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ORIGINAL ARTICLE

Atorvastatin improves erectile dysfunction in patients initially irresponsive to Sildenafil by the activation of endothelial nitric oxide synthase

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This study aimed at comparing the effects of atorvastatin and vitamin E on erectile dysfunction in patients initially irresponsive to sildenafil, with investigation into the underlying possible mechanisms. Sixty patients were randomly divided into three groups: the atorvastatin group received 80 mg daily, the vitamin E group received 400 IU daily and the control group received placebo capsules. Patients were examined both before and after 6 weeks of treatment for biochemical tests; Superoxide dismutase (SOD), glutathione peroxidase (GPO), C-reactive protein (CRP), interleukin-6 (IL-6), nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) and for erectile function tests; International index of erectile function (IIEF-5) scores and Rigiscan. Both atorvastatin and vitamin E showed a statistically significant GPO increase ($P < 0.05$) and a statistically significant IL-6 decrease ($P < 0.05$). Only atorvastatin showed a statistically significant increase in NO (15.19%, $P < 0.05$), eNOS (20.58%, $P < 0.01$), IIEF-5 score (53.1%, $P < 0.001$) and Rigiscan rigidity parameters ($P < 0.01$), in addition to a statistically significant decrease in CRP (57.9%, $P < 0.01$). However, SOD showed a statistically significant increase only after vitamin E intake (23.1%, $P < 0.05$). Both atorvatstain and vitamin E had antioxidant and anti-inflammatory activities. Although activating eNOS by atorvastatin was the real difference, and expected to be the main mechanism for NO increase and for improving erectile dysfunction. Atorvastatin, but not vitamin E, is a promising drug for sildenafil nonresponders.

INTRODUCTION

Since the discovery of sildenafil in 1998, much progress has been made in the treatment of erectile dysfunction (ED). Despite this revolution, the significant number of sildenafil nonresponders is a major challenge to all urologists.[1] Patients are considered to be sildenafil nonresponders, if they do not have sufficient erection for vaginal penetration after taking sildenafil.

Endothelial dysfunction is considered to be the main factor contributing to sildenafil treatment failure.[2] Sildenafil is unlikely to reverse endothelial dysfunction with reduced nitric oxide (NO) bioavailability.[3] Hence, oral medications that improve the endothelium, such as atorvastatin and vitamin E, are considered to be promising drugs for sildenafil combination, although the evidence-based support for these treatments remains limited and requires further investigations.[4] Therefore, this study aimed at comparing the effects of atorvastatin and vitamin E on patients with ED initially irresponsive to sildenafil, with investigation into the underlying possible mechanisms.

PATIENTS AND METHODS
Study design
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 October 2009 to May 2011. Sixty participants were enrolled in the study (Sample size of each group was calculated from Cohen's d tables[5] according to the desired study power(90%), the significance level (0.05) and the anticipated effect size(1.2), which was determined from previous clinical trials[6]). Their ages ranged from 40 to 60 years. Participants suffered from ED (as identified by The Sexual Health Inventory for Men (SHIM) score < 22) for at least 1 year, showing no response to sildenafil. Patients had normal cholesterol level as identified by an LDL-cholesterol level below 160 mg dl^{-1}. Patients were excluded if they had cardiovascular disorders (angina or myocardial infarction), hepatic disorders, malignancy history or diabetes.

Despite the fact that diabetes is a potential risk factor for endothelial dysfunction, diabetic patients were excluded from the study. This exclusion ensured that the improvement in ED is directly related to improving endothelial function, and there was no interference from neuronal aspects that might associate the endothelial dysfunction in diabetes or from improvement in insulin sensitivity that might accompany atorvastatin use in diabetic patients.[7] 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 three groups: group one consisted of 20 patients who received 80 mg capsules of atorvastatin daily for 6 weeks (Ator 80 mg, EIPECO Pharmaceutical Company, 10th of Ramaden City, Cairo, Egypt); group two consisted of 20 patients who received 400 IU capsules of vitamin E daily for 6 weeks (Vitamin E 400 IU, MEPACO Pharmaceutical Company,

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Anshas El-raml, Sharqia, Egypt); and Group 3 (Control group) consisted of 20 patients who received placebo capsules for 6 weeks. Participants were followed up every 2 weeks to ensure compliance, and to report any dropout or adverse effects.

Samples collection

About 10 ml of blood was taken from each patient both before and after the treatment course, and the blood sample was divided as follows: 5 ml of blood was transferred to polypropylene tubes and left at room temperature to clot, and then the serum was separated after centrifugation (Hettich EAB 12 Centrifuge, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) and stored at $-20\,^{\circ}$C. The other 5 ml of blood was transferred to polypropylene tubes containing EDTA. Plasma was then separated after centrifugation and stored at $-20\,^{\circ}$C. In addition, red blood cells (RBC) were separated gently to avoid hemolysis and were stored at $-20\,^{\circ}$C.

Biochemical tests

Nitric oxide. Plasma NO level was estimated by a colorimetric chemical method by measuring nitrite ($NO2-$) with Griess reagent, using vanadium chloride as a reducing agent to convert nitrate ($NO3-$) to $NO2-$.[8,9] A volume of 0.5 ml of serum sample was added to 1 ml of absolute alcohol and then centrifuged for 20 min at 3000 r.p.m. to precipitate unwanted proteins from the sample. Next, 0.5 ml of the prepared sample was added to 0.5 ml of vanadium chloride (0.8%) in 1 M HCl and to 0.5 ml of Griess reagent. After good mixing and incubation for 30 min at 37 °C, the absorbance was measured at 540 nm against deionized water. Different concentrations of sodium $NO2-$ were used to construct a standard curve.

Antioxidant activities. Superoxide dismutase (SOD) and glutathione peroxidase (GPO) were used to identify drug antioxidant activities. Plasma SOD activity was assayed using the colorimetric method[10] (Superoxide Dismutase Activity Colorimetric Assay Kit, Cell Biolab Inc.USA). The GPO activity of RBC lysate was assayed using the colorimetric method[11] (Glutathione Peroxidase Activity Colorimetric Assay Kit, Sigma-Aldrich Corp., St Louis, MO, USA).

Anti-inflammatory activities. C-reactive protein (CRP) levels and serum interleukin-6 (IL-6) levels were used to identify drug anti-inflammatory activities. Serum CRP was assayed using the Latex agglutination test[12] (CRP Latex Agglutination Kit, ProDia International, U.E.A. proDiagnostics GmbH, Germany). Serum IL-6 was assayed using the Enzyme-linked immunosorbent assay technique[13] (AviBion Human IL-6 Enzyme-linked immunosorbent assay Kit, Anibiotech Oy, Orgenium Laboratories Division Tiilitie 3, Vantaa, Finland).

Assay of NOsynthase. The activity of nitric oxide synthase (NOS) was assayed in RBC lysate. RBCs contain only functional endothelial NOS (eNOS) similar to that of the endothelium.[14] This eNOS can be used to measure penile eNOS. The activity of eNOS was assayed using validated colorimetric method[8,15] by measuring $NO2-$ concentrations (Ultrasensitive Colorimetric NOS Assay, Oxford Biomedical Research Inc. USA).

Erectile function tests

International Index of Erectile Function (IIEF-5). SHIM or IIEF-5 has been a reliable subjective method to assess erectile function, where participants were asked to answer a questionnaire composed of five main questions.[16] 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.

Rigiscan. Rigiscan (Rigiscan plus, Endocare Inc. MN, VA, USA) is a device that allows the measurement of the duration, rigidity and tumescence of the penis. The device provides a graphical and tubular display of base and tip penile rigidity, and tumescence data for interpretation by the physician. It uses loops that tighten slightly at discrete time intervals to measure and record data. A provocative method of Rigiscan operation was selected, in which Rigiscan recorded the baseline tumescence. Further, intracavernous injection of papaverine (60 mg) was used as an intervention to produce a one-time erection. Next, Rigiscan proceeded to measure event rigidity and tumescence.[17]

Statistical analysis

Values were presented as the mean ± s.d. using 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 after treatment. $P < 0.05$ was considered significant.

RESULTS

Participants' baseline data are listed in Table 1. Analysis of variance test revealed that there was no statistical significant difference between the three groups before starting intervention intake in either biochemical or clinical data ($P > 0.05$). Participants were classified according to their SHIM score. They showed a noticeable ED (SHIM score < 17), meaning that there were no mild cases of ED. During follow up, five participants were dropped out from the study and were replaced by new participants to maintain the study sample size. The higher dropout was observed in the atorvastatin group (3/5) owing to side effects, mainly severe muscle pain. After treatment course, the mean changes in biochemical findings and IIEF-5 score were presented in Table 2.

In comparison with the control group, the treated groups showed a statistically significant increase in GPO activity (49.95, $P < 0.05$ and 39.03%, $P < 0.05$ for atorvastatin and vitamin E, respectively). The increase in GPO activity after atorvastatin intake did not reveal any significant difference from that increase after vitamin E administration, indicating similar antioxidant activities (two sample *t*-test, $P > 0.05$). In addition, the treated groups showed a statistically significant decrease in IL-6 levels (42.3%, $P < 0.05$ and 76.14%, $P < 0.05$ for atorvastatin and vitamin E, respectively). The decrease in IL-6 levels after atorvastatin intake did not reveal any significant difference from that decrease after vitamin E administration, indicating similar anti-inflammatory activities (two-sample t-test, $P > 0.05$). Atorvastatin, but not vitamin E, showed a statistically significant decrease in CRP level (57.9%, $P < 0.01$), a statistically significant increase in NO level (15.19%, $P < 0.05$) Figure 1 and a statistically significant increase in NOS activity (20.58%, $P < 0.01$) Figure 2. Although the vitamin E group seemed to have a difference in NO level after treatment course, this difference did not reach the significant level (paired

Table 1. Participants' baseline data				
	Total (n = 60)	*Atorvastatin* (n = 20)	*Vitamin E* (n = 20)	*Placebo* (n = 20)
Age				
40 − 50	23(38.3%)	5 (25%)	10 (50%)	8 (40%)
50 − 60	37 (61.2%)	15 (75%)	10 (50%)	12 (60%)
ED duration				
1 − 5 years	13 (21.7%)	3 (15%)	5 (25%)	5 (25%)
> 5 years	47 (78.3%)	17 (85%)	15 (75%)	15 (75%)
Other complaints				
Hypogonadism[a]	20 (33.3%)	7 (35%)	5 (25%)	8 (40%)
Pelvic surgery	9 (15%)	4 (20%)	2 (10%)	3 (15%)
Smokers	13 (21.7%)	3 (15%)	6 (30%)	4 (20%)
Hypertension	23 (38.3%)	8 (40%)	9 (45%)	6 (30%)
ED degree[b]				
Mod./mild	40 (66.7%)	10 (50%)	14 (70%)	16 (80%)
Moderate	18 (30%)	9 (45%)	6 (30%)	3 (15%)
Severe	2 (3.3%)	1 (5%)	0 (0%)	1 (5%)

[a]Hypogonadism is confirmed by low testosterone levels. [b]The degree of ED was measured by IIEF-5 score.

Table 2. Changes in biochemical tests both before and after six weeks of treatment course in the three groups

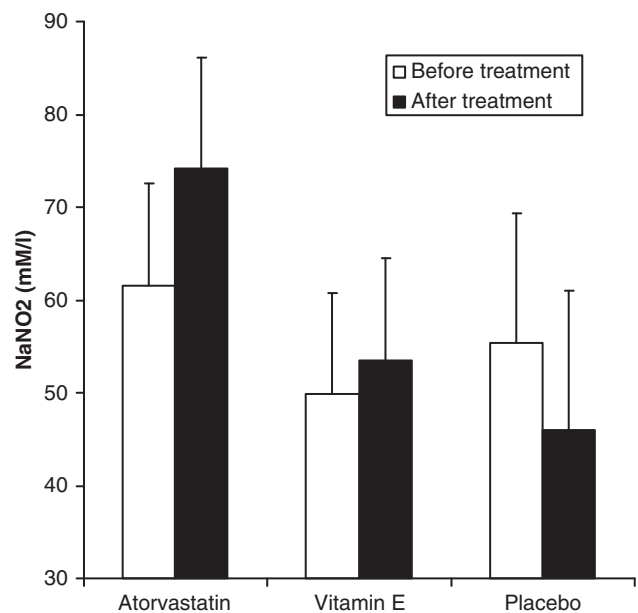|	Atorvastatin (n = 20)		Vitamin E (n = 20)		Placebo (n = 20)	
	Before treatment mean ± s.d.	After treatment mean ± s.d.	Before treatment mean ± s.d.	After treatment mean ± s.d.	Before treatment mean ± s.d.	After treatment mean ± s.d.
SOD	221.7 ± 63.3	233.7 ± 65.6	173.1 ± 60.8	213.1 ± 63.0*	200.1 ± 73.5	165.8 ± 64.3
GPO	10.85 ± 4.7	16.27 ± 8.6*	12.86 ± 4.44	17.88 ± 8.5*	9.24 ± 3.22	11.65 ± 6.7
CRP	20.55 ± 19	8.65 ± 6.82**	23.85 ± 42.5	30.40 ± 31.8	20.45 ± 23	22.15 ± 29
IL-6	55.8 ± 48.7	39.2 ± 36.7*	73.24 ± 38.4	41.58 ± 34.1*	53.5 ± 44.5	61.4 ± 37.3
NO	24.68 ± 4.7	28.43 ± 5.4*	20.56 ± 4.49	21.86 ± 3.63	22.5 ± 5.23	21.63 ± 4.5
NOS	61.5 ± 12.9	74.16 ± 17.7**	49.80 ± 11.9	53.53 ± 17.2	55.38 ± 17.5	46.01 ± 15.1
IIEF-5	11.85 ± 2.36	18.15 ± 1.69**	12.60 ± 2.04	12.75 ± 2.573	13.15 ± 2.34	13.4 ± 1.789

Abbreviations: CRP, C-reactive protein; GPO, glutathione peroxidase; IL-6, interleukin-6; IIEF-5, International index of erectile function; NO, nitric oxide; NOS, nitric oxide synthase. *($P < 0.05$), **($P < 0.01$).
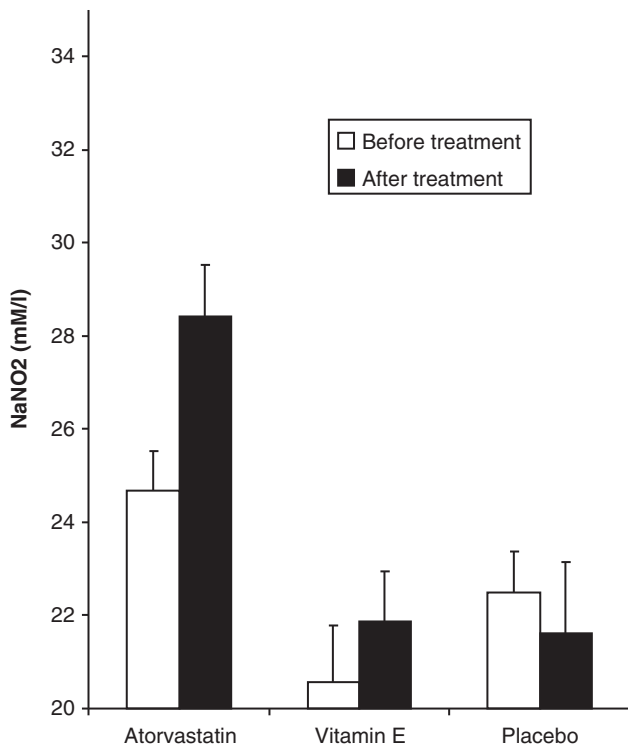
Figure 1. Mean values of serum NO level as indicated by sodium nitrite (NaNo2) (mM/l) both before and after treatment course in the three groups.

Figure 2. Mean values of NOS sctivity as indicated by NaNo2 (mM/l) both before and after treatment course in the three groups.

t-test, $P > 0.05$). On the other hand, SOD activity showed a statistically significant increase only after vitamin E intake (23.1%, $P < 0.05$).

The mean changes in participants' IIEF-5 scores were listed in Figure 3. Atorvastatin showed a statistically significant increase in IIEF-5 scores (53.1%, $P < 0.001$), but none of the participants showed a normal ED score (IIEF-5 score < 22). Furthermore, the changes in each question score of the IIEF-5 were presented in Figure 4. After atorvastatin administration, there was a significant increase in scores for question 2, question 3, question 4 and question 5 (paired t-test, $P < 0.05$). With regard to Rigiscan, atorvastatin revealed a statistically significant increase in average event rigidity at both the tip and base (16.6%, $P < 0.01$ and 17.2%, $P < 0.01$, respectively). Other Rigiscan parameters showed no statistically significant difference in the three groups ($P > 0.05$) Figure 5.
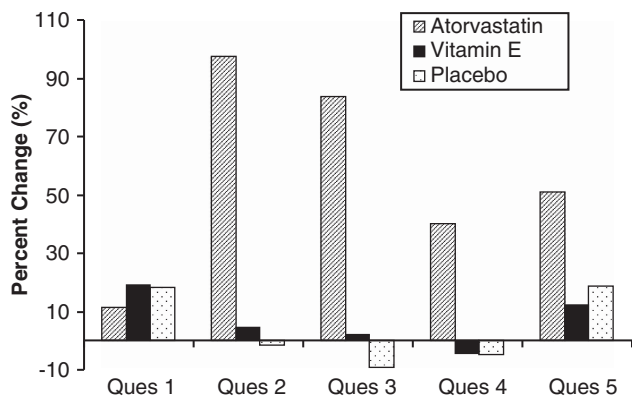
DISCUSSION

This clinical study investigated the mechanistic pathways that connect the endothelial effects of atorvastatin or vitamin E with the erectile function of sildenafil nonresponders. Using larger numbers of patients, this study reevaluated the hypothesis that men with ED resistant to sildenafil can be rescued with the addition of atorvastatin[18] or vitamin E. Participants had normal cholesterol in order to separate the lipid-lowering from the nonlipid-lowering (pleiotropic) effects of atorvastatin.[19]

Atorvastatin was chosen from statins despite the fact that rosuvastatin is much more effective in modifying the lipid profile,[20] because there was a reported interaction between rosuvastatin and sildenafil, which may result in Rhabdomyolysis and myopathy.[21] Further studies may be required to investigate the effects of rosuvastatin on erectile function of sildenafil nonresponders.

About one-third of participants showed low testosterone levels, which were in place of hypogonadism stratification. Sildenafil activity was compromised in low testosterone levels.[22] Androgen
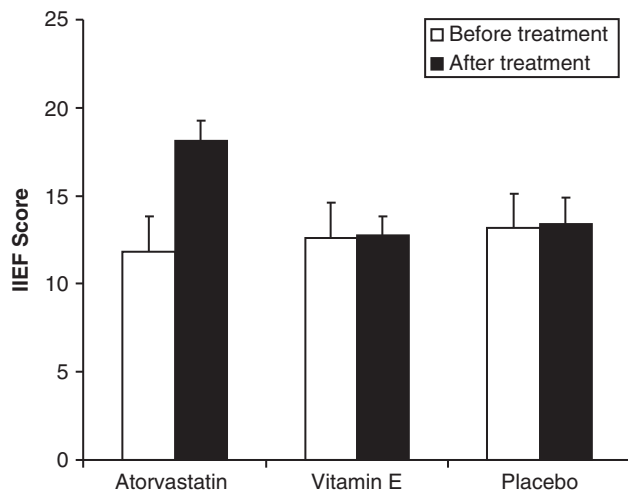
Figure 3. Mean values of IIEF-5 score both before and after treatment course in the three groups.

Figure 5. The percent change in average event rigidity and event tumescence more than baseline tumescence in the tip and the base both before and after treatment course in the three groups.
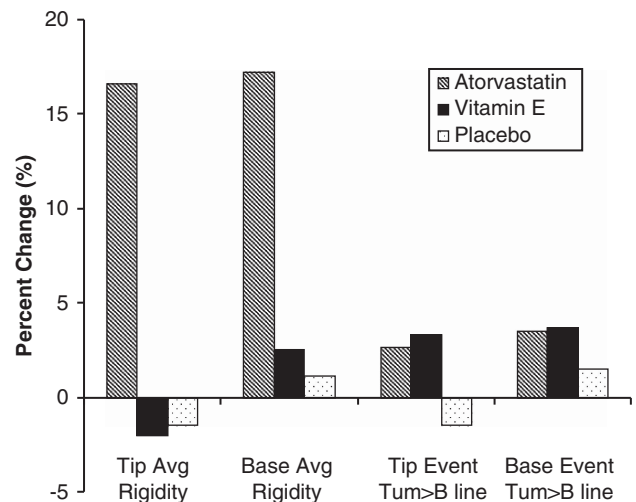

Figure 4. The percent change in IIEF-5 questions scores both before and after treatment course in the three groups.

roles in penile erection involved the regulation of the expression and activity of NOS isoforms in the corpus cavernosum. In castrated animals, testosterone or 5α-dihydrotestosterone administration restored the erectile response and NOS expression.[23] In addition, testosterone regulated phosphodiesterase (type) 5 activities. Castration was shown to reduce the expression and activity of PDE5, and androgen supplementation upregulated the expression and activity of PDE5.[24]

A study duration of 6 weeks was a sufficient period for atorvastatin or vitamin E to show response.[25,26] A daily dose of 80 mg of atorvastatin was required to enable the evaluation of the pleiotropic effects of statins.[19,27] On the other hand, a daily dose of 400 IU of vitamin E was considered to be the safest dose for therapeutic purposes.[28]

The present study showed the efficacy of atorvastatin in improving ED. As revealed from clinical findings, atorvastatin demonstrated a significant increase in IIEF-5 score, and improvement in both tip and base rigidity by Rigiscan. Clinical findings correlated with the change in biochemical results. Atorvastatin revealed a significant increase in NOlevel, which is the cornerstone for improving endothelial dysfunction. The term 'endothelial dysfunction' has now become synonymous with reduced NO.[29]

As indicated from results, atorvastatin had antioxidants activities (the increase in GPO), anti-inflammatory activities (the decrease in CRP and IL-6) and enhancement effects on NOS activity, in addition to increasing NO production and improving erectile function tests.In contrast, vitamin E only had antioxidants activities (the increase in SOD and GPO) and anti-inflammatory activities (the decrease in IL-6) without affecting NOS activity, NO level or erectile function tests. Taken together, these results indicated that increasing NOS activity was expected to be the major mechanism for NO increase and for improvement of both endothelial dysfunction and ED.

These clinical findings were in agreement with previous clinical trials that noticed the improvement of ED on adding atorvastatin to sildenafil therapy.[18] Treatment with 80 mg of atorvastatin daily for a period of 12 weeks improved the IIEF score by 7.8 in sildenafil nonresponders, supporting the hypothesis that vascular endothelial dysfunction contributes to ED. With regard to vitamin E, many animal and experimental studies showed the improvement in endothelial function and NO level on using vitamin E, as a combination therapy with sildenafil.[30,31] In contrast, this improvement failed to be established in clinical aspects.[32,33]

Previous preclinical animal studies supported the improvement in both erectile function and eNOS by atorvastatin through the inhibition of Rho/Rho-kinase contractile mechanisms of the penis. In experimental diabetes,[34] atorvastatin ameliorated the erectile response to the electrical stimulation of the cavernous nerve and normalized sildenafil effect on erectile function in diabetes through inhibiting RhoA/ROCK signaling and activating eNOS. In addition, in spontaneously hypertensive rats,[35] statins interfered with the RhoA/ROCK-mediated 'excitation–transcription coupling'. Atorvastatin inhibited RhoA membrane translocation and ROCK activity and restored penile erection. These effects were unrelated to the lipid-lowering activities of statins (Pleiotropic effects).

The significant increase in NO level after atorvastatin treatment was supported by earlier findings that showed that treatment with atorvastatin enhanced plasma NO concentrations, and increased the vascular activity to sildenafil in pharmacological researches.[36] However, vitamin E gave controversial results in earlier studies. Whereas some studies reported an increase in NO bioavailability,[37] others revealed no significant change or even a reduction in NO activity.[38]

Numerous studies reported the role for oxidative stress in reducing NO bioavailability. Oxidants such as superoxide anions (O_2-) react with NO to form peroxy NO2- radical, damaging

endothelial function. This explains why antioxidants may theoretically have a role in maintaining normal endothelial function.[39] Earlier studies confirmed the antioxidant activities of both vitamin E and atorvastatin. Regarding atorvastatin, it was found that atorvastatin interfered with the oxidation process, leading to the reduction of circulating markers of oxidation.[40] The effects of atorvastatin on reducing oxidative stress were significantly greater than other statins.[41] The antioxidant role of vitamin E was well established in earlier studies. Vitamin E is the major lipid-soluble antioxidant protecting cells against free-radical-mediated lipid peroxidation.[42]

The nonsignificant change in SOD activity did not interfere with atorvastatin antioxidant activities that were established by the significant increase in GPO activity. The reason might be that each type of antioxidant enzymes interacted with different types of oxidants.

Many studies confirmed the role of inflammation in reducing NO level. Endothelial function was impaired in the presence of inflammatory conditions.[43] Earlier studies supported the anti-inflammatory role of both atorvastatin and vitamin E on endothelial dysfunction. Regarding atorvastatin, it was observed that statins improved NO bioavailability by suppressing the expression of proinflammatory mediators in diabetic patients.[44] Furthermore, atorvastatin therapy was associated with inflammation decrease and an improvement in endothelial function in heart failure cases.[45] In addition, vitamin E had beneficial effects on diabetic nephropathy through selective reduction of inflammatory response.[46]

A possible explanation for this unexpected CRP result by vitamin E might be the limitation of latex agglutination test, which is semiquantitative in nature. This test depended on visual examination, leading to higher error rates than other quantitative methods, which mainly use the Enzyme-linked immunosorbent assay technique. Thus, the CRP test might be less sensitive.

NO synthase is responsible for NO formation in the endothelium. This study displayed that atorvastatin caused a significant increase in NOS activity, with subsequent induction of NO formation from the endothelium. The positive effects of atorvastatin on NO and NOS activity were the real difference between atorvastatin and vitamin E in biochemical results. Taken together, activating NOS by atorvastatin was expected to be the major mechanism responsible for increasing NO bioavailability.

The activity of NOS was measured in RBC lysate. RBCs contain only functional endothelial NOS and can be used as an indirect indication to penile eNOS.[11] Previous results failed to show any activities of either nNOS or inducible NOS in RBCs.[47] These atorvastatin effects on eNOS were in agreement with earlier findings that established the positive correlation between atorvastatin and eNOS activity. It was noted that eNOS functionality could be restored by statins with improvement in endothelial function.[28] In addition, combining low-dose atorvastatin with sildenafil augmented the myocardial infarct size limiting effects through enhancing penile P-eNOS expression.[48] In contrast, earlier studies gave conflicting results regarding the effects of vitamin E on eNOS. Most studies reported lacking activity on endothelial NOS.[49]

Clinical findings displayed that atorvastatin increased the IIEF-5 score and improved both tip and base rigidity by Rigiscan, indicating a real improvement in erectile function tests. Atorvastatin caused an increase in IIEF-5 by 6.3. Earlier clinical studies reported that atorvastatin showed a 7.8 increase in IIEF score[18] and a 3.87 increase in SHIM score[6] in patients with ED. However, vitamin E did not succeed in showing any significant change in these clinical tests.

The significant increase in IIEF-5 scores and favorable responses to questions 2, 3, 4 and 5 represented a clinically relevant success. Most participants experienced an improvement in erectile function within 4–6 weeks after taking atorvastatin as a daily maintenance dose. Despite the fact that it was not likely that all patients were accurate and uniform when recording IIEF-5 scores, this study allowed a preliminary conclusion to be considered.

The large increase in the scores of question 2 and question 3 suggested a specific positive action of atorvastatin in improving penis firmness (question 2) and erection maintenance ability (question 3). In addition, some patients taking atorvastatin achieved a response of 5 to some questions, but none of the participants regained normal erectile function (IIEF-5 score <22).

Mean Rigiscan parameters revealed a significant increase in both tip and base rigidity only after atorvastatin therapy. However, event duration or tumescence showed no significant change. Although the Rigiscan was not a good predictor of therapeutic response,[50] this uniform increase in rigidity parameters accompanied by the large increase in question 2 (penis hardness or rigidity) should be considered as a definite improvement triggered by atorvastatin administration.

The responsiveness to sildenafil was not evaluated at the end of intervention intake. This was a definite limitation of the study. Further studies may be required to evaluate the responsiveness to sildenafil after atorvastatin intake.

Testosterone level for hypogonadal participants was also evaluated at the end of the study, and testosterone deficiency was still present. Androgen replacement therapy was not started during the study period. This ensured that any improvement was directly related to the improvement of endothelial function by drug therapy and not by androgen therapy.

The current study concludes that activating eNOS is the main mechanism responsible for improving ED after atorvastatin administration in patients initially irresponsive to sildenafil. In addition, atorvastatin is a promising drug that can be used as salvage treatment for sildenafil failure. In addition, the present study discourages the claims that vitamin E can improve erectile function in clinical practice. However, larger controlled studies for longer durations may be needed to establish the real cost-effectiveness of such a combination.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript to be disclosed.

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