Biochemistry and Biophysics Reports 36 (2023) 101556

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Flickering of fetal erythrocytes membrane under gestational diabetes observed with dual time resolved membrane fluctuation spectroscopy

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A B S T R A C T

The membrane flickering of human fetal red blood cells (RBCs) affected by gestational diabetes mellitus (GDM) was studied with dual time resolved membrane fluctuation spectroscopy (D-TRMFS). This new technique is a modified version of the dual optical tweezers method that has been adapted to measure the mechanical properties of RBCs at two distant membrane points simultaneously. The micro-rheological parameters were obtained from direct membrane flickering measurements, followed by Fourier decomposition and cell membrane model adjustment. Our results show a significant decrease of 6.01 ± 1.19 nm in membrane fluctuations amplitude in healthy fetal, compared with healthy adult RBCs, meanwhile the amplitude in GDM cells increased 3.22 ± 1.10 nm compared with healthy fetal RBCs. Between GDM and healthy fetal RBCs, there are significant differences, especially in the bending modulus. Considering the mean of the two membrane points measured, the tension for GDM RBCs increased by 6.431 ± 3.57 (10^{-7} [N/m]) compared with healthy fetal RBCs, meanwhile, the bending was increased by 2.483 ± 0.58 (10^{-19} [J]) in GDM compared with healthy fetal RBCs. These results showed significant increment of 1.23 ± 0.07-fold and 3.29 ± 0.36-fold in tension and bending modulus in GDM, respectively. The strong impact of GDM on bending modulus could be associated with oxidative stress and lipid peroxidation, previously reported in fetal plasma of GDM cases.

1. Introduction

Gestational diabetes mellitus (GDM) is defined as any glucose intolerance first recognized during pregnancy [1]. GDM is diagnosed through the oral glucose tolerance test (OGTT) around the 22nd-24th week of pregnancy and usually resolves after childbirth. Importantly, GDM is associated with adverse outcomes for children and mothers, like fetal macrosomia, neonatal hypoglycemia, and a higher risk for metabolic and cardiovascular diseases [2,3]. Importantly, the health of the fetus is closely tied to the function of red blood cells (RBCs) or erythrocytes, which deliver oxygen and nutrients to developing organs and tissues [4]. Fetal RBCs play a crucial role in maintaining the health of the fetus during pregnancy, but in GDM these cells are exposed to hyperglycemia and hyperinsulinemia, suggesting alterations of plasma membrane integrity associated with oxidative stress [5]. Due to potential effects of oxidative stress on RBCs plasma membrane rheology, we hypothesized that GDM has significant impacts on RBCs membrane flickering.

To determine the micro-rheological parameters of human RBCs, with high spatial and temporal resolution, our team developed the dual time resolved membrane fluctuation spectroscopy (d-TRMFS) [6]. In this study, we report the first micro-rheological approach on fetal RBCs, at single cell level using this technique. Dual measurements are achieved using two orthogonally polarized laser beams at low optical power at two diametrically distant locations on the cell membrane, without the application of optical forces, and with similar or even higher resolution than other optical techniques [7]. The method is inspired by the time-resolved thermal fluctuation spectroscopy (TRMFS) of Betz [8] and the two-laser-beam optical tweezers for differential detection proposed by Moffitt and Bustamante [9]. This technique allows for the measurement at high resolution sampling over time of the fluctuation amplitudes that come from thermal flickering and cell metabolism. The d-TRMFS technique expands the possibilities of Betz et al. because it allows the observation of the structural dynamics of the RBCs beyond one single point in the membrane. Using a cell membrane model [10], dynamic properties can be obtained, mainly tension and bending modulus. By using this instrumentation, we gain insights into differences between adult and fetal RBCs and how GDM affects the membrane properties of fetal RBCs. This information may ultimately help to improve our understanding of fetal physiology and GDM and its impact on obstetrics and neonatal outcomes.

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https://doi.org/10.1016/j.bbrep.2023.101556
Received 11 September 2023; Received in revised form 5 October 2023; Accepted 7 October 2023

2. Material and methods

All experiments in this work conform to the principles outlined in the Declaration of Helsinki and were approved by the Ethics and Scientific Committee of Chilean National Health Service (approval code CEC-SSC: 21-04-24).

2.1. Sample preparation

Adult blood samples (10 μl) were obtained from three healthy

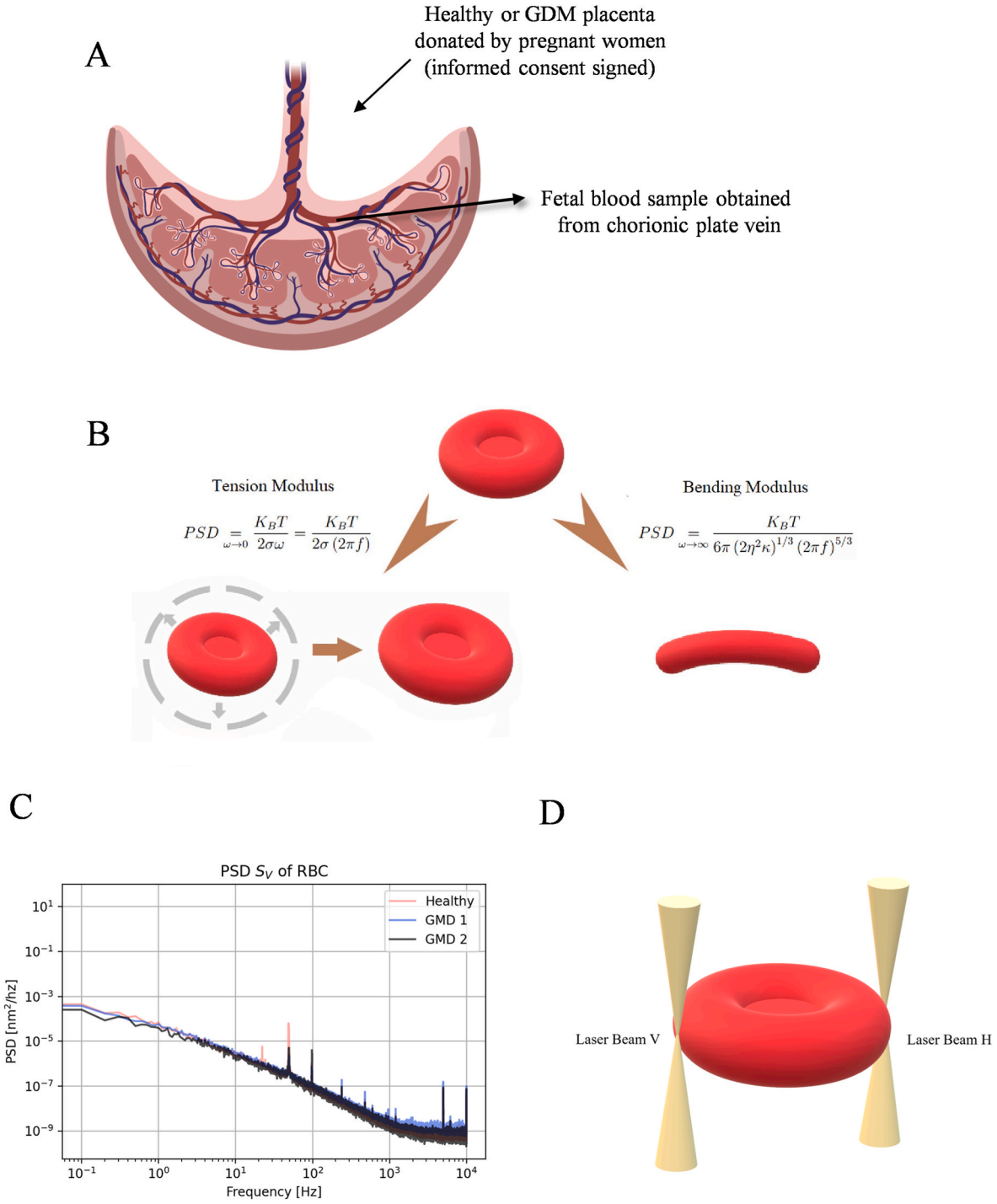

Fig. 1. Graphical model of methodology. A) This study analyzes fetal red blood cells derived from two mother groups: one group without any pathologies (healthy) and another group comprising mothers who developed gestational diabetes mellitus during pregnancy (GDM). Fetal blood samples were obtained from chorionic plate veins after delivery, avoiding contact with maternal blood. B) The analysis focuses on two aspects of deformability in red blood cells: Tension and Flexion. The deformability modulus is obtained by analyzing the power spectrum density where K_B is Boltzmann constant, T is the temperature in Kelvin, f is the frequency, σ is the tension modulus, and kappa is the κ bending modulus. C) The deformability module expressions are adjusted at lower frequencies to analyze tension and at higher frequencies to examine flexion. D) The membrane fluctuations of fetal red blood cells are measured using Dual-TRMFS spectroscopy. This method allows the researchers to measure the fluctuations at two different points on the cell membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

volunteers. Fetal blood samples (10 μl) were collected from chorionic veins of placenta, after delivery. Placentas were donated by pregnant women who had birth at *Hospital Guillermo* Grant *Benavente* of Concepción after signing informed consent (CEC-SSC 21-04-24). The blood samples were diluted (4/1000) in phosphate-buffered saline (PBS) solution [mM: 130 NaCl, 2.7 KCl, 0.8 Na_2HPO_4, 1.4 KH_2PO_4 (pH 7.4)] and analyzed in d-TRMFS system. RBCs groups were analyzed: "Healthy adult", consisting in 80 RBCs from healthy adults; "Healthy fetal", consisting in 60 fetal RBCs extracted from placenta of healthy mothers; and "GDM fetal", consisting in 130 fetal RBCs from placenta of mothers with GDM (see Fig. 1).

2.2. Instrumental methodology d-TRMFS

Data was collected through a spectroscopy technique based on d-TRMFS by placing two laser beams at two different points on the membrane of a red blood cell. In this way, metabolic vibrations of the erythrocyte membrane in addition to random thermal flickering affect the focalized beam path creating interference of the light beam [11]. Intensity variations are collected by two quadrant photodetectors (QPD) for each signal or spot S_V and S_H, independently. Here, the subscripts V and H indicate polarization state of the beams which is a mandatory condition to avoid probable cross interference of the light signal leading to false detections. In consequence, detected intensities variations at each QPD are proportional to these rapid membrane movements. Then, a simple procedure calibrates those values from millivolts to real distance in nano scale amplitude. The optics setup and calibration procedure are fully described by Tapia et al. [6], including description of software's tools for obtaining micro-rheological parameters. Additionally, the mathematics of the fluctuation membrane model for RBCs from Helfrich et al. [10] and Park [12] is also discussed. The setup has suffered minor upgrades from the previous report [6], mainly to optimize noise cancelation, new optics elements and protocol for sample handling from placenta to chamber preparation with cells in physiological solution. In summary, the technique has demonstrated capable resolution to differentiate changes in mechanical parameters of adult RBCs in the scale of ~ms temporal order and sub-nm order spatial resolution without mechanical interference. In the current report we vary the sample target incorporating fetal RBCs for the first time, in the context of GDM.

General data procedure is as follows; for each RBC, acquire 20 data files of 10 s at an acquisition rate of 20 kHz. This value corresponds to the average fluctuation of the membrane around its equilibrium position. Then, determine the average value per red blood cell. The final parameters magnitude is the mean of all RBCs measurements ± SD for each condition (see Table 1).

2.3. Statistic analysis

Results for amplitude and mechanical parameters are presented using mean values ± SD (in Table 1), to inform the variability of values in the dataset. For *post-hoc* analysis, results are expressed as values ± SEM to show the accuracy of our sample data to represents the whole population. A total of 270 RBCs were independently measured, 80 from adult and 190 from fetal samples. Data were tested by normality (Shapiro-wilk test) and was determined non-parametric distribution for all groups. Then, comparisons between groups were performed using the Mann–Whitney *U* test. $p < 0.05$ was considered statistically significant.

3. Results

The results were obtained by analysing micro-rheological parameters (amplitude, tension, and flexion) of the two beams, S_V and S_H, for three study groups (Healthy adult, Healthy fetal and GDM fetal) (see Table 1).

First, healthy fetal RBCs show significant reduction of amplitude, quantified with the two beams (Fig. 2A and B). The reduction in amplitude was 9.80 ± 2.2 nm (47.1% decrease) and 2.40 ± 1.4 nm (12.8% decrease) for S_V and S_H, respectively, in healthy fetal RBCs compared with healthy adult RBCs. There are no significative changes in tension (Fig. 2C and D) and bending (Fig. 2E and F) between healthy fetal and healthy adults RBCs (Table 1).

Table 1
Micro-rheological parameters of red blood cells analyzed with d-TRMFS spectroscopy. The values correspond to the mean of all RBCs measured ± standard deviation, for each signal or spot S_V and S_H, independently, and the mean of both. ***$p < 0.005$ vs healthy adult; *$p < 0.05$ vs healthy adult; $^{\dagger}p < 0.05$ vs healthy fetal;$^{\dagger\dagger}p < 0.01$ vs healthy fetal; $^{\ddagger}p < 0.005$ vs healthy fetal.

	Amplitude [nm]		
	S_V	S_H	Mean S_V-S_H
Healthy adult	20.85 ± 14.51	18.80 ± 7.81	19.83 ± 11.66
Healthy fetal	11.04 ± 9.01***	16.40 ± 8.58*	13.82 ± 9.16***
GDM fetal	14.21 ± 8.48†	19.64 ± 7.90††	17.04 ± 8.60‡
	Tension (10^{-6} [N/m])		
	S_V	S_H	Mean S_V-S_H
Healthy adult	4.027 ± 3.21	2.095 ± 1.84	3.075 ± 2.79
Healthy fetal	4.008 ± 3.98	1.578 ± 1.47	2.851 ± 3.28
GDM fetal	4.271 ± 3.46	2.697 ± 2.13‡	3.494 ± 2.98‡
	Bending (10^{-19}[J])		
	S_V	S_H	Mean S_V-S_H
Healthy adult	1.863 ± 2.17	0.978 ± 0.96	1.306 ± 1.48
Healthy fetal	1.157 ± 1.01	1.002 ± 0.76	1.082 ± 0.89
GDM fetal	4.349 ± 6.37††	2.841 ± 4.07†	3.565 ± 5.41‡

In GDM RBCs compared with healthy fetal RBCs, there are significant increases in amplitude (Fig. 3A and B), tension (Fig. 3C and D) and bending (Fig. 3E and F). In GDM, the amplitude increased 3.17 ± 1.4 nm (32.3%) for S_V (Fig. 3A) and 3.24 ± 1.3 nm (19.7%) for S_H (Fig. 3B); the tension not change for S_V (Fig. 3C) and increases 1.12 ± 0.3 (10^{-6} [N/m]) (70.9%) for S_H (Fig. 3D); and bending increased 3.19 ± 0.9 (10^{-19} J) (275.9%) for S_V (Fig. 3E) and 1.84 ± 0.6 (10^{-19} J) (137.5%) for S_H (Fig. 3F). Considering the average values for two spots (S_V and S_H), in GDM cells the tension increased 6.431 ± 3.57 (10^{-7} [N/m]) and bending increased 2.483 ± 0.58 (10^{-19} [J]), which means 22.6% and 229.4% of increments of these parameters, respectively (Table 1).

4. Discussion

This is the first study about the micro-rheological properties of fetal RBCs from healthy and GDM patients using the d-TRMFS technique. The main finding of this study is the increase of tension and bending modulus observed in fetal RBCs from GDM compared with healthy fetal RBCs. Besides, the technique lets differentiate between the adult and fetal RBCs, with significant differences in amplitude parameter.

In RBCs from cases of intrauterine growth restriction there is a significant increase of lipid peroxidation, associated with lower activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase [13]. The lipid peroxidation contributes to increased membrane stiffness and reduced membrane deformability [14]. Specifically, in GDM, the hyperglycemia and hyperinsulinemia during pregnancy are associated with oxidative injury for fetal RBCs [5]. The oxidative stress leads to the oxidation of haemoglobin and damage to the RBCs membrane, alongside ATP depletion and depression of energy metabolism [15]. In umbilical cord blood samples from GDM pregnancies, have been determined an increase of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-OH-dG), a marker of DNA oxidative damage, and glutathione (GSH), a marker of oxidative stress [16]. Additionally, the alterations in oxidant and antioxidant parameters of umbilical cord blood in GDM are associated with oxidative stress in placental tissue [17]. All this evidence suggests that the alterations in RBCs membrane in GDM could be related

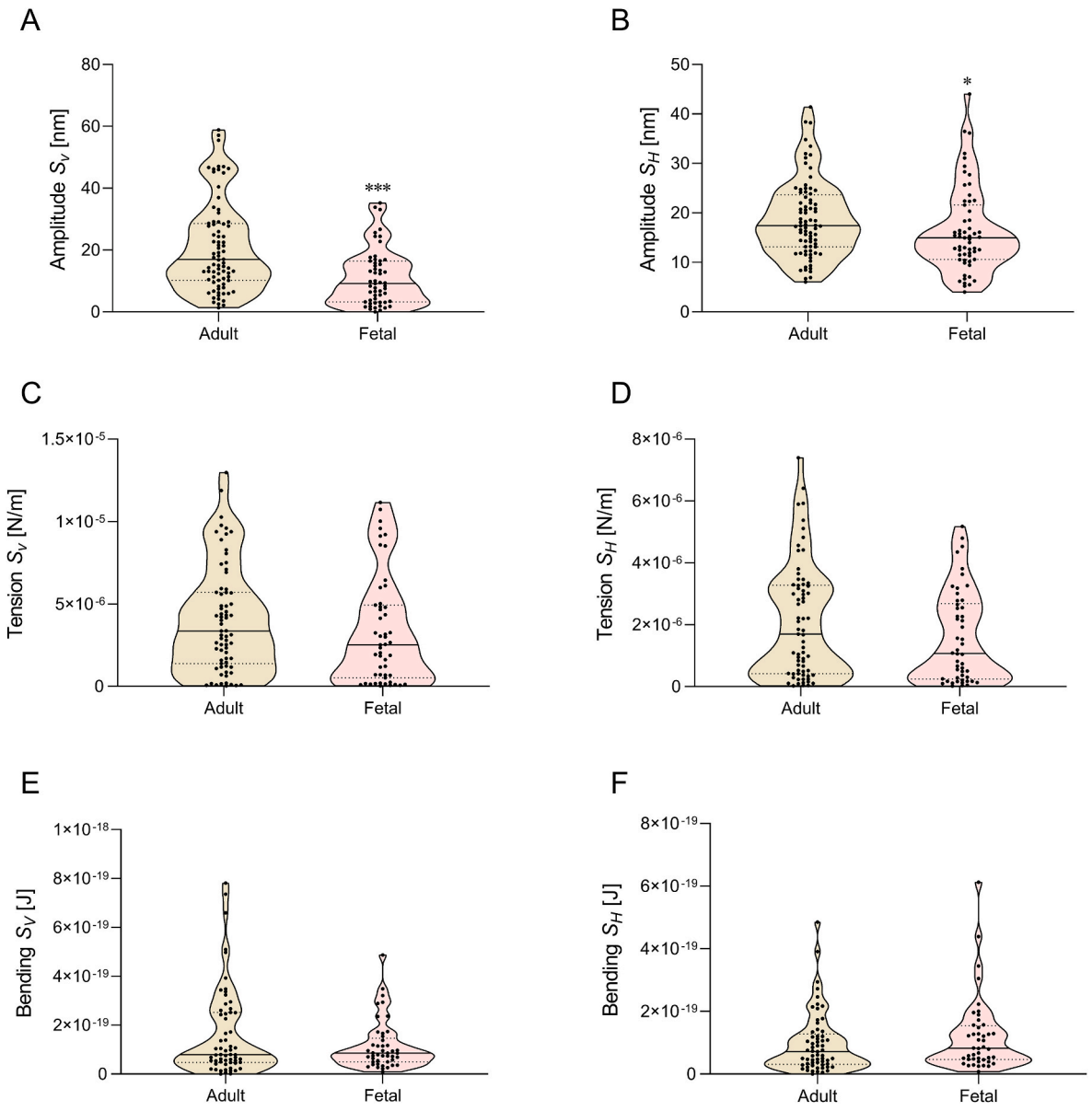

Fig. 2. Micro-rheological characterization of adult and fetal RBCs. Blood samples from adult and fetal blood were obtained and maintained at 37°C until d-TRMFS analysis. The parameter of amplitude (A, B), tension (C, D) and bending (E, F) were determined by each RBCs. Violin plots show the mean and distribution of all data alayzed. ***$p < 0.005$ vs healthy adult; *$p < 0.05$ vs healthy adult.

to oxidative stress in fetal plasma and lipid peroxidation. The lifespan of the neonatal RBCs is 60–90 days [18], so, the RBCs used in this study have exposed to several days to the fetal plasma characteristics, which also could be associated with higher neonatal ponderal index and decreased gestational age in GDM group (Table 2 in supplementary materials).

In micro-rheological parameters, the higher changes in GDM compared to healthy fetal cells was the increased bending modulus. In RBCs, the bending depends on the spectrin network tethered to the plasma membrane [19]. In adult RBCs from diabetic patients has been determined higher oxidation of spectrin, associated with a decrease in cell deformability [20]. The protein glycosylation causes irreversible oxidation of cysteine residues (cysteic acid, the final oxidation product of cysteine) in RBCs membrane and subsequently decreases deformability, leading to impaired microcirculation and tissue perfusion [20,21]. In a similar way, adult RBCs incubated with a high concentration of D-glucose induced a decrease in the bending modulus [6], suggesting that diabetes and hyperglycemia affect the bending and flexibility of RBCs. However, in GDM RBCs there was an increase in bending modulus, which could be associated with an adaptive response of fetal cells to the detrimental environment during GDM gestation, especially to the increase of oxidative stress. On the other hand, the nitric oxide-dependent S-nitrosation of membrane proteins of RBCs, results in significantly increased RBC deformability and reduced adhesion to cultured endothelial cells [22]. This finding correlates with the evidence that in fetal endothelium from GDM cases there is a higher synthesis of nitric oxide (NO) [23], which could be associated with the increase of bending modulus in the present study.

In summary, the findings underscore the significance of studying GDM and the potential impact of fetal RBC membrane alterations (or adaptations) on pregnancy outcomes. For further research, is suggesting comprehensive and multidisciplinary approach, focusing on the associated conditions of patients with GDM, like macrosomia and obesity.

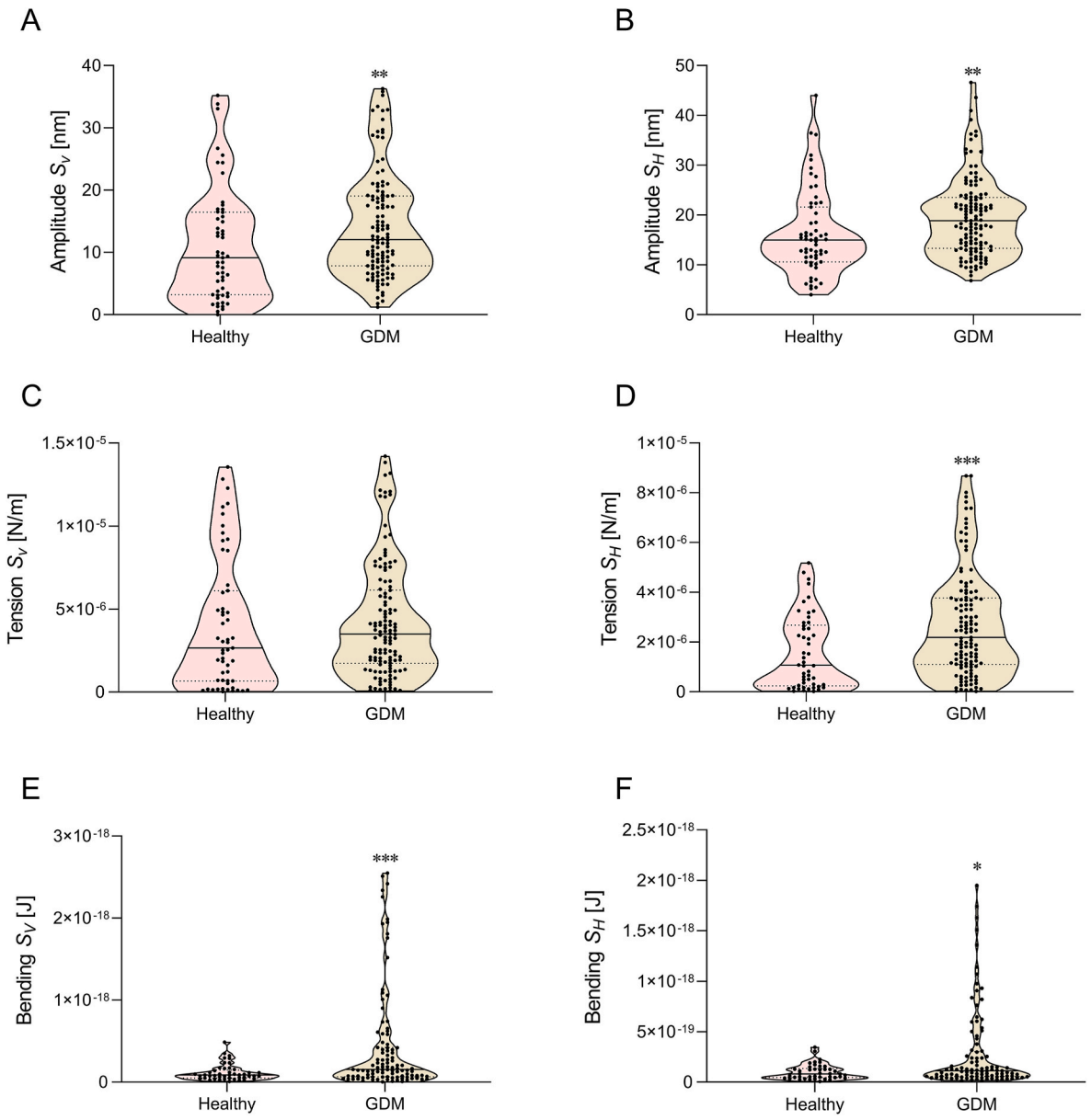

Fig. 3. Increase of tension and bending modulus in RBCs from GDM pregnancies. Blood samples from adult and fetal blood were obtained and maintained at 37°C until d-TRMFS analysis. The parameter of amplitude (A, B), tension (C, D) and bending (E, F) were determined by each RBCs. Violin plots show the mean and distribution of all data alayzed. ***p < 0.005 vs healthy fetal RBCs; **p < 0.01 vs healthy fetal RBCs; *p < 0.05 vs healthy fetal RBCs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

VRID-Multidisciplinario 2020000157MUL (U. De Concepción, Chile). Obstetrics and Gynecology Service, Hospital Regional Guillermo Grant Benavente, Concepción, Chile.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101556.

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