

Vitamin D₃, L-Arginine, L-Citrulline, and Antioxidant Supplementation Enhances Nitric Oxide Bioavailability and Reduces Oxidative Stress in the Vascular Endothelium – Clinical Implications for Cardiovascular System

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ABSTRACT

Background: Nitric oxide (NO) is a crucial signaling molecule which regulates the blood flow and prevents the adhesion of blood components to the vascular wall. A deficiency in bioavailable NO concentration is associated with the dysfunction of endothelial NO synthase (eNOS) and/or an increase in oxidative stress. The deficiency of bioavailable NO is a common denominator of several cardiovascular diseases, including diabetes, atherosclerosis, and hypertension. **Materials and Methods:** We used a nanomedical technology to elucidate the balance between bioavailable NO and oxidative stress (peroxynitrite ONOO⁻) in human umbilical vein endothelial cells (HUVECs) treated with a supplement containing L-arginine, L-citrulline, Vitamin D₃, and antioxidants. Nanosensors, with a diameter of 200–300 nm, are capable of measuring *in situ* NO and peroxynitrite (ONOO⁻) concentrations produced by single endothelial cells.

Results: The ratio of the concentration of cytoprotective NO [NO] to the concentration of cytotoxic peroxynitrite [ONOO⁻] was used to estimate the efficiency of eNOS. HUVECs incubated with L-citrulline, L-arginine, and Vitamin D₃ increased the [NO]/[ONOO⁻] ratio by 25%, while in the presence of antioxidants, the increase was 15%. The synergistic effect between the mix of L-arginine, L-citrulline, Vitamin D₃, and antioxidants was a favorable increase of the overall [NO]/[ONOO⁻] ratio by 50%.

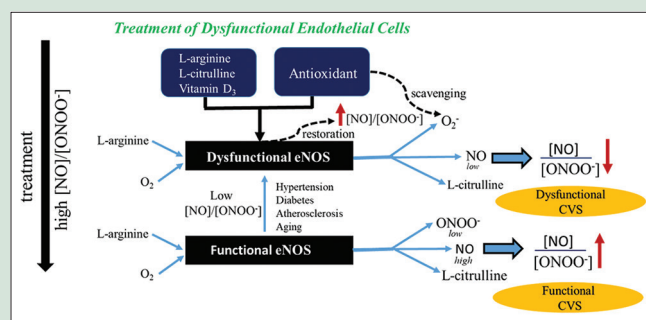
Conclusion: The findings of the study presented here clearly indicate that L-arginine, L-citrulline, and Vitamin D₃ can significantly alter the function of the endothelium and NO production, in a favorable manner, while pointedly reducing ONOO⁻ – the main component of oxidative stress. This effect can be significantly potentiated in the presence of antioxidants.

Key words: Antioxidant, endothelium, L-arginine, L-citrulline, nitric oxide, peroxynitrite, Vitamin D₃

SUMMARY

- Nanomedical studies were used to elucidate the role of a mixture of Vitamin D₃, L-arginine, L-citrulline, and several antioxidants in the improvement of nitric

oxide production and the reduction of oxidative stress in human endothelial cells. It appears that the combination of natural products can effectively improve endothelial function by about 50% and has shown that, on cellular models, it could potentially be used to improve the endothelial function in cardiovascular diseases.



Abbreviations Used: HUVECs: Human umbilical vein endothelial cells; O₂⁻: Superoxide; HBSS: Hank's balanced salt solution; EC: Endothelial cell; Cal: Calcium ionophore; CVD: Cardiovascular disease; eNOS: Endothelial nitric oxide synthase.

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INTRODUCTION

Nitric oxide (NO) is a gaseous molecule that is generated by the NO synthase (NOS) enzyme. NO is synthesized from two substrates: L-arginine (non-essential amino acid) and oxygen.^[1,2] This synthesis occurs through NOS in a five-electron transfer oxidation of L-arginine to L-citrulline. NOS is located in the membrane of endothelial cells, and its synthesis is stimulated by calcium flux.^[3,4] In the cardiovascular system, the calcium flux is triggered by a mechanical process (shear stress)^[5] and chemical stimuli such as acetylcholine, norepinephrine, angiotensin II, and many others.^[6,7]

NO can react rapidly with many biological components, including superoxide (O₂⁻), Fe (III) of hemoglobin, guanylate cyclase, and many others.^[8-10] Therefore, the measurement of reactive “free” NO is a challenging problem. In our laboratories, we are able to perform measurements of bioavailable NO produced by a single endothelial cell in different segments of the cardiovascular system, such as

capillary vessels, aorta, and heart. Maximal NO concentrations vary significantly, depending on the location of the endothelial cells – with the lowest concentrations in the small capillary (about 80 nM) and the highest in the endocardium of the heart (about 2.0 μM).^[11] The level of NO concentration depends largely on the velocity and type of blood flow (laminar vs. turbulent).^[12-14]

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There are two important functions of NO in the cardiovascular system: first, NO is a crucial signaling molecule which regulates the blood flow; second, it prevents the adhesion of blood components, as well as cells or bacteria, which may be present in the bloodstream. In the signaling process, NO released in the membrane of the endothelium diffuses to smooth muscle cells through cyclic guanosine monophosphate, which stimulates smooth muscle relaxation and increases the diameter of arteries, as well as the velocity and volume of the transportation of blood.^[15-17] In the brain, NO signaling also regulates blood flow through capillary vessels and is crucial for long-term memory.^[18,19]

NO, which diffuses through the luminal surface of endothelial cells, forms a thin protective layer that is essential in the prevention of adhesion. In the absence of luminal NO, red blood cells (RBCs), leukocytes, and platelets will be adsorbed on the surface of the endothelium, potentially forming a clot, which limits/prevents the flow of blood (which can lead to ischemia and stroke) and hinders further production of NO.^[15,20] An insufficient level of luminal NO is a trigger for stroke and atherosclerosis and can promote the adhesion of tumor cells, cholesterol, and β -amyloid.^[21,22]

A deficiency of NO in the cardiovascular system can be due to the low efficiency of NOS and/or high production of superoxide (O_2^-).^[8-10,23] Superoxide is the most efficient scavenger of NO to form peroxynitrite ($ONOO^-$). The rate of the reaction between NO and O_2^- is diffusion controlled. The reaction in each collision of NO with O_2^- produces $ONOO^-$.^[24,25] Superoxide and peroxynitrite are two of the major components of oxidative stress. The reaction of NO and O_2^- is faster than superoxide dismutase (SOD) that can dismutate O_2^- into H_2O_2 . Therefore, it is crucial for the maintenance of the cardiovascular system to produce biologically optimal levels of NO and low levels of O_2^- .

We found that a deficiency in bioavailable NO and/or an excess of O_2^- is a common denominator of several diseases^[12,13,26-30] such as hypertension, diabetes, stroke, aging, heart attack, and many other neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, epilepsy, and migraine.^[31-33] Therefore, it is our opinion that to prevent, treat, and hinder the progression of these diseases, it is absolutely essential to restore and maintain a high level of bioavailable NO and to reduce the level of oxidative stress.

Our laboratory was the first in the world to measure the production of NO in a single endothelial cell,^[34] *in vivo* in humans,^[35] and in the beating heart^[11,36] by utilizing nanomedical nanosensors with a diameter smaller than 300 nanometers (300×10^{-9} m). These studies elucidate NO and peroxynitrite concentrations using nanomedical sensors to measure NO and $ONOO^-$ release produced by human umbilical endothelial cells (HUVECs) in the presence of elevated concentrations of Vitamin D₃, L-arginine, L-citrulline, and several antioxidants.

MATERIALS AND METHODS

Cell culture

Human umbilical vascular endothelial cells (HUVECs) were isolated into primary cultures from female donors of Caucasian Americans (CAs). All cell culture donors were healthy, with no pregnancy or prenatal complications. The cultured cells were incubated in 95% air and 5% CO₂ at 37°C and passaged by an enzymatic procedure. The confluent cells (about 5×10^5 cells/35 mm dish) were placed with minimum essential media, containing 3 mM L-arginine and 0.1 mM (6 R) 5,6,7,8-tetrahydrobiopterin. Before experimental measurements, the cells were rinsed twice with HEPES buffer.

Chemicals

The supplement, named Cardio Miracle – which we will refer here as

CARDIO for simplification – was used as a model for these studies and was donated from evolution nutraceuticals. One serving size of CARDIO is 11.4 g and consists of the following components: L-arginine (3000 mg), L-citrulline (1000 mg), Vitamin D₃ (1500 IU), as well as a proprietary blend of antioxidants consisting of organic mushroom powder, grape seed extract, Orgen-B (guava extract), citrus lemon, mango extract, watermelon extract, hawthorn berry extract, turmeric powder, AstraGin (Panax), ginseng (root), *Astragalus membranaceus* (root), and pine bark extract. In addition, there is a proprietary blend of organic vegetables and fruits consisting of organic beet powder, organic carrot powder, organic coconut powder, organic pineapple powder, organic acerola powder, organic blueberry powder (790 mg), as well as a blend of fiber, flavors, prebiotics, and probiotics which include erythritol vegetable fiber blend (maize dextrin), organic acacia gum sunfiber (guar gum), citric acid, pomegranate extract, TASTEVA (stevia extract), Lactopore (*Bacillus coagulans*) (5695 mg).

Measurements of nitric oxide and $ONOO^-$

Measurements of NO or $ONOO^-$ were performed with electrochemical nanosensors with a diameter of 200–300 nm. Their design was based on previously developed and chemically modified carbon-fiber technology.^[34,37] Each of the nanosensors was made by depositing a sensing material on a carbon-fiber tip (length of 3–5 μ m and diameter of 100–300 nm). The fibers were sealed within a nonconductive epoxy and the electrically connected to copper wires with conductive silver epoxy. Electrically conductive film of polymeric n-type semiconductors Ni (II) tetrakis (2-methoxy-4-hydroxyphenyl) porphyrin or Mn (III) paracyclophanyl porphyrin was used for NO and $ONOO^-$ sensors, respectively.

Electrochemical measurements were performed using a Gamry III double-channel potentiostat. The electrochemical response was measured in amperometric mode, with a time resolution of about 1 μ s, using a three-electrode system with the nanosensor as a working electrode, silver/silver chloride as a reference electrode, and platinum as a counter electrode. The sensor was positioned in the proximity of the membrane of endothelium with the help of a remote-controlled micromanipulator (Sensapex). All sensors were calibrated using a standard solution of NO in a buffer (about 2.0 mM saturated solution of NO) or about 0.40 mM solution of $ONOO^-$.^[38]

Typical experiments

A controlled confluent layer of endothelial cells was placed in a cell dish (2 ± 5 μ m diameter) under a microscope. With the help of a remote-controlled micromanipulator, the sensor was positioned at a well-defined distance (2 ± 5 μ m) from the membrane of the endothelial cell. Utilizing a nano-injector, a stimulus of NO release was injected at a constant distance from the sensor. An amperometric curve (response) was then recorded, and the current was calibrated as concentrations versus time. At a potential of 0.6 V, it was typically about 2s after the injection that the maximum concentration was recorded.

RESULTS

Figure 1 shows a typical amperometric (concentration/current vs. time) response of the nanosensor to a standard concentration of NO. The amperometric curve was obtained about 50 ms after injection of NO standard solution to Hank's balanced salt solution (HBSS) buffer. As expected, after injection of NO, a rapid increase in concentration was observed, reaching a maximum of about 400 nmol/L after about 3s. After that, the NO concentration decreased due to its oxidation to NO_2^-/NO_3^- . Under this "environmental" condition, the half-life ($t_{1/2}$) of NO is about 6 s. After about 25 s, the NO concentrations dropped to

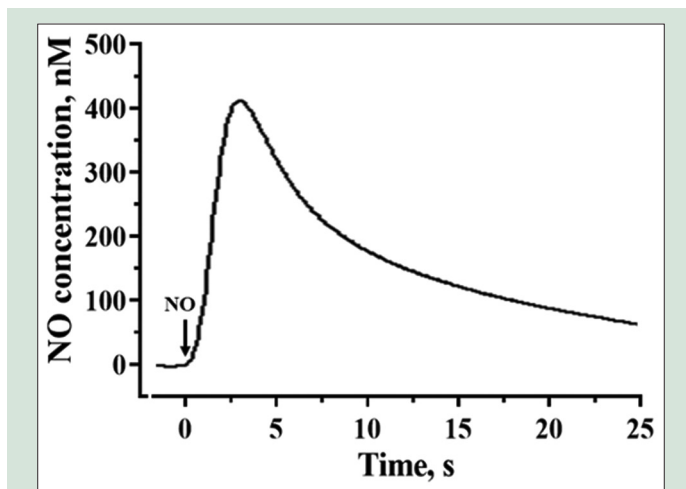


Figure 1: Amperometric curve showing response of nitric oxide sensor to 400 nM concentration of standard nitric oxide solution (Hank's balanced salt solution buffer)

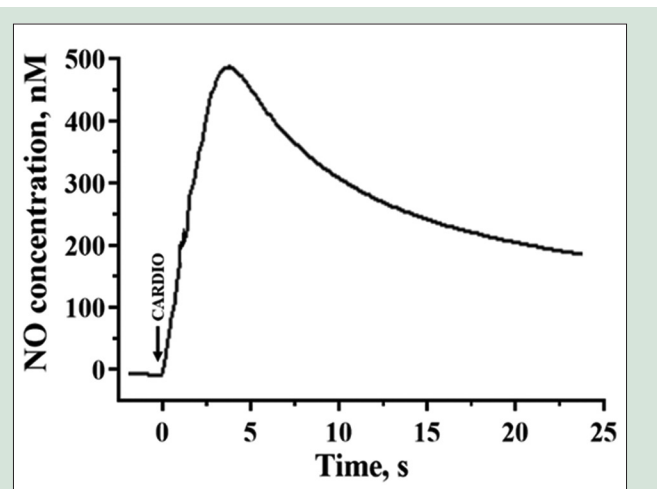


Figure 2: Amperometric curve showing response of nitric oxide sensor to 0.03% of CARDIO in HUVECs (Hank's balanced salt solution buffer)

about 50 nmol/L (about 10% of its original maximal level). This curve represents the dynamics of NO propagation, diffusion, and chemical transformation. The peak concentration is a characteristic value, representing the maximal concentration of “bioavailable” NO, and this concentration is crucial for the performance of any biological system involving NO as a messenger, as well as an inhibitor of adhesion. When concentrations are “too low” (most common) or “too high,” we observe this is a sign of pathological dysfunction of the NO-producing system.^[13]

Figure 2 depicts amperometric signals showing changes of NO concentration produced by endothelial cells after injection of CARDIO (0.03%). HUVECs of CAs in HBSS were used for this experiment. A maximal NO concentration of about 500 nmol/L was recorded about 2.5 s after the microinjection of CARDIO. The rate of NO release is moderate, about 20 nmol/L, per second. This rate of NO production, *in vitro*, is similar to the rate of production which we observed *in vivo*. The half-life of NO for this *in vitro* condition is about 10 s, which indicates that the level of oxidative stress produced during the NO release is rather low. This experiment is crucial for the characterization of the role of CARDIO in the cardiovascular system. CARDIO instantly stimulated NO release in the endothelium, and most importantly, the kinetics of NO production may be favorable for endothelial NO propagation and signaling.

The next experiment which we performed in this study was designed to show the long-term (hours) effect of CARDIO treatment on endothelial NO. In this experiment, the endothelial cells (CA) were treated with a constant concentration of CARDIO (0.03%, 100 nmol/L of D₃) for different periods of time, from 3 to 24 h. After each treatment, the cells were rinsed and placed in HBSS. NO release from the endothelium was stimulated with calcium ionophore (1.0 μM). Calcium ionophore induced calcium flux and stimulated NO release by endothelial NOS (eNOS). Stimulation with calcium ionophore produces NO concentrations which are close to the maximal levels that can be produced by endothelium under any given condition.

Figure 3 presents the data collected from these experiments. It is shown clearly that with the treatment by CARDIO, there is an increase in the efficiency of NO production by endothelium. After incubation of endothelial cells with 0.03% of CARDIO, NO concentration stimulated by calcium ionophore increased significantly by about 15%, as compared to control, untreated endothelium. After six hours of incubation with CARDIO, the NO bioavailability increases by about 30%. After further

incubation with CARDIO, a slight decrease in NO release was observed. However, the level of NO after 24 h of incubation with CARDIO was still significantly higher than control.

Using the nanosensors, we simultaneously measured the concentrations of NO, together with the concentrations of peroxynitrite (ONOO⁻). ONOO⁻ is a product of the fast reaction between NO and superoxide (O₂⁻). Peroxynitrite is a very strong oxidant and is one of the most destructive molecules in the biological milieu. The maximal concentration of ONOO⁻ produce by HUVECs and the ratio of NO concentration [NO] to ONOO⁻ concentration [ONOO⁻] are depicted in Figures 4 and 5. The incubation with CARDIO decreased [ONOO⁻] [Figure 4] and significantly increased the ratio of [NO]/[ONOO⁻] [Figure 5]. This ratio of [NO]/[ONOO⁻] was used as a marker of endothelial function. In normal, functional endothelium [NO]/[ONOO⁻] is 2–5. With a decrease in eNOS efficiency and endothelial function, the ratio also decreases. When the level of the ratio falls below 1.0, we observed significant endothelial and cardiovascular dysfunction.

The significant increase in the [NO]/[ONOO⁻] that was observed in the presence of CARDIO is a strong indicator of improvement in endothelial function and may also have a positive effect on the improvement of the cardiovascular system. In addition, [NO]/[ONOO⁻] increases with time of incubation with CARDIO.

The composition of CARDIO consists of two major groups. The first is L-arginine, L-citrulline, and Vitamin D₃ which can directly influence eNOS. The second is a group of antioxidants that may influence the level of oxidative stress. We tested these two groups separately on the release of NO, ONOO⁻, and the ratio of [NO]/[ONOO⁻] [Figure 6]. [NO]/[ONOO⁻] ratio was about 2.4 ± 0.14 at control. After incubation with CARDIO, the ratio increase significantly to about 3.7 ± 0.14. After incubation with L-arginine, L-citrulline, and Vitamin D₃, the ratio increased to 3.1 ± 0.15. The incubation of cells with antioxidants also increased [NO]/[ONOO⁻] to about 2.8 ± 0.13. The percentage of the improvement in [NO]/[ONOO⁻] ratio measured after incubation of different components of CARDIO versus placebo is shown in Figure 7. The most efficient improvement came through treatment with a mix of L-arginine, L-citrulline, and Vitamin D₃ (by about 25%), while improvement with antioxidants alone was only about 15%. However, a clear synergistic effect is shown with a combination treatment between the components of this group and the antioxidant group, CARDIO, with about a 50% overall improvement.

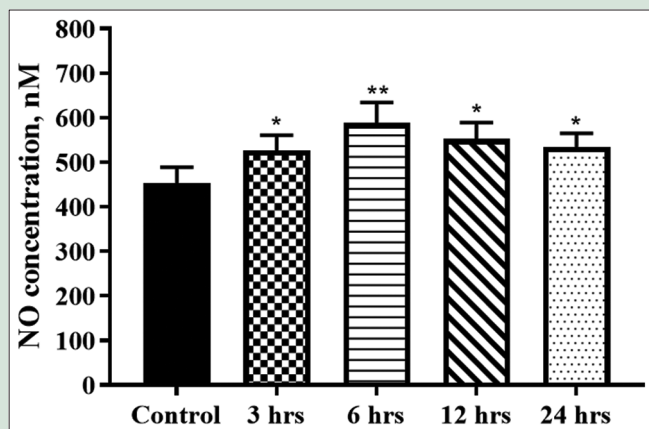


Figure 3: Maximal concentration of nitric oxide produced by HUVECs after stimulation with calcium ionophore (1.0 μ M) in Hank's balanced salt solution buffer. The cells were incubated with CARDIO 0.03% (100 nmol/L of D₃) for 3, 6, 12, and 24 h in serum-free media. * $P < 0.05$; ** $P < 0.005$ versus control group (unpaired, two-tailed Student's t -test). Data shown are the means \pm standard deviation, $n = 5$

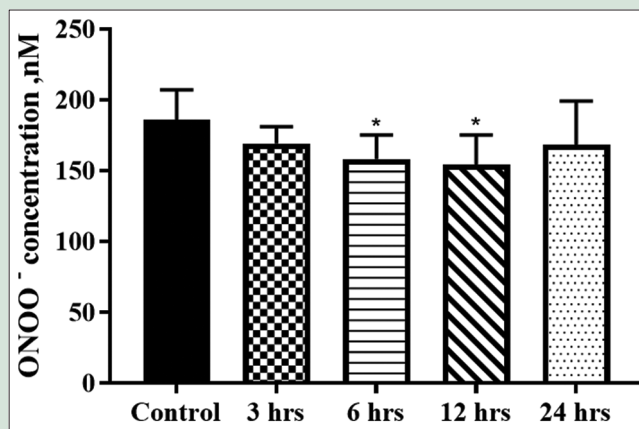


Figure 4: Maximal concentration of ONOO⁻ – produced by HUVECs (Caucasian American) after stimulation with calcium ionophore (1.0 μ M) in Hank's balanced salt solution buffer. The cells were incubated with CARDIO 0.03% (100 nmol/L of D₃) for 3, 6, 12, and 24 h in serum-free media. * $P < 0.05$ versus control group (unpaired, two-tailed Student's t -test). Data shown are the means \pm standard deviation, $n = 5$

DISCUSSION

One of the main observations in this study is that a mixture of L-arginine, L-citrulline, Vitamin D₃ and antioxidants significantly increases the concentration of bioavailable, cytoprotective NO that is produced in HUVECs. At the same time, the level of cytotoxic ONOO⁻ decreased. The increase in [NO] and the decrease in [ONOO⁻] improves the ratio between the two important signaling molecules in endothelial cells.

Using nanosensors, we measured the concentrations of NO and ONOO⁻ close to the surface of the membrane. We call this concentration the “surface concentration” of NO, and it is measured at a distance of about 5–10 μ m from the surface of the membrane of an endothelial cell, under static conditions and/or dynamic conditions, in the presence of blood flow. The surface concentration creates a gradient of concentrations, which is the main force for NO diffusion and propagation. This gradient of concentration is of prime importance in proper NO signaling which is crucial in the maintenance of the smooth muscle relaxation and the optimal diameter of blood vessels. A low gradient of NO will hinder the rate of diffusion in timing (signaling to slow) as well as the distance (short distance signaling), which can lead to the thickening of arteries and artery stiffness, which is associated with atherosclerosis and other cardiovascular diseases. Furthermore, at a low gradient, the NO concentration near the vascular wall is too low to penetrate or adhere. Our previous research of NO release and propagation clearly shows that it is not the total production of NO but the production of bioavailable NO (NO which survives at least 1–6 s in the biological environment) that is important in the proper function of the cardiovascular system.^[39-42]

NO signaling is crucial for smooth muscle relaxation, the regulation of blood, and long-form memory.^[18,19] In addition, NO is a molecule that prevents the adhesion of RBC, leukocytes, platelets, cells, bacteria, cholesterol, and β -amyloid to the surface of endothelial cells. Therefore, a sufficient concentration of bioavailable NO is needed to maintain the optimal blood flow in the vasculature. The deficiency in NO is not only caused by dysfunctional eNOS but also by superoxide production via NADPH oxidase; the molecule is also scavenged by superoxide (O₂⁻). NO reacts rapidly with O₂⁻ in a diffusion-controlled reaction ($k = 5 \times 10^9$) to produce another power

oxidant – peroxynitrite (ONOO⁻). It appears that every collision between NO and O₂⁻ molecules produces ONOO⁻. Both ONOO⁻ and O₂⁻ are the main components of oxidative/nitroxidative stress. It is well documented that oxidative/nitroxidative stress can seriously damage major components of the biological system (DNA, mitochondria, muscle cells, endothelial cells, etc.), leading to apoptosis and even cell death. The bottom line in preserving the function of the cardiovascular system is to maintain proper ratio between cytoprotective [NO] and cytotoxic oxidative stress [ONOO⁻].^[43]

Here, we have introduced several parameters which can accurately reflect the status of function in endothelial cells in both normal and diseased states. These parameters include the ratio of the maximal concentration of NO [NO] to the maximal concentrations of superoxide (O₂⁻) and/or peroxynitrite (ONOO⁻). This simultaneous, *in situ* measurements of these molecules is a powerful tool for determining the level of disease in the cardiovascular, as well as the effect of treatments on these diseases with various drugs. We have proven the efficiency and accuracy of our diagnostic tool, using several models (both animal and human) of diseases.^[27,30,44-47] We have proven our hypotheses that an improvement of NO production alone may not be sufficient enough for the improvement of a functional cardiovascular system. We found that the level of bioavailable NO must be recorded/measured simultaneously with the level of oxidative stress to have a precise diagnostic tool for the cardiovascular in health and disease.

In normal, functional endothelium, the ratio of [NO]/[ONOO⁻] is about 2.0–5.0, while in dysfunctional endothelium (diseased state), this ratio is below 1.0. In the studies presented here, we performed several experiments to make a list of preliminary estimations of how efficient and beneficial CARDIO could be in the enhancement of endothelial function and the efficiency of the cardiovascular system. These are preliminary experiments that have to be validated in animal models and clinical studies in humans but remain necessary to present the case.

The data presented here clearly indicate that the level of toxic ONOO⁻ produced by the endothelium decreased significantly (by 10%–15%) after treatment with CARDIO for up to 12 h. This is a significant finding. We hypothesized at this stage of research that a decrease in ONOO⁻ is due to a decrease in O₂⁻ and that decrease is due to

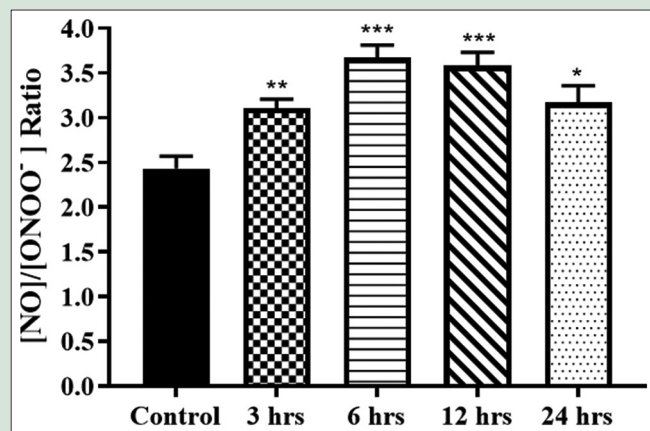


Figure 5: Ratio of nitric oxide to ONOO⁻ concentrations [NO]/[ONOO⁻] produced by HUVECs after stimulation with calcium ionophore (1.0 μM) in Hank's balanced salt solution buffer. The cells were incubated with CARDIO 0.03% (100 nmol/L of D₃) for 3, 6, 12, and 24 h in serum-free media. **P* < 0.05; ***P* < 0.005; ****P* < 0.0005 versus control group (unpaired, two-tailed Student's *t*-test). Data shown are the means ± standard deviation, *n* = 5

the action of Vitamin D₃, L-arginine, and L-citrulline, as well as several antioxidants contained in CARDIO. Having the maximal concentrations of NO and ONOO⁻ measured, we constructed a diagnostic plot for the action of CARDIO on the endothelium. These are the most important data from the study presented here [Figure 4].

CARDIO significantly improves endothelial function. After 6 h of incubation with CARDIO, maximal NO improved by more than 30%, compared to control, proving that CARDIO can enhance the production of bioavailable NO and simultaneously reduce the level of peroxynitrite. Therefore, the total improvement of [NO]/[ONOO⁻] by CARDIO is about 50%, with roughly 25% stemming from the combination of L-arginine, L-citrulline, and Vitamin D₃ and the remaining 15% coming from antioxidants. Therefore, a mixture of L-arginine, L-citrulline, and Vitamin D₃, together with antioxidants, shows not a simple additive but a strong synergistic effect on the endothelium.

Our long list of nanomedical studies of NO production clearly indicates that not only the absolute production of NO but also its bioavailability is the main factor in the regulation and efficiency of the cardiovascular system.^[9,16,23,24,27] We found that NO production by the endothelium is always accompanied by the production of O₂⁻. It has been known that NO is an extremely efficient scavenger of O₂⁻ (about 10 times better than SOD). Peroxynitrite, ONOO⁻, is the byproduct of the NO scavenging of O₂⁻. In normal/functional endothelium, the production of ONOO⁻ is relatively small, and at low concentration, cytotoxic ONOO⁻ is converted rapidly into non-toxic NO₃⁻. However, it has been established that in both *in vitro* and *in vivo* experiments, when ONOO⁻ is produced at high concentrations, it can initiate a cascade of highly toxic and oxidative radicals within the endothelium. This high level of ONOO⁻ has extremely negative effects that are observed in many cardiovascular and neurodegenerative diseases.

Apparently, the beneficial effect of CARDIO is based on two important pathways: L-arginine/NO pathway and the reduction of oxidative stress. L-arginine, L-citrulline, and Vitamin D₃ considerably enhance the production of bioavailable NO by improving the functionality of NOS. Antioxidants scavenge superoxide and prevent its reaction with NO, which decreases the overall production of ONOO⁻. The net effect of this is a substantial increase in [NO]/[ONOO⁻].

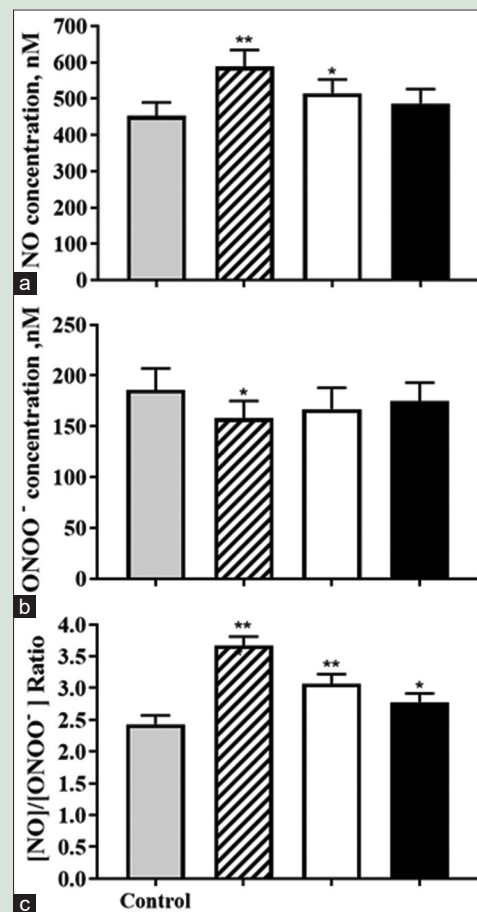


Figure 6: The maximal nitric oxide concentration (a), the maximal ONOO⁻ concentration (b) and the ratio of maximal nitric oxide concentration to maximal ONOO⁻ concentration (c) measured from HUVECs after stimulation with calcium ionophore (1.0 μM) in Hank's balanced salt solution buffer. The cells were incubated with 0.03% of different CARDIO components for 6 h in serum-free media: Control (without treatment (gray bars), (1) All components (dashed bars), (2) 3000 mg L-arginine, 1000 mg L-citrulline and 1500 IU vitamin D₃ (open bars), (3) antioxidants (solid bars). **P* < 0.05; ***P* < 0.005; versus control group (unpaired, two-tailed Student's *t*-test). Data shown are the means ± standard deviation, *n* = 5

Evidently, long-term treatment of endothelium with CARDIO improves endothelial function. The improvement of NO production in endothelial cells, *in vitro*, should have a significant effect on the function of the cardiovascular system and could be potentially used for the treatment and restoration of the function of a damaged cardiovascular system from the effects of hypertension, diabetes, atherosclerosis, and ischemia. However, this hypothesis must first be validated by *in vivo* studies in animal models.

CONCLUSION

Combined treatment of endothelial cells with Vitamin D₃, L-arginine, and L-citrulline improved vascular function as evidenced by increased NO bioavailability, reduced oxidative stress (ONOO⁻), and significantly increased the overall [NO]/[ONOO⁻] ratio. This effect is potentiated in a synergistic manner in the presence of the antioxidants. This effect may be of clinical importance for the restoration of dysfunctional endothelium that has been damaged by cardiovascular diseases.

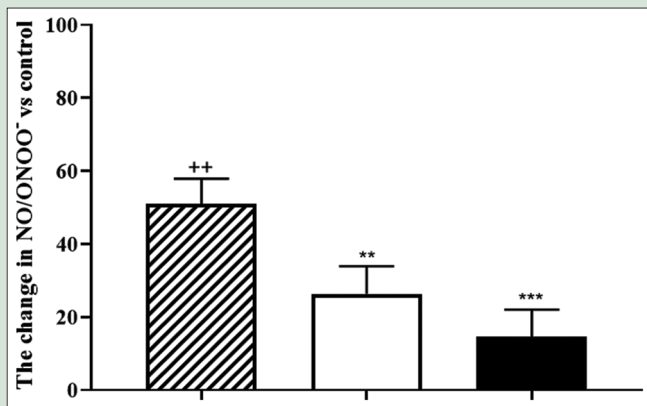


Figure 7: The relative changes (vs. untreated placebo) in the ratio of [NO]/[ONOO⁻] after incubation of HUVECs with 0.03% of different CARDIO components for 6 h in serum-free media. [NO] and [ONOO⁻] maximal concentration was measured after stimulation with calcium ionophore (1.0 μM); total CARDIO (dashed bars), L-arginine, L-citrulline, and Vitamin D₃ (open bars), antioxidants (solid bars). ***P* < 0.005, ****P* < 0.0005 versus total CARDIO; ++*P* < 0.005 versus antioxidant group only (unpaired, two-tailed Student's *t*-test). Data shown are the means ± standard deviation, *n* = 5

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Conflicts of interest

There are no conflicts of interest.

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