was disclosed between two groups at different ozone concentrations.

As to the median SO<sub>2</sub> level, it was elevated only in the 160 µg/mL samples when compared with the 40 µg/mL samples (*P*<0.05) in AD group. In addition, the SO<sub>2</sub> median level was notably lower in 40 µg/mL samples but higher in 80 µg/mL and 160 µg/mL samples in AD group compared with control group (all *P*<0.05).

### The effect of ozone-AHT on antioxidant effect

As presented in Table 3, the MDA median levels were similar in samples exposed to 0 µg/mL, 40 µg/mL and 80 µg/mL ozone in both two groups (all *P*>0.05). However, when exposed to 160 µg/mL, the MDA median level was obviously elevated compared to that in samples with other ozone concentrations (all *P*<0.05). In addition, the MDA median level was increased

in AD group when exposed to 0 µg/mL and 80 µg/mL ozone compared with control group (*P*=0.028 and *P*=0.012, respectively).

The median levels of SOD in both two groups increased in samples exposed to 40 µg/mL, 80 µg/mL and 160 µg/mL compared to that in samples exposed to 0 µg/mL (all *P*<0.05). However, the SOD median level in 160 µg/mL samples was markedly higher when compared that in the 40 µg/mL and 80 µg/mL samples in both two groups (all *P*<0.001). In addition, the SOD median level was declined in AD group than control group at 40 µg/mL and 80 µg/mL ozone concentrations (*P*=0.006 and *P*=0.012, respectively).

### The effect of ozone-AHT on Fhb level

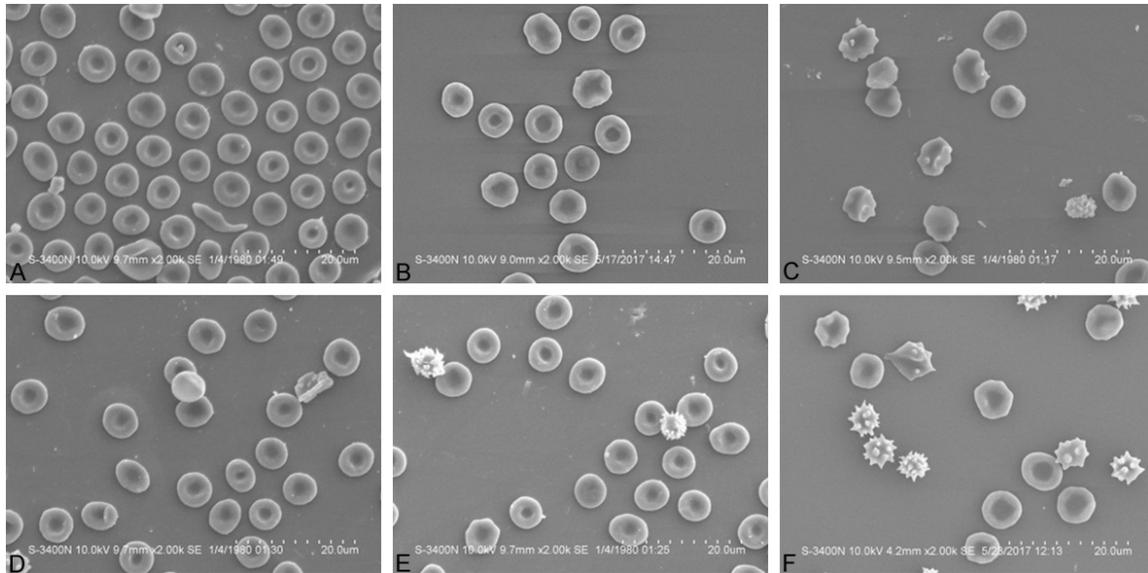
The Fhb level in AD group and control group only increased in samples at 80 µg/mL and 160 µg/mL ozone concentrations compared to samples at 0 µg/mL ozone concentration (all *P*<0.001). Additionally, in AD group, the Fhb level in samples with 160 µg/mL ozone concentration was higher compared with that in samples with 40 µg/mL and 80 µg/mL ozone

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**Table 5.** The malformation percentages of the erythrocyte in different Ozone concentrations

Concentrations	0 µg/mL	80 µg/mL	160 µg/mL	P value
Malformation percentages of erythrocyte (%)				
AD group	3.78 (2.87-5.21)	5.65 (4.62-8.96)	13.24 (10.43-15.26) <sup>a,b</sup>	<0.001
Control group	5.23 (2.72-6.45)	5.36 (3.62-7.53)	10.32 (7.97-16.24) <sup>a,b</sup>	<0.001
P value	0.425	0.482	0.223	

Data was presented as median (25<sup>th</sup>-75<sup>th</sup>). Comparison among different concentrations was determined by Kruskal-Wallis H rank sum test, and a Dunn-Bonferroni test for post hoc comparisons. Comparison between AD group and Control group was determined by Wilcoxon rank sum test.  $P < 0.05$  was considered significant. <sup>a</sup>Ozone concentrations 0 µg/mL vs 80 µg/mL or 160 µg/mL are significantly different ( $P < 0.05$ ), <sup>b</sup>Ozone concentrations 80 µg/mL vs 160 µg/mL are significantly different ( $P < 0.05$ ). AD, aortic dissection.



**Figure 1.** The malformation percentages of the erythrocyte when exposed to the different ozone concentrations by SEM. In control Healthy erythrocytes presented uniform and circular biconcave shapes at 0 µg/mL ozone concentration (A), and the malformation percentage did not change when the cells exposed to the ozone concentration at 80 µg/mL (B). The malformation percentage was elevated when the cells exposed to the ozone concentration at 160 µg/mL (C). In AD group, the malformation percentage slightly increased compared with control group at 0 µg/mL ozone concentration (D). However, no malformation percentage change was observed when the blood exposed to 80 µg/mL ozone (E), the malformation percentage was risen when the blood exposed to 160 µg/mL ozone (F) ( $P < 0.05$ ). Bar = 10 µm, original magnification = ×2000. Comparison between two groups was determined by t test.  $P < 0.05$  was considered significant.

concentrations (all  $P < 0.001$ ). And in control group, the Fhb level increased in 160 µg/mL samples compared with that in the 40 µg/mL samples ( $P < 0.001$ ). In addition, Fhb levels were elevated in AD group compared with that in control group at all ozone concentrations (all  $P < 0.001$ ) (Table 4).

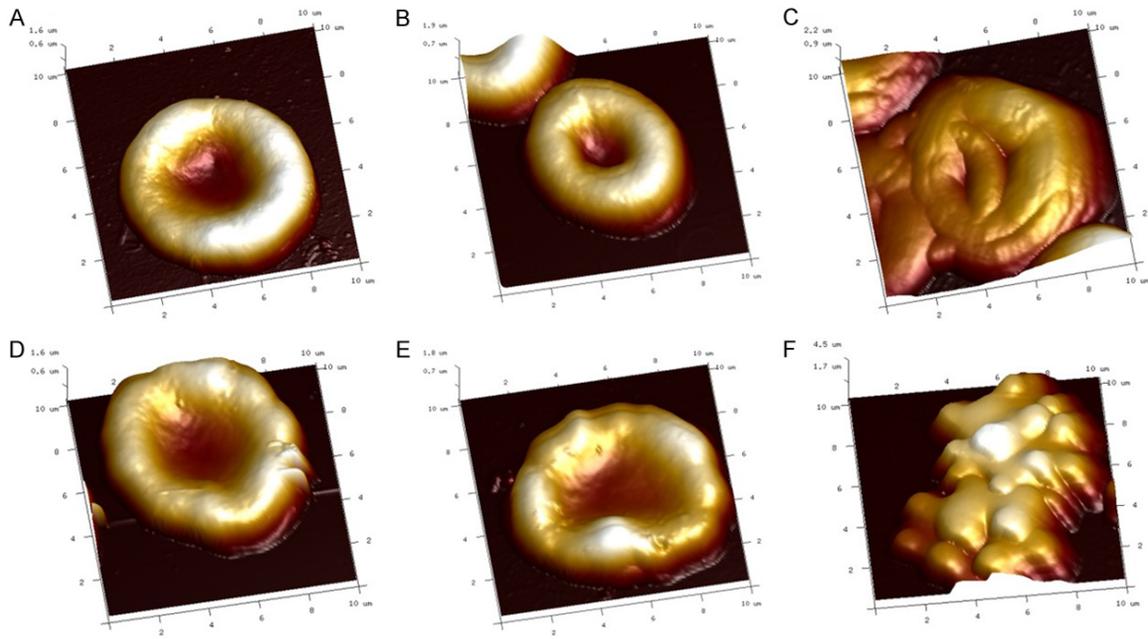
### *The malformation of erythrocyte under different ozone concentrations*

As shown in Table 5, the malformation percentage of the erythrocyte increased when samples were exposed to ozone with concentration 160 µg/mL compared with that in samples at 0 µg/

mL and 40 µg/mL ozone concentration (all  $P < 0.001$ ). No difference was found between AD group and control group in regard to different ozone concentrations ( $P = 0.425$ ,  $P = 0.482$  and  $P = 0.223$ , respectively).

As shown in Figure 1, in control group, the healthy erythrocytes presented uniform and circular biconcave shapes (Figure 1A), and there was no malformation percentage increase when the cells exposed to the ozone concentration at 80 µg/mL (Figure 1B), while the malformation percentages increased when the cells exposed to the ozone concentration at 160 µg/mL compared to that of cells exposed to 0 µg/

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**Figure 2.** The morphological change of the erythrocyte when blood exposed to the ozone by AFM 3D image. In the control group, the erythrocyte was a biconcave shape and with regular cell surface lattice structure (A), there was no morphological change when the erythrocyte exposed to 80  $\mu\text{g}/\text{mL}$  ozone (B). However, the morphology changed when the ozone concentration reached 160  $\mu\text{g}/\text{mL}$  compared to that at 0  $\mu\text{g}/\text{mL}$  ozone concentration (C). In the AD-group, the shape remained to exhibit double concave disc (D). There was no worse change of erythrocyte exposed to ozone concentration at 80  $\mu\text{g}/\text{m}$  (E), but the morphology changed greatly when the cell exposed to 160  $\mu\text{g}/\text{mL}$  ozone (F). Scanning area: 10  $\mu\text{m}\times 10\ \mu\text{m}$ . Comparison between two groups was determined by t test.  $P<0.05$  was considered significant.

mL and 80  $\mu\text{g}/\text{mL}$  ozone concentration (**Figure 1C**) ( $P<0.05$ ).

In AD group, the malformation percentage was slightly increased compared with control group ( $P>0.05$ ) at 0  $\mu\text{g}/\text{mL}$  ozone concentration (**Figure 1D**). However, there was no malformation percentage change when the blood exposed to 80  $\mu\text{g}/\text{mL}$  ozone (**Figure 1E**), the malformation percentage was risen when the blood exposed to 160  $\mu\text{g}/\text{mL}$  ozone (**Figure 1F**) ( $P<0.05$ ).

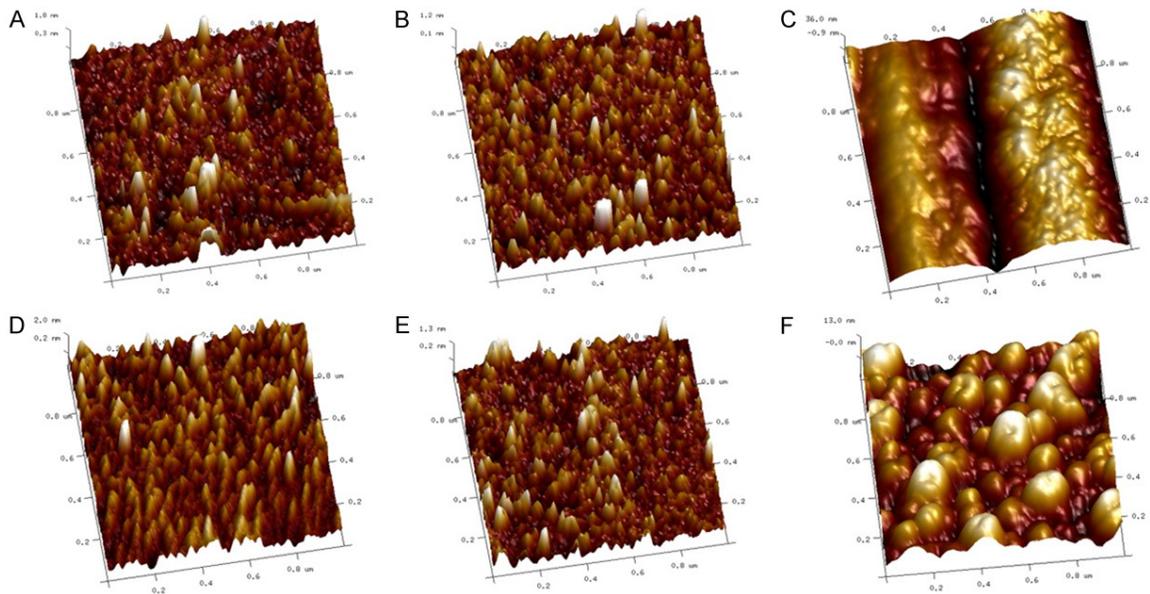
### *The morphology change of erythrocyte under different ozone concentrations*

As presented in **Figure 2A**, in the control group, the erythrocyte was a biconcave shape and with regular cell surface lattice structure, there was no morphological change when the erythrocyte exposed to 80  $\mu\text{g}/\text{mL}$  ozone (**Figure 2B**), however, the change happened when the ozone concentration reached 160  $\mu\text{g}/\text{mL}$  compared to that of samples exposed to 0  $\mu\text{g}/\text{mL}$  ozone concentration (**Figure 2C**). In the AD-group, although some abnormal changes were ob-

served on the cells, the shape remained to exhibit double concave disc (**Figure 2D**). There was no worse change when cell exposed to ozone concentration at 80  $\mu\text{g}/\text{m}$  (**Figure 2E**), but the morphology changed largely when the cell exposed to 160  $\mu\text{g}/\text{mL}$  ozone (**Figure 2F**).

As presented in **Figure 3**, the cell membrane was relatively smooth and intact, which was formed by strengthening different sizes of concave and convex surfaces, like ears of corn with uniform density, and the gaps between uplifts of concave depth were relatively equally distributed under ozone concentration at 0  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  (**Figure 3A, 3B**). While, different sizes of protrusions appeared on cell membrane surface, the surface concavity and convexity were enhanced and disoriented, and the surface roughness was increased under ozone concentration at 160  $\mu\text{g}/\text{mL}$  (**Figure 3C**). Similar 3D images of erythrocytes under ozone concentrations at 0  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  were observed in AD group (**Figure 3D, 3E**). However, as presented in **Figure 3F** obvious protrusions appeared on cell membrane surface under ozone concentration at 160  $\mu\text{g}/\text{mL}$ .

## Ozonated autohemotherapy in AD patients



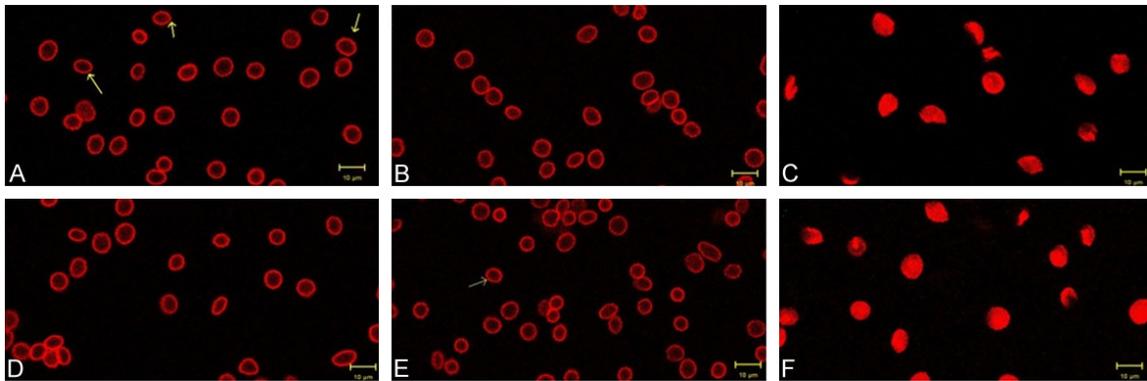
**Figure 3.** The morphological change of the erythrocyte when blood exposed to the ozone by AFM 3D image. The cell membrane was smooth and intact, like ears of corn with uniform density under ozone concentration at 0 µg/mL (A) and 80 µg/mL (B). However, the surface concavity and convexity were enhanced and disoriented, and the surface roughness was increased under ozone concentration at 160 µg/mL (C). Similar 3D images of erythrocytes under ozone concentrations at 0 µg/mL (D) and 80 µg/mL (E) were observed in AD group. However, obvious protrusions appeared on cell membrane surface under ozone concentration at 160 µg/mL (F). Scanning area: 1 µm×1 µm. Comparison between two groups was determined by t test.  $P < 0.05$  was considered significant.

**Table 6.** Erythrocyte membrane surface parameters measurement results

Concentrations	0 µg/mL	80 µg/mL	160 µg/mL	P value
<b>D (µm)</b>				
AD group	7.76 (7.72-7.79)	7.79 (7.76-7.91)	7.51 (7.41-7.61) <sup>a,b</sup>	<0.001
Control group	7.67 (7.50-7.79)	7.65 (7.58-7.84)	7.47 (7.27-7.72)	0.121
P value	0.078	0.006	0.597	
<b>H (µm)</b>				
AD group	1.57 (1.50-1.64)	1.69 (1.56-1.73)	1.51 (1.48-1.54) <sup>b</sup>	<0.001
Control group	1.64 (1.54-1.73)	1.68 (1.54-1.76)	1.54 (1.29-1.78)	0.290
P value	0.208	0.871	0.935	
<b>Depth (µm)</b>				
AD group	1.31 (1.29-1.33)	1.35 (1.29-1.41)	1.27 (1.25-1.29) <sup>b</sup>	<0.001
Control group	1.33 (1.25-1.45)	1.43 (1.33-1.46)	1.33 (1.25-1.36) <sup>b</sup>	0.026
P value	0.378	0.081	0.033	
<b>Rq (nm)</b>				
AD group	17.15 (12.55-19.53)	17.20 (12.78-20.98)	71.75 (65.45-76.40) <sup>a,b</sup>	<0.001
Control group	8.45 (7.73-10.60)	9.39 (8.27-10.73)	68.59 (64.75-72.48) <sup>a,b</sup>	<0.001
P value	<0.001	<0.001	0.256	
<b>Ra (nm)</b>				
AD group	9.80 (9.30-14.48)	10.77 (9.52-14.17)	41.75 (37.38-45.55) <sup>a,b</sup>	<0.001
Control group	5.58 (3.63-7.87)	5.03 (3.78-7.40)	41.58 (39.26-44.96) <sup>a,b</sup>	<0.001
P value	<0.001	<0.001	0.957	

Data was presented as median (25<sup>th</sup>-75<sup>th</sup>). Comparison among different concentrations was determined by Kruskal-Wallis H rank sum test, and a Dunn-Bonferroni test for post hoc comparisons. Comparison between AD groups and C group was determined by Wilcoxon rank sum test.  $P < 0.05$  was considered significant. <sup>a</sup>Ozone concentrations 0 µg/mL vs 80 µg/mL or 160 µg/mL are significantly different ( $P < 0.05$ ), <sup>b</sup>Ozone concentrations 80 µg/mL vs 160 µg/mL are significantly different ( $P < 0.05$ ). AD, aortic dissection; D, diameter; H, height; Depth, concave depth; Rq, root-mean-square roughness; Ra, roughness.

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**Figure 4.** The spatial distribution of F-actin in erythrocytes revealed by fluorescent probes of phalloidin-rhodamine when cells exposed to different concentration ozone. The erythrocyte membrane was relatively complete under ozone concentration at 0  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  in the control group (A, B) and the AD group (D, E). Erythrocyte membrane was not complete, complete red fluorescence ring were not seen in cells, and the area of fluorescent dye in each erythrocyte was increased in samples exposed to ozone concentration at 160  $\mu\text{g}/\text{mL}$  in control group (C) and AD group (F). Bar = 10  $\mu\text{m}$ , original magnification =  $\times 2000$ . Comparison between two groups was determined by t test.  $P < 0.05$  was considered significant.

As listed in **Table 6**, no morphological parameter changed in both AD group and control group when samples exposed to 80  $\mu\text{g}/\text{mL}$  ozone. However, when samples exposed to 160  $\mu\text{g}/\text{mL}$  ozone, the diameter (D) in AD group, root-mean-square roughness (Rq) and roughness (Ra) in both two groups predominantly changed compared with samples under 0  $\mu\text{g}/\text{mL}$  ozone (all  $P < 0.001$ ). Additionally, the D and H in AD group, as well as depth (concave depth), Rq and Ra in both two groups changed under 160  $\mu\text{g}/\text{mL}$  ozone compared to 0  $\mu\text{g}/\text{mL}$  ozone (all  $P < 0.001$ ). Meanwhile, the D under 80  $\mu\text{g}/\text{mL}$  ozone, Depth under 160  $\mu\text{g}/\text{mL}$  ozone, Rq and Ra under 0  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  ozone were different in AD group compared to control group (all  $P < 0.05$ ).

### *The spatial distribution of F-actin in erythrocytes*

The erythrocytes had obvious red fluorescence rings under laser scanning confocal microscope, and the erythrocyte membrane was relatively complete under ozone concentration at 0  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  (**Figure 4A, 4B, 4D, 4E**). In addition, erythrocyte membrane was not complete, some cells did not form a complete red fluorescence ring, and the area of fluorescent dye in each erythrocyte was increased in the cell cytoplasm in samples exposed to ozone concentration at 160  $\mu\text{g}/\text{mL}$  (**Figure 4C, 4F**).

### Discussion

Ozone-AHT, as an emerging type of ozone therapy, is a very popular and controversial thera-

peutic technology using the ozonated blood for the treatment of patients in recent years. As one of the most newly developed ozone therapies, the procedure of Ozone-AHT is conducted through transferring the ozonated blood, which is obtained from the same patient and prepared by the injection of different concentrations of ozone, to patients with various diseases such as gout and acute cerebral infarction [8, 12, 13]. In order to explore the usefulness of Ozone-AHT in AD patients, we conducted this *in vitro* experiment as a preliminary study, in which the effect of ozone on the hypoxia, hemolysis and morphological changes of erythrocytes of blood samples obtained from AD patients and healthy controls.

In our study, the Ozone-AHT markedly increased the  $\text{PO}_2$  and  $\text{SO}_2$  of blood samples obtained from AD patients, which was in consistent with a previous study [13]. Moreover, in our study, the results showed that samples were more accessible to  $\text{PO}_2$  of 400 mmHg when the ozone concentration was 40  $\mu\text{g}/\text{mL}$ , which satisfied the requirement of clinical needs, indicating the oxygenation effect of ozone was powerful *in vitro*. Bocci et al demonstrate that the correlation of oxygenation effect of ozone on human blood is dose-response association, which means that ozone is benefit to human blood at low level but harmful at high level [14]. An *in vivo* experiment conducted in rat models reveals that ozone-AHT protects kidney from ischemia and an improvement of the oxygen consumption of the rat kidney is reported [15].

However, no study has been done for exploring the mechanism of ozone-AHT for AD patients or for verifying its clinical efficacy and safety in clinical practice, as a preliminary *in vitro* experiment we firstly discovered good effect of ozone-AHT on increasing the oxygenation in AD blood samples. And this effect observed in our study largely came from that ozone was a strong oxidizing agent and has been found to be capable of supplying energy and oxygen to blood and tissues. As another aspect of efficacy evaluated in our study, the MDA level did not change in blood samples exposed to 40  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  ozone, however the SOD level was greatly improved under ozone concentrations at 40  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$ . Emerging number of studies have clearly shown that erythrocytes are protected by plasmatic antioxidants when exposed to ozone concentrations at therapeutic doses, which is claimed to be the doses between 10  $\mu\text{g}/\text{mL}$  and 80  $\mu\text{g}/\text{mL}$  [16, 17]. Furthermore, the elucidation of the mechanisms might be that ozone may be active in antioxidant system [18, 19]. In view that oxidative stress contributes to the progression of AD, the effects of ozone administration on SOD and MDA levels may indicate that ozone-AHT has the potential of preventing progression of AD [20]. Although the age of AD patients is older than the control group, which may result in the  $\text{PO}_2$ ,  $\text{SO}_2$  and antioxidant effect in the elderly is poorer than the younger, due to that aging is correlated with cardiovascular and cerebral vascular degeneration, which may lead to relatively reduced oxygen saturation. However, it is reported that the oxygen saturation and antioxidant in the elderly compared with younger population may be limited [21, 22].

Additionally, recent studies indicate that the cytokines are stimulated in AD patients, which may lead to the progression of AD [23]. Interestingly, ozone is reported to be correlated with increased levels of inflammatory cytokines in other diseases, however, those are most inflammation related diseases [24, 25]. Although the association of inflammatory cytokines with AD is not well established yet, the association of ozone with inflammatory cytokines in AD might need further investigation.

For the assessment of hemolysis, the Fhb levels at different ozone concentrations were evaluated, and the data showed that the Fhb slightly increased when the blood exposed to differ-

ent ozone concentrations, but in the control group, all the values were in normal range (10~50 mg/L). This means that the slight hemolysis caused by therapeutic ozone doses is acceptable. The dissection itself may induce red cells damage due to disorder of blood coagulation system and hemodynamic, which could explain why the basic value of Fhb of AD-group was higher than control group [26].

Most previous studies aims to investigate the physicochemical and oxidative stress mechanisms of the effect of ozone on multiple diseases, while the studies which aim to observe the microscopic morphological changes of erythrocyte are few. According to previous reports, erythrocyte membrane surface ultrastructure changes when the cell is damaged under non-physiological situation [27, 28]. In particular, membrane surface roughness (Ra and Rq) is more sensitive to changes in membrane-skeleton integrity which could be conjectured that the damage of erythrocyte can be detected at the nanometer level. In our study, the cell membranes of erythrocytes under therapeutic ozone doses (10-80  $\mu\text{g}/\text{mL}$ ) were relatively smooth and intact, and the surface roughness did not obviously change, therefore therapeutic ozone seemed to be unharmed to erythrocytes. In addition, some cell shapes and ultrastructure parameters changed when the erythrocyte exposed to the overdosed ozone (160  $\mu\text{g}/\text{mL}$ ), and heterogeneous features of size, shape were presented, such as acanthocytes, echinocytes, leptocyte et al, implying that the overdosed ozone could cause damage to erythrocytes. So far, some researchers suggest that it might be resulted from that the membrane proteins, skeleton construction and cell mechanical stability are damaged by excessive oxidative stress [29, 30].

The erythrocyte membrane skeleton mainly composes of actin and spectrin, presenting a flexible discoid shape in three-dimensional network [31]. It plays a critical role in diverse physiological processes including cell morphological changes, motility and migration. Our data showed that ozone at concentration of 160  $\mu\text{g}/\text{mL}$  promoted the F-action to be in the depolymerization state, destroyed the integrity of cytoskeleton protein. And the erythrocyte membrane was not complete and couldn't form a complete fluorescent ring, therefore, fluorescent dyes entered in the cytoplasm. Fortunately,

although the integrity of the erythrocyte membrane was destroyed at over dosed ozone concentration, we didn't find the similar change in the cells at therapeutic ozone concentrations. However, the damage mechanism remains unclear.

There were some limitations in this study: (1) the sample size was relatively small, further study should enlarge the sample size; (2) this was a preliminary study, which only investigated the effect of ozone on the blood samples obtained from AD patients, thus a clinical study using the ozone-AHT treating AD patients should be performed to further validate our results; (3) the effect of ozone on the levels of inflammatory cytokines in the blood samples from AD patients was not investigated in our study, therefore further studies could evaluate the effect of ozone on the inflammatory cytokine expression in AD patients to further explore their correlations.

In conclusion, Ozone improves oxygen content and reduces oxidative damage in blood from AD patients, and therapeutic dose ozone do not induce hemolysis and morphology change of erythrocytes.

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### Disclosure of conflict of interest

None.

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