NanoScience Technology, Volume 4 (2022), pp: 17-25

NanoScience Technology

Journal homepage: https://jnanoscitec.com

The investigation of hemoglobin in hypertension; spectroscopic and thermodynamic analysis

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Abstract

Hypertension is a disease with a significant risk factor for heart failure and stroke. One of the main reasons behind heart failure and stroke in hypertensive patients is the reduction of erythrocytes deformability. An influential factor in determining erythrocyte deformability is the erythrocyte's cytoplasmic viscosity. The majority of erythrocyte cytoplasm is composed of hemoglobin. This report investigates structural and thermodynamical hemoglobin changes in induced-hypertensive rats using circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC) techniques. The obtained results from hypertensive cases reveal increased flexibility and a reduction of compactness in hemoglobin structures. Additionally, the availability of heme groups and hydrophobic residues in hemoglobin structures decreased. This process led to a lack of oxygen binding to heme groups. Furthermore, hemoglobin aggregation occurs and causes an increase in cytoplasmic viscosity and a decrease in erythrocyte deformability.

Keywords: Hypertension, Hemoglobin, Erythrocyte's deformability

© Article info: Accepted by: 2 May 2022, Published by: 17 may 2022.

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Highlights

- Biophysical parameters of hemoglobin in hypertensive rats were assessed.
- The compactness of α-helix secondary structure of hemoglobin is decreased.
- The hemoglobin tertiary structure becomes more flexible and looser.
- Hydrogen bond and Van der Waals forces of hemoglobin structure are attenuated.
- Instability and aggregation phenomenon in hemoglobin structure are observed.

1. Introduction

Hypertension is one of the leading causes of cardiovascular diseases [1-3]. It was estimated that hypertension causes 54% of strokes and 47% of ischemic heart diseases [1]. Hypertension would increase blood viscosity, decrease erythrocyte deformability, and cause changes in blood rheological properties [3-5]. The decrease in deformability of erythrocytes leads to stroke and heart failure. As the capillaries' diameters are approximately half of the erythrocytes' diameter, erythrocytes' deformability property performs the most crucial role in passing erythrocytes through capillaries [6-8].

The erythrocytes' deformability is the characteristic property of the viscoelastic erythrocytes. It is characterized via three factors: the erythrocyte geometry, the structure of the erythrocyte membrane cortex, and the cytoplasmic viscosity—the cytoplasmic viscosity is associated with hemoglobin concentration cytoplasm [7]. Therefore, the increase of hemoglobin concentration or cytoplasmic viscosity leads to the reduction of erythrocyte deformability. As a result, the increase in hemoglobin concentration is directly correlated to hypertension. Furthermore, another consequence of hypertension is a decrease in blood oxygen levels known as hypoxia [9, 10]. This phenomenon is due to the change in conformation of porphyrin in hemoglobin structure; consequently, hypertension alters and reduces the efficiency of oxygen binding [11].

Hemoglobin with a molecular weight of 64 KD is a heterotetrametric protein with two subunits: α_2 and β_2, that has tetrahedron arrangement [12, 13]. Hemoglobin subunits also include the heme groups bound with the oxygen molecule. The heme groups are composed of iron atoms in the center of porphyrin rings, and iron atoms in the ferrous state (Fe^{+2}) are bound to oxygen molecules from the porphyrin structure [12, 14]. Moreover, most hemoglobin residues preferably form α-helix structures in which they are connected via non-helical conformation [15]. In the hemoglobin sequence, several positions of amino acids have been conserved during evolution, which implies their crucial roles in hemoglobin function [16-18]. Furthermore, they are closely associated with heme groups and oxygen molecules. Accordingly, hemoglobin structure is altered to different conformations, including oxyhemoglobin and deoxyhemoglobin. Oxyhemoglobin conformation is when oxygen molecules are connected to heme groups, a loose form of hemoglobin. On the other hand,

hemoglobin conformation in the absence of oxygen molecules is a tight structure called deoxyhemoglobin [19-21].

Therefore, to further assess the hemoglobin changes in hypertension, structural and thermodynamical studies of hemoglobin were carried out in experimentally induced hypertension by the chronic administration of L-NAME in a group of rats. Thus, in this report, the structural analysis of hemoglobin in hypertensive rats compared to normal rats was investigated by spectroscopic methods, including UV-visible, circular dichroism (CD). Furthermore, in hypertensive rats, the differential scanning calorimetry (DSC) method was used to evaluate hemoglobin changes in thermodynamic parameters.

2. Materials and Methods

2.1 Animal

In this report, 48 male Wistar rats (200-250 g) are supplied from the Pasture institute and maintained in an animal laboratory under standard conditions (12-hour day, 12-hour night). The animals were divided into two series of healthy and induced hypertension rats. Each of the series included three age groups (2, 4, and 6 weeks old). The experimental induced hypertension protocol was conducted by chronic administration of L-NAME in drinking water at a concentration of 0.4 mg/ml for four weeks and 0.25 mg/ml continuously for another two weeks [22, 23]. Then, systolic blood pressure was measured using a tail-cuff Electro Sphygmomanometer (PE 300, Narco). Subsequently, healthy and induced hypertension rats were anesthetized by urethane and were exsanguinated. Finally, the samples were collected in sodium citrate tubes to inhibit blood coagulation [24, 25].

2.2 Isolation and Purification of hemoglobin

Erythrocytes were initially separated by centrifugation at 3000 rpm for 20 min at 4°C, after that the resultant sediment as erythrocyte pellet was resuspended in saline phosphate buffer with pH=7.4 and was washed three times by centrifugation at 10000 rpm for 15 min at 4°C. Subsequently, erythrocyte pellets were lysed with cold distilled water. In the next step, the sample was centrifuged at 15000 rpm for 15 min at 4°C. Then, ammonium sulfate 20% solution was added to the supernatant containing hemoglobin and stayed in a static state for 15 min at room temperature. After that, the sample tube was centrifuged at 14000 rpm for 60 min followed by collecting the supernatant which is then purified hemoglobin. Finally, purified hemoglobin was dialyzed in 50 mM phosphate buffer in pH= 7.4 for 48 hours at 4 ˚C.

2.3 Electrophoresis of purified hemoglobin

The electrophoresis technique approved the purification of hemoglobin. It was run on sodium dodecyl sulfate-polyacrylamide gel (12%) for 1 hour. The gel was stained with Coomassie blue for 20 min.

2.4 Concentration measurement of hemoglobin

Measuring the concentration of purified hemoglobin was performed using the Bradford protocol. In brief, a standard curve initially was drawn and the 50 µl diluted samples were stained with 1 ml coomassie blue, and the absorption was recorded at λ595 nm [26].

2.5 UV spectroscopy

To investigate the heme group qualification of hemoglobin in hypertensive rats relative to normal rats, multiwavelength UV-visible spectroscopy was used. Absorbance spectra (from 300 nm to 700 nm) of hemoglobin solutions (0.5 mg/ml) were recorded by Agilent Carry 100, spectrophotometer.

2.6 Far-UV circular dichroism (CD) Spectropolarimetry

Secondary structures of hemoglobin for all groups of hypertension and normal rats were studied by far-UV CD spectropolarimeter (model JASCO J-715) in the wavelength range from 200 to 250 nm. All samples were diluted in 0.2 mg/ml concentration with 50 mM phosphate buffer pH= 7.4. Finally, the results were analyzed by JASCO J-715 software. Moreover, the results of CD spectra of hemoglobin were characterized in the form of molar ellipticity, as shown in equation (1):

$$[\theta] = \frac{\theta \times 100\ \mathrm{MRW}}{cl}$$

In which [θ] (degree.cm2.dmol-1) is referred to ellipticity, c and l are referred to protein concentration

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

	Normal rat			Induced hypertensive rat		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
Weight (gr)	274	292	323	262	280	296
blood pressure (cmHg)	9	10	11	12	15	13
Heart rate (bpm)	400	385	370	365	300	350

(mg/ml) and light path length, respectively. MRW= mean residual.

2.7 Near-UV circular dichroism (CD) Spectropolarimetry

Near-UV CD spectra reflect the environments of phenylalanine, tyrosine, and tryptophan amino acids and thus give information about the tertiary structure of hemoglobin. CD spectra in 2 mg/ml concentration of phosphate buffer pH=7.4 for all groups of hemoglobin samples were carried out on JASCO J-715 in the wavelength range of 250- 320 nm. Eventually, the noise of the data was smoothed and analyzed by JASCO J-715 software. Furthermore, molar ellipticity values of CD spectra were obtained through the above formula.

2.8 Thermodynamic study of hemoglobin

Thermodynamic investigations of hemoglobin of normal and hypertension samples were performed by a differential scanning calorimeter, TA instrument, and Nano DSC III. Moreover, the hemoglobin solution was prepared at a concentration of 0.4 mg/ml in 50 mM phosphate buffer at pH =7.4. The samples from 20 to 80 ^{o}C were heated in a DSC instrument at a constant heating rate of 1 °C/min without the presence of air bubbles. Analysis of thermogram was carried out on micro origin software [27].

3. Results and discussion

3.1 Blood pressure measurement

According to Table 1, the results showed that induced hypertensive rats generally had higher blood pressure and lower heart rate than normal ones, respectively. In previous reports, blood pressure is increased by L-NAME administration reversibly. In fact, in the presence of L-NAME, nitric oxide production is inhibited, which leads to the closure of vessels and the increment of systolic blood pressure [28-31].

To evaluate the effectiveness of L-NAME, blood pressure of normal and induced hypertensive rats was measured.

3.2 Electrophoretic analysis of purified hemoglobin

The purified hemoglobin is analyzed by the sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis technique. Figure 1 shows the electrophoretic pattern of the hemoglobin protein. The bright bands illustrate all groups of normal and hypertensive rats, including 2, 4, and 6 weeks of L-NAME administration from left to right, respectively, and the proteins ladder at the end of the lane.

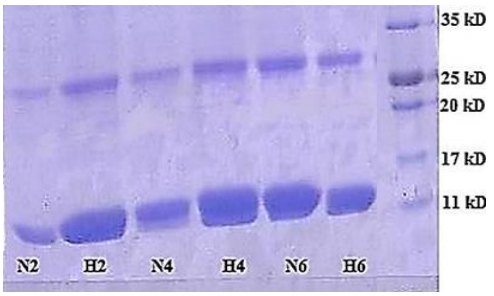

Figure 1. Electrophoresis of hemoglobin in normal and hypertensive rats Left to right: hemoglobin protein in normal and hypertensive rats after 2, 4 and 6 weeks of L-NAME administration respectively (N: Normal and H: Hypertension).

3.3 Multiwavelength UV-Visible spectroscopy of hemoglobin

Results of UV-Visible spectra of hemoglobin were demonstrated in three groups of hypertensive rats (2, 4, and 6 weeks of administration of L-NAME)

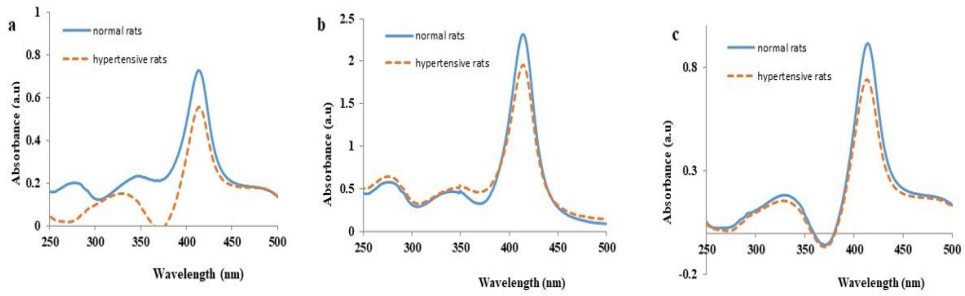

Figure 2. UV-Visible spectra of hemoglobin in normal and hypertensive rats after 2, 4 and 6 weeks administration of L-NAME from a to c, respectively.

compared to normal groups (Figures 2 a-c). According to UV-Visible spectra, the hemoglobin absorbance at wavelengths of 280 nm, 340 nm, and 418 nm are related to tyrosine and tryptophan, π-π* electron transition, and ultimately heme groups in the soret region, respectively.

Figure 2a displays hemoglobin absorbance spectrum after 2 weeks of L-NAME administration, accordingly, the absorbance of 340 nm and 418 nm were decreased and the peak at 340 nm was shifted to a lower wavelength. Moreover, the absorbance intensity at 280 nm disappeared entirely. In figure 2b, hemoglobin absorbance in 4 weeks of L-NAME administration slightly decreased to 418 nm. On the other hand, the absorbance intensities at 280 nm and 340 nm were not changed. Figure 2c illustrates the hemoglobin absorbance after 6 weeks of L-NAME administration that the absorbance peak at 418 nm was reduced. However, the absorbance intensity at 340 nm was not changed. Besides, absorbance peaks at 280 nm disappeared in both hypertensive rats and normal rats. The results of UV-Visible spectroscopy of hemoglobin showed a decrease in hemoglobin absorbance intensity in hypertensive rats at 418 nm. Considering that 418 nm wavelength is related to the heme groups, it can be said that the availability of heme groups was decreased. The absorbance intensity of hemoglobin of hypertensive rats at 280 nm disappeared, representing a lack of access to tryptophan residues. Moreover, the absorbance intensity at 340 nm was detected which represented the doxy conformation of hemoglobin. According to previous reports, the absorbance peak at 340 nm is attributable to hemoglobin structures and the protonation of the heme groups. The heme group in Fe^{+3} states is bound to oxygen, and the absorbance peak at 340 nm is not observed. [12, 32]. The absorbance peak of hemoglobin at 340 nm in hypertensive rats after two weeks of L-NAME administration was shifted to the shorter wavelength. This is implied the changes in hemoglobin structure in hypertension.

3.4 Secondary structure analysis of hemoglobin

Figures 3a to c show the CD spectra of hemoglobin related to the changes of α-helix secondary structure in

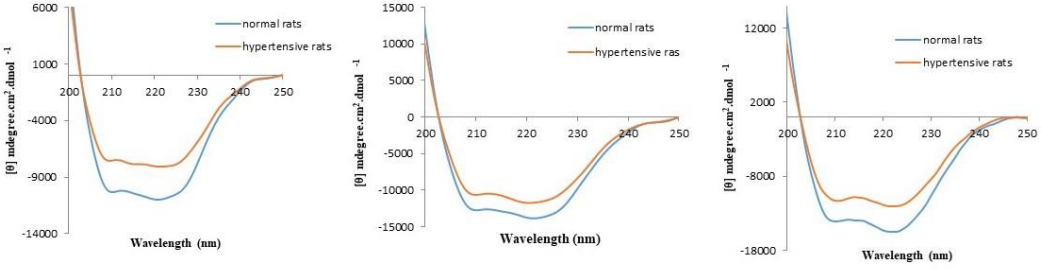

Figure 3. Far UV- CD spectra of hemoglobin in normal and hypertensive rats after 2, 4 and 6 weeks administration of L-NAME from a to c, respectively.

hypertensive rats after 2,4 and 6 weeks of L-NAME administration compared to normal rats at phosphate buffer pH= 7.4. Molar ellipticity intensities of hemoglobin in all groups of hypertensive rats were generally decreased.

The helical content in the secondary structure of the protein is evaluated using Far UV-CD spectra. Therefore, the α-helix structure is detected by the CD technique at 222 nm. According to previous reports, most amino acids of hemoglobin tend to form α-helix structures and each subunit is organized into several α-helix structures that are connected to non-helical secondary structures [32, 33]. In the current experiment, the CD spectra displayed the α-helix structure of hemoglobin at 222 nm. Moreover, the results of CD spectra indicated that molar ellipticity intensities of hemoglobin in hypertensive rats compared to normal rats were decreased. Thus, the level of α-helix structure of hemoglobin in hypertension was attenuated. It seems that the compactness of α- helix structures in hypertensive rats is reduced.

3.5 Tertiary structure analysis of hemoglobin

The local changes of hemoglobin tertiary structure around aromatic amino acids are obtained by the Near-UV CD method. Moreover, the absorbance peaks of phenylalanine, tyrosine, and tryptophan are respectively characterized in wavelength ranges of 250- 270 nm, 270- 290 nm, and 280-300 nm. The molar ellipticity results of hemoglobin related to tertiary structure changes in 3 groups of hypertensive rats compared to normal rats at phosphate buffer pH= 7.4 for 2 mg/ml constant concentration are shown in figures 4a-c. The hemoglobin molar ellipticity of hypertensive rats after 2, 4, and 6 weeks of L-NAME administration in comparison with normal rats at the range of 250-270 nm was decreased generally. Therefore, the changes in tertiary structure are occurred in around phenylalanine. Also, the changes of molar ellipticity at ranges of 270- 290 nm and 280- 300 nm in hypertensive rats after 2, 4, and 6 weeks of L-NAME administration relative to normal rats were not observed considerably. It can be said the tertiary structure of hemoglobin around tyrosine and tryptophan was not changed much.

The results of changes in hemoglobin tertiary structure in hypertension indicate a decrease in molar ellipticity around phenylalanine residues [34, 35]. It seems that the flexibility of hemoglobin tertiary structure around phenylalanine residues is increased. According to the previous reports, phenylalanine 43 in the α-chain of hemoglobin directly contacts the heme group [36]. Besides, a decrease in molar ellipticity intensity near 260 nm is related to the changes in the optical activity of heme groups in α and β chains of hemoglobin [37, 38]. It can be said that the tertiary structure of hemoglobin surrounding the phenylalanine attributable to heme groups in hypertension is loose and flexible which leads to the reduction of heme moiety availability.

3.6 Irreversible thermal denaturation of hemoglobin

Figures 5a-c indicate a thermogram of hemoglobin at 50 mM phosphate buffer pH= 7.4 for all groups of hypertensive and normal rats comparatively. The figures clearly show melting temperature (Tm) and the transition phase of hemoglobin. Therefore, thermodynamical investigation results of hemoglobin for all groups of hypertensive and normal rats are provided with hemoglobin Tm and ΔH values. In general, in all groups, Tm and ΔH values in

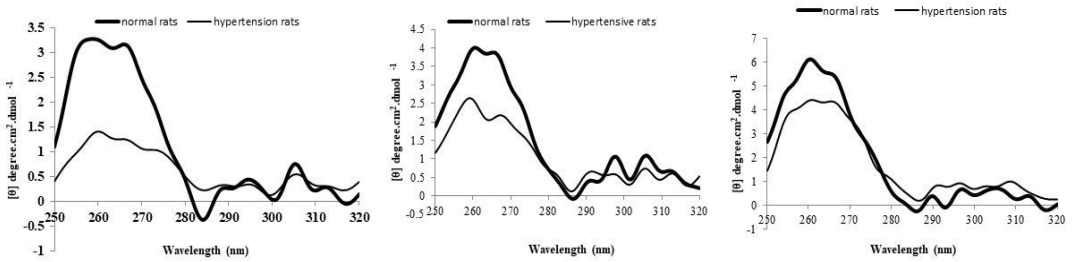

Figure 4. Near UV- CD spectra of hemoglobin in normal and hypertensive rats after 2, 4 and 6 weeks administration of L-NAME from a to c, respectively.

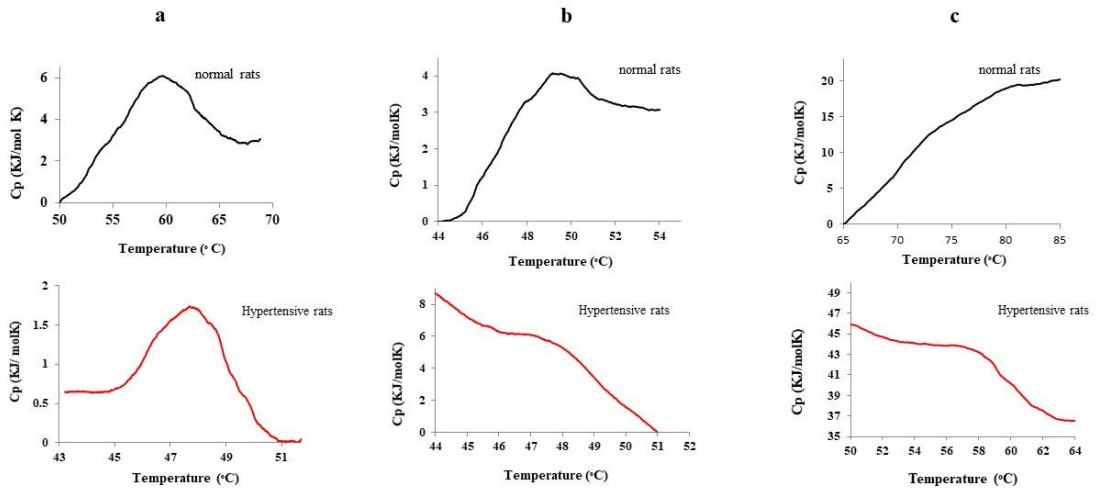

Figure 5. DSC thermograms of hemoglobin of normal and hypertensive rats (up to down) respectively. a) after 2 weeks of L-NAME administration, b) after 4 weeks of L-NAME administration c) after 6 weeks of L-NAME administration.

hypertensive rats compared to normal rats are considerably decreased (Table 2) (Figures 5a-c). Moreover, the decrease of Tm and ΔH values in hypertensive rats after 2 weeks of L-NAME administration, hemoglobin aggregation was observed (Figure 5a) (Table 2). Therefore, the ΔH value of hemoglobin denaturation is slightly decreased in hypertensive rats after 4 weeks of L-NAME administration relative to normal rats (Table 2) (Figure 5b). However, a significant reduction of Tm and ΔH values is observed in hypertensive rats after 6 weeks of L-NAME administration compared to normal rats (Table 2) (Figure 5c).

Thermodynamical studies of hemoglobin display a decrease in melting temperature (Tm) and enthalpy (ΔH) value in hypertensive rats compared to normal rats. According to the previous reports, Tm and ΔH values are related to the numbers of van der Waals forces and hydrogen bonds in protein structure. Thus, it decreases the hemoglobin structure's van der Waals forces and hydrogen bonds in hypertension. Furthermore, it implies that the secondary and tertiary structures of hemoglobin in hypertensive rats are less compacted and more flexible than in normal rats. It seems the hemoglobin stability in hypertension is decreased, leading to phase transition at lower temperatures. The results of hemoglobin thermal analysis are in accordance with the CD spectra results. Moreover, the phenomenon of hemoglobin aggregation in all groups of hypertensive rats, especially after two weeks of L-NAME administration was observed. Reduction of compression and increment of the flexibility of hemoglobin structures in hypertension leads to aggregation phenomenon. Subsequently, it may be the reduction of blood oxygen level in terms of the unavailability of oxygen to the heme groups in hemoglobin. According to previous reports, the hemoglobin concentration is increased in hypertension which reduces the ability of erythrocyte's deformability [39]. It can be concluded, the process of hemoglobin aggregation in hypertension, causes an

Table 2. Thermodynamical parameters of hemoglobin for normal and hypertensive rats after 2, 4 and 6 weeks of L-NAME administration (N: Normal and H: Hypertension).

Groups DSC data	N2	H2	N4	H4	N6	H6
Tm (˚C)	59.8	48	49.1	47.6	75	58.1
ΔH (KJ/mol)	33.5	4.46	6.43	2.05	56.2	19.9

increase in the cytoplasmic viscosity. Ultimately, in the current experiment, all results confirm each other.

4. Conclusion

In conclusion, the present study confirms that the structure of hemoglobin in hypertension is altered. Thus, this problem disrupts the hemoglobin functionality. In hypertensive cases, the compactness of the α-helix structure of hemoglobin is decreased, and the flexibility of the tertiary structure around phenylalanine is increased. Subsequently, hydrophobic groups such as tryptophan and heme groups form a hydrophobic package that reduces their accessibility. So, oxygen binding to heme groups is inhibited, and the blood oxygen level is reduced. Furthermore, the structural changes of hemoglobin in hypertension cause hemoglobin aggregation, an increase in cytoplasmic viscosity, and a decrease in erythrocytes deformability.

Conflict of interest

The authors declare that they have no conflict of interest.

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