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Simple Method for Measuring Endothelial Nitric Oxide Synthase Activity in Clinical Researches

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ABSTRACT

Objectives: This study aimed to evaluate a new simple colorimetric method for the assay of endothelial nitric oxide synthase activity in clinical researches and to compare between the role of vanadium chloride and nitrate reductase enzyme in the assay method.
Methods and materials: The new method involved using RBCs lysate to measure eNOS of the endothelium, using NADPH recycling system to enable the colorimetric measurement of eNOS, and using vanadium chloride to converting nitrate to nitrite. The method repeated again using nitrate reductase then vanadium results were compared with nitrate reductase results. The clinical study involved sixty patients who were randomly divided into two groups; group one received atorvastatin 40 mg daily, group two (control group) received placebo capsules. Patients were examined both before and after six weeks for endothelial nitric oxide synthase (eNOS) and for international index of erectile function-5 (IIEF-5) scores.
Results: In comparison to nitrate reductase, vanadium showed no significant difference in eNOS activity indicating similar role and activity. However, In comparison to control group, atorvastatin showed a significant increase in eNOS (48.16% for vanadium, and 44.3% for nitrate reductase, $P<0.05$). Also, atorvastatin showed a significant increase in IIEF-5 score (39.16%, $P<0.05$) and there was a significant correlation between eNOS and IIEF-5 score ($P<0.05$).
Conclusion: The study recommended this method for assaying eNOS using RBCs lysate, NADPH recycling system and vanadium chloride. This method is simple, reliable, and suitable for routine use in clinical researches.

INTRODUCTION

Nitric oxide (NO) is considered the most important signaling molecule in the vasculature. Nitric oxide is produced by a group of enzymes called nitric oxide synthases (NOS). These enzymes catalyze the production of NO and L-citrulline from L-arginine, O_2, and NADPH derived electrons (Moncada and Higgs, 2006). Mammalian systems contain three isoforms of the enzyme: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Endothelial NOS is the major source of the bioavailable NO in the vasculature (Knowles and monkanda, 1994). Endothelial nitric oxide synthase plays a role in the control of vascular tone, insulin secretion, and in the regulation of cardiac function and angiogenesis.

The assay for eNOS is required for evaluating endothelial function. Moreover, measuring the change in eNOS activity is needed for evaluating the effect of drug on endothelial function in clinical researches (Delker *et al.*, 2010). Up till now, finding a simple method for the assay of eNOS is a major challenge to clinical researchers. The difficulties in assaying eNOS include that tissue sample for eNOS from the endothelium cells of heart, muscle, or penile tissue is only applicable for animal researches and notsuitable for clinical researches. In addition, the traditional method for measuring eNOS activity is the radiochemical assay that measures the conversion of L-[3H] arginine to L-[3H]citrulline (Bredt and Schmidt, 1996).

This method is expensive and requires both the regulation of radioactive materials and the ability to handle radioactive materials and to dispose them. Also, it requires special measurement equipment which may not be affordable by many labs. In addition, NADPH can interfere in the assay by quenching the fluorescence produced (Fernández-Cancio *et al.*, 2001).

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Recently, the use of a NADPH recycling system allows NOS to be assayed spectrophotometrically in biological fluids, and cell lysate (Ghigo *et al.,* 2006). This colorimetric method measures the accumulation ofNOS stable degradation product nitrite and nitrate followed by reduction of nitrate to nitrite by nitrate reductase and estimation of nitrite using Griess Reagent (Schmidt and Kelm, 1996). Nitrite and nitrate represent the final products of nitric oxide (NO) oxidation pathways and their hematic concentrations are frequently assessed as an index of systemic NO production.

Unfortunately, this method cannot differentiate between the three isoforms of NOS and the result obtained will be the overall NOS activities of the three isoforms. Consequently, eNOS should be either separated first before assaying or finding a sample source which contain only eNOS similar to that of the endothelium without interference from the other two isoforms (nNOS and iNOS). In addition, this colorimetric method use nitrate reductase enzyme which require sensitive condition for maintenance increasing the cost of the assay method (Ghigo *et al.,* 2006). So, finding a suitable chemical alternative may make the assay more suitable for laboratory practice.

Therefore, this study aimed to evaluate a new simple colorimetric method for the assay of endothelial nitric oxide synthase activity in clinical researches using therapeutic intervention with atorvastatin "drug proven to increase eNOS" in patients with erectile dysfunction (La Vignera *et al.,* 2012). Moreover in this study, we will compare the results using vanadium chloride with nitrate reductase results to test the reliability of the method.

MATERIALS AND METHODS

The protocol for this study was approved by the National Research Ethics Committee of Tanta University, Egypt. This study is a blinded randomized investigational study conducted in the Urology Center of Urology Department in Tanta University Hospital started from March 2015 toJune2015. Sixty participants were enrolled in the study. Their ages ranged from 40 to 55 years. Participants suffered from ED (as identified by the international index of erectile function score <22) for at least 1 year (Cappelleri and Rosen, 2005). Patients were excluded if they had a history of hepatitis B, hepatitis C, bilharziasis, or abnormal laboratory values in ALT (alanine transaminase) and AST (asparatate transaminase), or a history of malignancy within the previous 5 years.

Eligible patients gave their written, informed consent. After signing a consent form, patients were interviewed for complete history and clinical examinations, which were carried out by qualified physicians from Urology Department, Tanta University Hospital. Participants were randomly divided using stratification method into two groups: group one consisted of 30 patients who received 40mgcapsules of atorvastatin daily for six weeks (Ator 40 mg, EIPECO Pharmaceutical Company, 10th of Ramaden City, Cairo, Egypt); group two consisted of 30 patients who received placebo capsules for six weeks. Participants were followed up every two weeks to ensure compliance and to report any drop out.

SAMPLE COLLECTION

Blood samples were taken both before starting and after the end of intervention. About 5 ml of blood was withdrawn from each participant and transferred to polypropylene tubes and left at room temperature to clot then serum was removed after centrifugation of the clotted blood at 3000 g for 10 min (Hettich EAB 12 Centrifugation, Germany) leaving red blood cells that were handled gently to avoid hemolysis and stored at -20 ºC.

CHEMICALS

Vanadium Chloride(VCl3), Sulphanilamide, N (1 naphthyl) ethylenediamine dihydrochloride (NEDD), sodium nitrite, EDTA, NADP, glucose 6 phosphate, L-arginne, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 50 mU/mL Nitrate Reductase and 40 mU/ml G6PD (from Saccaromyces cerevisiae) were all purchased from Sigma Aldrich CO. Solid vanadium chloride was stored in dark place.

PREPARATION

Vanadium Chloride (VCl3) was prepared in 1 M HCL (0.8 % w/v), Sulphanilamide was prepared in 5% HCl (2% w/v) (Griess Reagent part 1), and NEDD was prepared in H2O (0.1% w/v)) (Griess Reagent part 2). Sodium nitrite standard stock was obtained by preparing concentration of 500 mM/L. Reaction buffer consisted of 50mM of HEPES plus 0.5mM EDTA adjusted to pH 7.2. In addition, NADPH recycling system includes two parts. NADPH part 1 consisted of $NADP^+$ 0.2mM, glucose 6 phosphate 0.17 mM and L-arginne 360 µM, while NADPH part 2 consisted of Glucose 6 phosphate dehydrogenase 40 mU/ml.

PRINCIPLE OF THE METHOD

This method depends on three main points:

1- **The use of RBCs lysate as the sample source for measuring of eNOS**

Red blood cells contain only functional eNOS while nNOS and iNOS failed to be represented in RBCs (Kleinbongard *et al.,* 2006; Kanias *et al.,* 2013). Therefore, no need for separation of specific isoform of NOS and RBCs represents aunique source of eNOS like that of the endothelium in our body without interference from the other two isoform.

2- The use of NADPH recycling system

NADPH recycling system allows eNOS to operate continuously for production of NO. The quantity of the produced NO indirectly express the activity of eNOS. In this recycling

system, NADPH is renewed using glucose 6 phosphate dehydrogenase enzyme which convert $NADP^+$ to NADPH allowing NOS to operate linearly for hours producing nitric oxide derived nitrate and nitrite (Ghigo *et al.*, 2006). Both nitrate and nitrite should be monitored.

3- **The use of vanadium chloride**

Vanadium chloride is used to reduce nitrate ($NO3^-$) to nitrite ($NO2^-$) in a more simple way than nitrate reductase (Miranda *et al.*, 2001). Finally, Griess reagents are added to convert nitrite into a deep purple azo compound. Photometric measurement of absorbance due to this azochromophore accurately determines nitriteconcentration (Schmidt and Kelm, 1996).

Procedures for measuring eNOS activity of RBCs lysate using either vanadium chloride or nitrate reductase

A-Measuring eNOS Activity of RBCs lysate using Vanadium Chloride

1- Red blood cells lysate was first prepared by adding 2 ml distilled Water to 0.5 ml of RBCs and centrifugation at 3000 g for 10 min.

2- To 0.5 ml of lysate supernatant, 1 ml of absolute alcohol was added and centrifuged for 20 min at 3000 g to precipitate unwanted proteins.

3- To 0.5 ml of prepared sample, 1 ml of Reaction buffer, 50 µl of NADPH part 1 and 50 µl NADPH Part 2 were added, mixed well and incubated for 6 hours at 37^o C then chilled on ice for 5 min.

4- To 0.5 ml of previous mixture, 0.5 ml Vanadium Chloride was added and mixed well. Then, 0.25 ml of Sulphanilamide (Griess Reagent part 1)and 0.25 ml of NEDD(Griess Reagent part 2)was added, mixed well and incubated for 30 min at 37oC.

5- Absorbance was read at 540 nm against deionized water UV visible spectrophotometer.

Calculation

The standard curve for sodium nitrite was constructed by plotting the absorbance for different standard concentrations against their concentrations. The concentration of sample sodium nitrite was determined from the standard curve after measuring absorbance corresponding to prepared concentrations.

B: Measuring eNOS Activity of RBCs lysate using nitrate reductase

1- Red blood cells lysate was first prepared by adding 2 ml distilled Water to 0.5 ml of RBCs and centrifugation at 3000 g for 10 min.

2- To 0.5 ml of lysate supernatant, 1 ml of absolute alcohol was added and centrifuged for 20 min at 3000 g to precipitate unwanted proteins.

3- To 0.5 ml of prepared sample, 1 ml of Reaction buffer, 50 µl of NADPH part 1 and 50 µl NADPH Part 2 were added, mixed well and incubated for 6 hours at 37^o C then chilled on ice for 5 min.

4- To 0.5 ml of previous mixture, 0.5 ml nitrate reductase was added and mixed well. Then, 0.25 ml of Sulphanilamide (Griess Reagent part 1)and 0.25 ml of NEDD (Griess Reagent part 2)was added, mixed well and incubated for 30 min at 37oC.

5- Absorbance was read at 540 nm against deionized water UV visible spectrophotometer.

Calculation

The standard curve for sodium nitrite was constructed by plotting the absorbance for different standard concentrations against their concentrations. The concentration of sample sodium nitrite was determined from the standard curve after measuring corresponding to prepared concentrations.

CLINICAL TEST (INTERNATIONAL INDEX OF ERECTILE FUNCTION)

International Index of Erectile Function (IIEF-5) is measured both before and after atorvastatin intake. International Index of Erectile Function has been considered a reliable subjective method to assess erectile function, where participants were asked to answer a questionnaire composed of five main questions (Cappelleri and Rosen, 2005). The answer to each question was given a score from 1 to 5. The possible scores for the IIEF-5 ranged from 5 to 25, and ED was classified into five categories on the basis of these scores: severe, moderate, mild to moderate, mild and no ED.

STATISTICAL ANALYSIS

Values were presented as the mean ± S.D. using Minitab (Minitab release 15, Pine Hall Road, State College, PA, USA). Paired t-test was used to assess any significant difference between each group before and after treatment course. One-way analysis of variance test and two-sample t-test was used to assess any significant difference between groups. Correlation test between IIEF-5 score and eNOS activity was assessed by spearman correlation test. $P<0.05$ was considered significant.

Results

Participants' baseline data were presented in Table (1). During follow up, seven participants were dropped out from the study due to side effects mainly severe muscle pain and were replaced with other participants. Two sample t-test revealed that there was no statistical significant difference between the two groups before starting intervention intake in either eNOS or IIEF-5 score ($P>0.05$). Participants were classified according to their IIEF-5 score.

The mean changes of endothelial nitric oxide synthase activity before and after six weeks of treatment course were listed in Table (2) and Figure (1). After atorvastatin intake, there was a statistically significant increase in eNOS activity using vanadium

chloride by 48.16 % (paired t-test, p<0.05). In addition, there was a statistically significant increase in eNOS activity using nitrate reductase by 44.3 % after atorvastatin intake (paired t-test, p<0.05). Two sample t-test showed that there was no significant difference in eNOS activity after atorvastatin intake using either nitrate reductase or vanadium chloride (two sample t-test, p>0.05).

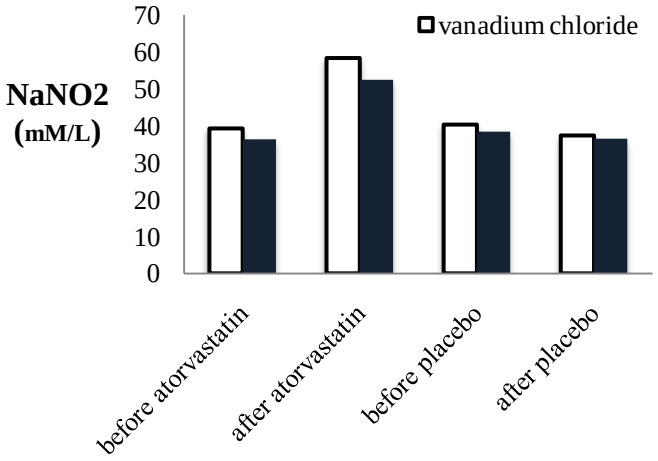

Fig. 1: Mean values of endothelial nitric oxide synthase (eNOs) level as indicated by sodium nitrite ($NaNo_2$) (mM/L) using vanadium chloride and nitrate reductase before and after six weeks of treatment course. No significant difference between vanadium chloride and nitrate reductase in all groups (paired t-test, p> 0.05), data are presented as mean ± SD

The mean changes of IIEF-5 score before and after six weeks of treatment course were presented in Table (2) and Figure (2). There was a statistically significant increase in IIEF score by39.16% after atorvastatin intake (paired t-test, p<0.05).

Figure (3) showed the correlation between IIEF-5 score and eNOS activity using vanadium after atorvastatin intake. There was a significant correlation between IIEF score and eNOS after atorvastatin intake, where r= 0.49 (Spearmen correlation test, p< 0.05).

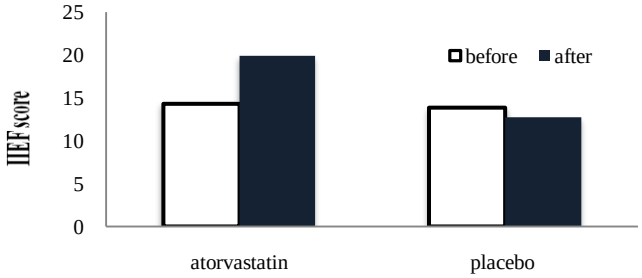

Fig. 2: Mean values of international index of erectile function (IIEF-5) score both before and after six weeks of treatment course. *significant difference compared with their respective value before atorvastatin intake (paired *t-test*, p< 0.05), data are presented as mean ± SD

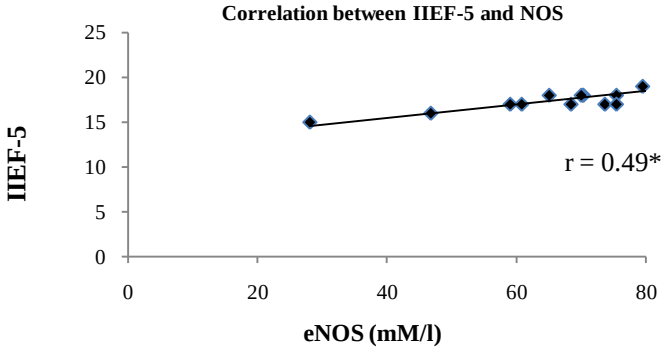

Fig. 3: Correlation between eNOS and IIEF-5 after atorvastatin intake. *significant correlation between IIEF-5 score eNOS after atorvastatin intake (spearman correlation test, P<0.05)

Table 1: The baseline date of the participants.

	Participants (Total n=60)	Atorvastatin (n=30)	Placebo (n=30)
Age			
40-45 years	9(15%)	4(13.3%)	5(16.6%)
45-50 years	24(40%)	10(33.3%)	14(46.6%)
50-55 years	27(45%)	16(53.3%)	11(36.6%)
Concurrent Disorders			
Diabetes			
Obesity	18(30%)	8(26.6%)	10(33.3%)
Smoke	21(35%)	12(40%)	9(30%)
	15(25%)	6(20%)	9(30%)
ED degree			
Mild	6(10%)	2(6.6%)	4(13.3%)
Moderate/mild	24(40%)	14(46.6%)	10(33.3%)
moderate	15(25%)	7(23.3%)	8(26.6%)
severe	15(25%)	7(23.3%)	8(26.6%)

ED erectile dysfunction, ED classification is according IIEF-5 score

Table 2: The mean changes in endothelial nitric oxide synthase (eNOs) level and IIEF-5 score both before and after six weeks of treatment course.

	Before atorvastatin intake	After atorvastatin intake	% change	Before placebo intake	After placebo intake	% change
eNOS Using Vanadium chloride	39.36±10.54	58.32*±13.27	48.1%	40.36±9.51	37.32±11. 34	-7.6%
eNOS Using Nitrate Reductase	36.40±11.96	52.55*±12.40	44.3%	38.40*±10.06	36.55*±12.11	-5%
IIEF-5 score	14.3±4.3	19.9*±4.6	39.16%	13.8±3.5	12.75±3.9	-7.1%

* Significant difference compared with their respective values before atorvastatin intake (paired *t-test*, p< 0.05)

To our knowledge, this is the first clinical study that provides a simple method for assaying eNOS in clinical research depending on the following main three points. First, using RBCs lysate as an indicator for eNOS from endothelium. Second, the colorimetric assay of eNOS using NADPH recycling system. Third, using vanadium chloride instead of nitrate reductase to convert nitrate to nitrite. This method is simple and has high accuracy without using expensive instruments or complex procedures.

In addition, this study compared role of vanadium chloride with the role of nitrate reductase in the test. The results of this study showed that vanadium chloride has a similar activity like nitrate reductase in reducing nitrate to nitrite and can be used in routine laboratory practice confirming the reliability of the vanadium chloride method.

Also, this method showed that the changes in eNOS had a strong correlation with the improvement in clinical effects indicated by the improvement in IIEF-5 score after atorvastatin intake. This indicates that our simple method can be used to evaluate the changes in endothelial dysfunction and its related disorders like erectile dysfunction.

In order to obtain a sample source that only represents eNOS of the endothelium without interference from the other two isoforms, RBCs is the ideal solution for this case. RBCs only contain eNOS similar to that of the endothelium and can be used as an indirect sample source to measure eNOS of the endothelium (Kanias *et al.*, 2013). In recent studies, functional eNOS has been detected in erythrocytes, offering a possibility that erythrocytes are indeed NO generators and can contribute to the intravascular NO pool and regulate physiologically relevant mechanisms (Ozuyaman *et al.,* 2008). Kleinbongard provided evidence that RBCs from humans express an active and functional endothelial-type NOS (eNOS), which is localized in the plasma membrane and the cytoplasm of RBCs. The red blood cell (RBC) endothelial nitric oxide synthase (eNOS) regulates intrinsic erythrocyte rheological properties, such as membrane deformability, assuring the presence of a functional eNOS (Kleinbongard *et al.,* 2006).The NOS-dependent conversion of l-arginine in RBCs is comparable to that of cultured human endothelial cells. The eNOS-like protein of RBCs is localized in the cytoplasm with activity and regulatory mechanisms resembling those of endothelium-derived eNOS (Kleinbongard *et al.,* 2006).

In addition, RBC-NOS resembles endothelium-derived eNOS in that it is specifically stimulated by the substrate l-arginine, it is sensitive to common NOS inhibitors, and its activity depends on the intracellular calcium level (Fulton *et al.,* 2001). Recently, computational modeling (Chen *et al.,* 2009) simulated NO production from eNOS in erythrocytes and its transport through an arteriole. It was found that the expression level of eNOS in erythrocytes is similar to that in endothelium, and researchers can predict a level NO in the vascular smooth muscle from this RBCs source. The colorimetric assay of NOS was first reported by Stuehr et al. by measuring the synthesis of nitrite/nitrate in the cytosol of activated murine macrophages over a 3-h period (Stuehr *et al.,* 1989). At the same time, hundreds of researchers have reported the use of the radiochemical assay. The expected reason for the rare use of the colorimetric NOS assay in cell lysates lies in the difficulty of providing an adequate supply of NADPH for a prolonged period of time due to its susceptibility to auto-oxidation in the presence of O_2 at physiological pH (Bredt and Schmidt, 1996).

Recently, Ghigo et al made a NADPH Recycling system using glucose 6-phosphate dehydrogenase (G6PD) enzyme. This helps supply adequate NADPH amount for prolonged time and make the reaction rate is a linear function of the enzyme concentration giving the opportunity to measure NOS by a colorimetric method through measuring nitric oxide the end product of NOS reaction (Ghigo *et al.,* 2006).

In aqueous solution, NO is converted to nitrate and nitrite (Schmidt and Kelm, 1996). NO-derived nitrite and nitrate must be measured. Spectrophotometric quantization of nitrite using Griess Reagent is straightforward, but does not measure nitrate. So this requires a nitrate reduction to nitrite prior to quantization of nitrite using Griess Reagent (Marzinzig *et al.,* 1997).Nitrate reduction to nitrite can be accomplished by specific nitrate reductases or by the use of different reducing metals like cadmium or vanadium (Ignarro *et al.,* 1987).

Bacterial nitrate reductase enzyme catalyzes the reduction of nitrate by NADPH as shown $NO3^- + NADPH + H^+ \rightarrow NO2^- + NAPD^+ + H2O$

This method has been widely used in NOS determination in biological samples. However, nitrate redcutase has the disadvantages that nitrate reductase requires caution in dealing as a biological protein (Gillam *et al.,* 1993). Also, NADPH interferes with subsequent Griess Reaction. This later interference can be avoided by using lactate dehydrogenase (LDH) to decompose NADPH after nitrate reduction is complete. The use of LDH increases the cost, time of the assay (Verdon *et al.,* 1995). Other disadvantages of the enzymatic method include incomplete nitrate reduction at high concentration range and batch variations related to enzyme activity changes in different preparations and during storage (Granger *et al.,* 1991).

On the other hand, the reduction of nitrate by vanadium (III) chloride is a promising option. Vanadium chloride does not need the use of LDH to remove excess NADPH saving time, efforts, and cost making the method more suitable for routine laboratory practice. This assay is sensitive to 0.5 uM $NO3^-$ and is suitable for a variety of fluids including cell culture media, serum, and plasma (Miranda *et al.,* 2001). Also, vanadium is inexpensive and will not show variations in activity due to batch variations.

Previous research claimed that metals like cadmium can replace nitrate reductase. But, in Comparing vanadium with cadmium, the vanadium reduction does not bias the results. The vanadium method does not need a specially trained technician and an expensive auto-analyser system required for cadmium assay. Also, cadmium is highly toxic (García-Robledo *et al.,* 2014). However, there are few precautions on using vanadium. First, the instability of vanadium solution which requires frequent checks

and limits shelf-life when provided in a reagent kit format. Second, being a spectrophotometric procedure, samples must not be turbid and should not show absorbance at 540 nm. Both of these characteristics may be corrected for the samples by filtration pretreatment (Sun *et al.*, 2003).

The strong correlation between eNOS activity and IIEF-5 score confirm that eNOS is the main mechanism in improving endothelial dysfunction in patient with ED and that our method is valuable in evaluating the changes in endothelial dysfunction and clinical progress to its related disorders. Erectile dysfunction (ED) is caused by endothelial dysfunction (Sullivan *et al.*, 2001). In these patients, the decrease eNOS activity of the vascular endothelium results in decreased formation of NO, which is the main messenger that induces arterial and corporal vasodilation in the corpus cavernosum (Kendirci *et al.*, 2006; Robinson *et al.*, 2006).

Recently, many studies confirmed the role of Statin in the improvement of endothelial function (Liao and Laufs, 2005). These effects of statins are, in part, mediated by an effect on eNOS, because these effects can be inhibited by eNOS inhibitors (John *et al.*, 1998). A previous study showed that treatment with atorvastatin increased plasma NO concentrations (Förstermann and Sessa, 2012), which was consistent with the outcomes in the most recent trial (El-Sisi *et al.*, 2013). This manifestation could be explained by the upregulation of eNOS expression (Kureishi *et al.*, 2000).

CONCLUSION

In conclusion, this study recommends a simple and reliable colorimetric method for measuring eNOS in clinical research. The RBCs lysate can be used as a sample source to measure eNOS of the endothelium and NADPH recycling system enables the colorimetric measurement of eNOS. Also, vanadium chloride is preferred to nitrate reductase in converting nitrate to nitrite for routine laboratory practice. The clinical study showed that the discussed method is valuable in evaluating the changes in endothelial dysfunction and clinical progress in its related disorders. However, larger controlled studies for longer duration may be needed in the future.

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