Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 81 (2018) 475–481

www.jcma-online.com

Original Article

The changes of red blood cell viscoelasticity and sports anemia in male 24-hr ultra-marathoners

Che-Hung Liu [a,b], Yen-Fang Tseng [c], Jiun-I Lai [d,e], Yin-Quan Chen [c], Shih-Hao Wang [f,g,h], Wei-Fong Kao [i,j], Li-Hua Li [k,l], Yu-Hui Chiu [a,b,j,*], Chorng-Kuang How [e,m], Wen-Han Chang [a,b]

[a] *Department of Emergency Medicine, Mackay Memorial Hospital, Taipei, Taiwan, ROC*
[b] *Department of Medicine, Mackay Medical College, New Taipei City, Taiwan, ROC*
[c] *Institute of Biophotonics, National Yang-Ming University, Taipei, Taiwan, ROC*
[d] *Division of Medical Oncology, Department of Oncology, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[e] *School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[f] *Department of Recreation and Leisure Industry Management, College of Management, National Taiwan Sport University, Taoyuan, Taiwan, ROC*
[g] *Department of Physical Medicine and Rehabilitation, Chang Gung Memorial Hospital, Chiayi, Taiwan, ROC*
[h] *Department of Emergency Medicine, Dali Tzu Chi Hospital, Chiayi, Taiwan, ROC*
[i] *Department of Emergency & Critical Care Medicine, Taipei Medical University Hospital, Taipei, Taiwan, ROC*
[j] *Department of Emergency, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC*
[k] *Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[l] *School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei Taiwan, ROC*
[m] *Department of Emergency Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*

Received May 17, 2017; accepted July 28, 2017

Abstract

Background: In endurance sports, stress, dehydration and release of chemical factors have been associated with red blood cell (RBC) alterations of structure and function, which may contribute to sports anemia, a well-observed phenomenon during long-distance running. Until now, the investigation of the changes of viscoelastic properties of RBC membrane, a decisive factor of RBC deformability to avoid hemolysis, is lacking, especially in an Oriental population.

Methods: nineteen runners were prospectively recruited into our study. Hematological parameters were analyzed before and immediately after the 2015 Taipei 24H Ultra-Marathon Festival, Taiwan. Video particle tracking microrheology was used to determine viscoelastic properties of each RBC sample by calculating the dynamic elastic modulus G′(f) and the viscous modulus G″(f) at frequency f = 20 Hz.

Results: Haptoglobin, RBC count, hemoglobin, hematocrit, mean cell hemoglobin, plasma free hemoglobin and unsaturated iron-binding capacity values of the recruited runners showed a statistically significant drop in the post-race values. Blood concentration of reticulocyte and ferritin were significantly higher at post-race compared with pre-race. 15 out of the 19 runners had a concurrent change in the elastic and the viscous moduli of their RBCs. Changes in the elastic and the viscous moduli were correlated with changes in the RBC count, hemoglobin and hematocrit.

Conclusion: Viscoelasticity properties, the elastic modulus G′(f) and the viscous modulus G″(f) of RBCs are associated with endurance exercise-induced anemia.

Keywords: Clinical sports medicine; Red blood cell; Sports anemia; Ultra-marathon; Viscoelastic properties

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author: Dr. Yu-Hui Chiu, Department of Emergency Medicine, Mackay Memorial Hospital, 92, Section 2, Zhongshan North Road, Taipei 104, Taiwan, ROC.

E-mail address: yuhui7786@gmail.com (Y.-H. Chiu).

https://doi.org/10.1016/j.jcma.2017.09.011

1. Introduction

Exercise associated anemia, or decrease in red blood cell (RBC) levels, is a widely observed phenomenon during long-distance running.[1–3] Hemolysis has been proposed as a mechanism for exercise associated anemia, with numerous hematological abnormalities being described in runners after strenuous exercise. Studies supporting the phenomenon of exercise induced hemolysis report decrease in haptoglobin and increase in free circulating plasma hemoglobin levels, reticulocytes, and increased ferritin levels.[3–5] Several recent studies have reported conflicting results reporting a modest presence of hemolysis by measuring similar physiological parameters with various methods.[6–8] The proposed term "Foot strike hemolysis", defined as a shearing force causing RBC corpuscular destruction resulting from the athlete's feet striking on hard surfaces frequently, has also been called into question.[4,6] While the causes of fluctuations in RBC levels are far from settled, it is generally accepted that RBC levels do change during prolonged exercise.[9] Whether or not this is purely secondary to fluid status changes, or do RBC characteristics (such as half-life, rheology, size and shape) play a role in determining RBC levels, is still largely unknown.

During high intensity endurance sports, the body undergoes dehydration and release of chemical factors into the blood stream. This phenomenon as well as changes in rheology (increased blood flow and speed) can theoretically alter with RBC structure and function.[10–12] Blood viscosity has been reported no changes after endurance sports such as cycling.[13] Other studies report structural changes in RBC membrane skeleton following marathon running.[14,15] Altered RBC osmotic resistance and increased susceptibility to chemical and physical stress after prolonged endurance exercise have also been reported in different studies.[16] These evidence prompted us to hypothesize that microscopic changes in RBC properties such as viscosity and elasticity may be present in athletes following endurance sports.

We thus designed an experimental study to specifically assess the viscoelastic properties of RBC in runners before and after a 24-hr ultra-marathon.

2. Methods

2.1. Study design and population

Twenty-five experienced male ultra-marathon runners participating in the event known as the 2015 Taipei 24H Ultra-Marathon Festival, in Taipei, Taiwan volunteered for this study. Approval was obtained from the TMU- Joint Institutional Review Board (201309022). All subjects provided written consent to participate in the study. The competition began at 3 pm February 13, 2015 and ended at 3 pm on February 14, 2015. Ultimately, the data of 19 male runners were included in the analysis; One runner who had history of anemia (hemoglobin <13 g/dL), 1 runner who didn't run more than 2/3 of the average kilometers of all finishers and 4 runners who didn't finish the 24-h race were excluded. All runners ran around a 668-m park trail, and they were permitted to rest and to ingest water and food freely. Before the competition, all runners were required to complete a questionnaire for demographic data and information on medical and training history. The body weight of each of the 19 subjects was measured 30 min before and immediately after the race.

2.2. Laboratory assessment

Using sterile techniques, blood (20 mL) was drawn from an antecubital vein from each subject 1 week before and immediately after the race. All specimens were refrigerated and transported to the laboratory within 4 h of sampling. Red blood cell (RBC) count, hemoglobin, hematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width, coefficient of variation (RDW-CV) and reticulocyte were performed on a Coulter LH 750 Hematology Analyzer (Beckman Coulter, Miami, FL, USA), which was based on impedance detection for counting and sizing the blood cells. The evaluated plasma haptoglobin was assayed by the rate nephelometry on an Immage 800 Analyzer (Beckman Coulter, Fullerton, CA, USA). Free plasma hemoglobin was tested with a HemoCue Plasma/Low Hb System (HemoCue, Lake Forest, CA, USA) utilizing the azide-methemoglobin method. Ferritin levels were determined by an Architect I-2000 Analyzer (Abbott Diagnostics, Abbott Park, IL, USA), which used a chemiluminescent microparticle immunoassay. Iron and unsaturated iron-binding capacity (UIBC) were measured on a Modular E170 Analyzer (Roche Diagnostics, Mannheim, Germany) using an electrochemiluminescence immunoassay.

2.3. Viscoelastic analysis

Particle Tracking Microrheology (PTM) has been established as an important tool for the study of the viscoelasticity of living cells for more than a decade[17–24]; however, only a few studies deal with RBC viscoelasticity measurements by PTM.[21,23] On the measurements of RBC viscoelasticity, the experimental results may vary by one to two orders of magnitude depending on the specific approach to measure either the global stretching, bending, or torsional modes of the RBC deformations or local membrane fluctuation.[17,18] In our VPTM approach, we used fluorescent nanoparticles attached to the RBC membrane to probe the local RBC membrane fluctuation to deduce the elastic and the viscous moduli (via the Generalized Stokes–Einstein equation),[17,21,22] which are expected to be dominant by the viscoelasticity of the RBCs spectrin network underneath the lipid bilayer.[23] Specifically, Video particle tracking microrheology (VPTM) was used to track, record, and analyze the Brownian motion of 100 nm diameter fluorescence polystyrene beads attached to the membrane of the RBC samples to deduce the dynamic elastic modulus $G'(f)$ and the viscous modulus $G''(f)$ of each RBC sample, for frequency (f) in the range of 0.2 Hz–100 Hz. The sample preparation and the experimental procedures were as

follow. 100 μL of blood sample was taken from the Vacutainer and mixed with 1000 μL of 0.9% saline in an Eppendorf tube and then centrifuged at 3000 rpm at 4 °C for 10 min to separate the RBCs from the whole blood. 20 μL of Poly-L-Lysine coated fluorescent beads (diameter = 100 nm, fluorescence excitation/emission peaks: 580/605 nm) were added to 500 μL saline and agitated with an ultrasonic cleaner for 20 min to avoid beads aggregation. The two saline solutions (500 uL containing the beads and 1 μL containing RBCs), were mixed, and agitated in a rotator for 30 min to allow the beads to bind to the RBCs. A small drop (10 μL) of the sample of RBCs with beads (~3–7 beads per RBC) attached to their outer membranes was transferred to a sample chamber of an inverted epifluorescence microscope (Nikon Eclipse Ti, Tokyo, Japan), equipped with an oil immersion objective lens (100×, N.A = 1.45) and a CCD camera (Hamamatsu, Hamamatsu, Japan, OHCA-Flash 4.0) to track and record the Brownian motion of beads on the RBCs membrane for 5 s. From the 2-dimensional projection of the trajectory x(t) and y(t) of the Brownian motion of each bead, the mean square displacement MSD of each bead as a function of the lag time (τ), and the dynamic elastic modulus G′(f) and the viscous modulus G″(f) of the RBC membrane, for frequency (f) in the range of 0.2 Hz to 100 Hz, were deduced, via a standard mathematical model and algorithm.[17,20,21]

2.4. Statistical analysis

Descriptive results were reported as median (IQR, interquartile range). The plasma hematological and biochemical values immediately post-race were compared to the pre-race values using Wilcoxon signed-ranks test. The values of the elastic modulus G′(f) and the viscous modulus G″(f) at f = 20 Hz of each RBC from each individual runner before and after the ultra-marathon event were also compared by Wilcoxon signed-ranks test. The Spearman's rank correlation coefficient was used to evaluate the association of hematological changes and their association with viscoelastic moduli differences. Commercially available statistical software (SPSS version 21.0, IBM Corp., Armonk, NY, USA) was used for statistical analysis. Differences were considered to be statistically significant when 2 tailed $p < 0.05$.

3. Results

During the competition, the ambient temperature ranged from 17.4 to 23.4 °C, the relative humidity was 39–59%, and the wind speed ranged from 1.1 to 4.6 m/s (data provided by the Taiwan Central Weather Bureau). There were 19 male runner participants (median, 45 years) who finished the 24-h ultra-marathon race. Their demographic data are summarized in Table 1.

Hematological parameters and RBC viscoelastic properties of these subjects throughout this 24-h ultra-marathon are shown in Table 2. In terms of hematological parameters, the values of body weight, haptoglobin, plasma free hemoglobin, RBC count, hemoglobin, hematocrit, MCH and UIBC showed statistically significant decreased between immediate post-race compared to pre-race results. Reticulocyte and ferritin increased at the immediate post-race values. No significant changes were observed in MCV, MCHC, RDW-CV and iron immediately post-race compared with pre-race levels.

Table 1
Demographics of 24-h ultra-marathoners (n = 19).

Parameter	Median (IQR)
Age (years)	45 (38–54)
Weight (kg)	65.1 (62.4–71.4)
Height (m)	1.72 (1.68–1.75)
BMI (kg/m^2)	22.8 (20.8–24.1)
Running marathons (years)	4 (2–8)
Running ultra-marathons (years)	2 (1.5–3)
Training distance (n)	
<40 km/week	4
40–100 km/week	13
>100 km/week	2
Best marathon score (min)	231.50 (200.75–241.25)
This ultramarathon score (km)	153.64 (134.27–167.00)

IQR = interquartile range.

As for RBC viscoelastic properties at f = 20 Hz, our statistical analysis of elastic modulus G′(f) and the viscous modulus G″(f) for 19 male ultra-marathoners before and after the race is summarized in Table 2, and shown graphically in Fig. 1 in box and whisker plots. On the average over the 19 male runners, the differences in the values of both the elastic and the viscous moduli before versus after the race are statistically insignificant. As a control experiment, we did measure the viscoelasticity of RBC samples (from a single volunteer) *in vitro* under different saline osmolarity (of 250 mOSM, 300 mOSM, and 350 mOSM), the results (not shown) are comparable with the corresponding values of the RBC samples from the ultra-marathon runners, given in Fig. 1.

Since the particle tracking microrheology method allows us to determine the elastic modulus and the viscous modulus of individual RBC samples, we further examined our data and compared the effect of the 24-h run on the elastic and the viscous moduli for each individual runner. The results are summarized in Fig. 2 in a 2-D plot with the fractional changes (i.e., post-race value − pre-race value/pre-race value) in the elastic modulus G′(f) and the viscous modulus G″(f) for the 19 male runners. Fig. 2 reveals that of the data set (ΔG′/G′, ΔG″/G″) associated with 19 runners, 15 (79%) lie in the vicinity of a straight line (the dash line in Fig. 2) with a slope of 0.33. That means for each individual, the fractional change ΔG′/G′ bears a positive correlation with ΔG″/G″. Specifically, of these 15 runners, 7 exhibited an increase in both the elastic modulus and the viscous modulus of their RBC membranes after the run, while 8 exhibited a decrease in both.

To further investigate the role of RBC viscoelastic properties in the physiological context of ultra-marathoners in Table 3, we performed Spearman's ranked coefficient analysis between the viscoelastic properties and biochemical parameters in these 2 groups with concurrent changes in elastic modulus G′(f) and the viscous modulus G″(f). The associated RBC parameters and viscoelastic moduli for each subject are

Table 2
Hematological and RBC viscoelastic parameters throughout the ultra-marathon race (total subject, n = 19).

Parameter	Median (IQR)		*p*	Normal range
	Pre-race	Post-race		
Weight (kg)	65.1 (62.4–71.4)	64.2 (59.0–70.6)	**0.000**	
Osmolality (mosm/kgH_2O)	289 (285–292)	306 (301–308)	**0.000**	
CBC counts				
RBC (x 10^6/μL)	4.66 (4.55–5.12)	4.60 (4.48–4.73)	**0.006**	4.6–6.1
Hgb (g/dL)	14.8 (13.9–15.3)	14.2 (13.8–14.5)	**0.001**	13–17
Hct (%)	42.7 (41.8–44.9)	41.5 (40.9–42.7)	**0.001**	39–52
MCV (fL)	91.1 (87.2–94.5)	90.5 (86.7–94.1)	0.337	80–99
MCH (pg)	30.7 (30.2–32.3)	30.3 (29.9–32.1)	**0.011**	26–34
MCHC (g/dL)	34.2 (34.0–34.7)	34.2 (33.6–34.6)	0.367	33–37
RDW-CV (%)	13.1 (12.8–13.6)	13.2 (12.8–13.7)	0.887	11.5–14.5
Reticulocyte (%)	1.12 (0.98–1.58)	1.35 (1.11–1.82)	**0.020**	0.5–2.5
Hemolysis markers				
Haptoglobin (mg/dL)	76.6 (47.5–101.6)	19.1 (4.0–52.6)	**0.000**	30–200
Plasma Hgb (g/dL)	0.03 (0.03–0.07)	0.02 (0.02–0.03)	**0.007**	0–3
Iron Panel				
Ferritin (ng/mL)	110.4 (58.4–151.4)	158.3 (68.1–202.8)	**0.000**	21–274
Iron (ug/dL)	98 (71–113)	100 (77–152)	0.056	35–200
UIBC (ug/dL)	209 (181–232)	191 (130–210)	**0.001**	191–269
Viscoelastic properties				
Elastic Modulus (Pa)	74.8 (60.9–106.4)	68.6 (57.6–103.7)	0.798	0–100
Viscous Modulus (Pa)	41.7 (35.8–48.8)	42.9 (34.7–47.3)	0.984	0–100

IQR = interquartile range; CBC = complete blood cell; RBC = red blood cell; Hgb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration; RDW-CV = red cell distribution width, coefficient of variation; UIBC = unsaturated iron-binding capacity.
$p < 0.05$ is considered significant, and is highlighted in bold.

presented in Table 4. We found that in the "increased" group (defined as post race values increased when comparing to pre-race), the changes in the elastic modulus and viscous modulus were strongly negatively correlated with changes in the anemia indicators, RBC count, hemoglobin and hematocrit. In the "decreased" group, decrease in the viscous modulus was strongly positively correlated with changes in RBC count and hematocrit.

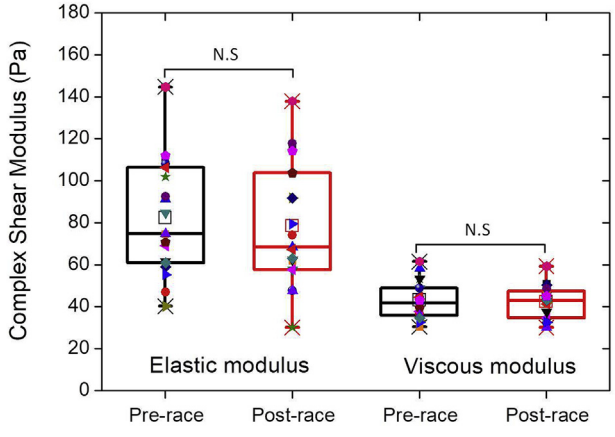

Fig. 1. Box plot of membrane elastic modulus and viscous modulus (both at 20 Hz) of RBCs from 19 male runners before and after the 24-hour ultra-marathon. The lower and the upper boundaries of the box represent Q1 (25 percentile) and Q3 (75 percentile) of the data, respectively; we denote IQR = Q3 − Q1, and indicate Q1 − 1.5 IQR by the lowest horizontal line, and Q3 + 1.5 IQR by the highest horizontal line; the symbol "□" and the horizontal bar inside the box represent the mean and the medium, respectively.

4. Discussion

Pre-race hemoglobin and hematocrit values were within the normal ranges, suggesting the runners were well hydrated and not suffering from anemia before the race. The three indicators of anemia, RBC count, hemoglobin and hematocrit significantly decreased after the 24-h ultra-marathon race. In hemolysis, intravascular destruction of erythrocytes releases free hemoglobin forming stable complexes with haptoglobin to inhibit its oxidative activity, which causes haptoglobin levels to be decreased.[3,8,15] Our findings showed all the 19 runners had significant decrease of haptoglobin at the immediate post-race time point. Moreover, the reticulocyte levels, a reflection of the release of an increased of red blood cell production, also statistically increased in our data. It has been previously proposed that exercise-induced hemolysis results in increase in iron levels.[25,26] In our study, we observed a trend (but insignificant) in iron increase ($p = 0.056$) and a significant increase of ferritin increase ($p = 0.000$) immediately post-race, which are in line with Robach's study of an extreme mountain ultra-marathon.[8] Moreover, the results of iron elevation and UIBC depression ($p = 0.001$) immediately after the 24-h ultra-marathon race were similar to our previous study with a rebound raise of iron and drop of TIBC in the 24-h timepoint measurement after a 100-km ultra-marathon.[3] Our data, although far from conclusive, provides additional information regarding the phenomenon of whether hemolysis is present in long distance running sports.

Our study on RBC viscoelastic properties showed that 7 runners in the "increased" group had increased viscosity (or

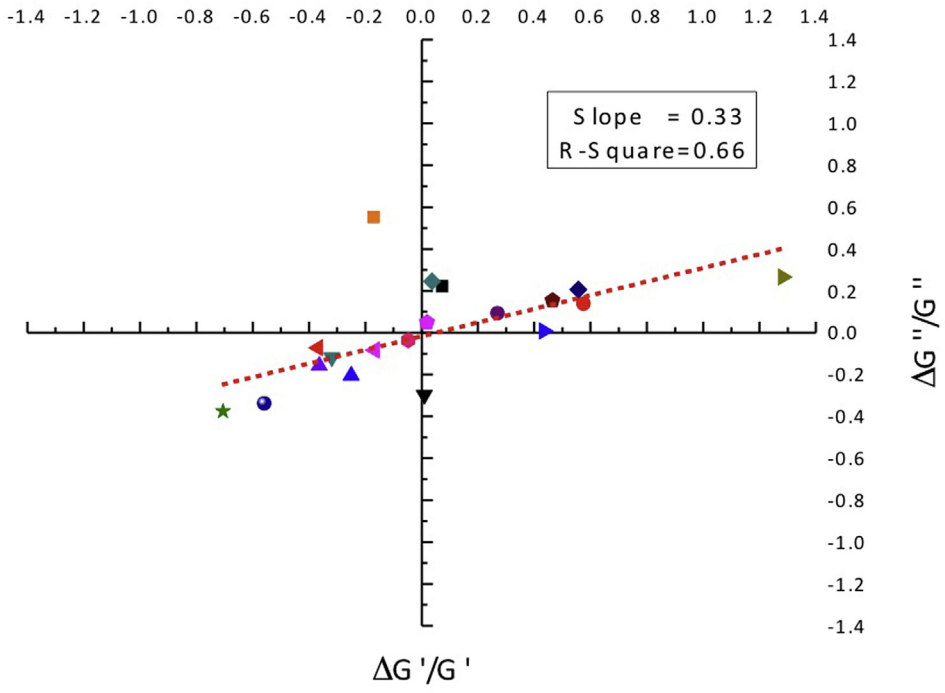

Fig. 2. The effect of the 24-hr ultra-marathon on the elastic and the viscous moduli for each individual runner summarized in the form of a 2-d plot where the fractional change (i.e., post-race value − pre-race value/pre-race value) in elastic modulus ($\Delta G'/G'$) is plotted along the horizontal axis, and the corresponding fractional change in viscous modulus ($\Delta G''/G''$), is plotted along the vertical axis for the 19 male runners. The dash line represents the linear least square fit of the data.

less fluidicity) after the run, while the 8 runners in the "decreased" group showed the opposite trend. Although the viscoelasticity (or deformability) and pathophysiology of RBCs have been studied and reported from the perspective of different diseases,[10,27] most of the molecular mechanisms remain unclear. It is remarkable that 15 out of the 19 runners (79%) had a concurrent change in elastic and viscous modulus (both increased or both decreased). This implicates that there

Table 3
Subjects' demographic/hematological parameters and their association with viscoelastic moduli differences.

Parameter	G′_Ri n = 7 (p; rs)	G′_Rd n = 8 (p; rs)	G″_Ri n = 7 (p; rs)	G″_Rd n = 8 (p; rs)
Age (years)	NS	NS	NS	NS
Weight_R (kg)	NS	NS	NS	NS
Training distance (km)	NS	NS	NS	NS
This ultra-marathon score (min)	NS	NS	NS	NS
Osmolality_R	NS	NS	NS	NS
RBC_R	**0.014; −0.857**	NS	**0.014; −0.857**	**0.004; 0.881**
HgB_R	**0.023; −0.821**	NS	**0.023; −0.821**	NS
Hct_R	**0.023; −0.821**	NS	**0.003; −0.929**	**0.015; 0.810**
MCV_R	NS	NS	NS	NS
MCH_R	NS	NS	NS	NS
MCHC_R	NS	NS	NS	NS
RDW-CV_R	NS	NS	NS	NS
Reticulocyte_R	NS	NS	NS	NS
Haptoglobin_R	NS	NS	NS	NS
Plasma HgB_R	NS	NS	NS	NS
Ferritin_R	NS	NS	NS	NS
Iron_R	NS	NS	NS	**0.037; 0.738**
UIBC_R	NS	NS	NS	NS

Ri = ratio between pre- and post-race value in "increased" group; Rd = ratio between pre- and post-race value in "decreased: group; R = ratio; rs = Spearman rank correlation coefficient; NS = not statistically significant; RBC = red blood cell; Hgb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration; RDW-CV = red cell distribution width, coefficient of variation; UIBC, unsaturated iron-binding capacity.
$p < 0.05$ is considered significant, and is highlighted in bold.

Table 4
Associated RBC parameters and viscoelastic moduli of each subject (n = 19).

Subject	G′(f)_b (Pa)	G′(f)_a (Pa)	Ratio	G″(f)_b (Pa)	G″(f)_a (Pa)	Ratio	RBC_b ($\times10^6$/μL)	RBC_a ($\times10^6$/μL)	Hgb_b (g/dL)	Hgb_a (g/dL)	Hct_b (%)	Hct_a (%)
I_1	47.02	74.06	+58%	36.63	41.68	+14%	4.2	4.08	13.8	13.4	40	38
I_2	40.21	91.81	+128%	33.33	42.19	+27%	5.1	4.73	15.5	14.4	45	42
I_3	59.02	91.87	+56%	41.73	50.33	+21%	4.8	4.60	14.9	14.1	44	42
I_4	70.81	103.74	+46%	39.65	45.80	+15%	4.7	4.52	15.2	14.2	45	42
I_5	92.67	117.72	+27%	44.46	48.56	+9%	4.6	4.58	13.8	13.9	42	42
I_6	55.36	79.44	+44%	32.43	32.68	+1%	4.6	4.50	13.9	13.5	40	40
I_7	112.01	114.14	+2%	43.07	45.15	+5%	5.5	5.54	15.5	15.5	46	47
D_1	91.45	68.60	−25%	58.20	46.25	−21%	4.6	4.42	15.2	14.5	44	42
D_2	84.62	57.63	−32%	48.69	42.94	−12%	4.7	4.63	13.8	13.8	41	40
D_3	69.04	57.53	−17%	37.83	34.69	−8%	4.6	4.52	14.3	13.7	42	41
D_4	144.73	137.96	−5%	61.49	59.29	−4%	4.7	4.65	15.1	15.2	44	44
D_5	101.97	30.13	−70%	53.34	33.31	−38%	5.2	4.94	15.3	14.5	45	43
D_6	108.12	47.72	−56%	48.83	32.35	−34%	4.5	4.30	14.8	14.3	43	41
D_7	74.84	47.62	−36%	35.79	30.17	−16%	5.1	5.10	15.7	15.7	47	46
D_8	106.35	67.25	−37%	44.62	41.41	−7%	4.5	4.47	14.5	14.4	42	42
N_1	109.33	117.32	+7%	41.62	50.88	+22%	4.9	4.87	13.9	13.8	42	41
N_2	74.39	61.77	−17%	30.45	47.28	+55%	4.8	4.48	14.6	13.5	42	39
N_3	61.68	62.31	+1%	53.30	37.49	−30%	5.1	4.72	15.6	14.2	45	41
N_4	60.90	63.13	+4%	34.65	43.15	+25%	4.2	4.60	14.8	14.7	42	43

I = increased group; D = decreased group; N = not lie in the vicinity of the red line in Fig. 2 with a slope of 0.33; G′(f) = elastic modulus; G″(f) = viscous modulus; RBC = red blood cell; Hgb = hemoglobin; Hct = hematocrit; b = pre-race; a = post-race.

might be an underlying relationship between the exercise-induced anemia and the RBC viscosity and elasticity. It is intriguing to fathom that changes in viscoelastic properties in RBC might lead to changes in half-life or promote hemolysis. Indeed, changes in viscoelastic properties in RBC have been linked to vulnerability to stress and shortened RBC life span.[28] Our study provides a tantalizing hint that microscopic properties of RBC may play a role in exercise associated anemia and rheological changes.

We acknowledge that there are some limitations in our study. The relatively small sample size and the observational design limit the overall strength of the conclusions. Due to the limited number of cases available in this study, more detail biochemical and biophysical analysis (in conjunction with the viscoelasticity measurements) with a much larger number of samples will be required to elucidate the molecular mechanisms involved. On the other hand, ultra-marathon is an exclusive sports with far fewer participants compared to regular marathon races, and we believe our study, albeit a small sample size, is of noteworthy importance. Second, we did not follow-up the subjects' hematological tests and viscoelastic properties later than the 24-h measurement, and therefore were not able to assess the long-term effects of the 24-h race on these ultra-marathoners. It can be hypothesized, however, that the changes in viscoelasticity are either reversible, or is too mild to cause global pathological situations in athletes. Since RBCs have a half-life of 3 months, even irreversible changes to RBC will only last for the lifetime of the affected cell: newly produced RBC should theoretically have unaltered characteristics. A final limitation is that only male runners were recruited in our study, in consideration of sex differences in hemogram data. Therefore our data would not be representative of any potential underlying sex related physiological changes related to this event.

In conclusion, running a 24-hr ultra-marathon will induce changes in hematological parameters and change the RBC viscoelastic properties. In most runners, viscous and elastic moduli are concurrently either increased or decreased, hinting an underlying relationship between RBC viscoelastic properties and long distance endurance sports.

Acknowledgments

We thank all the ultra-marathon runners who participated in this study and all the doctors, nurses, and emergency medical technicians who provided professional care at this ultra-marathon race. We also express gratitude to our colleagues at Soochow University and the Chinese Taipei Association of Ultra Runners, all of which assisted at the ultra-marathon event. We deeply appreciate Arthur Chiou, Professor, Institute of Biophotonics, National Yang-Ming University, Taipei, Taiwan to organize our viscoelastic analysis. We further feel an immense gratitude to Yen-Kuang Lin, Research Fellow, Biostatistics Center, Taipei Medical University, Taipei, Taiwan to help our statistical analysis. Finally, this study was supported by Mackay Memorial Hospital, Taiwan (MMH10574/106128).

References

1. Mairbaurl H. Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Front Physiol* 2013;**4**:332.
2. Dang CV. Runner's anemia. *JAMA* 2001;**286**:714–6.

3. Chiu YH, Lai JI, Wang SH, How CK, Li LH, Kao WF, et al. Early changes of the anemia phenomenon in male 100-km ultramarathoners. *J Chin Med Assoc* 2015;**78**:108−13.
4. Telford RD, Sly GJ, Hahn AG, Cunningham RB, Bryant C, Smith JA. Footstrike is the major cause of hemolysis during running. *J Appl Physiol* 2003;**94**:38−42.
5. Dickson DN, Wilkinson RL, Noakes TD. Effects of ultra-marathon training and racing on hematologic parameters and serum ferritin levels in well-trained athletes. *Int J Sports Med* 1982;**3**:111−7.
6. Lippi G, Schena F, Salvagno GL, Aloe R, Banfi G, Guidi GC. Foot-strike haemolysis after a 60-km ultramarathon. *Blood Transfus* 2012;**10**:377−83.
7. Christensen DL, Espino D, Infante-Ramirez R, Brage S, Terzic D, Goetze JP, et al. Normalization of elevated cardiac, kidney, and hemolysis plasma markers within 48 h in Mexican Tarahumara runners following a 78 km race at moderate altitude. *Am J Hum Biol* 2014;**26**:836−43.
8. Robach P, Boisson RC, Vincent L, Lundby C, Moutereau S, Gergelé L, et al. Hemolysis induced by an extreme mountain ultra-marathon is not associated with a decrease in total red blood cell volume. *Scand J Med Sci Sports* 2014;**24**:18−27.
9. Lombardi G, Lanteri P, Fiorella PL, Simonetto L, Impellizzeri FM, Bonifazi M, et al. Comparison of the hematological profile of elite road cyclists during the 2010 and 2012 GiroBio ten-day stage races and relationships with final ranking. *PLoS One* 2013;**8**:e63092.
10. Connes P, Simmonds MJ, Brun JF, Baskurt OK. Exercise hemorheology: classical data, recent findings and unresolved issues. *Clin Hemorheol Microcirc* 2013;**53**:187−99.
11. Reinhart WH, Bärtsch P, Straub PW. Red blood cell morphology after a 100-km run. *Clin Lab Haematol* 1989;**11**:105−10.
12. Reinhart WH, Stäubli M, Straub PW. Impaired red cell filterability with elimination of old red blood cells during a 100-km race. *J Appl Physiol* 1983;**54**:827−30.
13. Simmonds MJ, Connes P, Sabapathy S. Exercise-induced blood lactate increase does not change red blood cell deformability in cyclists. *PLoS One* 2013;**8**:e71219.
14. Jordan J, Kiernan W, Merker HJ, Wenzel M, Beneke R. Red cell membrane skeletal changes in marathon runners. *Int J Sports Med* 1998;**19**:16−9.
15. Yusof A, Leithauser RM, Roth HJ, Finkernagel H, Wilson MT, Beneke R. Exercise-induced hemolysis is caused by protein modification and most evident during the early phase of an ultraendurance race. *J Appl Physiol* 2007;**102**:582−6.
16. Smith JA, Kolbuch-Braddon M, Gillam I, Telford RD, Weidemann MJ. Changes in the susceptibility of red blood cells to oxidative and osmotic stress following submaximal exercise. *Eur J Appl Physiol Occup Physiol* 1995;**70**:427−36.
17. Wirtz D. Particle-tracking microrheology of living cells: principles and applications. *Annu Rev Biophys* 2009;**38**:301−26.
18. Weihs D, Mason TG, Teitell MA. Bio-microrheology: a frontier in microrheology. *Biophys J* 2006;**91**:4296−305.
19. Chen YQ, Kuo CY, Wei MT, Wu K, Su PT, Huang CS, et al. Intracellular viscoelasticity of HeLa cells during cell division studied by video particle-tracking microrheology. *J Biomed Opt* 2014;**19**:011008.
20. Chen YQ, Su PT, Chen YH, Wei MT, Huang CH, Osterday K, et al. The effect of enterohemorrhagic E. coli infection on the cell mechanics of host cells. *PLoS One* 2014;**9**:e112137.
21. Wei MT, Latinovic O, Hough LA, Chen YC, Ou-Yang HD, Chiou A. *Optical-tweezers-based microrheology of soft materials and living cells. Handbook of photonics for biomedical engineering*. 2014. p. 1−20.
22. Gómez-González M, Del Álamo JC. Two-point particle tracking microrheology of nematic complex fluids. *Soft Matter* 2016;**12**:5758−79.
23. Chen YQ, Liu YS, Liu YA, Wu YC, Del Álamo JC, Chiou A, et al. Biochemical and physical characterizations of mesenchymal stromal cells along the time course of directed differentiation. *Sci Rep* 2016;**6**:31547.
24. Turlier H, Fedosov DA, Audoly B, Auth T, Gov NS, Sykes C, et al. Equilibrium physics breakdown reveals the active nature of red blood cell flickering. *Nat Phys* 2016;**12**:513−9.
25. Latunde-Dada GO. Iron metabolism in athletes−achieving a gold standard. *Eur J Haematol* 2013;**90**:10−5.
26. Buchman AL, Keen C, Commisso J, Killip D, Ou CN, Rognerud CL, et al. The effect of a marathon run on plasma and urine mineral and metal concentrations. *J Am Coll Nutr* 1998;**17**:124−7.
27. Parthasarathi K, Lipowsky HH. Capillary recruitment in response to tissue hypoxia and its dependence on red blood cell deformability. *Am J Physiol* 1999;**277**:H2145−57.
28. Bracci R, Martini G, Buonocore G, Talluri B, Berni S, Ottaviani MF, et al. Changes in erythrocyte properties during the first hours of life: electron spin resonance of reacting sulfydryl groups. *Pediatr Res* 1988;**24**:391−5.