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Experimental evaluation of erythrocyte proteins membrane skeleton in hypertension

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Abstract

Reduced erythrocyte deformability is one of the important causes for stroke and heart failure in hypertensive patients. Deformability is a biophysical feature of erythrocyte determined by membrane cortex structure. Proteins of membrane skeleton such as spectrin, actin, band 4.1, ankryn and transmembrane protein Band 3 play a more important role than other membrane components in determining erythrocyte deformability. However, changes in proteins of erythrocyte membrane skeleton in hypertension have not been investigated so far. Therefore, in this report, calorimetric and spectroscopic investigations of the proteins of erythrocyte membrane skeleton are investigated in hypertensive rats and normal animals. Several methods were applied including differential scanning calorimetry (DSC), circular dichroism spectropolarimeter (CD), fourier transform infrared (FTIR). Our results showed that proteins of erythrocyte membrane skeleton, especially spectrin and Band 3 protein in hypertensive rats, are more compact than in normal rats. These changes are likely to have considerable effect on the membrane phospholipids and will increase their rigidity, which will ultimately reduce erythrocyte deformability.

Keywords: hypertension, deformability of erythrocyte, protein of membrane skeleton, CD, DSC

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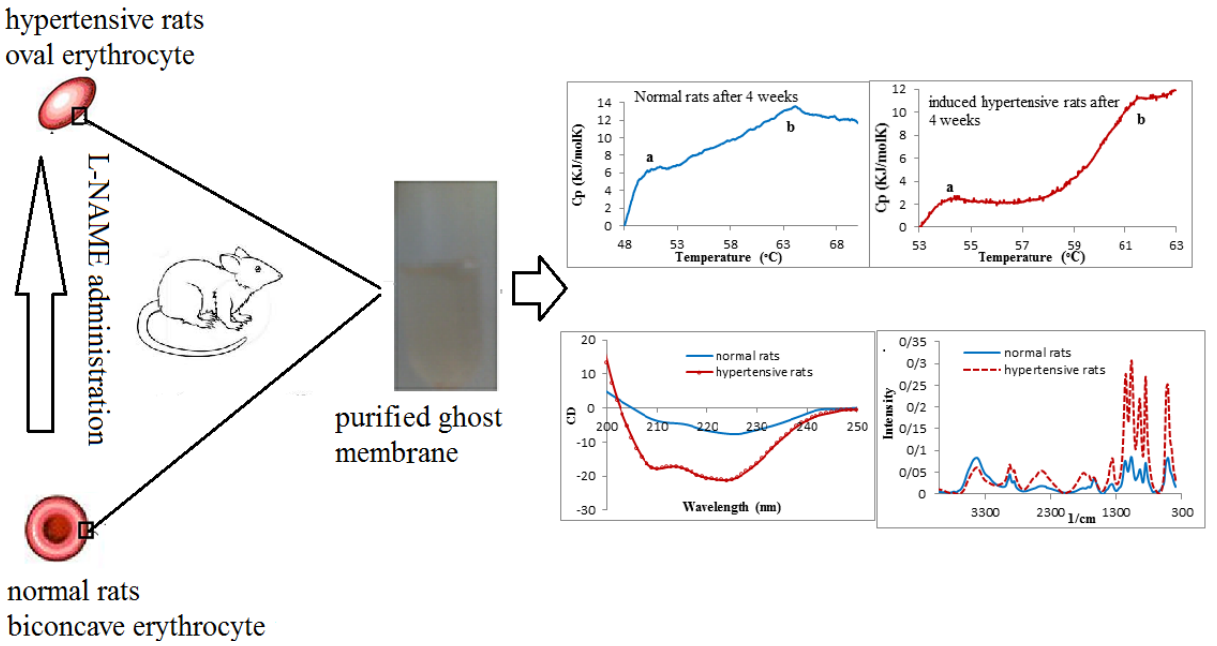

Highlights

- Protein stability of the erythrocyte membrane skeleton in hypertension was assessed.
- In hypertension, melting temperatures (T_m) of spectrin and Band 3 protein increased.
- The bonding of lipid hydrocarbon chains in erythrocyte membrane enhanced.

1. Introduction

Hypertension is a serious universal issue that is growing significantly. It is a chronic disease known as an important risk factor of cardiovascular diseases [1-3]. In hypertension, changes in rheological parameters are associated with increased blood viscosity, erythrocyte aggregation and reduction of erythrocyte deformability [3, 4]. Among these, deformability performs the key role in passing erythrocyte through the capillaries because the erythrocyte diameter is greater than the capillaries diameter [5, 6]. Therefore, reduction of erythrocyte deformability in hypertension leads to erythrocyte rupture during passage through the capillaries and this, in turn, may result in stroke and heart diseases [7].

Deformability is measured by various techniques including an optical tweezer, micropipette aspiration and atomic force microscopy (AFM) [8]. Chabanel et al. have found that erythrocyte deformability is decreased in hypertensive rats [9]. Also, previous studies using AFM illustrated that erythrocyte in hypertensive patients has been changed from biconcave to oval shape which represented a reduction of erythrocyte deformability [10]. Even though the reduction of erythrocyte deformability in hypertension has previously been reported by different techniques, there is limited knowledge about the subcellular (molecular) mechanism such as structural changes of membrane proteins and lipids related to reduced erythrocyte deformability [8].

Proteins of membrane skeleton (POMS) are the major factors determining erythrocyte deformability [11, 12]. The POMS consist of spectrin, actin, Band 4.1 and ankyrin. Spectrin is a large heterodimer protein with α and β subunits which is more abundant than other POMS. It is indirectly linked to proteins of Band 4.1 and Band 3 via actin and ankyrin, respectively [13-16]. On the other hand, the POMS interact with membrane phospholipids which preserve biconcave shape and flexibility of erythrocyte. According to the previous studies, intermolecular and intramolecular interactions of these proteins adjust flexibility and viscoelastic properties of membrane structure [17].

In this study, the structural and thermodynamic changes of POMS in hypertension induced by chronic administration of L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) are investigated. To evaluate the changes in thermodynamic parameters of POMS in hypertensive animals, calorimetric studies of POMS were carried out by differential scanning calorimetry (DSC). Structural changes of POMS in hypertension are studied by circular dichroism (CD) spectropolarimeter. Fourier transform infrared (FTIR) spectroscopy is used to assess the changes of membrane lipids in hypertensive rats relative to normal rats.

2. Materials and Methods

2.1 Animal

In this report, 48 male Wistar rats (200-250 g) were maintained at standard conditions (12-hour day, 12-hour night) in the animal laboratory. The animals were divided into 2 series of normal and induced-hypertension. Each series included three age groups (2, 4 and 6 weeks old). Induced hypertension was conducted by chronic administration of L-NAME in drinking water at a concentration of 0.4 mg/ml for 4 weeks and then 0.25 mg/ml for another 4 weeks [18, 19]. According to previous reports, low concentrations of L-NAME do not have any effects on erythrocyte deformability and erythrocyte membrane structure [20, 21]. Therefore, L-NAME is not expected to have any direct effects on POMS in this experiment.

Systolic blood pressure was measured using a tail-cuff Electro Sphygmomanometer (PE 300, Narco). Data on weight, blood pressure and heart rate were expressed as means ± standard deviation of the mean. Then, normal and induced hypertension rats were anesthetized and exsanguinated. In order to inhibit blood coagulation, the blood samples were collected in sodium citrate tubes.

2.2 Purification of erythrocyte and ghost membranes

Figure 1 shows the schematic representation of purification of erythrocyte and ghost membranes. Erythrocyte was initially separated by centrifugation at 3000 rpm for 20 min at 4°C. The erythrocyte pellet was then resuspended in phosphate-buffered saline (PBS) pH=7.4 and washed by centrifugation at 10000 rpm for 15 min at 4°C for 3 times. Subsequently, erythrocyte pellets were lysed by cold distilled water. The samples were then centrifuged at 15000 rpm for 15 min at 4°C and the supernatant was removed. The process of washing was repeated 4 times and most of the hemoglobin was removed. Finally, purified colorless

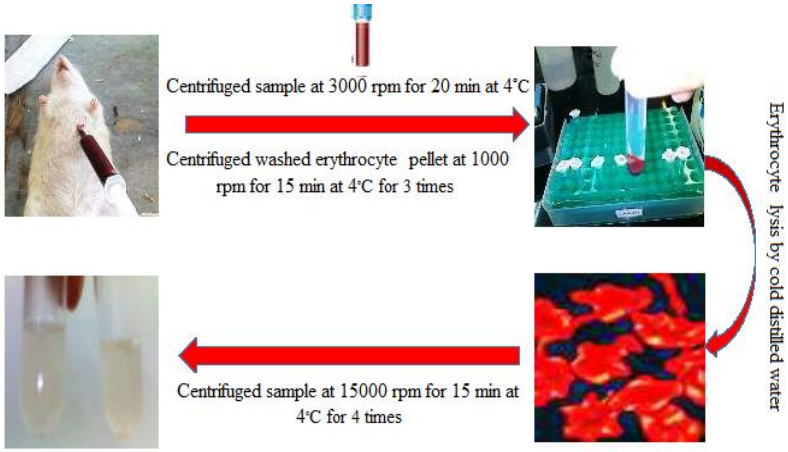

Figure 1. Steps of erythrocyte and ghost membrane purification.

ghost membranes were suspended in 20 mM phosphate buffer pH=7.4 [22].

2.3 Determining ghost membrane proteins by polyacrylamide gel electrophoresis

Erythrocyte ghost membrane proteins were characterized by electrophoresis on gradient polyacrylamide gel (5% - 15%) for 1 hour [23]. The gel was stained with Coomassie Blue for 20 min.

2.4 Concentration measurement of erythrocyte ghost membranes proteins

The concentration of erythrocyte ghost membranes was evaluated according to Bradford protocol. Briefly, a standard curve was initially prepared from BSA protein (bovine serum albumin) and the 50 µl diluted samples were stained with 1 ml Coomassie Blue. The absorbance of the samples was measured at λ_{595} nm [24].

2.5 Thermal analysis of ghost membrane proteins by differential scanning calorimeter

Thermodynamic studies of POMS of normal and hypertension groups were performed with a differential scanning calorimeter (TA instruments, Nano DSC III). Diluted samples were prepared in 0.6 mg/ml, 20 mM phosphate buffer (pH 7.4). The sample cell was filled by protein solution and the reference was filled by the buffer, without air bubbles. The heating rate was 1 °C/min. The thermogram was analyzed using Micro Origin Software.

2.6 Structural assessment of erythrocyte ghost membrane proteins by CD Spectropolarimetry

The secondary structure of POMS for all groups of hypertensive and normal rats was studied with JASCO J-715 spectropolarimeter in the wavelength range of 200-250 nm. All samples were diluted (0.2 mg/ml concentration) in 0.2 M phosphate buffer (pH 7.4). Data were smoothed and analyzed by JASCO J-715 software. Each CD Spectropolarimetry experiment was performed 3 times and the data were represented as means and standard deviations.

2.7 Analysis of lipid content of erythrocyte membrane by FTIR spectroscopy

To study erythrocyte membrane lipid changes in hypertension, infrared spectra of erythrocyte (0.5 mg/ml concentration in phosphate buffer, pH 7.4) for four groups of hypertension and normal rats were recorded by FTIR Nicolet IR-100 in the frequency range of 900-4000 cm^{-1}.

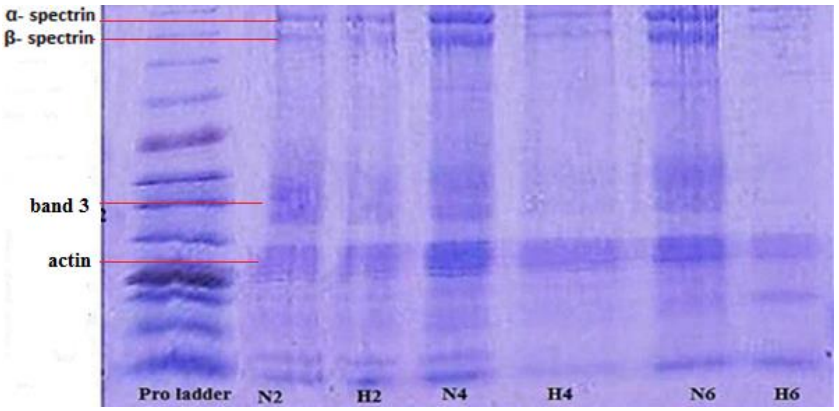

Figure 1. Electrophoresis of erythrocyte ghost membranes in normal and hypertensive rats. Left to right: protein ladder, proteins of ghost membranes in normal and hypertension after 2, 4 and 6 weeks administration of L-NAME (N: Normal and H: Hypertension).

2.8 Statistical analysis

The GraphPad Prism V6.0 (GraphPad Software, San Diego, CA) was used for the statistical analysis of data. Differences in blood pressure and heart rate were assessed by a repeated measure two-way ANOVA with a Bonferroni post-test. Results were represented as mean ± standard deviation. P-values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1 Blood pressure measurement

According to previous reports, L-NAME administration increases blood pressure reversibly. Nitric oxide production is inhibited by L-NAME, which leads to the closure of vessels and increased systolic blood pressure [14, 33-36]. The blood pressure of both normal and hypertensive rats was measured. The results showed high blood pressure ($p < 0.001$) and low heart rate ($p < 0.001$) in hypertensive rats compared to their normal counterparts. Blood pressure was significantly raised ($p < 0.01$) and heart rate reduced ($p < 0.01$) after four weeks of L-NAME administration. After 6 weeks administration, the blood pressure of induced-hypertensive rats was higher than normal rats ($p < 0.001$) (Table 1) Also, blood pressure after six weeks is lower than 4 weeks, this might be due to the reduced L-NAME concentration in the 6th week [25, 26].

3.2 Electrophoretic analysis of ghost membrane proteins

In Figure 2, the bright bands show the ghost membrane proteins for all groups of normal and hypertensive rats (2, 4 and 6 weeks of L-NAME administration from left to right). The bright bands of α and β spectrin with molecular weights of 240 and 245 kDa, Band 3 at 80 kDa and actin are marked. [27-30].

Table 1. Experimentally measured blood pressures of normal and hypertensive rats

	Normal rat			**Induced hypertensive rat**		
	2 weeks n=8	4 weeks n=8	6 weeks n=8	2 weeks n=8	4 weeks n=8	6 weeks n=8
Weight (gr)	274±2.4	292±4.5	323±3.9	262±2.7	280±4.3	296±4.3
blood pressure (cmHg)	9.3±0.5	10.3±0.5	11.3±0.5	12.3±0.5	15.3±0.5	13.3±0.5
Heart rate (bpm)	400±2	385±3	370±2	365±2	300±3	350±2

SDS-PAGE analysis of erythrocyte membrane proteins shows that α and β spectrin in hypertensive rats is remarkably lower compared with normal rats. Actin showed only a slight decrease. Moreover, after 6 weeks of L-NAME administration, Band 3 protein was not observed. In previous studies, Band 3 protein is reduced in hypertensive patients [31], which is consistent with the current experiment.

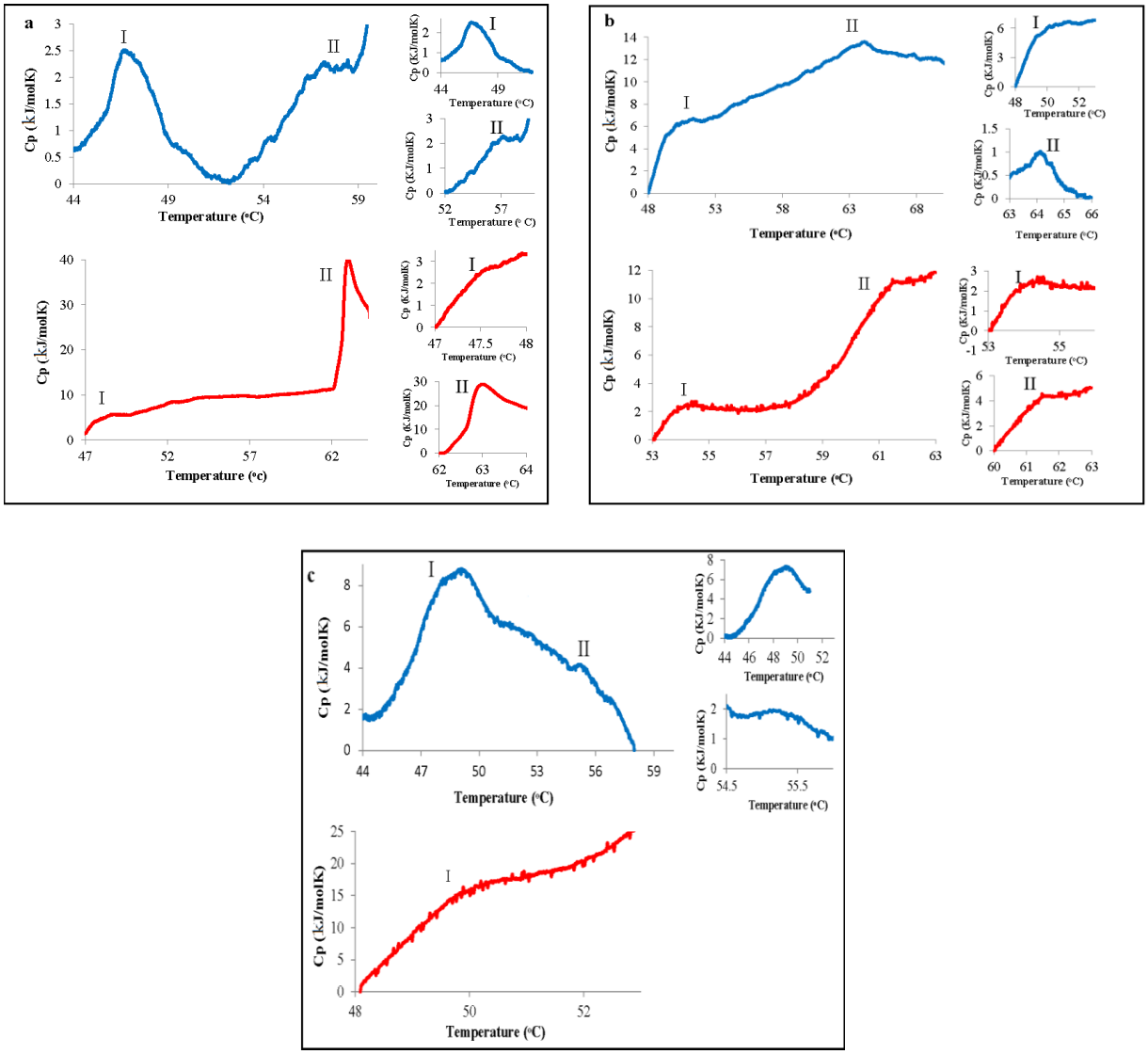

Figure 2. a) DSC thermograms of erythrocyte ghost membrane proteins of normal and hypertensive rats after 2 weeks of L-NAME administration from top to bottom, respectively, insert pictures: I) Spectrin and II) Band 3 protein. b) DSC thermograms of erythrocyte ghost membrane proteins of normal and hypertensive rats after 4 weeks of L-NAME administration from from top to bottom respectively, insert pictures: I) Spectrin and II) Band 3 protein. c) DSC thermograms of erythrocyte ghost membrane proteins of normal and hypertensive rats after 6 weeks of L-NAME administration from top to bottom respectively, insert pictures: I) Spectrin and II) Band 3 protein.

3.3 Irreversible thermal denaturation of erythrocyte ghost membrane proteins

DSC of α and β spectrin and Band 3 protein in 20 mM phosphate buffer, pH 7.4 in all groups of hypertensive and normal rats are shown in Figure 3 a-c. V.L. Shnyrov, et al. reported that rat erythrocyte ghost membranes in similar experimental conditions clearly showed two peaks. The lower temperature transition is for spectrin and the other transition is attributable to Band 3 protein [22, 23, 32]. In the current experiment, the figures show the temperature transition of spectrin and Band 3 (I and II). Figure 3a. shows ghost membrane proteins for hypertensive and normal rats after 2 weeks of L-NAME administration. The melting temperatures (T_m) of spectrin and Band 3 protein in hypertensive rats increased (Table 2). Figure 3b shows the DSC thermogram after four weeks of L-Name administration. The spectrin T_m of hypertensive rats is increased; whereas Band 3 protein T_m decreased (Table 2). Figure 3c shows that the melting temperature of spectrin in hypertensive rats after 6 weeks of L-NAME administration increased; while the peak for Band 3 protein disappeared (Table 2). Denaturation of spectrin and Band 3 protein in POMS indicated an increase in the melting temperature (Tm) in all groups of hypertensive rats in comparison with normal rats. This increase in stability is attributed to an increase in the abundance of Van der Waals forces in the spectrin structure [33] in hypertensive rats than in normal rats. Moreover, the Tm value of spectrin increased much more after 4 weeks of L-NAME administration than after 2 and 6 weeks, probably the cause of higher blood pressure at 4 weeks (Table 1). Tm of Band 3 protein is at its maximum level after two weeks of L-NAME administration and disappears after 6 weeks in accordance with the electrophoretic results in Figure 2.

3.4 Secondary structure analysis of erythrocyte ghost membrane proteins

CD spectra of erythrocyte ghost membrane proteins or POMS (in phosphate buffer, pH 7.4) of the 3 groups of hypertensive and normal rats were analyzed. The ellipticity intensity of POMS is displayed in Figures 4 (a-c). The structural changes of POMS at 222 nm that are related to α- helix secondary structure are shown in Figure 4a. In Figure 4a, the ellipticity intensity after 2 weeks of L-NAME administration is increased. For the period of 4-6 weeks, as shown in Figures 4(b-c), considerable changes were observed around 230 nm, especially in Figure 4c, representing an increase of β-turn structure [34].

CD spectra of erythrocyte ghost membrane proteins showed the α-helix structure of POMS at λ_{222} [35]. CD data showed that the ellipticity intensity of POMS in hypertensive rats increased. This implies that POMS have gained a compact structure. Besides, there was less change of ellipticity intensities in the hypertensive rat after 6 weeks of L-NAME administration, indicating a decrease in the secondary structure in POMS. These results are in accordance with the DSC results in this study.

3.5 Analysis of the structure of erythrocyte membrane lipids

FTIR spectra of erythrocyte in all groups of hypertensive and normal rats are shown in Figures 6 a-c. The main absorbance bands for the α-helix and β-sheet secondary structure of proteins (rat erythrocyte) consist of 1645-1660 cm^{-1} and 1530-1680 cm^{-1}, NH stretch vibrations at 3400 cm^{-1}, C-C vibrations at 978 cm^{-1} and CH vibrations at regions 2800-2900 cm^{-1}. Figure 6a shows structural changes of erythrocyte in hypertensive rats after 2 weeks of L-NAME administration, in comparison to normal rats. Considerable enhancement is observed in the intensity of C-C vibrations; while the intensity of the NH vibration band did not change. The intensity of the α-helix, β-sheet and C-H bands increased slightly. The FTIR spectrum of hypertensive rats after 4 weeks of administration is displayed in Figure 6b. The intensity of the C-C vibration band increased much more than the other bands. The intensity band at 3400 cm^{-1} decreased and did not change at 1530 cm^{-1}. Fig. 6c illustrates the erythrocyte FTIR spectrum of hypertensive rats after 6 weeks of L-NAME treatment. In this Figure, the band intensity in most of the regions including 978 cm^{-1}, 1530-1686 cm^{-1}, 1645-1660 cm^{-1} and 2800-2900 cm^{-1} considerably increased, but the intensity of the band at 3400 cm^{-1} did not change. FTIR showed increased intensity for hypertensive rats in most regions but especially the C-C vibration band.

Table 2. DSC melting temperature of erythrocyte membrane skeletal proteins in normal and hypertensive rats after 2, 4 and 6 weeks of L-NAME administration (N: Normal and H: Hypertension).

	Melting temperature (Tm) (°C)	
	Spectrin*	Band 3 protein*
N2	46.7±0.03	56.25±0.03
H2	47.45±0.03	63.04±0.03
N4	49.27±0.03	64.12±0.03
H4	53.90±0.03	61.48±0.03
N6	48.14±0.03	55.55±0.03
H6	49.89±0.03	-

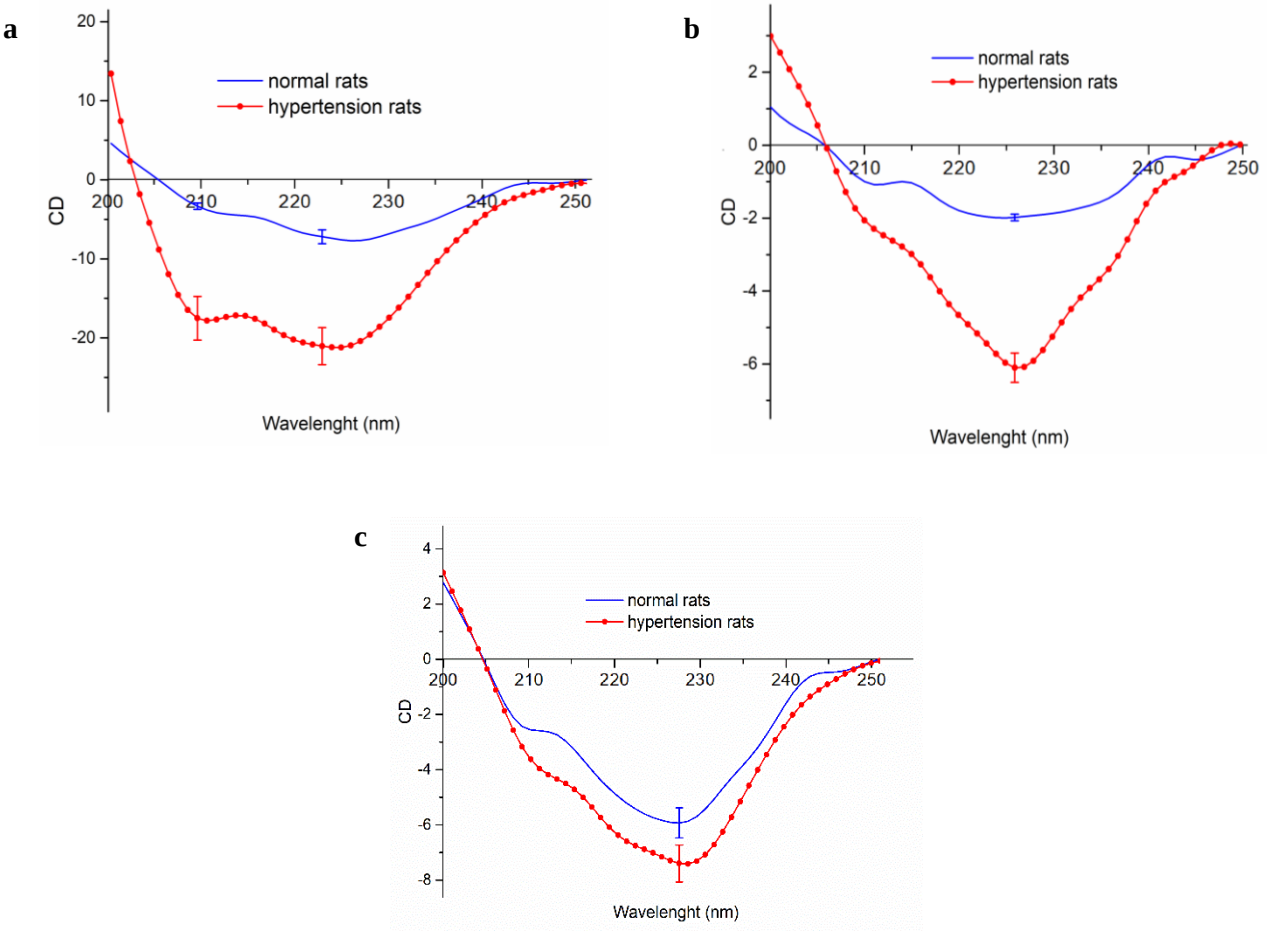

Figure 4. CD spectra of erythrocyte ghost membrane proteins in normal and hypertensive rats after 2, 4 and 6 weeks administration of L-NAME from a to c, respectively. The peak values are presented as means with standard error bars.

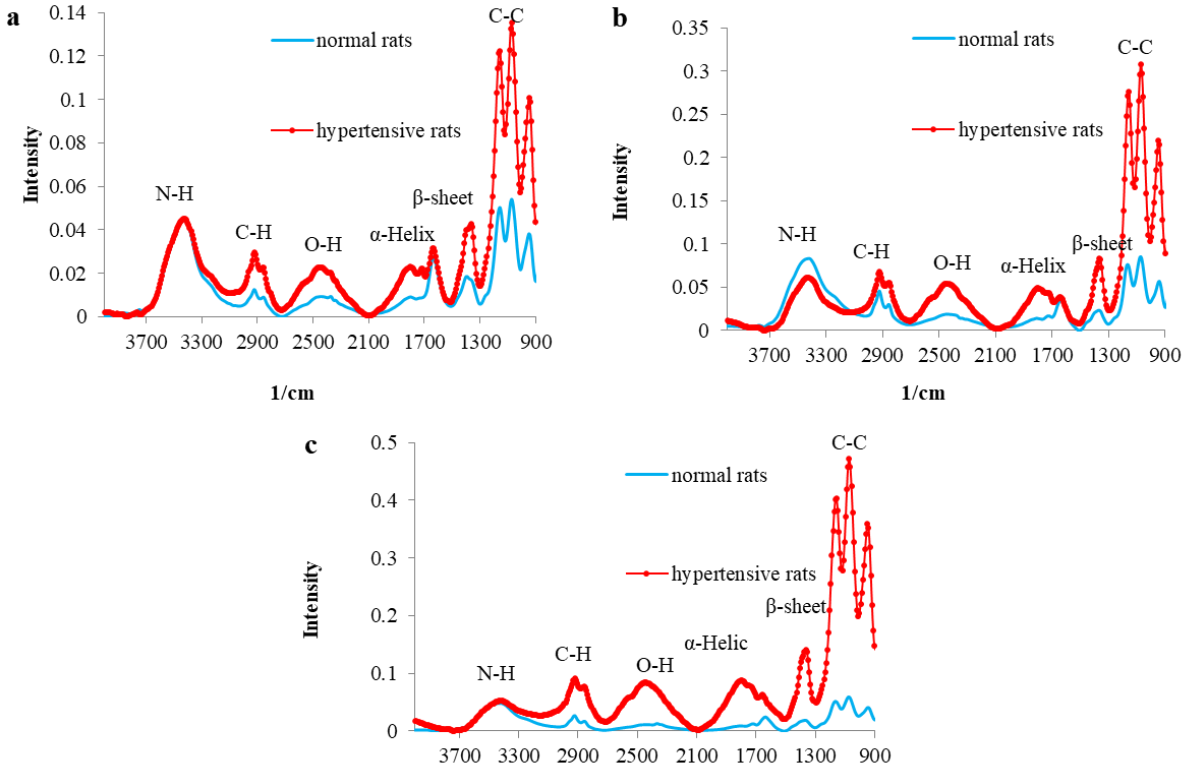

Figure 5. FTIR spectra of erythrocyte in normal and hypertensive rats after 2, 4 and 6 weeks administration of L-NAME, from a to c, respectively

This is related to the lipid component of the membrane, which gained a structure of higher compactness and rigidity. According to previous reports, the lipid components of erythrocyte membranes alter hypertension, leading to decreased fluidity [36, 37]. On the other hand, the organization of membrane lipids is determined by POMS. Phospholipid interactions of erythrocyte membrane are also enhanced in hypertension which leads to decreased flexibility and deformability of erythrocyte.

4. Conclusion

The results of this investigation confirmed that the numbers of Van der Walls forces in spectrin and Band 3 protein structures are increased in hypertensive rats and this leads to increased compactness and stability of structures. Also, the secondary structures of erythrocyte membrane skeletal proteins in hypertensive rats became more compact. Subseqently, the structure of the Band 3 protein was destroyed. This process affected the organization of membrane lipids and enhanced the chemical bonds of phospholipids chains. As a result, the rigidity of the erythrocyte membrane increased in hypertensive rats and their deformability decreased, giving a brittle structure when passing through capillaries.

Conflict of interest

The authors declare that they have no conflict of interest.

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